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ORIGINAL ARTICLE

Biodiversity and chemotaxonomy of *Preussia* isolates from the Iberian Peninsula

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Abstract This work documents 32 new *Preussia* isolates from the Iberian Peninsula, including endophytic and saprobic strains. The morphological study of the teleomorphs and anamorphs was combined with a molecular phylogenetic analysis based on sequences of the ribosomal rDNA gene cluster and chemotaxonomic studies based on liquid chromatography coupled to electrospray mass spectrometry. Sixteen natural compounds were identified. On the basis of combined analyses, 11 chemotypes are inferred.

Keywords *Preussia* · Chemotypes · Mass spectrometry · Secondary metabolites

Introduction

The combination of geo-climatic factors that influence the Iberian Peninsula have shaped an extraordinary variety of habitats. These privileged areas for biodiversity studies have great richness in flora and fauna, where endemic and singular plants are likely to be present. Although more than 10,000 fungal species have been described in Spain (Moreno-Arroyo 2004), most of them were mushrooms, leaving this environment open to other exhaustive fungal studies. Very few examples of fungal endophytes have been described from the Iberian Peninsula, suggesting that a large number of new fungal species will be discovered (Collado et al. 2002; Oberwinkler et al. 2006; Bills et al. 2012).

Members of the Sporormiaceae are widespread and, despite that they are most commonly found on various types of animal dung, they can also be isolated from soil, wood, and plant debris. Fungi of Sporormiaceae form dark brown, septate spores with germ slits, and include approximately 100 species divided into ten genera, including the recently described genera *Forliomyces* and *Sparticola* (Phukhamsakda et al. 2016) and *Chaetopreussia*, *Pleophragmia*, *Preussia*, *Pycnidiophora*, *Sporormia*, *Sporormiella*, *Sporormiella*, *Sporormiella*, *Sporormiella*, and *Westerdykella*. Among these, *Sporormiella* and *Preussia* are particularly species-rich (Barr 2000).

The genus *Preussia* was erected by Fuckel (1866) to include bitunicate ascomycetes with non-ostiolate, globose to subglobose ascomata, 8-spored, broadly clavate or subglobose asci, and ascospores with germ slits that are mostly surrounded by a gelatinous sheath. *Preussia* species are isolated from soil, wood, or plant debris. Later, *Sporormiella* was defined to include coprophilous bitunicate ascomycetes with ostiolate perithecioid ascomata and cylindrical to cylindric-claviform asci (Ellis and Everhart 1892). In 1961, Cain (1961) reviewed the genus *Preussia*, included new coprophilous species, and, accordingly, broadened the ecological concept of the genus. von Arx (1973) highlighted that the presence or absence of ostioles may vary with the growth conditions, indicating that this morphological character could not be considered as a valid taxonomic criterion. In 2009, a

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systematic analysis on the phylogenetic relationships based on four loci (ITS, 28S, 18S, and β-tubulin) proposed 12 new *Preussia* combinations (Kruys and Wedin 2009). Nevertheless, recent publications maintain the genera *Preussia* and *Sporormiella* (Doveri and Sarrocco 2013).

Previous studies identified 33 *Preussia* species from the Iberian Peninsula. *Preussia intermedia* was the first species cited by Urries (1932), followed by *P. dakotensis* cited in a study of ascomycetes of the Iberian Peninsula and the Balearic Islands (Unamuno 1941). Lundqvist (1960) reported four additional species of *Preussia* (*P. lageniformis*, *P. longispora*, *P. megalospora*, and *P. minima*) in a report on coprophilous ascomycetes from northern Spain. Later reported species were *P. pascua* (de la Torre 1974), *P. australis*, *P. grandispora*, *P. vexans* (Barrasa and Moreno 1980), *P. clavispora* (Guarro et al. 1981), *P. thypharum* (Guarro Artigas 1983), *P. cylindrospora*, *P. dubia*, *P. heptamera*, *P. irregularis*, *P. leporina*, *P. ovina*, *P. teretispora*, *P. pyriformis* (Barrasa 1985), *P. capybarae*, *P. cymatomera*, *P. systenospora* (Soláns 1985), *P. tenerifae* (von Arx and Van der Aa 1987), *P. splendens* (Sierra López 1987), *P. fleischhakii* (Barrasa and Checa 1989), *P. affinis* and *P. funiculata* (Valldosera and Guarro 1990), and *P. mediterranea* (Arenal et al. 2007). All previously cited species were isolated from dung except *P. mediterranea*, which was isolated from the plant *Cistus albidus*. More recently, the hairy species *Sporormiella octomegaspora* was isolated from deer dung in Andalusia (Doveri and Sarrocco 2013).

Coprophilous fungi play an important ecological role in decomposing and recycling nutrients from animal dung. They have the ability to produce a large array of bioactive secondary metabolites (Sarrocco 2016). Bioactive secondary metabolites produced by these fungi are typically involved in defense mechanisms against other competing microbes (Bills et al. 2013). Most of these bioactive compounds are antifungals, such as australifungin, an inhibitor of the sphingolipid synthesis (Hensens et al. 1995), preussomerins, inhibitors of the ras farnesyl-protein transferase (Weber et al. 1990), and zaragozic acids, potent inhibitors of squalene synthase (Bergstrom et al. 1995).

Bioactive secondary metabolites produced by *Preussia* species such as 7-chloro-6-methoxymellein, hyalopyrone, leptosin, cissetin, or microsphaeropsone A are also produced by other fungi, while auranticins, australifungin, zaragozic acid B, terezines, and sporminarins are known to be produced exclusively by *Preussia* sp. (Table 1).

The purpose of this study was to review the fungal biodiversity of *Preussia* species from environmental samples of the Iberian Peninsula and characterize occurring chemotypes. The biodiversity of *Preussia* endophytes isolated from plants in arid zones from the south of Spain and a small number of strains from soils and herbivore dung were compared with *Preussia* strains from Arizona desert plants (Massimo et al. 2015) and other Sporormiaceae obtained from public collections.

Materials and methods

Isolation, culturing, and morphology

Nine areas, including Mediterranean and Eurosiberian regions, were surveyed. Different plant species, characteristic of each geographic region, soil, and animal dung were sampled. Standard indirect techniques were performed to isolate plant endophytes: plant specimens such as stems or leaves were cut into 5-mm^2 fragments. Their surface was disinfected by serial immersion in 95% ethanol (30 s), 25% bleach (1.25% NaClO) (1 min), and 95% ethanol (30 s). Ten sterilized fragments were aseptically transferred to corn meal agar (CMA) and supplemented with streptomycin sulfate (50 mg/mL) and oxytetracycline (50 mg/mL) (Bills et al. 2012). Soil fungi were obtained following a particle filtration method (Bills et al. 2004). Coprophilous fungi were isolated directly from perithecia developed on animal dung after incubation in moist chambers.

