

Molecular phylogeny and re-assessment of some *Scleroderma* spp. (Gasteromycetes)

by

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Abstract

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The fungal genus *Scleroderma* is cosmopolitan in temperate and tropical regions and forms ectomycorrhizal associations with a wide range of forest trees. To delimit phylogenetic *Scleroderma* species and identification, 43 basidiomes were chosen of different geographical origins and their rDNA internal transcribed spacer (ITS) were sequenced. Phylogenetic analyses of these sequences together with additional GenBank sequences identified 11 taxa. A strong phylogenetic pattern was observed related to a character of their basidiospore ornamentation.

Keywords: Boletales, ecology, ectomycorrhizal fungi, gasteromycete, *Scleroderma*, taxonomy, phylogenetic species.

Introduction

Scleroderma was first introduced by Persoon (1801) with 11 species. The first *S. pistillare* L.: Pers. is now placed in *Podaxis* Desv. The second *S. herculeum* Pers. is placed in *Pisolithus* (Alb. & Schwein). The third, *S. carcinomarlis* L., is considered a synonym of *Podaxis pistillaris* (L.: Pers.) Morse. and fourth and fifth *S. tinctorius* Mich.: Pers. and *S. arbizus* Pers. respectively are considered at present to represent the same species, now placed in *Pisolithus*. This leaves a remaining six species of which *S. aurantiacum* Pers. and *S. citrinum* Pers. are now considered the same (Guzmán, 1970), under the latter name, *S. verrucosum*

Resumen

Phosri, C., Martín, M.P., Watling, R., Jeppson, M. & Sihanonth, P. 2009. Filogenia molecular y reevaluación de algunas especies de *Scleroderma*. *Anales Jard. Bot. Madrid*. 66S1: 83-91 (en inglés).

Las especies del género *Scleroderma* son cosmopolitas y forman ectomicorrizas con un amplio rango de hospedantes, tanto de zonas templadas como tropicales. Para delimitar las especies filogenéticas y la identificación de las mismas, se han seleccionado 43 basidiomas, de diferente origen geográfico, y se han secuenciado las regiones de transcripción interna del ADN ribosómico nuclear. Los análisis filogenéticos de estas colecciones, junto con secuencias obtenidas del GenBank, permiten identificar 11 especies, agrupadas en tres clados relacionados con el tipo de ornamentación de la basidióspora.

Palabras clave: Boletales, ecología, especies filogenéticas, gasteromicete, hongo ectomicorrízico, *Scleroderma*, taxonomía.

(Vaill.) Pers. and *S. spadiceum* Schaeff.: Pers., with this last species considered a *nomen confusum* (Guzmán, 1970), *S. cepa* Pers. and *S. cervinum* (L.) Pers. *S. cervinum* undoubtedly is referable to the ascomycetous genus *Elaphomyces* (Guzmán, 1970). As presently understood *Scleroderma* is restricted to Persoon's *S. citrinum* and *S. verrucosum* consortia. At least 25 species is classified within the Sclerodermataceae (Hawksworth & al., 1995; Sims & al., 1995).

The characters which have been extensively used to separate the species within the genus are the thickness and the scaliness of the peridium and whether the basidiome is stalked or not, and if the former the structure of the stipe. In the case of *S. polyrbizum* (J.F. Gmel.)

Pers. the shape of the basidiome in old age is also important. These characters at a later date were combined with the size of the basidiospores and their ornamentation and in the latter case whether the ornamentation is composed of isolated spines or warts or forms a reticulum. The spines may be narrow or broadly based or the reticulum complete and very distinctive or incomplete. Vaillant (1727) in '*Botanicon Parisiensis*' was probably the first author to recognize a species of *Scleroderma*, viz. *S. citrinum* in the phrase '*Lycoperdon ornati colores ad basim rugosum*' but even after that date confusion between the puff-balls and the earth-balls persisted to which Fries (1821) blamed Micheli in his lumping.

Many publications include species of *Scleroderma* but it was Šebek (1953) who first attempted a consolidation of the known facts based on Czechoslovakian collections. Although this author gave an excellent account of the history of the genus his interpretation of the specific names was very different to that accepted by the classical authors and that in current use in other European countries. It was not until 1970 that the genus was fully monographed (Guzmán, 1970) and his concepts have generally stood the test of time. Guzmán (1970) also gave a good historical account of the genus and the various fungi assigned to it over the years.

Guzmán (1970) divided the genus into three subgenera, those species with spiny basidiospores (*Aculeatispora*), those with subreticulate basidiospores (*Sclerangium*) and those with distinctly reticulate basidiospores (*Scleroderma*). Fries (1821) stated 'still doubt the possibility of an easy solution of the taxonomic position of the species in the genus *Scleroderma* which had been very negligently worked at the time' – fide Šebek (1953). Although much work has been carried out since this statement was made there are still some problems in the circumscription of some constituent taxa, viz. *S. verrucosum* (Bull.: Pers)/*S. cepa* Pers.: Pers and *S. bovista* Fr. and its relatives especially the recently described *S. septentrionale* Jeppson from Northern Europe.

