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Molecular phylogenetic assessment of the genus *Scutellinia* (Pezizales: Pyronemataceae)

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ABSTRACT

The genus *Scutellinia* consists of cup fungi belonging to the *Pyronemataceae* (Ascomycota). It comprises more than 100 species with a worldwide distribution. Species delimitation is based on morphological characteristics of the ascocarps, such as spore size, form and ornamentation as well as size and morphology of hairs that typically cover the external surface of the ascoma. These characters are, however, highly variable and species recognition challenging. In this paper, ribosomal DNA traits such as the internal transcribed spacer and the large subunit ribosomal gene were sequenced in several *Scutellinia* samples and, along with all available sequences on public database, used to improve the knowledge about taxonomy and phylogeny of this genus. Based on sequence similarity and trees topology, about 50 phylogenetic species were recognized and their phylogenetic relationships disclosed.

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Introduction

Pyronemataceae Corda is the largest and most heterogeneous family of *Pezizales* J. Schröt., encompassing 78 genera and approximately 660 currently recognized species (Kirk et al. 2008). However, the taxonomy of *Pyronemataceae* has been extremely controversial on several issues and while the positioning of many genera within the family has recently been resolved (Laessøe and Hansen 2007; Perry et al. 2007), the species recognition problem has attracted much less attention (Tamm et al. 2010; Guevara-Guerrero et al. 2012).

The genus *Scutellinia* (Cooke) Lambotte includes cup fungi belonging to the *Pyronemataceae* (*Ascomycota* Caval.-Sm.). The genus was established by Cooke (1879), as a subgenus of *Peziza* Dill. ex Fr., for discomycetes with 'setose' hairs and sessile apothecia. Lambotte (1887) raised the group to generic level. *Scutellinia* sp.pl. are recognized by the presence of shield-like red or orange apothecia, globose to ellipsoidal ascospores with or without various wall ornamentations, and stiff, long, blackish-brown hairs that arise from inner cells of the ectal excipulum. However, species delimitation is challenging because morphological characters are quite variable and frequently overlap among closely related species. Therefore, many taxonomic studies of the genus *Scutellinia* have been performed over years, and several species and varieties were described from different geographic areas revealing that this genus is cosmopolitan (Lambotte 1887; Seaver 1928; Le Gal 1953; Dennis 1955; Gamundi 1956; Denison 1959; Gamundi 1960; Le Gal 1966; Moravec 1974; Gamundi 1975; Waraitch 1977; Schumacher 1979; Dennis 1981; Kullman 1982; Dissing and Sivertsen 1983; Kaushal et al. 1983; Lohmeyer and Häffner 1983; Breitenbach and Kränzlin 1984; Cao and Moravec 1988; Hallgrimsson and Schumacher 1990; Korf and Zhuang 1991; Schumacher 1993; Meihua and Hongwei 1996; Moravec 1996; Yao and Spooner 1996; Cho et al. 1997; Matočec 1998; Hansen and Knudsen 2000; Matočec 2000; Douanla-Meli and Langer 2005; Bogacheva and Kullman 2006; Calonge 2006; Medardi 2007; Han et al. 2010; Lantieri 2010; Olariaga and Hansen 2011; Jeannerot 2011a, 2011b, 2011c; Choi et al. 2013; Dzhagan and Scherbakova 2013; Izquierdo-San Agustín et al. 2013; Radu 2013; Zhuang 2013; Jeannerot 2019; Keles 2019). At current time searching the Species Fungorum database (http://www.speciesfungorum.org/Names/Names.asp) 231 records were reported for the genus *Scutellinia* and, among these, 122 were currently recognized as valid *Scutellinia* species.

Among the characters considered for species identification, hair morphology and ascospore shape and ornamentation have been especially emphasized to delineate identification keys, taxonomic groups and phylogenetic relationship among species (Denison 1959; Svrček 1971; Korf 1972; Le Gal 1974; Moravec 1974; Schumacher 1979; Kullman 1982; Schumacher 1990; Yao and Spooner 1996; Greaves 2017; Jeannerot 2019). However, although several classification schemes have been

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proposed, species delimitation and infrageneric subdivision within *Scutellinia* remained problematic.

Studies based on molecular phylogenetic approaches have been applied at the species level to members of the *Pyronemataceae*, e.g. *Otidea* (Pers.) Bonord. (Liu and Zhuang 2006), *Chaetothiersia* Perry and Pfister (Perry and Pfister 2008) and *Geopora* Harkn. (Tamm et al. 2010), but only a few *Scutellinia* species have been included in large level studies (Perry et al. 2007; Hansen et al. 2013). In this study, a large set of *Scutellinia* samples were analyzed based on the comparison of the sequences of the internal-transcribed spacer region (ITS) and the large subunit (LSU) ribosomal gene. The aims were (i) evaluating the degree of genetic diversity to improve the taxonomic delineation and attribution of *Scutellinia* species, (ii) providing the first view of infra-generic phylogenetic relationship in this genus based on molecular data.

Materials and methods

Sample source

The *Scutellinia* samples considered in this study were mainly collected in Italy. Voucher specimens of 79 examined samples were deposited at the herbarium of Perugia Unversity (PERU, Table 1). Several type specimens were also obtained from the PRM herbarium of National History Museum, mycological department, Praga, Czech Republic (https://botany.natur.cuni.cz/cevnate/prc/fungi.php?lang=en) and from the TAAM mycological collection of the Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences (https://kogud.emu.ee/?p=2&id=9&lang=eng) and were included as reference samples (Table 1). Several additional specimens were also obtained from PRM and TAAM collections, but, since reliable molecular data were not be obtained, these were excluded from this study.

Morphological analyses

The specimens were examined considering the following morphological characters according to Schumacher (1990): ascospore shape and ornamentations (sculpturing), marginal hair length and width, marginal hair shape at the base (presence of bifurcations). Microscopic study using a compound microscope (Leica DM 1000, Leica Microsystems Ltd, Germany) was conducted on dried specimens and, where available, from fresh collections. The primary mounting media used were water, Melzer's reagent, and cotton blue in lactic acid (Schumacher and Kohn, 1985). Micrographs were taken with True Chrome HD II S (T-EsseLab) digital video camera. Biometric data were acquired according to Schumacher (1990), using the program ISCapture (Tucsen Photonics Co., Ltd). At least 20 ascospores, asci, paraphyses and hairs from each ascoma were measured, and length and width ranges were recorded. Spore measurements excluded ornaments, which were measured separately. Spore statistics were based on measurements of 20 spores from each specimen: Lm = mean length, Wm = mean width and Qm = Lm/Wm. The extreme values are given in parentheses. Only asci with mature spores were measured.