Isolates were cultured on 2% malt agar (MEA), CMA, oat meal agar (OMA, Difco™), and synthetic nutrient agar (SNA; Nirenberg 1976) to study their macroscopic and microscopic characteristics. Microscopic features were evaluated by observing the structures in 5% KOH. Axenic strains were preserved as frozen suspensions of conidia, ascospores, or sterile mycelium in 10% glycerol at −80 °C. Strains are currently maintained in the Fundación MEDINA culture collection (http://www.medinadiscovery.com). ID coding, geographical origin, isolation substrata, and GenBank accession numbers of their rDNA gene sequences are listed in Table 2.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from aerial mycelia of strains grown on malt-yeast extract agar (Bills et al. 2012). DNA fragments containing the ITS1–5.8S–ITS2 (ITS) and the initial 600 nucleotides of the 28S rDNA gene (28S) were amplified with the 18S3 (5′-GATGCCCTTAGATGTTCTGGGG-3′) (Bills et al. 2012) and NL4 primers (O'Donnell 1993). Polymerase chain reaction (PCR) amplifications followed standard procedures (5 min at 93 °C, 40 cycles of 30 s at 93 °C, 30 s at 53 °C, and 2 min at 72 °C), using the Taq DNA polymerase (QBiogene™ Inc.), following the manufacturer-recommended procedures. Amplification products (0.1 mg/mL) were sequenced with the Big Dye Terminator Cycle Sequencing Kit® (Applied Biosystems™), also following manufacturer recommendations. Each PCR product was sequenced bidirectionally with the same primers that were used for the PCR reactions. Partial sequences obtained during sequencing reactions were assembled with the GeneStudio® software (GeneStudio™ Inc., Georgia). Sequences of the complete ITS1–5.8S–ITS2–28S region or independent ITS and partial 28S rDNA sequences were

Table 1 Bioactive secondary metabolites reported from *Preussia* species

Compounds	Species	Major nutrient sources	Fermentation state	Biological activity	References
Asterric acid	*Preussia* sp.	Rice-based media	Solid	Endothelin binding inhibitor	Talontsi et al. (2014)
Auranticins A, B	*P. aurantiaca*	Rice-based media	Solid	Antifungal and antibacterial	Poch and Gloer (1991)
7-Chloro-6-methoxymellein	*P. affinis*	Corn starch and molasses	Liquid	Antifungal	McGahren and Mitscher (1968)
Antibiotic FR 173945	*P. aemulans*	Starch and peach power	Liquid	Antifungal	Sato et al. (1998)
Antibiotic WF 15604A	*P. minima*	Starch acid and cotton flour	Liquid	Antifungal	Hatori et al. (2004)
Australifungin	*P. australis*	Corn-based media	Solid	Cytotoxic	Hensens et al. (1995)
$C_{28}H_{42}N_4O_8$	*P. minimoides*	Corn meal and sucrose	Liquid	Antifungal	Clapp-Shapiro et al. (1998)
Cissetin	*Preussia* sp.	Rice-based media	Solid	Antibacterial	Talontsi et al. (2014)
Cryptosporiopsin	*P. affinis*	Corn starch and molasses	Liquid	Antifungal	McGahren et al. (1969)
Culpin	*Preussia* sp.	Soluble starch and glucose	Liquid	Antifungal and antibacterial	Robinson et al. (1988)
Cyperin	*P. fleischhakii*	Potato dextrose broth	Liquid	Phytotoxic	Weber and Gloer (1988)
Hyalopyrone	*P. teretispora*	Soy flour medium	Liquid	Phytotoxic	Wang et al. (1995)
Leptosin A, C	*P. typharum*	Cheerios breakfast cereal	Solid	Cytotoxic	Du et al. (2014)
Preussiadin A, B	*P. typharum*	Cheerios breakfast cereal	Solid	Cytotoxic	Du et al. (2014)
Microsphaeropsone A	*P. minima*	Rice-based media	Solid	Antifungal	Xiong et al. (2014)
Preussiafuran A, B	*Preussia* sp.	Rice-based media	Solid	Cytotoxic	Talontsi et al. (2014)
Preussin	*Preussia* sp.	Soluble starch and glucose	Liquid	Antifungal	Johnson et al. (1989)
Preussochromone A, B, C, D	*P. africana*	Rice-based media	Solid	Cytotoxic	Zhang et al. (2012)
Preussomerin A, E, D, G, B	*P. isomera*	Potato dextrose broth	Liquid	ras Farnesyl-protein	Weber and Gloer (1991)
Similin A, B	*P. similis*	Soybean and dextrose	Liquid	Antifungal	Weber et al. (1992)
Spiropreussione	*Preussia* sp.	Wheat bran and glucose	Liquid	Cytotoxic	Chen et al. (2009)
Sporminarin A, B	*P. minimoides*	Rice-based media	Solid	Antifungal	Mudur et al. (2006)
Sporostatin	*Preussia* sp.	Glucose and peptone broth	Liquid	Cytotoxic	Kinoshita et al. (1997)
Sporovexin A, B, C	*P. vexans*	Potato dextrose broth	Liquid	Antifungal and antibacterial	Soman et al. (1999)
Terezine A, B, C, D	*P. teretispora*	Soy flour medium	Liquid	Antifungal and antibacterial	Wang et al. (1995)
Zaragozic acid B	*P. intermedia*	Cerelose and cottonseed	Liquid	Antifungal	Bergstrom et al. (1995)

compared with sequences deposited at GenBank® or the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/) by using the BLAST® application.

Phylogenetic analysis

Species and genus affinities were inferred in a Bayesian analysis by using the Markov chain Monte Carlo (MCMC) approach with MrBayes 3.01 (Ronquist and Huelsenbeck 2003). To improve mixing of the chains, four incrementally heated simultaneous Monte Carlo Markov chains were run over 2×10^6 generations. Hierarchical likelihood ratio tests with the MrModeltest® 2.2 software (Nylander 2004) were used to calculate the Akaike information criterion (AIC) of the nucleotide substitution models. The model selected by the AIC for the alignment was GTR + I + G, which is based on six classes of substitution types, a portion of invariant alignment positions, and mean substitution rates, varied across the remaining positions according to a gamma distribution. Priors used for the MCMC processes were followed by a Dirichlet distribution for the substitution of rates and nucleotide frequencies, and a unification of the rate parameter for the gamma distribution. The MCMC analysis used the following parameters:

Table 2 *Preussia* strains included in the phylogenetic analysis (newly isolated strains from the Iberian Peninsula are in **bold**)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
Forliomyces uniseptata	MFLUCC 15-0765 (ex-type)	*Spartium junceum*	Spain	KU721772	KU721762	Phukhamsakda et al. (2016
Preussia aemulans	CBS 287.67	Soil	The Netherlands	DQ468017	DQ468037	Arenal et al. (2007)
Preussia aemulans	CBS 318.81	Soil	The Netherlands	KX710218		–
Preussia africana	S17 (holotype)	*Viburnum tinus* leaf	Tenerife, Spain	AY510418	AY510383	Arenal et al. (2005)
Preussia africana	**CF-279770**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710221**		–
Preussia africana	**CF-098213**	***Erica araganensis***	**Spain, Lugo**	**KX710222**		–
Preussia africana	S14	Zebra dung	South Africa	AY510417	AY510382	Arenal et al. (2005)
Preussia africana	DN136	*Ramalina calicaris*	China	JQ031265		Zhang et al. (2012)
Preussia africana	S15	Zebra dung	South Africa	AY510421	AY510385	Arenal et al. (2005)
Preussia africana	S12	Goat dung	Tanzania	AY510420	AY510384	Arenal et al. (2005)
Preussia cymatomera	CBS 396.81	*Juniperus communis*	Switzerland	KX710252		–
Preussia flanaganii	CBS 112.73 (isotype)	Sandy soil	Mexico	AY943061		–
Preussia fleischhakii	CBS 565.63	Soil	Germany	GQ203761	GQ203721	Kruys and Wedin (2009)
Preussia fleischhakii	CBS 361.49	Man's nail	The Netherlands	DQ468018	DQ468038	Arenal et al. (2007)
Preussia funiculata	UPS:Huhndorf et al. 2577 (F)	Porcupine dung	USA	GQ203762	GQ203722	Kruys and Wedin (2009)
Preussia isomera	CBS 415.82	–	Venezuela	KX710243		–
Preussia isomera	NBRC 30581	Soil	Nepal	NBRC 03058101		–
Preussia isomera	CBS 388.78	Cow dung	Venezuela	GQ203763	GQ203723	Kruys and Wedin (2009)
Preussia isomera	CBS 671.77	–	Japan	KX710241		–
Preussia mediterranea	S23 (holotype)	*Cistus albidus* leaf	Spain, Caceres	DQ468022	DQ468042	Arenal et al. (2007)
Preussia mediterranea	S30	*Alnus glutinosa* leaf	Spain, Caceres	DQ468023	DQ468043	Arenal et al. (2007)
Preussia mediterranea	S34	*Daphne gnidium* leaf	Spain, Caceres	DQ468025	DQ468045	Arenal et al. (2007)
Preussia mediterranea	S31	*Quercus suber* leaf	Spain, Caceres	DQ468024	DQ468044	Arenal et al. (2007)
Preussia mediterranea	S22	*Quercus ilex* leaf	Spain, Guadalajara	DQ468021	DQ468041	Arenal et al. (2007)
Preussia minimoides	MEXU 26355	*Hintonia latiflora*	Mexico	KF557658	KF557659	Leyte-Lugo et al. (2013)
Preussia minimoides	NRRL 37629	*Trametes hirsutum*	Hawaii	GU183123		Mudur et al. (2006)
Preussia minimoides	S10	Pig dung	Argentina	AY510423	AY510388	Arenal et al. (2005)
Preussia minimoides	S18	*Prunus lusitanica*	Canary island	AY510422	AY510387	Arenal et al. (2005)
Preussia persica	CBS 117680	Barley dead leaf	Iran	GQ292750	GQ292752	Asgari and Zare (2010)
Preussia polymorpha	CBS 117679 (holotype)	Barley dead leaf	Iran	GQ292749	GQ292751	Asgari and Zare (2010)
Preussia sp.	ELV3.2	*Eustrephus latifolius*	Australia	JN418773	KF269206	Mapperson et al. (2014)
Preussia sp.	ELV3.11	*Eustrephus latifolius*	Australia	JN418774	KF269205	Mapperson et al. (2014)
***Preussia* sp.**	**CF-095571**	***Pinus pinaster***	**Spain, Lerida**	**KX710247**		–
	CF-155367					–
Preussia sp.	WO-009	Soil	Spain	KX710239		–
Preussia sp.	CF-209171	Kudu	South Africa	KX710223		–
***Preussia* sp.**	**CF-277787**	**Soil**	**Spain, Granada**	**KX710262**		–
***Preussia* sp.**	**CF-277801**	**Soil**	**Spain, Granada**	**KX710260**		–
***Preussia* sp.**	**CF-277817**	**Soil**	**Spain, Granada**	**KX710251**		–
***Preussia* sp.**	**CF-277822**	**Soil**	**Spain, Granada**	**KX710220**		–
***Preussia* sp.**	**CF-277849**	**Soil**	**Spain, Granada**	**KX710263**		–
***Preussia* sp.**	**CF-277856**	**Soil**	**Spain, Granada**	**KX710253**		–
***Preussia* sp.**	**CF-277965**	**Soil**	**Spain, Granada**	**KX710261**		–
***Preussia* sp.**	**CF-279766**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710250**		–
***Preussia* sp.**	**CF-279773**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710256**		–
***Preussia* sp.**	**CF-282341**	***Dittrichia viscosa***	**Spain, Granada**	**KU295582**		**González-Menéndez et al.** (2016)
***Preussia* sp.**	**CF-285370**	***Anabasis articulata***	**Spain, Almeria**	**KX710254**		–
***Preussia* sp.**	**CF-285378**	***Salsola oppositifolia***	**Spain, Almeria**	**KX710249**		–
***Preussia* sp.**	**CF-285772**	***Phragmites australis***	**Spain, Granada**	**KX710255**		–
Preussia sp.	SNP008	*Larrea tridentata*	USA	KP335214		Massimo et al. (2015)
Preussia sp.	SNP034	*Simmondsia chinensis*	USA	KP335239		Massimo et al. (2015)
Preussia sp.	SNP057	*Parkinsonia microphylla*	USA	KP335262		Massimo et al. (2015)
Preussia sp.	SNP156	*Phoradendron californicum*	USA	KP335359		Massimo et al. (2015)
Preussia sp.	SNP164	*Larrea tridentata*	USA	KP335365		Massimo et al. (2015)
Preussia sp.	SNP208	*Larrea tridentata*	USA	KP335402		Massimo et al. (2015)
Preussia sp.	SNP220	*Larrea tridentata*	USA	KP335411		Massimo et al. (2015)
Preussia sp.	SNP223	*Larrea tridentata*	USA	KP335414		Massimo et al. (2015)
Preussia sp.	SNP232	*Larrea tridentata*	USA	KP335422		Massimo et al. (2015)
Preussia sp.	SNP235	*Parkinsonia microphylla*	USA	KP335425		Massimo et al. (2015)
Preussia sp.	SNP252	*Simmondsia chinensis*	USA	KP335440		Massimo et al. (2015)
Preussia sp.	SNP301	*Parkinsonia microphylla*	USA	KP335487		Massimo et al. (2015)
Preussia sp.	SNP309	*Simmondsia chinensis*	USA	KP335495		Massimo et al. (2015)
Preussia sp.	SNP334	*Larrea tridentata*	USA	KP335519		Massimo et al. (2015)
Preussia sp.	SNP392	*Larrea tridentata*	USA	KP335569		Massimo et al. (2015)
Preussia sp.	SNP408		USA	KP335584		Massimo et al. (2015)