The present article attempts to examine the broad division of *Scleroderma* using molecular techniques and to try and resolve the differences experienced whilst examining material of the taxa noted above. Included in this work are collections of the type species of the genus, *S. citrinum*. Two members of the subgenus *Sclerangium* i.e. *S. sinnamariense* Mont. and *S. polyrhizum* from Thailand and North America respectively are added to the analysis.

Materials and methods

Fungal Isolates

The *Scleroderma* samples used in this work were either fresh, cultured or herbarium specimens. They were collected from a variety of geographical origins (Table 1). Basidiomes for each collection were retained in the herbarium at the Biology Programme, Faculty of Science & Technology, Pibulsongkram Rajabhat University, Thailand. Cultures obtained from basidiomata were grown and maintained on Modified Melin Norkrans' (MMN) medium (Marx, 1981) at 30 °C with subculturing every 1-2 months. Herbarium specimens were provided by BCN, E, and A.D. Parker Herbarium in Wisconsin for comparison with those collections cultured. Basidiospores ornamentation was analysed using scanning electron microscopy (SEM). Samples were air-dried and sputter-coated with gold and examined with JEOL JSM-840 scanning electron microscope.

Molecular methods

Samples for DNA extraction were excised from fresh or dry basidiomes or the outer part of mycelium agar culture. To avoid contamination by other fungi, tissues were taken from the inner part of the basidiome. DNA was isolated from each sample with the E.Z.N.A. fungi DNA miniprep kit (Omega Biotech, GA, USA) as described by Martín & García-Figueres (1999). The ITS regions were amplified with Ready-To-Go™ PCR Beads (GE healthcare Life Sciences, NJ, USA) (Martín & Winka, 2000). Primer pairs ITS1F/ITS4B and ITS1/ITS4 were used for amplification of the ITS regions including the 5.8S of the ribosomal RNA gene cluster, as described by White & al. (1990) and Gardes & Bruns (1993). When no products were obtained with these primer pairs, ITS2 and ITS3 (White & al. 1990) were used in different combinations with the primers mentioned above, to amplify the two ITS regions separately. A negative control was prepared in each series of amplifications in order to detect possible contamination in the reagents. The PCR was performed using a PE 9700 DNA thermocycler (Perkin Elmer Applied Biosystems, MA, USA). The cycling parameters were five cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Before initiating the cycling parameters, an initial denaturation at 94 °C for 5 min was done. PCR products were analysed by electrophoresis on 2% agarose gels

Table 1. List of *Scleroderma* and *Pisolithus* sp. collections.