Species identification was performed according to the current literature and available identification keys, however some specimens were not reliably identified. All morphological and biometrical data acquired in this study were reported in Table S1.

Molecular analysis

Genomic DNA was isolated from a small part (few mg) of a single ascocarp for each of the specimen collected and the Internal Transcribed Spacer (ITS) and a fragment of the Large Subunit (LSU) of the rDNA region were analyzed by polymerase chain reaction (PCR). The ITS region was amplified using the primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) according to Pagiotti et al. (2011). The LSU was amplified with LR0R and LR3 primers according to Vilgalys and Hester (1990). PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and double strand sequencing was performed using the BigDye Terminator Cycle Sequencing Kit, Version 3.1, according to the supplier's instruction. Sequences were run on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed and edited with Bio Edit software (Hall 1999). All the ITS and SSU sequences were deposited in GenBank and accession numbers are provided in Table 1. The sequences were checked against the GenBank database using BLASTn to verify the successful amplification of *Scutellinia* sp.pl. and to exclude PCR contamination. The ITS and LSU sequences relative to *Scutellinia* sp.pl. and some phylogenetically related genera such as *Cheilymenia* Boud., *Wilcoxina* Chin S. Yang & Korf, *Sphaerosporium* Schwein. and *Kotlabaea* Svrček, were downloaded from GenBank. The samples PM513 and P1743 identified as *Melastiza* sp. were used as outgroup. Multiple sequence alignments (MSA) were performed with MAFFT using the L-INS-i parameters setting (Katoh et al. 2005). Phylogenetic trees were inferred with maximum-likelihood (ML) method using RaxML software (Stamatakis 2014) using the CIPRES Science Gateway V. 3.3 web service (https://www.phylo.org/portal2/login). Rapid bootstrapping, GTRGAMMA distribution model and empirical base frequency options were used. To evaluate if closely related specimens can be considered different phylogenetic species, we assessed the ITS sequence dissimilarity (p-distance or PSD) among samples based on pairwise sequence alignment (Table S2) using the software MEGA X (Kumar et al. 2018).

Results and discussion

Sequencing and characterization of ITS and LSU regions

Successful PCR amplification and sequencing were obtained for 77 (ITS) and 66 (LSU) specimens, among these, 17 were type specimens (Table1). More specifically 12 were obtained from the PRM Museum and 5 from the TAAM Collection. The non-type specimen TAAM066041 was also included.

Table 1. List of specimens considered in this study.