Table 2 (continued)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
		Phoradendron californicum				
Preussia sp.	SNP419	*Simmondsia chinensis*	USA	KP335595		Massimo et al. (2015)
Preussia sp.	SNP420	*Simmondsia chinensis*	USA	KP335596		Massimo et al. (2015)
Preussia sp.	SNP437	*Simmondsia chinensis*	USA	KP335613		Massimo et al. (2015)
Preussia sp.	SNP458	*Parkinsonia microphylla*	USA	KP335634		Massimo et al. (2015)
Preussia sp.	SNP459	*Larrea tridentata*	USA	KP335638		Massimo et al. (2015)
Preussia subticinensis	CBS 443.9	–	France	KX710258		–
Preussia subticinensis	CBS 125.66	–	Germany	KX710259		–
Preussia subticinensis	**CF-278595**	**Soil**	**Spain, Granada**	**KX710257**		–
Preussia terricola	CBS 317.65	*Musa sapientum*	Honduras	GQ203765	GQ203725	Kruys and Wedin (2009)
Preussia terricola	CBS 527.84	Elephant dung	Tanzania	GQ203764	GQ203724	Kruys and Wedin (2009)
Preussia typharum	CBS 107.69	Deer dung	Japan	GQ203766	GQ203726	Kruys and Wedin (2009)
Preussia typharum	718249	Degraded organic matter	USA	JX143871		
Preussia typharum	NBRC 32847	Unidentified plant	Iraq	NBRC 03284701		–
Preussia typharum	**CF-085890**	**Microbial mats**	**Spain, Tarragona**	**KX710219**		–
Preussia vulgaris	UPS:Strid 18884	Hare dung	Sweden	GQ203767	GQ203727	Kruys and Wedin (2009)
Sparticola junci	MFLUCC 15-0030 (ex-type)	*Spartium junceum*	Spain	KU721775	KU721765	Phukhamsakda et al. (2016)
Sparticola forlicesenica	MFLUCC 14-1097 (ex-type)	*Spartium junceum*	Spain	KU721773	KU721763	Phukhamsakda et al. (2016)
Sporormiella affinis	UPS:Lundqvist 17739-j	Rabbit dung	Denmark	GQ203770	GQ203730	Kruys and Wedin (2009)
Sporormiella alloiomera	UPS:Lundqvist 21345-p	Goat dung	Norway	GQ203771	GQ203731	Kruys and Wedin (2009)
Sporormiella antarctica	CBS 222.89	Soil	Norway	KX710224		–
Sporormiella australis	S5	Gazelle dung	South Africa	AY510411	AY510376	Arenal et al. (2005)
Sporormiella australis	**CF-285375**	***Launaea arborescens***	**Spain, Almeria**	**KU295583**		**González-Menéndez et al.** (2016)
Sporormiella australis	NBRC 101144	Soil	Israel	NBRC 11731401		–
Sporormiella australis	S7	Zebra dung	South Africa	AY510413	AY510378	Arenal et al. (2005)
Sporormiella australis	**CF-091932**	***Tamarix canariensis***	**Spain, Almeria**	**KX710240**		–
Sporormiella australis	UPS:Lundqvist 20884-a	Rabbit dung	France	GQ203773	GQ203732	Kruys and Wedin (2009)
Sporormiella australis	S6	Gazelle dung	Namibia	AY510412	AY510377	Arenal et al. (2005)
Sporormiella bipartis	UPS:Lundqvist 17250-a	*Lagopus muta* dung	Sweden	GQ203774	GQ203733	Kruys and Wedin (2009)
Sporormiella borealis	UPS:Lundqvist 16745-c	Horse dung	Romania	GQ203775	GQ203734	Kruys and Wedin (2009)
Sporormiella dakotensis	UPS:Thulin 2570-g	Cow dung	Ethiopia	GQ203776	GQ203735	Kruys and Wedin (2009)
Sporormiella dubia	UPS:Strid 19562-G	Horse dung	Iceland	GQ203777	GQ203736	Kruys and Wedin (2009)
Sporormiella grandispora	S37	*Phragmites communis*	Madrid, Spain	DQ468032	DQ468052	Arenal et al. (2007)
Sporormiella heptamera	UPS:Lundqvist 3090-b	Horse dung	Sweden	GQ203778	GQ203737	Kruys and Wedin (2009)
Sporormiella intermedia	UPS:Kruys 304	Cow dung	Sweden	GQ203779	GQ203738	Kruys and Wedin (2009)
Sporormiella intermedia	S1	Elk dung	USA	AY510415	AY510380	Arenal et al. (2005)
Sporormiella intermedia	CF-208569 WQ-056	Dung	Spain	KX710225		–
Sporormiella intermedia	**CF-279774**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710226**		–
Sporormiella intermedia	**CF-209155**	***Lithodora diffusa***	**Portugal, Algarve**	**KX710227**		–
Sporormiella intermedia	S4	Goat dung	Greece	AY510416	AY510381	Arenal et al. (2005)
Sporormiella intermedia	S3	Goat dung	Greece	AY510414	AY510379	Arenal et al. (2005)
Sporormiella intermedia	UAMH 7460	*Populus tremuloides*	Canada	DQ468020	DQ468040	Arenal et al. (2007)
Sporormiella irregularis	UPS:Lundqvist 16568-f	Cow dung	Hungary	GQ203780	GQ203739	Kruys and Wedin (2009)
Sporormiella isabellae	S25 (holotype)	Leaf litter	Puerto Rico	AY510424	AY510389	Arenal et al. (2005)
Sporormiella leporina	UPS:Richardson MJR93/04	Spruce grouse dung	Canada	GQ203782	GQ203741	Kruys and Wedin (2009)
Sporormiella leporina	UPS:Lundqvist 19873-a	Hare dung	Sweden	GQ203781	GQ203740	Kruys and Wedin (2009)
Sporormiella lignicola	**CF-282334**	***Dittrichia viscosa***	**Spain, Granada**	**KX710231**		–
Sporormiella lignicola	CBS 363.69	Rabbit dung	The Netherlands	GQ203783	DQ384098	Kruys and Wedin (2009)
Sporormiella lignicola	**CF-282002**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710232**		–
Sporormiella lignicola	**CF-282345**	***Dittrichia viscosa***	**Spain, Granada**	**KX710233**		–
Sporormiella lignicola	**CF-279765**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710234**		–
Sporormiella lignicola	**CF-097553**	***Viscum album***	**Spain, Guadalajara**	**KX710235**		–
Sporormiella lignicola	**CF-121346**	***Erica australis***	**Spain, Caceres**	**KX710236**		–
Sporormiella lignicola	**CF-279767**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710237**		–
Sporormiella lignicola	CF-214984 WQ-124	Dung	Spain	KX710238		–
Sporormiella lignicola	**CF-090241**	***Genista umbellata***	**Spain, Almeria**	**KX710230**		–
Sporormiella longisporopsis	UPS:Lundqvist 16551-g	Rabbit dung	Hungary	GQ203784	GQ203742	Kruys and Wedin (2009)
Sporormiella megalospora	UPS:Kruys 305	Cow dung	Sweden	GQ203785	GQ203743	Kruys and Wedin (2009)
Sporormiella minima	S13	Gazelle dung	Namibia	AY510426	AY510391	Arenal et al. (2005)
Sporormiella minima	CF-160935	Elephant dung	India	KX710243		–
Sporormiella minima	CF-066028	Vegetation	Dominican Republic	KX710244		–
Sporormiella minima	**CF-279768**	***Retama sphaerocarpa***	**Spain, Granada**	**KX710245**		–
Sporormiella minima	CF-215748 WQ-140	Dung	Spain	KX710246		–
Sporormiella minima	NBRC 32842	Garden soil	India	NBRC 03284201		–
Sporormiella minima	CBS 524.50	Goat dung	Panama	DQ468026	DQ468046	Arenal et al. (2007)
Sporormiella minima	UPS:Lundqvist 17212-a	Cow dung	Sweden	GQ203786	GQ203744	Kruys and Wedin (2009)