Name	DNA isolation code	Herbarium No.	Origin	Collection date	Genbank Accession No.
<i>Pisolithus</i> sp.	PISOL3	BCN-MPM2676	Catalonia, Spain	-/10/1997	FM213365
<i>Scleroderma areolatum</i>	ARESC1_1	E00278288	Sheboygan county, WI., USA	05/09/1998	FM213351
<i>S. areolatum</i>	ARESC1_2	E00278290	Sheboygan county, WI., USA	05/09/1998	FM213352
<i>S. areolatum</i>	ARESC1_3	E00278286	Dane county, WI., USA	08/09/2001	FM213353
<i>S. bovista</i>	BOVSCL_1	BCN-MPM1989	Catalonia, Spain	12/07/1995	FM213340
<i>S. cf. bovista</i>	BOVSCL_2	BOVSCL_2	Richland county, WI., USA	03/10/1993	FM213339
<i>S. cepa</i>	CEPSCL_1	BCN-MPM2603	Catalonia, Spain	20/10/1996	-
<i>S. cepa</i>	CEPSCL_2	BCN-MPM2525	Catalonia, Spain	26/11/1995	FM213354
<i>S. cepa</i>	CEPSCL_3	BCN-MPM2526	Catalonia, Spain	01/05/1995	-
<i>S. cepa</i>	CEPSCL_4	E00278292	Dane county, WI., USA	08/09/2001	-
<i>S. cepa</i>	CEPSCL_5	E00278296	Adam county, WI., USA	21/09/2001	FM213355
<i>S. cepa</i>	CEPSCL_6	MJ1926	V. Tunhem, Sweden	30/8/1985	-
<i>S. citrinum</i>	CITSCL_1	E00278300	Adam county, WI., USA	20/09/1985	FM213344
<i>S. citrinum</i>	CITSCL_2	-	Dane county, WI., USA	22/09/2001	FM213345
<i>S. citrinum</i>	UNSC1_2	SCL3	Dawyck, Scotland	28/01/2002	FM213333
<i>S. citrinum</i>	UNSC1_3	SCL5	Dawyck, Scotland	28/01/2002	FM213334
<i>S. citrinum</i>	UNSC1_4	SCL7	Dawyck, Scotland	28/01/2002	FM213335
<i>S. michiganense</i>	MICSCL_1	E00278306	Sheboygan county, WI., USA	14/09/1991	FM213346
<i>S. michiganense</i>	MICSCL_2	E00278311	Sheboygan county, WI., USA	05/09/1998	FM213347
<i>S. michiganense</i>	MICSCL_3	E00278309	Waukesha county, WI., USA	30/08/1992	FM213348
<i>S. polyrhizum</i>	POLSCL_1	E00278315	Grant county, WI., USA	23/09/2001	FM213349
<i>S. polyrhizum</i>	POLSCL_2	E00278313	Richland county, WI., USA	10/10/2000	FM213350
<i>S. cf. septentrionale</i>	SEPSCL_1	E00278318	Adam county, WI., USA	14/10/2000	FM213337
<i>S. septentrionale</i>	SEPSCL_2	J. Nitare (12.9.1986)	Norrbottnen, Sweden	12/09/1986	FM213336
<i>S. septentrionale</i>	SEPSCL_3	A.D. Parker (2.10.1997)	Lone Rock, WI., USA	02/10/1997	FM213338
<i>S. sinnamariense</i>	SINSCL_1	SCLK4	Hot, Chiang Mai, Thailand	28/01/2002	FM213356
<i>S. sinnamariense</i>	SINSCL_2	SCLP3	Hot, Chiang Mai, Thailand	28/01/2002	FM213357
<i>S. sinnamariense</i>	SINSCL_3	SCLN	Hot, Chiang Mai, Thailand	28/01/2002	FM213358
<i>S. sinnamariense</i>	SINSCL_4	SCLY5	Hot, Chiang Mai, Thailand	28/01/2002	FM213359
<i>S. sinnamariense</i>	SINSCL_5	SC1	Hot, Chiang Mai, Thailand	28/01/2002	FM213360
<i>S. sinnamariense</i>	SINSCL_6	SCLD1	Hot, Chiang Mai, Thailand	28/01/2002	FM213361
<i>S. sinnamariense</i>	SINSCL_7	SINSCL_7	Muang, Yasothon, Thailand	12/08/2001	FM213362
<i>S. sinnamariense</i>	SINSCL_8	SINSCL_8	Pranomprai, Roi Et, Thailand	22/08/2001	FM213363
<i>S. sinnamariense</i>	SINSCL_9	SINSCL_9	Muang, Yasothon, Thailand	24/08/2001	FM213364
<i>Scleroderma</i> sp.	UNSC1_5	E00278667	Juneau county, WI., USA	25/09/1993	FM213342
<i>Scleroderma</i> sp.	UNSC1_6	E00278668	Adam county, WI., USA	14/10/2000	-
<i>Scleroderma</i> sp.	UNSC1_7	-	Hot, Chiang Mai, Thailand	11/12/2001	FM213343
<i>Scleroderma</i> sp.	UNSC1_8	MJ6006	Orgovány, Hungary	01/11/2002	FM213341
<i>Scleroderma</i> sp.	UNSC1_1	SCB	Hot, Chiang Mai, Thailand	28/01/2002	-
<i>S. verrucosum</i>	VERSC1_1	BCN-MPM1737	Catalonia, Spain	16/11/1993	-
<i>S. verrucosum</i>	VERSC1_2	BCN-MPM2523	Catalonia, Spain	31/10/1995	-
<i>S. verrucosum</i>	VERSC1_3	BCN-MPM2529	Catalonia, Spain	06/11/1995	-
<i>S. verrucosum</i>	VERSC1_4	BCN-MPM2605	Catalonia, Spain	29/09/1996	AJ629886

on 1 × Tris-acetate buffer (Sambrook & al., 1989) and visualized by staining with ethidium bromide. Products were cleaned directly using E.Z.N.A. Clean kit (Omega Biotech) or from the gel using QIAQuick PCR purification kit (QIAGEN, Chatsworth, CA). All samples were sequenced in both directions. The primers used for sequencing were ITS1, ITS2, ITS3 and ITS4. Aliquots of the purified products were mixed separately with the direct and reverse primers before sending to the Secugen company (Madrid, Spain) for sequencing. When weak PCR products were obtained, the products were cleaned from the gel using QIAquick Gel (QIAGEN) cloned with pGEM®-T Easy Vector Systems (Promega, Madison, WI) and purified with QIAprep Spin Mini prep (QIAGEN). To confirm that the correct product had been inserted, 2 µl of the purified sample was digested with *Eco* RI following the instructions of the manufacture. Both strands were sequenced separately using vector specific primers T7 and M13 in the Secugen company.