Perumyc ID	Original identification	Date	Provenance	GenBank accession no.		Holotype description
PM432	*S. trechispora*	2013	C. Italy (Città di Castello, Umbria)	MW540894	MW540978	
PM434	*S. trechispora*	2013	C. Italy (Città di Castello, Umbria)	MW540895	MW540979	
PM435	*S.* sp.	2013	C. Italy (Città di Castello, Umbria)	MW540896	MW540980	
PM439	*S. barlae*	2014	C. Italy (Città di Castello, Umbria)	MW540898	MW540982	
PM441	*S. barlae*	2014	C. Italy (Città di Castello, Umbria)	MW540899	MW540983	
PM442	*S. barlae*	2014	C. Italy (Città di Castello, Umbria)	MW540900	MW540984	
PM443	*S. barlae*	2014	C. Italy (Città di Castello, Umbria)	MW540901	MW540985	
PM444	*S. barlae*	2014	C. Italy (Città di Castello, Umbria)	MW540902	MW540986	
PM445	*S.* sp.	2013	C. Italy (Montemartano, Umbria)	MW540903	MW540987	
PM446	*S. trechispora*	2013	N. Italy (Bolzano, Trentino Alto Adige)	MW540904	–	
PM447	*S. trechispora*	2013	C. Italy (Bevagna, Umbria)	MW540905	MW540988	
PM454	*S. barlae*	2014	C. Italy (Opi, Abruzzo)	MW540906	MW540989	
PM455	*S. trechispora*	2014	C. Italy (Monte S. Vicino, Marche)	MW540907	MW540990	
PM456	*S. vitreola*	2014	C. Italy (Piobbico, Marche)	MW540908	MW540991	
PM457	*S. umbrorum*	2014	C. Italy (Coste di Contradarno, Abruzzo)	MW540909	MW540992	
PM458	*S. trechispora*	2014	N. Italy (Braies, Trentino-Alto Adige)	MW540910	MW540993	
PM459	*S. trechispora*	2014	N. Italy (Braies, Trentino-Alto Adige)	MW540911	MW540994	
PM460	*S. setosissima*	2014	N. Italy (Braies, Trentino-Alto Adige)	MW540912	MW540995	
PM461	*S. setosissima*	2014	N. Italy (Braies, Trentino-Alto Adige)	MW540913	MW540996	
PM462	*S. vitreola*	2014	C. Italy (Pietracamela, Abruzzo)	MW540914	MW540997	
PM463	*S. cejpii*	2014	C. Italy (Rocca S. Maria, Abruzzo)	MW540915	MW540998	
PM464	*S. trechispora*	2014	C. Italy (Rocca S. Maria, Abruzzo)	MW540916	MW540999	
PM465	*S. vitreola*	2014	C. Italy (Rocca S. Maria, Abruzzo)	MW540917	MW541000	
PM466	*S. setosissima*	2014	N. Italy (Braies, Trentino Alto Adige)	MW540918	MW541001	
PM467	*S. superba*	2014	N. Italy (Braies, Trentino Alto Adige)	MW540919	MW541002	
PM469	*S. barlae*	2014	C. Italy (Rocca S. Maria, Abruzzo)	MW540920	MW541003	
PM2854	*S. erinaceus*	2016	C. Italy (Città di Castello, Umbria)	MW540921	MW541004	
PM471	*S. trechispora*	2007	C. Italy (Rocca S. Maria, Abruzzo)	MW540922	MW541005	
PM472	*S. minor*	2001	N. Italy (Forca Rossa, Veneto)	MW540923	MW541006	
PM473	*S. minor*	1994	N. Italy (Forcella Venegia, Trentino Alto Adige)	MW540924	MW541007	
PM476	*S. cejpii*	1987	N. Italy (Val Gares, Veneto)	MW540927	–	
PM477	*S. trechispora*	2008	N. Italy (Melere, Veneto)	MW540928	–	
PM481	*S. umbrorum*	1984	N. Italy (Caviola, Veneto)	MW540929	–	
PM483	*Parascutellinia* sp.	1987	N. Italy (Stelvio, Trentino-Alto Adige)	MW540930	MW541010	
PM484	*S. scutellata*	2015	N. Italy (Stelvio, Trentino-Alto Adige)	MW540931	MW541011	
PM485	*S. scutellata*	1984	N. Italy (Caviola, Veneto)	MW540932	MW541012	
PM486	*S. patagonica*	2001	N. Italy (Fuciade, Trentino-Alto Adige)	MW540933	MW541013	
PM487	*S. ceijpii*	2014	N. Italy (Sedico,Veneto)	MW540934	MW541014	
PM488	*S.* sp.	2015	N. Italy (Val venegia, Trentino-Alto Adige)	MW540935	MW541015	
PM489	*S. umbrorum*	2003	N. Italy (Passo delle Selle, Trentino-Alto Adige)	MW540936	MW541016	
PM490	*S.* sp	2009	N. Italy (Cornuda, Veneto)	MW540937	MW541017	
PM491	*S.* sp.	2000	N. Italy (Passo delle Selle, Trentino-Alto Adige)	MW540938	MW541018	
PM492	*S.* sp.	2001	N. Italy (Fuciade, Trentino-Alto Adige)	MW540939	–	
PM493	*S.* sp.	1987	N. Italy (Mezzano, Trentino-Alto Adige)	MW540940	MW541019	
PM496	*S.* sp.	1986	N. Italy (Mezzano, Trentino-Alto Adige)	MW540941	–	
PM497	*S. vitreola*	1999	N. Italy (Mezzano, Trentino Alto Adige)	MW540942	–	
PM498	*S. setosissima*	1999	N. Italy (Mezzano, Trentino-Alto Adige)	MW540943	MW541020	
PM1939	*S.* sp.	2017	C. Italy (Ceppo, Abruzzo)	MW540944	MW541021	
PM1940	*S. erinaceus*	2017	C. Italy (Ceppo, Abruzzo)	MW540945	MW541022	
PM1941	*S.* sp	2017	C. Italy (Ceppo, Abruzzo)	MW540946	MW541023	
PM1942	*S.* sp.	2017	C. Italy (Ceppo, Abruzzo)	MW540947	MW541024	
PM1743	*Melastiza cornubiensis*	2016	N. Italy (Mandre, Veneto)	MW540948	MW541025	
PM1744	*S. trechispora*	2016	N. Italy (Col di Foglia, Veneto)	MW540949	MW541026	
PM1745	*S. subhirtella*	2016	N. Italy (Faè, Veneto)	MW540950	MW541027	
PM1746	*S. trechispora*	2016	N. Italy (Binatega, Veneto)	MW540951	MW541028	
PM1747	*S. umbrorum*	2016	N. Italy (Passo di Giau, Veneto)	MW540952	MW541029	
PM1748	*S. superba*	2016	N. Italy (Mandre, Veneto)	MW540953	MW541030	
PM992	*S.* sp.	2015	N. Italy (Carezza, Trentino-Alto Adige)	MW540954	MW541031	
PM1204	*S. colensoi*	2013	C. Italy (Collestrada, Umbria)	MW540955	MW541032	
PM513	*Melastiza* sp.	2015	C. Italy (Castel Rigone, Umbria)	MW540956	MW541033	
PM2189	*S. crucipila*	2018	N. Italy (Stelvio, Trentino-Alto Adige)	MW540957	MW541034	
PRM147265	*Lachnea hrabanovi* (lectotype)	1948	Czech Republic (Hrabanov, Lysà)	MW540958	–	*S. hrabanovii* (Velen.) Svrček, *Česká Mykol.* 31(2): 70 (1977)
PRM147295	*Lachnea pilati* (holotype)	1924	Czech Republic (Bohemia, Mnichovice)	MW540959	–	*S. pilatii* (Velen.) Svrček, *Česká Mykol.* 25(2): 84 (1971)
PRM150963	*Lachnea lusatiae var. macrospora* (holotype)	1948	–	MW540960	MW541035	*S. macrospora* (Svrček) Le Gal, *Bull. Tr. Soc. myc. Fr.* 80:123 (1964)
PRM150995	*Lachnea ginttlii* (lectotype)	1971	–	MW540961	MW541036	*S. gintlii* (Velen.) Svrček, *Česká Mykol.* 25(2): 83 (1971)
PRM610126	*S. subcervorum (*holotype*)*	1981	Czech Republic (Bohemia)	MW540962	MW541037	Česká Mykol. 25(2): 86 (1971)
PRM616842	*S. subhirtella (*holotype*)*	1971	Czech Republic (Bohemia)	MW540963	MW541038	Česká Mykol. 25 (2): 85 (1971)
PRM628978	*S. fimicola (*holotype*)*	1968	Czech Republic (Bohemia)	MW540964	–	Česká Mykol. 22(1): 39 (1968)
PRM710003	*S. pseudoumbrarum (*holotype*)*	1971	Ilidža near Sarajevo (Jugoslavia)	MW540967	MW541040	Česká Mykol. 25(4): 199 (1971)
PRM724261	*S. peloponnesiaca (*holotype*)*	1974	Greece	MW540968	–	Česká Mykol. 28(1): 20 (1974)
PRM724262	*S. parvispora (*holotype*)*	1974	Greece	MW540969	MW541041	Česká Mykol. 28(1): 23 (1974)
PRM830017	*S. furcatopila (*holotype*)*	1984	Central Africa	MW540970	MW541042	Česká Mykol. 37(4): 245 (1984)
PRM668618	*S. minutella* (holotype)	1969	Czech Republic (Bohemia)	–	MW541039	Česká Mykol. 23(3): 156-159 (1969)
TAAM003594	*S. scutellata var macrosculpturata* (holotype)	1982	Soviet Union	MW540971	MW541043	Scripta Mycol., Tartu 10: 99 (1982)
TAAM042082	*S. heterosculpturata* (holotype)	1977	West Siberia	MW540972	–	Folia cryptog. Estonica 7: 4 (1977)
TAAM048870	*S. caucasica* (holotype)	1978	Russia (Krasnodar)	MW540973	MW541044	Folia cryptog. Estonica 10: 6 (1978)
TAAM066675	*S. scutellata* var *discreta* (paratype)	1982	Soviet Union	MW540975	MW541046	Scripta Mycol., Tartu 10: 100 (1982)
TAAM096480	*S. pseudoumbrarum* var *kurilensis* (holotype)	1982	Soviet Union	MW540976	–	Scripta Mycol., Tartu 10: 82 (1982)
TAAM066041	*S. vitreola*	–	–	–	MW541045	–