Table 2 (continued)

Species	Strain code[a]	Substrate	Origin	GenBank accession numbers[b]		Reference
				ITS	28S	
Sporormiella minima	NBRC 8595	Soil	–	NBRC -00859501		–
Sporormiella minima	S26	Leaf litter	USA	AY510427	AY510392	Arenal et al. (2005)
Sporormiella minima	S21	Rhinoceros dung	South Africa	AY510425	AY510390	Arenal et al. (2005)
Sporormiella minima	CF-209022 WQ-064	Dung	Spain	KX710248		–
Sporormiella minipascua	UPS:Kruys 306	Cow dung	Sweden	GQ203787	GQ203745	Kruys and Wedin (2009)
Sporormiella muskokensis	NBRC 8539	Soil	–	NBRC 00853901		–
Sporormiella octomera	UPS:Huhndorf et al. 2579	Porcupine dung	USA	GQ203788	GQ203746	Kruys and Wedin (2009)
Sporormiella pilosella	S38	*Quercus ilex* twigs	Guadalajara Spain	DQ468033	DQ468053	Arenal et al. (2007)
Sporormiella pulchella	UPS:MJR67/01, #216605	Rabbit dung	USA	GQ203789	GQ203747	Kruys and Wedin (2009)
Sporormiella septenaria	UPS:Espigores 00036	Sheep dung	Argentina	GQ203790	GQ203748	Kruys and Wedin (2009)
Sporormiella similis	**CF-285357**	***Asparagus horridus***	**Spain, Almeria**	**KX710228**		**–**
Sporormiella similis	S19	Undetermined dung	USA	AY510419	AY510386	Arenal et al. (2005)
Sporormiella similis	CBS 804.73	Saline dessert soil	Kuwait	DQ468028	DQ468048	Kruys and Wedin (2009)
Sporormiella similis	CF-210023 WQ-023	Dung	Spain	KX710229		–
Sporormiella vexans	UPS:23.VIII.1995, Andersson	Moose dung	Sweden	GQ203793	GQ203751	Kruys and Wedin (2009)
Sporormiella tenerifae	CBS 354.86	Rabbit dung	Tenerife	GQ203794	GQ203752	Kruys and Wedin (2009)
Verruculina enalia	CF-090068	Soil	Singapore	KX710217		–
Westerdykella dispersa	CBS 297.56	*Phlox drummondii*	USA	GQ203797	GQ203753	Kruys and Wedin (2009)
Westerdykella ornata	CBS 379.55 (holotype)	Mangrove mud	Mozambique	AY943045	GU301880	Schoch et al. (2009)
Westerdykella purpurea	NBRC 9428 (ex-type)	Soil	–	LC146766		–

[a] *CBS* CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; *CF* Fundación MEDINA Private Fungal Collection, Granada, Spain; *MFLUCC* Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; *NBRC* Biological Resource Center, National Institute of Technology and Evolution, Tokyo, Japan; *NRRL* Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, Illinois, USA; *UAMH* University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada; *UPS* The Museum of Evolution Herbarium, Sweden

[b] Accession numbers of sequences newly generated in this study are indicated in **bold**. *28S* Large subunit of the nrDNA; *ITS* internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA

sampling frequency = 100; first 1000 trees were discarded before the majority rule consensus tree was calculated.

In addition, the maximum likelihood (ML) method and ultrafast bootstrap support values for the phylogenetic tree were assessed calculating 1000 replicates with IQ-TREE software (Nguyen et al. 2015). All parameters were estimated by the software [the TIM2e + I + G4 model of nucleotide substitution was selected, assuming the shape parameter of the Invar + Gamma distributed substitution rates (gamma shape alpha = 0.4917) to accommodate rate variations among sites and an estimation of nucleotide frequencies as A = 0.25, C = 0.25, G = 0.25, and *T* = 0.25]. Aligned sequence data and phylogenetic trees were deposited in TreeBASE (SN 20908) http://purl.org/phylo/treebase/phylows/study/TB2:S20908

Preparation of extracts and metabolomic analysis

Thirty-seven fungal strains (23 Iberian isolates plus 14 *Preussia* strains from public collections) were grown in duplicate in two culture media with different carbon and nitrogen sources (MMK2 and YES media; González-Menéndez et al. 2014). Extracts generated from submerged fungal cultures were analyzed by low-resolution mass spectrometry (LR-MS) in the range of positive *m*/*z* for each extract. Four sets of *m*/*z* data ranging from 150 to 1500 Da were generated for each culture. The differential chemotypes in the crudes were identified using a matrix that correlated the intensity of each *m*/*z* per strain and a multivariate statistical analysis using Bionumerics® (Applied Maths™). The resulting dendrogram, built on a similarity matrix based on the *m*/*z* signals according to the Pearson correlation coefficient (see the supplementary material) and unweighted pair group method with arithmetic mean (UPGMA) allowed the identification of differential secondary metabolites and chemotypes among the studied species.

Chemical profiles were performed and compared to our internal proprietary databases for the identification of known secondary metabolites by low-resolution LC-LRMS (UV signal, retention time, and fragmentation patterns) against 950 standards and high-resolution LC-HRMS (retention time and accurate mass) against 835 standards (González-Menéndez et al. 2016; Pérez-Victoria et al. 2016). In addition, the compounds that were not identified from the database of standards were isolated by semi-preparative HPLC. Their predicted molecular formulas were confirmed by LC-ESI-HRMS/MS and compared to the entries in the Chapman & Hall Dictionary of Natural Products (v25.1) in order to identify compounds already described in the literature.

Results

Phylogenetic analysis and morphological observations

DNA fragments consisting of 465–485 bp (ITS) and 584–587 bp (28S) were obtained for the sequenced *Preussia* isolates. The different runs of the Bayesian analyses that were performed and ML analyses yielded the same topology (TreeBASE SN 20908). The consensus phylogenetic tree of 32 isolated strains with 104 GenBank™ sequences of representative strains including endophytic *Preussia* strains isolated recently from plants of the Arizona desert (Massimo et al. 2015) showed a very similar topology to the phylogenetic tree obtained previously by Kruys and Wedin (2009). Overall, all the *Preussia* strains are grouped in a single cluster that accommodates numerous, monophyletic, statistically supported subclades of both algorithms (posterior probability values = 95–100%/maximum likelihood bootstrap >70%). The only exception was the clade containing the strains of *P. minima*, *P. persica*, *P. isabellae*, and *P. mediterranea* that, despite the lack of support by Bayesian analyses, was well-supported by ML bootstrap (98%).