Sequences analysis: Sequences from each sample was assembled to obtain the consensus sequence of the ITS1, 5.8S and ITS2 nrDNA using Navigator™ Sequence comparison software (Perkin Elmer Applied Biosystem) or Sequencher (Gene Codes Corporation Inc, Ann Arbor, Michigan, USA). Multiple sequences alignment of the ITS regions from different collection with selected *Scleroderma* ITS sequences obtained from GenBank DNA database (<http://www.ncbi.nlm.nih.gov/entrez/>) i.e. *S. bovista* (AB211267 & AB099901), *S. cepa* (DQ453694) and *S. citrinum* (AY935514) were performed using the SEQAPP software (Perkin Elmer Applied Biosystem). The alignment was optimized visually. Alignment gaps were indicated as “_” and ambiguous nucleotides were marked as “N”.

Phylogenetic analyses: The alignment was analysed using the programme PAUP 4.0b10 (Swofford, 2003) and MrBAYES 3.0 (Huelsenbeck & Ronquist, 2001). *Pisolithus* sp. (Martin & al., 2002, clade of *Pisolithus* sp.4) was used as outgroup taxon as this species is closely related to *Scleroderma* (Binder & Bresinsky 2002). First a parsimony analysis under a heuristic search was conducted to analyse the phylogenetic relationships between the *Scleroderma* samples. Gaps were treated as missing data. The tree branch robustness was estimated by bootstrap (BS) analysis (Felsenstein, 1985) employing 10K replicates, using the fast-step option. The starting branch lengths were obtained using the Roger-Swofford approximation method and the starting trees for branch swapping were obtained by stepwise addition. The

tree bisection-reconnection (TBR) branch-swapping algorithm was used with the Multrees options in effect. The data were further analysed using a Bayesian approach (Huesenback & al., 2000; Larget & Simon, 1999). The posterior probabilities (PP) were approximated by sampling trees using a MCMC method. The Bayesian analysis was performed assuming the general time reversible model (Rodriguez & al., 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G). A run with 2M generation starting with a random tree and employing 12 simultaneous chains was executed. Every 100th tree was saved into a file. The log-likelihood scores of sample points were plotted against the number of generations using TRACER 1.0 (<http://evolve.zoo.ox.ac.uk/software.html>) to determine that the stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist, 2001). The initial 1K trees were discarded as a burn-in before calculating PPs. Using the “sumt” command of MrBAYES, the majority-rule consensus tree was calculated from 19K trees sampled after reaching likelihood convergence to calculate the PPs. The phylogenetic tree was drawn using Treeview (Page, 1996) and edited in Adobe Illustrator CS3.

Results

The sequences obtained have been deposited within EMBL (<http://www.ebi.ac.uk/embl>) with the accession numbers indicated in Table 1. It was not possible to obtain any sequences for some isolates since no PCR products were visualized from the agarose gel. From some collections (e.g. CEPSC6 and SINSCL6) three similar sequences were obtained after cloning, but only one was included in the alignment. The new sequences of this study were aligned with four from the GenBank. A new *Pisolithus* sp. sequence was included as outgroup (Table 1). Sequences from two collections (UNSCL1 and CEPSC5) were shorter and/or with weak peak in the electropherogram. These two sequences were excluded from the alignment; however, the sequence fragment of UNSCL1 was identical to SINSCL8 and the sequence fragment of CEPSC5 was identical to CEPSC6 and UNSCL7. The final alignment has a total of 38 sequences with 892 nonambiguously aligned sites. 641 characters were constant, 81 variable characters were parsimony-uninformative and 170 characters were parsimony-informative. Under a heuristic search 100 parsimonious trees were obtained with a length of 410 steps, CI = 0.7439, RI = 0.9147 and RC = 0.6805. In the consensus tree (Fig. 1)

two main clade appeared; clade (I) and (II) respectively. These clades comprised 11 major terminal clades (a-k). Those *Scleroderma* collections with spiny basidiospores were grouped together in sub-clade IA and those collections with sub-reticulate basidiospores appeared together within sub-clade IB. The collections with reticulate spores were grouped together in clade II. The Bayesian ITS analysis revealed basically the same topology as the parsimo-

nious consensus tree in relation to the two main clades (tree not shown) with strong BS support (bold branches in Fig. 1).