BLAST analysis of ITS sequences showed close similarity with *Scutellinia* sp. sequences for most of the samples considered with few exceptions. Some specimens in fact showed the highest BLAST similarity with other genera of *Pyronemataceae*: The sample PM2189 [identified as *S. crucipila* (Cooke & W. Phillips) J. Moravec] showed the best match (97% of identity) with the ITS sequences of *Cheilymenia stercorea* (Pers.) Boud. (DQ491500, MH930238). The sample PM483 showed high similarity (99% of sequence identity) with *Parascutellinia fuckeli* Donadini & Svrček (MT273640), and about 84% of sequence identity with *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf (KU061020) and several other undetermined *Wilcoxina* sp. sequences (e.g. JN704821, HM146893, KU245943, KU245942). The ITS sequences of the samples PM2854 and PM1940 both identified as *S. erinaceus* (Schwein.) Kuntze showed the best matches with *S. erinaceus* (MK575465) but with a low similarity (87% of identity), and with *Sphaerosporium lignatile* Schwein. (MN749373). Blast results of the LSU region for these specimens showed similar results: the PM2189 LSU sequence produced the best match (100% sequence identity) with some *Cheilymenia* species such as *C. crucipila* (DQ220320, DQ220321) *C. sclerotiorum* T. Schumach. (KC012671, DQ220324), *C. stercorea* (DQ220323) and *C. vitellina* (Pers.) Dennis (EU940117, DQ220325). The LSU sequence of PM483 showed about 84–85% of sequence identity with several sequences of genus *Wilcoxina* such as *W. mikolae* (DQ220468). The PM1940 and PM2854 LSU sequences showed the best match with *Sphaerosporium lignatile* (MN749494, MN749495), *Miladina lecithina* (Cooke) Svrček (DQ220371, DQ220372), *Lamprospora crechqueraultii* (P. Crouan & H. Crouan) Boud. (KC012698) and *Ramsbottomia asperior* (Nyl.) Benkert & T. Schumach. (DQ220408) but with 94-95% of sequence identity only. The ITS sequences obtained from type specimens of *S. macrospora* (Svrček) Le Gal (PRM150963) also showed the best BLAST match (identity ranging from 96 to 99%) with sequences classified as *Cheilymenia* such as *C. pulcherrima* (P. Crouan & H. Crouan) Boud. (MH859871) and *C. stercoraria* (KX592795). Other similar sequences belonged to *Scutellinia* sp. pl. (KU556557, KU556583, JF908716, AF072091) and *Kotlabaea* sp. pl. (KU534714, GU055660, GU055642, JX545195). Likewise, the most similar LSU sequences (98-99% of sequence identity) to PRM150963 were those of *C. pulcherrima* (MH871658, MH878398), *C. stercoraria* (Velen.) J. Moravec (KX592807), *C. fimicola* (Bagl.) Dennis (DQ220322), *Byssonectria deformis* (P. Karst.) U. Lindem. & M. Vega (DQ220356) and some uncultured *Kotlabaea* sp.pl. (GU055660, GU055642).

Molecular phylogenetic delimitation of Scutellinia species

To better compare the sequenced sample and to assess the phylogenetic relationship with all available sequences, 146 ITS sequences related to *Scutellinia* were retrieved from GenBank and included in the MSA to end up with 223 sequences. Similarly, 63 LSU sequences were downloaded to obtain an MSA of 129 sequences.

Phylogenetic analysis confirmed the position of specimens of controversial attribution in the basal clades of ITS tree (clades I, II, and III) and of LSU tree (Figures 1 and S1). More specifically, the sample PM483 clustered in the ITS clade I and appear phylogenetically closely related to species in the *Wilcoxina - Trichophaea hybrida* (Sowerby) T. Schumach. clade described by Tedersoo et al. (2015), indicating an erroneous identification as *Scutellinia* sp. Similar results were obtained with LSU sequences (Figure S1). The sample PM2189 identified as *S. crucipila* was included in the clade II of ITS tree (Figure 1). This species was considered belonging to *Scutellinia* by Moravec (1984) and by Schumacher (1990), however, Yao and Spooner (1996) later questioned this attribution, on the basis of morphological traits such as stellate hairs and the absence of guttulate ascospores and considered it belonging to the genus *Cheylimenia* (*C. crucipila*). According to our analysis, the specimen PM2189 belonged to the *Cheilymenia-Byssonectria* P. Karst. lineage described by Lindemann (2015). The ITS sequence of this specimen, in fact, closely matched that of *C. stercorea*. Similarly, the LSU tree (Figure S1) showed high similarity of PM2189 with *C. crucipila*, *C. stercorea*, *C. sclerotiorum* T. Schumach. and *C. vitellina* (Pers.) Dennis. Therefore, this analysis confirmed the interpretation of Yao and Spooner (1996) that *S. crucipila* should, indeed, be ascribed to the genus *Cheilymenia*.