In detail, the ITS/28S rDNA tree revealed 19 clades named according to Kruys and Wedin (2009) (Fig. 1): (a) the clades "Sparticola", "Forliomyces", and "Westerdykella" were supported as previously shown in other phylogenetic studies (pp = 100%/bs = 100%) for each clade (Phukhamsakda et al. 2016); (b) the "Megalospora" clade grouped *Preussia* sp. SNP235, *Preussia* sp. (CF-277965 and CF-277801), *Preussia* sp. (CF-277787 and CF-277849), *P. terricola*, *Sporormiella megalospora*, and *P. polymorpha* with high statistical support (pp = 100%/bs = 100%); (c) the "Sporminula" clade with *P. cymatomera*, *P. pilosella*. *P. longisporopsis*, *P. grandispora*, *P. tenerifae*, and *Sporormia subticinensis* was not supported (pp = 87%/bs = 84%); (d) the highly supported "Vexans" clade, including the species *P. affinis*, *P. heptamera*, *P. octomera*, and *P. vexans*, clustered with the monospecific "Leporina" clade (pp = 100%/bs = 100%); (e) *P. dubia*, *P. irregularis*, and *P. muskokensis* cluster in the highly supported "Irregularis" clade (pp = 100%/bs = 100%); (f) the "Preussia" clade grouped seven species, *P. fleischhakii*, *P. flanaganii*, *P. alloiomera*, *P. thypharum*, *P. funiculata*, *P. vulgaris*, and *P. aemulans*, with strong support (pp = 100%/bs = 100%), clearly distinguished from the *Preussia* sp. strains SNP459 and SNP392 (pp = 100%/bs = 100%), and *P. septenaria* and *Preussia* sp. CF-282341 (pp = 100%/bs = 100%); (g) a main branch that contained the five statistically well-supported clades "Africana", "Intermedia", "Similis", "Lignicola", and "Australis" (pp = 100%); (h) relatedness of the two monospecific clades "Isomera" and "Minimoides" was supported by (pp = 100%/bs = 100) and (pp = 97%/bs = 93%), respectively; and (i) relatedness of the "Isabellae", "Minima", and "Mediterranea" complex, including the *Preussia* sp. strains CF-285378, SNP309, SNP057, SNP220, and SNP156, was supported by a 98% of bootstrap but not by Bayesian analyses, with the posterior probability value of 66%.

Based on their phylogenetic position, 14 of our isolates could be identified as *P. grandispora*, *P. subticinensis*, *P. typharum*, *P. funiculata*, *P. africana*, *P. intermedia*, *P. similis*, *P. australis*, and *P. minima*, all of which have been previously collected in the Iberian Peninsula. Their tentative phylogenetic position was verified following the methodology described by Arenal et al. (2004, 2005). Nine strains from different plants were morphologically and phylogenetically identified as *P. lignicola* (Fig. 2), a species that has not previously been cited from the Iberian Peninsula. Isolates currently not identifiable at the species level and distributed in the new clades were selected for morphological studies in order to compare them with other phylogenetically related *Preussia* species.

The asci and ascospore morphology of CF-277856 resembled that of *P. cymatomera* (Soláns 1985). *Preussia* sp. CF-277801 showed compact asci and four-celled, biseriately arranged ascospores showing parallel and diagonal germ slits extending over the entire spore length. *Preussia* sp. (CF-285378) showed similar colony morphologies, ornamental hyphae, and peridial cells as *P. isabellae* and *P. minima*. On the other hand, strains CF-155367, CF-279766, CF-279733, and CF-277817 only developed non-sporulating, darkly pigmented, and septate mycelium. A phoma-like anamorph was seen in CF-282341 and CF-209171 and a chrysosporium-like anamorph in CF-277787. The first report of a chrysosporium-like anamorph associated with a *Preussia* species was reported by Asgari and Zare (2010), who described the anamorphic state of *P. polymorpha*. Prior to this study, only *Phoma* sp. had been reported as anamorphs of *Preussia* species (de Gruyter et al. 2013).

Dereplication of known compounds and identification of chemotypes

The LC-HRMS dereplication of fungal extracts by comparison with more than 900 microbial natural product standards (González-Menéndez et al. 2016) identified 32 known compounds. Among them, we could identify seven compounds known to be produced by *Preussia* sp., including australifungin, australifunginol, asterric acid, preussomerins A and B, and sporminarins A and B. Twenty-three compounds previously described in other distantly related taxa were also identified, including altersetin, antibiotic FR 198248, bisdechlorodihydrogeodin, brefeldin A, brevianamide F, calbistrin A, chloro-6-methoxymellein, cis-4-hydroxy-6-deoxyscytalone, citrinin, cytochalasin F or B, curvicollide A, 11-deacetoxywortmannin, equisetin, funicone, globosuxanthone A, 2-(2-hydroxy-5-