The first sub-clade (IA) branched into 3 terminal clades (a, b & c). Clade a (BS 90% /PP1.0) consisted of *S. cepa* (CEPSCL6), a GenBank sequence under *S. cepa* (DQ453694) and one unidentified sample (UNSCL7) whilst one *S. cepa* collection (CEPSCL2) and one *S. verrucosum* (VERSCL4) are formed to-

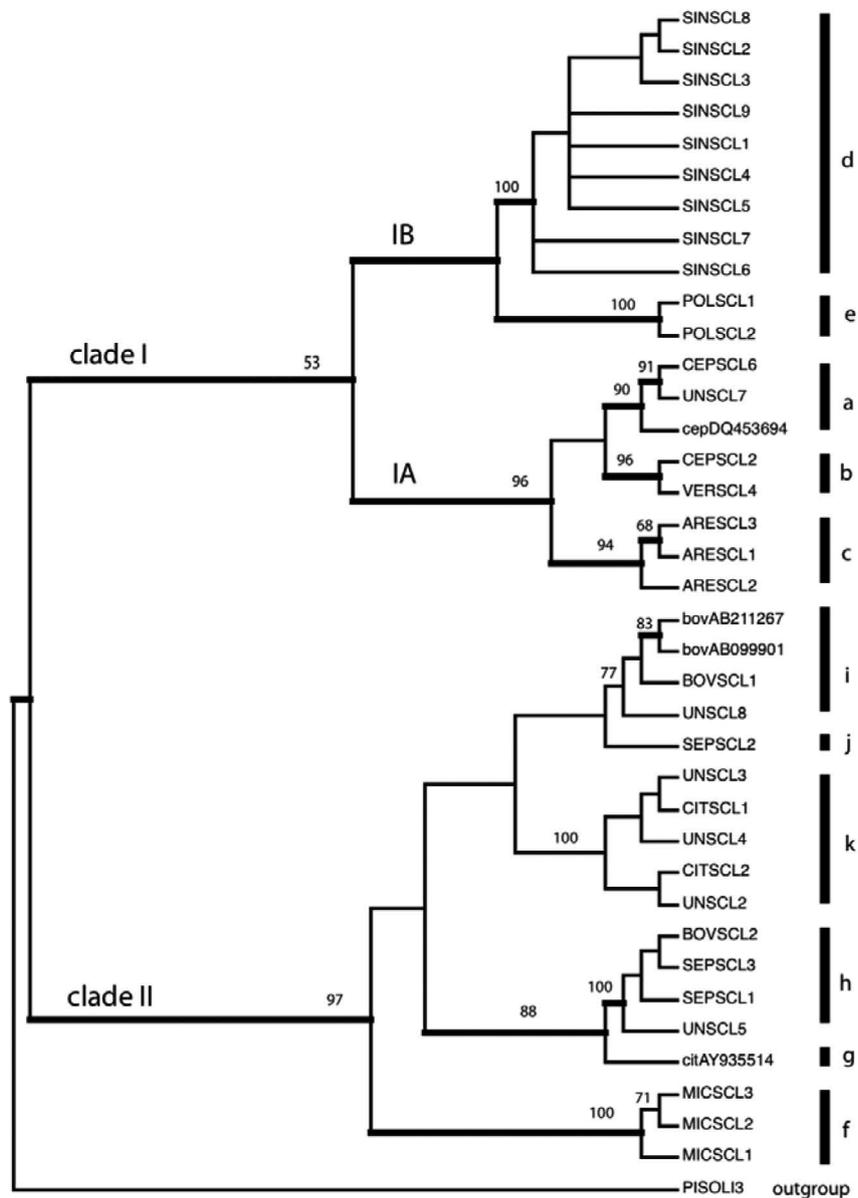


Fig. 1. Strict consensus tree of the 100 ITS trees obtained from heuristic search in the parsimony analysis. Bootstrap support values (10,000 replications) above 50% are shown at the nodes. Branches in bold print indicate significant statistical support in the Bayesian analysis (posterior probability >0.85). The 34 *Scleroderma* new sequences generated in this study are labeled with the isolation DNA code indicated in Table 1. The 4 sequences retrieved from the GenBank are indicated with the accession numbers. *Pisolithus* sp. was included as outgroup.

gether in clade b (BS 96% /PP0.99). All the *S. areolatum* samples are well separated in clade c with good supported (BS 94% /PP1.00). However, the relationship between these taxa involved in this subclade IA is not well resolved since the clades a and b are not well

supported and are sister groups in the parsimony tree (lesser than 50% BS).

Sub-clade IB representing another two terminal clades. One clade (d) contained all *Scleroderma* described as *S. sinnamariense* from Thailand with significant