The samples PM2854 and PM1940 were morphologically ascribed to *S. erinaceus* (Table S1). The ITS sequences of these samples showed similarity with MK575465 and MT386345 also classified as *S. erinaceus* but with a low similarity (about 83% of identity, Table S2) suggesting that *S. erinaceus* is a species complex with at least two phylogenetic species (Figure 1). Within the same clade the sequence MT386365, classified as *S. setosa* (Nees) Kuntze in Genbank, need to be reviewed. All these samples are placed in a basal clade in both ITS (Clade III) and LSU trees, and showed low phylogenetic affinity with other *Scutellinia* species. In both trees, the most closely related genus was *Spaerosporium lignatile*, but with low ITS similarity (about 75% of identity). This result was in agreement with the morphology of this species that is different from *Scutellinia*. These results also confirmed those of Perry et al. (2007) that, on the basis of LSU sequence, placed *S. erinaceus* near the genus *Kotlabaea* and at the root of other *Scutellinia* sp. pl. Therefore, these data suggested that *S. erinaceous* did not belong to the genus *Scutellinia*. Indeed, *S. erinaceus* shows some morphological features divergent from those of *Scutellinia* sp. pl. such as the yellowish color of apothecium. However, more species sampling and sequencing are needed to better clarify phylogenetic position of *S. erinaceous* and determine whether it must be included in *Kotlabaea* or it may represent a different genus.

Considering the nested nodes of the ITS tree, five major clades IV, V, VI, VII and VIII can be identified (Figure 1). In the most basal clade IV are grouped the type specimens of *S. macrospora* (PRM150963) and other sequences labeled as *Scutellinia* sp. retrieved from GenBank. The ITS sequences of these specimens are also similar to specimens labeled as *Kotlabaea* sp. and *Cheilymenia* sp. (e.g. *C. pulcherrima* and *C. stercoraria*). Similar patterns resulted from the LSU tree. According to Lindemann et al. (2015) the genus *Cheilymenia*

Figure 1. Phylogenetic tree based on ITS sequences. The specimens sequenced in this study were reported in bold. Asterisk indicates type specimens from PRM or TAAM herbarium. Numbers near the branches indicates bootstrap values.

is polyphyletic. In fact, based on LSU and Ef1a sequences, these authors hypothesized that *C. stercoraria* not belonged to *Cheilymenia* but, rather, it was more related to *Scutellinia*. Our analysis confirmed the similarity with some samples classified as *Scutellinia*. However, further investigations are necessary to determine whether this group of species should be considered or not within the genus *Scutellinia*.

The clade V may represent the root of the *Scutellinia* lineage. Within this clade, at least four phylogenetic species (Ps1–Ps4) can be identified. The Ps1 included the specimen MK019113 labeled as *Scutellinia* sp. that showed >10% divergence from the other sequences of this clade. The Ps2 was represented by the type specimen of *S. furcatopila* J. Moravec (PRM830017). The closely related ITS sequences belonged to the unknown samples MZ808266, MT386358, MT156530, which shared with PRM830017 about 92% of sequence identity, thus these samples can be considered a different species (Ps3). Few other *Scutellinia* sequences retrieved from GenBank but not classified at specific level belong to the same sub-clade. Their ITS sequence were near identical to each other but showed >3% (13–15%) divergence with respect to the others in the sub-clade, thus likely they represent a different phylogenetic species (Ps4). The phylogenetic position of PRM830017 is not congruent in the ITS and LSU trees, as in this last it is placed close to the *S. cejpii* group. This may be due to the less phylogenetic resolution of LSU and species sampling compared to ITS.

The clade VI contains three main subclades A, B, and C where, based on sequence similarity, 13 putative phylogenetic species can be identified (Ps5–Ps17). Within the sub-clade A, several samples identified as *S. cejpii* (Velen.) Svrček and *S. hirta* (Schumach.) Cooke, which showed similar ITS sequences (identity >97%) are grouped. Therefore, this analysis confirmed that these two species should be considered synonyms as suggested by Svrček (1971). In the same clade, the sequence from the type of *S. caucasica* Kullman & Raitv. (TAAM048870) was included. Although reported as a valid species according to index fungorum, *S. caucasica* showed high sequence similarity with *S. cejpii* (>97%) and thus, should be considered conspecific. The high similarity of TAAM048870 with *S. cejpii* samples was also confirmed by the LSU analysis. Phylogenetic analyses performed here are in agreement with the observations made by H.A. Huijser and T. Schumacher for TAAM048870 holotype as shown in the original documentation preserved in the TAAM herbarium (Figure S2). LSU tree also showed that sample DQ220418 identified as *S. geneospora* (Berk.) Kuntze belonged to the *S. cejpii* species complex, however further detailed analyses are needed, as the ITS sequence was not available for this species. Three additional sequences from GenBank belonged to the same clade: JF908722, MH930303, and KR673635. The sequence JF908722 identified as *S. ampullacea* (Limm.) Kuntze should be renamed as it showed >98% sequence similarity with *S. hirta*/*S. cejpii* samples. All these sequences were indicated as Ps7 in the ITS tree. The samples MH930303 and KR673635 showed about 93% similarity with each other and more than 4% of divergence from Ps7. Although KR673635 was labeled as *S. hirta*, these likely represent two phylogenetic species (Ps5 and Ps6). According to the ITS differences (>3%) and tree topology, the sub clade B included at least six different phylogenetic species (Ps8–Ps13). The Ps8 was represented by the type specimens of *S. heterosculpturata* Kullman & Raitv (TAAM042082). The Ps9 may represent *S. marginata* Gamundí according to the GenBank record; The Ps10 included the undetermined samples MK351733, collected in Korea according to GenBank record and specimens PM1939 and PM1941. These two samples are of uncertain classification but are close to *S. nigrohirtula* (Svrček) Le Gal or *S. kerguelensis* (Berk.) Kuntze according to morphological features (Table S1). The Ps11 included three unclassified samples MT386359, MN653027, and MT386348. The Ps12 included the samples PM467 and PM1748 that were ascribed to *S. superba* (Velen.) Le Gal according to their morphology (Table S1). Finally, other two unclassified *Scutellinia* sequences (MT386354 and MT386363) may represent a further phylogenetic species (Ps13). In the LSU tree, the samples PM1939, PM1941, PM467, and PM1748 are phylogenetically related to DQ220420 identified as *S. pennsylvanica* (Seaver) Denison according to the Genbank record.