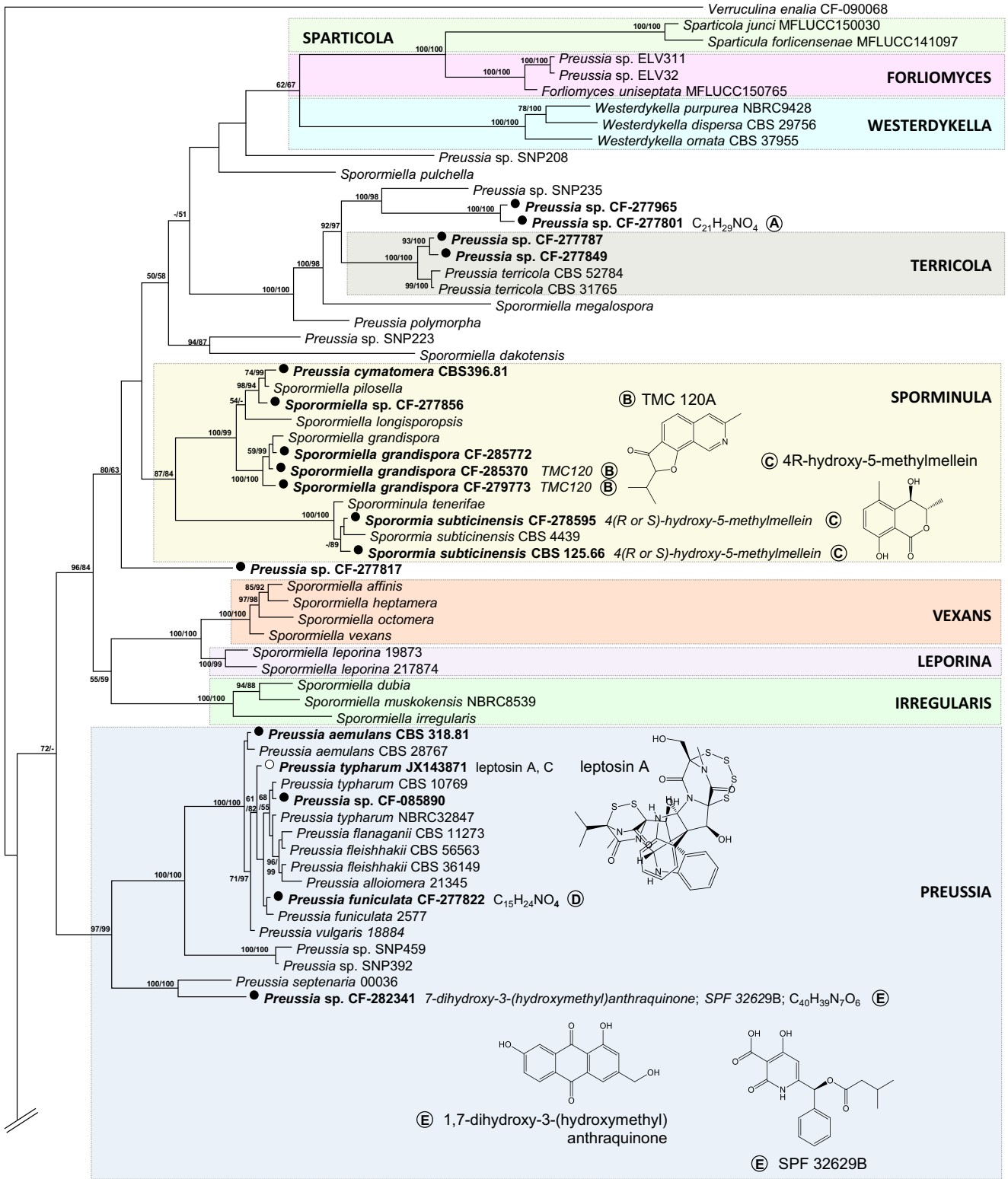

Fig. 1 Consensus tree from Bayesian phylogeny inferences based on ITS/28S sequences of selected *Preussia* species and related genera. The symbol ● identifies strains from the Iberian Peninsula isolated in this study and the symbol ○ indicates producers of compounds described in the literature. The most relevant compounds, including those newly identified in this study, are printed next to their producing taxa. Differential chemotypes are identified by **A**–**K**. Clade probability values/maximum likelihood bootstrap values are indicated respectively at the branches. Values <50 are designated by –. *Verruculina enalia* CF-090068 was used as an outgroup

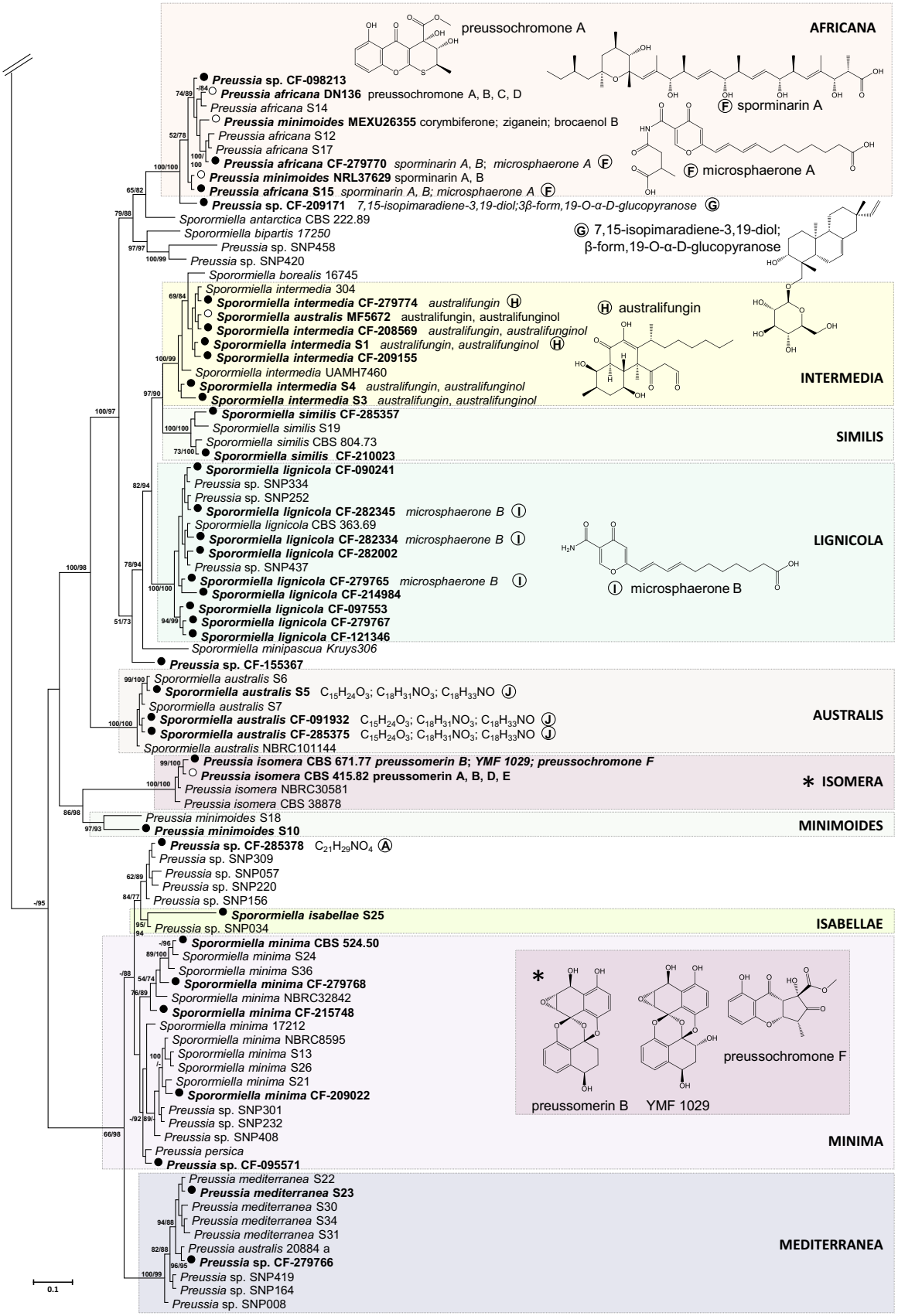

Fig. 1 (continued)