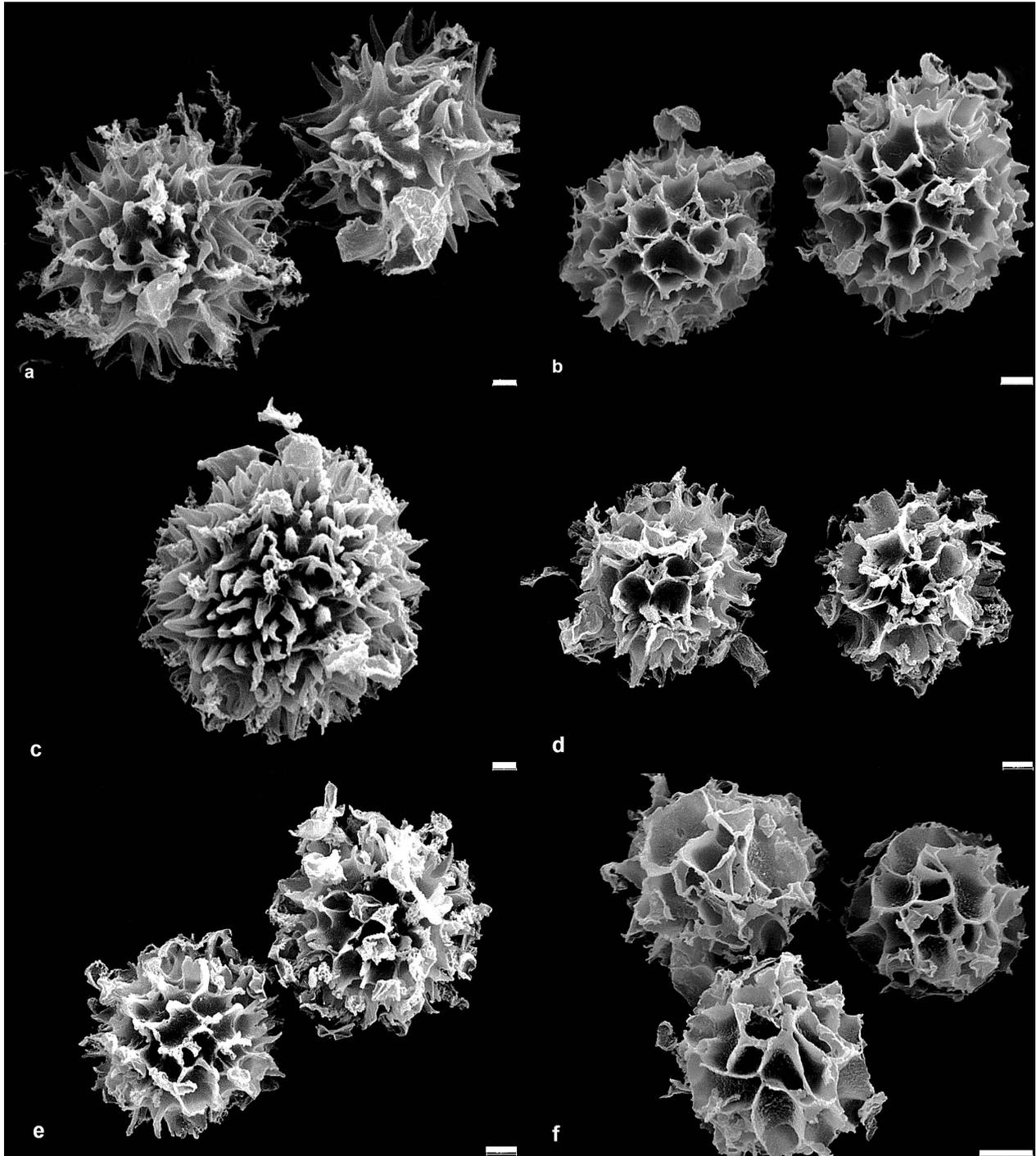


Fig. 2. Basidiospores of selected *Scleroderma* collections displaying their ornamentation confirmed subgenera proposed by Guzmán (1970). **a**, spiny ornamentation of *S. areolatum* (ARESC2, bar = 1 μ m); **b**, reticulate ornamentation of *S. bovista* (BOVSCL2, bar = 2 μ m); **c**, spiny ornamentation of *S. cepa* (CEPSCL5, bar = 1 μ m); **d**, **e**, reticulate ornamentation of *S. citrinum* (CITSCL1, bar = 2 μ m and CITSCL2, bar = 2 μ m, respectively); **f**, reticulate ornamentation of *S. michiganense* (MICSCL1, bar = 5 μ m).