The sub-clade C included several ITS sequences, many of them identified as *S. colensoi* Massee ex Le Gal. These sequences showed a sequence identity ranging from about 86–99%, thus likely representing a species complex (Figure 1, Ps14–Ps17). In particular, among these, the sample PRM1024 (Ps15) was classified as *S. colensoi* but showed only 86 to 89% of sequence identity with the other sequences. In the same clade, the type specimen (TAAM003594) of *S. scutellata var. macrosculpturata* Kullman & Raitv. (Kullman, 1982) shared high similarity (about 95% of sequence identity) with several sequences of *S. colensoi*. LSU tree also showed that this sample belonged to the *S. colensoi* species complex. According to species fungorum *S. scutellata var. macrosculpturata* is considered a synonym of *S. scutellate* (L.) Lambotte. However, in agreement with our phylogenetic analyses, TAAM003594 in the TAAM herbarium was annotated as *S. colensoi* Massee ex Le Gal (T. Schumacher, Figure S3). Most of the samples in the ITS clade VI are grouped together also in the LSU tree, although the differentiation of sub-clades is less clear than in the ITS tree.

The clade VII included nine major sub-clades (D-N). Five putative phylogenetic species (Ps18–Ps22) can be identified in the sub-clade D. The Ps18 included KY462576 and MT386361, both unclassified specimens. The sample JF908718, identified as *S. vitreola* Kullman (Ps19) was close to the type specimen of *S. peloponnesiaca* J. Moravec (PRM724261) as they showed 97,9% of sequence identity, thus species assignment of this sample needs to be revised. This is in agreement with the fact that *S. vitreola* was likely represented by specimens in the sub-clade L (Ps35). The Ps20 included the type specimen of *S. hrabanovi* (Velen.) Svrček (PRM147265) and MT386347 labeled in Genbank as *S. scutellata*. The *S. hrabanovi* (Ps20) sample appears distinct from *S. peloponnesiaca* in the ITS tree, however these two type specimens were very close as they share 97.7% of sequence identity. Therefore, further analyses are needed to ascertain if these two type specimens represent two good species. A fourth putative

phylogenetic specie (Ps21) may represent a species complex as it included the type specimen of *S. pseudoumbrarum* J. Moravec (PRM710003) and samples (PM486 and JF908720), classified as *S. patagonica* (Rehm) Gamundí. The sample PM457, also showing a similar ITS sequence, was ascribed to *S. umbrorum* (Fr.) Lambotte although its identification remains uncertain as it was immature. These results were in agreement with morphology as *S. patagonica* belong to the *S. umbrorum* (= *S. umbrarum*) complex which grouped taxa with similar and hardly distinguishable morphological characters (Moravec 1974; Paal et al. 1998). In fact, according to several Authors, the species *S. pseudounbrarum* is uncertain and can be regarded as a synonym of *S. umbrorum* (Schumacher 1990; Jeannerot 2019). It is worth noting that specimens referred to *S. umbrorum* (PM489, PM457, PM481 and PM1747) and to species morphologically similar such as *S. patagonica* (Ps21), the type of *S. pseudoumbraum* (Ps21), *S. parvispora* J. Moravec (Ps40), *S. subhirtella* Svrček (Ps50) and the type of *S. pseudoumbrarum* var. *kurylensis* Kullman (= *S. umbrorum*) (Ps49) were dispersed into different clades of the ITS tree. Therefore, according to this analysis, *S. umbrorum*-like morphotypes appear to represent phylogenetically unrelated species. However, further studies are needed for this group, as we not examined the type of *S. patagonica* and the type of *S. umbrorum* is not available (see Moravec 1974).

A fifth group consisted of several sequences (AY220816–AY220821), from specimens collected in Korea and classified as *S. superba* according to GenBank records, but morphological descriptions were not available. These samples, likely represent a phylogenetic species (Ps22). However, our phylogenetic analysis suggested that their species attribution needs to be further investigated because, as reported above, the samples PM467 and PM1748 (Ps12), showing ITS sequences unrelated to those of Ps22, can be also ascribed to *S. superba* according to morphology (Table S1).

The sub-clade E is represented by the sample AR290 (Ps23) only. Unfortunately, after DNA isolation, this sample was lost and morphological analysis could not be performed. The sub-clade F includes several sequences retrieved from GenBank. These sequences were distributed in tree groups that according to the similarity level (about 88–93% of sequence identity between groups) may represent three putative phylogenetic species (Ps24, Ps25 and Ps 26). Differently from Ps24 and Ps25 that included unclassified specimens only, several samples grouped under Ps26 were labeled as *S. scutellata*. Samples identified as *S. scutellata* were also in the sub-clade I and L, however we hypothesized that these last specimens may not represent the true *S. scutellata* as they not clustered all together but were close to other *Scutellinia* species such as *S. pilati* (Velen.) Svrček, *S. cervorum* (Velen.) Svrček, *S. gintli* (Velen.) Svrček, *S. vitreola* (see below). In the sub-clade G were grouped several samples mostly classified as *S. nigrohirtula* (Ps27) according to the GenBank record, but additional data were not available for these specimens. In the sub-clade H, two phylogenetic species can be identified: the Ps28 includes the samples PM490 and PM445 closely related to the type of *S. fimicola* J. Moravec (PRM628978), the Ps29 included four samples that, based on morphology, can be classified as *S. setosissima* Le Gal (Table S1). The sub-clade I includes five putative phylogenetic species (Ps30–Ps34). The Ps30 includes the specimen MZ919167 only, labeled as *S. crinita* (Bull.) Lambotte according with Genbank record. However, it needs to be revised as several other specimens labeled as *S. crinita* clustered elsewhere in the ITS tree (subclade L). The Ps31 was represented by PM1942 and the type specimen of *S. gintlii* (PRM150995). The samples PM481 and PM1747 (Ps32) were morphologically similar to *S. umbrorum* (Table S1).

The Ps33 included several samples with similar ITS sequences (similarity >99%) that were morphologically ascribed to *S. scutellata* (Table s1) and the samples DQ411492 and MH930391, also classified as *S. scutellata* according to the GenBank record. The Ps34 included the specimen FJ235141and the type specimen of *S. pilati* (PRM14725). It is worth mentioning that phylogenetic species hypothesized in the sub-clade I, although suggested by the ITS tree topology, need to be confirmed by additional studies as these shared high similarity (95-98% of sequence identity).