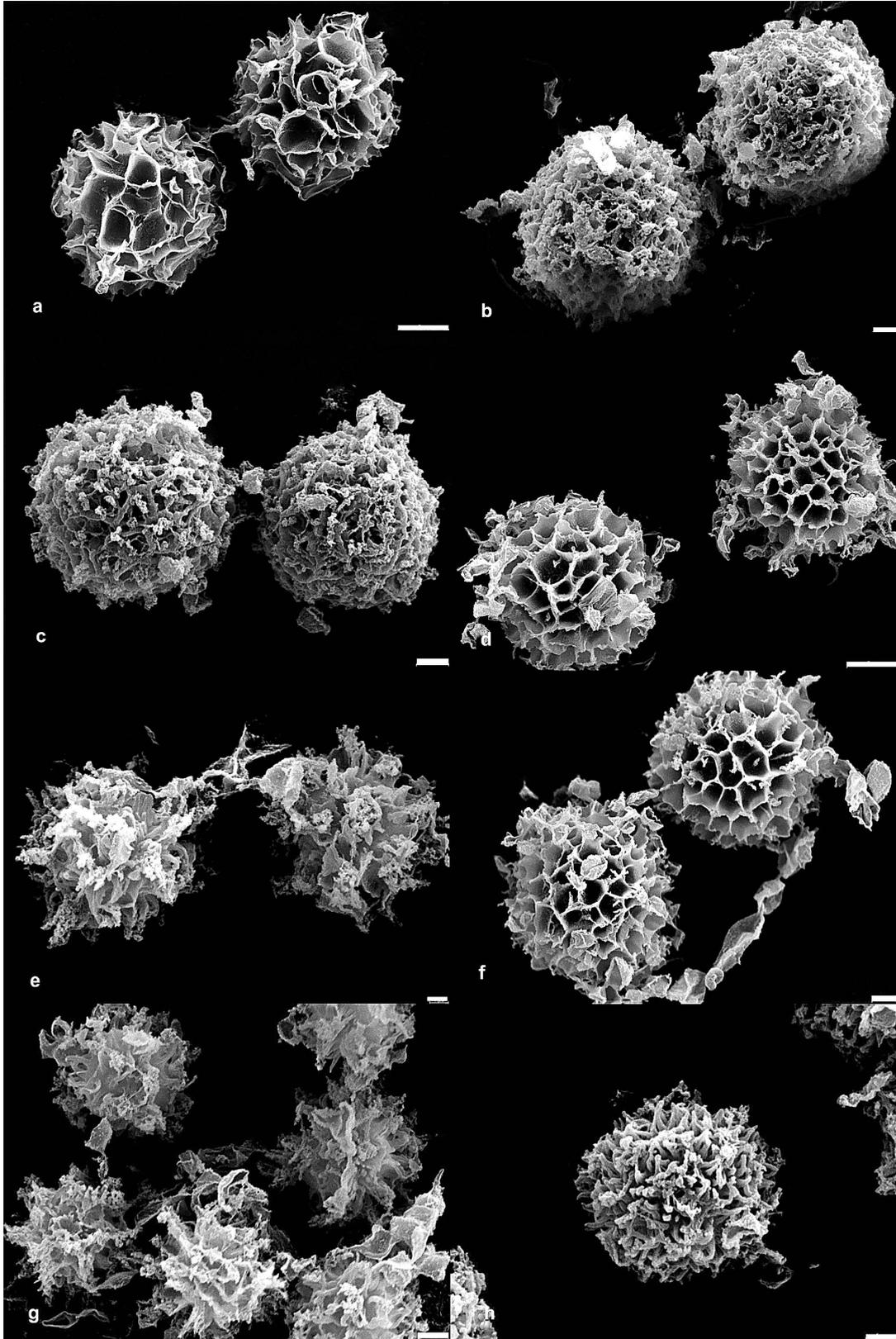


Fig. 3. Basidiospores of selected *Scleroderma* collections displaying their ornamentation confirmed subgenera proposed by Guzmán (1970). **a**, reticulate ornamentation of *S. michiganense* (MICSCL2, bar = 5 μ m); **b, c**, sub-reticulate ornamentation of *S. polyrhizum* (POLSCL1, bar = 2 μ m & POLSCL2, bar = 2 μ m, respectively); **d**, reticulate ornamentation of *S. cf. septentrionale* (SEPSCL1, bar = 5 μ m); **e & g**, sub-reticulate ornamentation of *S. sinnamariense* (SINSCL7, bar = 1 μ m & SINSCL9 bar = 2 μ m, respectively); **f**, reticulate ornamentation of *Scleroderma* sp. (UNSCCL5, bar = 2 μ m); **h**, spiny ornamentation of *Scleroderma* sp. (UNSCCL7, bar = 1 μ m).

support (BS 100% /PP 1.00). In another terminal clade (e), *S. polyrhizum* from North America grouped together with the Thai collections in a well supported clade (BS 100%/PP 1.00). These two terminal clades have sub-reticulate basidiospores.

Clade II included the collections with reticulate basidiospores. The clade ramified into several moderately to well-supported branches and terminal clades. It identified at least 6 main groups (f-i). The very well supported clade f (BS100%/PP 1.00) that includes three identical sequences of *Scleroderma michiganense* Guzmán (MICSCL1, 2 & 3) is the sister group of the rest of the collection in the parsimony analyses. However, in both analyses, the relationships within some clades are not well resolved. Apparently, the two species which are currently identified as *S. bovista* (BOVSCL in the trees) and *S. septentrionale* Jeppson (SEPSCL) appear in different terminal clades of the phylogenetic trees (Clades h, i & j).

Clade h consisted of *Scleroderma* described as *S. bovista* (BOVSCL2), *S. septentrionale* (SEPSCL1 & SEPSCL3), and *Scleroderma* sp. (UNSCL5). All samples came from the Great Lakes regions (North America) whilst a Genbank sequence described as *S. citrinum* from Spain (AY935514) was formed as a separated branch in clade g. Two *Scleroderma* sequences described from Japan as *S. bovista* (AB211267 & AB099901), one *S. bovista* (BOVSCL1) from Spain and *Scleroderma* sp. (UNSCL8) from Hungary formed clade i, that was not well resolved in the parsimony analysis (BS 77%). This clade is the sister group of a sample of *S. septentrionale* from Sweden (SEPSCL2); although this relation has not BS support (less 50%). Moreover, in the Bayesian tree, SEPSCL2 grouped into clade i, also with low support (PP 0.62).

The *Scleroderma citrinum* from Scotland (UNSCL2, 3, 4) appeared along with *S. citrinum* (CITSCL 1 & 2) from North America in clade (k) with strongly supported values in both Bayesian (PP 1.00) and MP (BS 100%) analyses.

Discussion

The information gleaned from the present sample of *Scleroderma* spp. confirms the robustness of the three subgenera proposed by Guzmán (1970). However, what is demonstrated is that even from the small sample of species examined during the present study there are still problems of identification within the genus. The classical species, *S. areolatum* Ehrenb.(c), *S. bovista* (i), *S. cepa* (a), *S. citrinum* (k), *S. polyrhizum* (e), *S. sinnamariense* (d) and *S. verrucosum* (b) form coherent groups along with the more recently described species *S. michiganense* (f) and *S. septentrionale* (j).

However, two units need to be reassessed in the future, a species close to *S. bovista* as understood in Europe which apparently has been found in North America and which includes material identified as both *S. bovista* and *S. septentrionale* (terminal clade h), and a sequence obtained from GenBank. CitAY935514 from Genbank was collected in Spain (Ruiz-Díez & al., 2006) and identified as *S. citrinum* (g) which it is clearly not. The North American collections need to be carefully examined within a revision of other specimens from the Great Lakes region but this is something outside the scope of this present study. However, as a result of this study it is suggested that the North American group (terminal clade h) might represent western collections of *S. meridionale* Demoulin & Malençon. This is a South European and North African taxon with reticulate basidiospores and pseudostipe which is characterised by a thick, golden yellow, furfuraceous peridium and a transversely furrowed and cracked pseudostipe. The peridium sometimes splits in a star-shaped way and thus shows similarities with some populations of *S. bovista* when overmature. It is apparently associated with evergreen oaks and *Pinus* spp. (Calonge 1998). Demoulin (1974) recognized *S. meridionale* from the Great Lakes region of North America and from coastal areas of North Carolina. According to Guzmán & Ovrebo (2000), however, the same large *Scleroderma* of the Great Lakes sand dunes is identical with *S. septentrionale*, a species growing in sand-dune habitats, resembling *S. bovista* but with a quite distinct morphology. In the molecular analysis in the present study it is included in terminal clades i & h in the tree.

From the above alignments it has been demonstrated that *Scleroderma polyrhizum* is immersed within the main body of *Scleroderma* despite the fact that it had been separated out in a distinct genus. The conclusions of Guzmán (1970) are therefore supported that *Sclerangium* Lev. is a synonym of *Scleroderma*. *Sclerangium* was erected (Léveillé, 1848) to host *S. polyrhizum* which he considered quite distinct in the stellate dehiscence of the mature basidiome and retention of the exposed spore-mass centrally resembling that of an earthstar (*Geastrum*). This is undoubtedly the same reason why the epithet for *S. geaster* Fr. was erected but this species is generally agreed to be conspecific with *S. polyrhizum*. Massee (1889) also saw a difference when he erected the genus *Stella* for *S. americana* Mass., a synonym of *S. stellatum* Berk. (= *Sclerangium brasiliense* Henn. & *Nepotatus stellatus* Lloyd and *Scleroderma bermudense* Coker but not *Lycoperdon stellatum* = *Astraeus*).

Scleroderma polyrhizum must be considered the type of the genus *Sclerangium* and therefore by default sg. *Sclerangium* (Lev.) Guzmán as defined by Guzmán (1970). Thus *S. polyrhizum* based on two collections from North America and *S. sinnamariense* based on nine different analyses of the same population sit neatly between the two subgenera mentioned earlier.

S. sinnamariense is widespread in the tropics commonly occurring in Thailand, Malaysia, Indonesia and the Philippines. It is also known from Fiji, Central America, New Guinea, etc. (Guzmán, 1970; Watling & Sims, 2004). It has been found with the so-called *Astraeus hygrometricus* (Pers.) Morgan and *Pisolithus arhizus* (Pers.) Rauschert. One of us (RW) judging from the subtle differences in macromorphology of *S. sinnamariense* that it will prove to be a complex of taxa. In the present study only a single population was used to anchor the study.

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