In the sub-clade L, several samples with similar ITS sequences (>98% of identity) were grouped. Although the high similarity, three phylogenetic species can be hypothesized among these samples, on the basis of tree topology. The Ps35 was represented by three sequences retrieved from GenBank, either unclassified (MF230412 or MK351710) or labeled as *S. scutellata* (MZ159479), and four specimens examined in this study, identified as *S. vitreola* (Table S1). The Ps36 included the type of *S. subcervorum* Svrček (PRM610126) and a sample from GenBank identified as *S. crinita*. According to Svrček (1971) *S. subcervorum*, differ, from *S. cervorum* (Velen.) Svrček only for few characteristics such as the hairs of the apothecium that were shorter. Moreover, *S. cervorum* is a synonym of *S. crinita* according to index fungorum. Therefore, our phylogenetic analysis was in agreement with the fact that these two species share similar morphological features. Unfortunately, we could not get a reliable nucleotide sequence of the *S. subcervorum* type specimen from the PRM herbarium. Finally, several sequences retrieved from GenBank, all labeled as *S. crinita* were grouped together (Ps37). Like in the sub-clade I most of the specimens in the subclade L share high levels of ITS sequence similarity. Therefore, phylogenetic species definition must be considered with caution. Several samples in the subclades I, and L were ascribed to *S. scutellata*. However, as reported above, these samples may not belong to *S. scutellata* and their taxonomical attribution need further dedicated studies. Unfortunately, it was impossible to analyze the type specimen of *S. scutellata*. The species of the ITS clade VII are grouped together also in the LSU tree. However, a further subdivision in sub-clades is not evident due to the lower phylogenetic resolution of LSU and less species sampling compared to the ITS.

The Clade VIII includes five sub-clades (M, N, O, P, Q, Figure 1). In the sub-clade M, according to the ITS similarity, four putative phylogenetic species can be identified (Ps38–Ps41). The Ps38 included two similar ITS sequences KY462643 and MT386352, both classified as *Scutellinia* sp. The Ps39 was represented by the sample JF908712. According to Osmundson

et al. (2013), this sample belonged to *S. legaliae* Lohmeyer & Häffner, but morphological description was not provided. Unfortunately, we were unable to find morphotypes that could be reliably identified as *S. legaliae* or to analyze the type specimen. The Ps40 included the type of *S. parvispora* (PRM724262) and the samples PM435 and PM492, which consequently might be ascribed to this species. The Ps41 grouped several samples sharing morphological characters that clearly matched those of *S. trechispora* (Berk. & Broome) Lambotte. ITS sequences were clustered in three groups suggesting the existence of three species. However, sequence similarity was >97% (Table S2). Therefore, considering also the common morphological features, we interpreted these samples as belonging to the same phylogenetic species with a certain level of intraspecific variability. The specimens MT273643 and MT273645 classified as *S. rotundisperma* Donadini and *S. trechisperma* f. *nigromarginata* Donadini can be also considered belonging to *S. trechispora* as ITS sequences share from 97 to 99% of similarity with *S. trechispora* samples (Figure 1, Table S2). The sub-clade N included the phylogenetic species Ps42 and Ps43. The Ps42 included the sample PM489 identified as *S. umbrorum* and two samples JF908715 and JF908721 retrieved from GenBank identified as *S. subhirtella* and *S. heterosculpturata* Kullman & Raitv., respectively. However, as reported above, species attribution of *S. umbrorum* morphotypes can be misleading due to the variability of *S. umbrorum* species complex. Moreover, the type specimens of *S. subhirtella* (Ps50), and *S. heterosculpturata* (Ps8) were in the sub-clade Q and B, respectively. Therefore, species attribution of samples belonging to Ps42 remains undefined and more samples are needed to evaluate this taxon. In the LSU tree, the sample PM489 form a cluster with the sample KC012704 identified as *S. subhirtella*, but the limited species sampling not allowed clarifying the phylogenetic position of this taxon. The Ps43 included the samples PM472 and PM473 identified as *S. minor* (Velen.) Svrček, the undetermined sample PM491, and the sample JF908714 classified as *S. barlae* (Boud.) Maire. These last sequences showed >97.3% of identity with *S. minor* samples and might be renamed accordingly. The LSU tree confirmed the similarity of these tree samples and their differentiation from PM489. In the sub-clade O, the phylogenetic species Ps44 included the unclassified sequences AY220822 and AY220823 and the type specimen of *S. scutellata* var. *discreta* Kullman & Raitv. (TAAM06675). According to index fungorum, this last is not a valid species as it was a synonym of *S. crinite* (Bull.) Lambotte. This raise a question whether *S. crinita* should be associated with this type specimen or with Ps37 belonging to sub-cade L. The sub-clade P included the sequences PM493 and MT273642, which represent two phylogenetic species (Ps45 and Ps46). Unfortunately, species name was not available in the Genbank record for MT273642 and PM493 was an immature specimen that cannot be reliably classified. The Ps47 included sequences with >97% of sequence identity, all clearly identified as *S. barlae* based on their morphology. The sample MT273644, classified as *S. trechisperma* f. *micropilifera* Donadini, was also included within this clade. Finally, the sub-clade Q included at least three hypothetical phylogenetic species (Ps48–Ps50). The Ps48 was represented by the unclassified *Scutellinia* species MT386350. The Ps49 was represented by the type specimen of *S. pseudoumbrarum* var. *kurylensis* Kullman (TAAM096480) that according to index fungorum is a synonym of *S. umbrorum,* and by the unclassified specimen MT38635. The Ps50 included the type specimen of *S. subhirtella* (PRM616842) that clustered with some other sequences identified as *S. subhirtella* including the sample PM1745. It is worth noting that most of the species in the clade VIII have globose or sub-globose ascospores, except samples belonging to the Ps40, the sample PM489 and those in the sub-clade Q that showed spores approximately ellipsoid.

Toward a natural classification of Scutellinia

Several studies, aimed at defining the phylogenetic relationships within the genus *Scutellinia* based on morphological characters, have been previously conducted (Svrček 1971; Le Gal 1974; Moravec 1974; Kullman 1982; Schumacher 1990). Over the years, *Scutellinia* classification was refined by including new discovered species and by performing several adjustments consisting of the creation of new groups (sections) and/or by reassigning species to the existing section following different criteria. The main species classification schemes were summarized in supplementary Table S3. Svrček (1971) based on spore morphology and hair length defined eight sections: *Globisporae*, *Levisporae*, *Pseudotrechisporae*, *Pseudocheilymeniae*, *Minutae*, *Hirtulae*, *Pilosae* and *Scutellinia*. This classification scheme was partially modified by Moravec (1974). More specifically, this author questioned the validity of the *Globisporae* as many *Scutellinia* species often showed both globose and ellipsoid spores. Moravec gave high importance to sporal ornamentation and proposed renaming the *Globisporae* into *Armatosporae* and to include in this section species with spiny spore only. Accordingly, he moved species with verrucose spores (*S. trechispora* and *S. paludicola* (Boud.) Le Gal) to the section *Hirtulae*. Le Gal (1974), also considered the form of spores irrelevant for a natural classification of *Scutellinia* species, and made a revision considering hair characteristics. Due to the high variability within the species, he does not considered the length but other characters of the hairs, such as the size of basal part, the thickness of the external walls, presence and arrangement of the septa and, with less importance, hairs density in the marginal part of the ascocarp, the so-called marginal hairs. Based on these characters he defined a first group characterized by thick hairs and numerous septa (unfortunately the name of this first group is missing). By contrast, a second group (section), named *Setosae*, was characterized by hairs with thinner walls, showing little or no septa. The two additional sections of *Hirtae* and *Trechispore* were considered related to the *Setosae* as these share similar hair morphology. The section *Barbatae* accommodate species of tropical origin with flexuous hairs of irregular diameter. The other two sections were those of *Ampullaceae* with evident hairs septa and *Scutelliniae* showing tapered hairs tip.

Kullman (1982) made a further revision of the classification scheme of Svrček (1971). Here, spore ornamentation was considered as having an essential taxonomic value and six types were defined. These ranged from types with discrete ornamentation (aculeate and tuberculate) to types (microverrucose, verrucose, macroverrucose and reticulate) with various

degrees of confluence and fusion of ornamentation elements. By considering these ornamentation types, a classification scheme consisting of four sections: *Levisporae*, *Globisporae*, *Hirtulae* and *Scutellinia* was proposed. More specifically, the section *Hirtulae* was revised by including the former sections of *Pilosae*, *Minutae* and *Pseudocheilymeniae* of Svrček (1971).

Finally, Schumacher (1990) in a monographic study of genus *Scutellinia* recognized 45 species. By using a parsimony approach and a series of cladistics and numerical analyses based on various combinations of characters of spore and hairs, he defined two subgenera and several sections. The two subgenera were named *Scutellinia* with long marginal hairs differentiated from basal hairs and *Legaliae* showing marginal and basal hairs of similar length.

Within the subgenus *Scutellinia*, *S. geneospora*, *S. trechispora* and *S. rotundisperma* have been considered to have each unique characters and thus belonging to three monotypic sections named *Geneospermae*, *Rotundispermae* and *Globisporae*, respectively. The sections *Reticulatae*, including species with reticulate spore and *Scutellinia*, a large heterogeneous group accommodating species not sharing character with the other sections also belonged to *Scutellinia* subgenus. The subgenus *Legalia*, included two sections: *Legalia* and *Minutae*. *Legalia* has been divided into two series with different spore morphology: *Legalia* with globose or sub-globose, aculeate or tuberculate spores (e.g. *S. legalie*, *S. barlae*, *S. minor*) and *Sublaevisporae* with ellipsoid tuberculate spores. Similarly, the section *Minutae*, previously introduced by Svrček (1971), included two series: *Minutae* with crestate or tuberculate spores, and the monotypic group of *Pseudotrechisporae*.

By observing the ITS tree topology, the species considered in this study were grouped in four main clades (V, VI, VII and VIII). Therefore, the subdivision of *Scutellinia* into two subgenera based on the length and morphology of marginal hairs as proposed by T. Schumacher (1990) was not supported by molecular phylogenetic analysis performed in this study. This suggests that the length of marginal hairs does not represent a reliable phylogenetic trait to reconstruct phylogenetic relationships among *Scutellinia* groups. We can hypothesize that differences in air length and distribution in the ascocarps are not ancestral characters but may represent derived traits likely arising as an adaptation to various environments. Considering the four main ITS clades only the clade VIII appears to include species sharing common morphological characters. In fact, most of the specimens in this clade showed spherical spores with a free-spines type of ornamentation. However, besides the specimens retrieved from GenBank, for which no morphological descriptions were available, some exceptions are clearly present in this clade, regarding specimens analyzed in this study: i.e. the samples belonging to Ps40, Ps42 and those in the sub-clade Q that are characterized by ellipsoidal spores. However, this partially agrees with the interpretation of Moravec (1974), which considered spore form less important than spore ornamentation. Accordingly, the clade VIII appear to includes species belonging to Moravec's sections *Armatosporae* and *Hirtulae* which are characterized by sporal ornamentation consisting mainly of free-spines or warts.

Conclusions

In conclusion, this work, far from being exhaustive, may help to clarify the taxonomic position of some problematic species. In particular, it was shown that the morphological examination often leads to errors in species attribution. In fact, several inconsistencies have been detected in species attribution concerning the samples present in the Genbank. Similarly, species attribution of some samples examined in this study was not always supported by molecular analysis, confirming how reliable identification is often difficult due to the extreme variability and overlapping of morphological characters. Overall, based on an examination of the ITS tree, 50 putative *Scutellinia* phylogenetic species were identified. Moreover, it represents the first attempt to study phylogenetic relationships within the genus *Scutellinia* based on molecular data. The phylogenetic reconstruction showed inconsistencies with the infra-generic classification schemes previously proposed on the basis of morphological traits. Therefore, many characters that in the past were taken into consideration for infra-generic classification of *Scutellinia* sp. pl., although useful for species identification, revealed indeed little utility for evaluating phylogenetic relationships.

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Author contributions

All authors contributed to the study conception and design. Sample collection was performed by G. Bistocchi and A. Arcangeli. Morphological analyses were performed by P. Angelini, G. Bistocchi and A. Arcangeli. Molecular analyses were performed by A. Rubini and P. Angelini. Phylogenetic analyses were performed by A. Rubini. The first draft of the manuscript was written by A. Rubini and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The sequences generated in this study are deposited in GenBank (https://www.ncbi.nlm.nih.gov) under the accession numbers given in Table 1.

Multiple sequences alignments were deposited at Treebase (https://treebase.org/treebase-web/home.html) under the accession no. S29147. Voucher specimens were deposited in the mycological herbarium of University of Perugia (PERU).

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