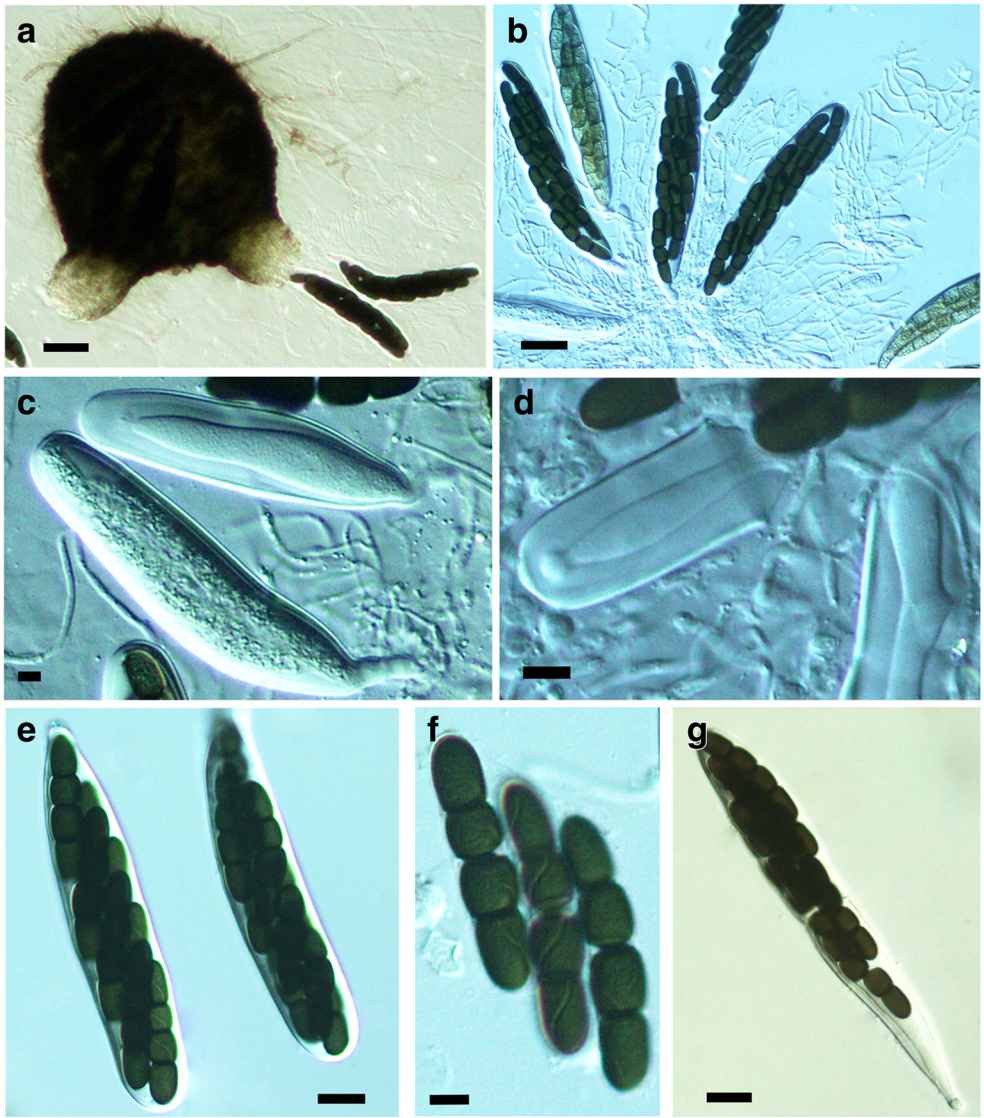

Fig. 2 *Preussia lignicola* CF-279765. **a** Ascoma formed on CMA. **b**–**e**, **g** Mature and immature asci. **f** Ascospores showing germ slits. Scale bars = 80 μm (**a**), 35 μm (**b**), 10 μm (**c**, **d**, **f**), 20 μm (**e**), 25 μm (**g**)

methoxyphenoxy)acrylic acid, 3-hydroxymellein, palmarumycin C15, penicillic acid, phomasetin, rugulosin, ulocladol, and waol A.

A metabolite profiling approach was also applied for the characterization of the 23 Iberian isolates and the 14 strains from public collections, encompassing 22 different species of *Preussia*. Mass spectrometry (MS) metabolite profiles from two different liquid media conditions were compared. The mass to charge ratio (*m*/*z*) and intensity of ionized molecules allowed the identification of known compounds and chemotypes characterizing different species of the genus.

The dendrogram obtained after multivariate statistical analysis of these profiles proved the relationships between the different strains (supplementary Fig. 1). Four strains (CBS 318.51, CF-277787, CF-279766, and CF-155367) belonging to four *Preussia* species presented profiles with a low number of metabolites and 70% similarity with the unfermented control media profile. Several sterile isolates that could not be identified produced chemical profiles closely related to other *Preussia* species. For example, strain CF-091932 showed a compound profile similar to that of one of the *P. australis* strains. This analysis grouped 12 strains in five monophyletic clades, where they clustered with *P. australis*, *P. lignicola*, *P. cymatomera*, *P. grandispora*, and *P. subticinensis*, regardless of their geographical origin or isolation source. Three of the five strains of *P. minima*, CF-095571, CF-206340, and CF-215745, clustered with *Preussia* sp. CF-277801 and *P. minimoides* S10, with 80% similarity. The two other strains of *P. minima*, CF-209022 and CF-279768, clustered with *P. mediterranea* S23, *P. isomera* CBS 671.77, *P. isabellae* S25, and two plant isolates (CF-285357 and CF-285378). In addition, both clades clustered together with *P. africana* S15 and *P. intermedia* S3, with 75% similarity. Finally, two strains with phoma-like

anamorphs (CF-209171 and CF-282341) were positioned outside the central clade, suggesting that they present different metabolic profiles (supplementary Fig. 1).

On the basis of the similarity matrix, we could determine compounds characteristic for each cluster. No specifically characterizing compounds were seen in 11 species (17 strains). Sixteen secondary metabolites were found to present good signal to noise ratios and could be used as differential compounds of the *Preussia* species analyzed. The presence or absence of a given compound, or a combination of more than one of these molecules, permitted the establishment of 11 different chemotypes (A–K) that grouped 21 of the studied strains into nine *Preussia* species (chemotypes C–K) (Fig. 1). Six species-specific secondary metabolites were identified for *P. subticinensis* (chemotype C), *Preussia* sp. CF-282341 (E), *P. africana* (F), *Preussia* sp. CF-209171 (G), *P. lignicola* (I), and *P. australis* (J). The "Grandispora" clade was characterized differentially by the compounds $C_{15}H_{18}O_3$, TMC120, and $C_{15}H_{27}NO_4$ (chemotype B). The "Subticinensis" clade presented differentially 4-hydroxy-5-methylmellein (chemotype C). The "Africana" clade showed $C_{15}H_{18}O_3$, $C_{16}H_{12}O_7$, and microsphaerone A (chemotype F). All members of the "Lignicola" clade were characterized as producers of TMC120, microsphaerone B, and $C_{21}H_{29}NO_4$ (chemotype I). The "Australis" clade (chemotype J) included $C_{15}H_{24}O_3$, $C_{18}H_{33}NO$, and $C_{18}H_{31}NO_3$ producers (see Tables 3 and 4).

Discussion

Although a comprehensive classification requires extensive efforts to recollect, culture, and phylogenetically characterize the full range of predominantly coprophilous *Preussia* species, our study has focused mainly on endophytic *Preussia* strains from Spain and Portugal, and only few were isolated from soil and dung.

Fungal endophytes form a very diverse group composed mostly of phylogenetically unrelated ascomycetes (Arnold 2007; Rodriguez et al. 2009). There have been many reports on endophytic species of *Preussia* isolated from different plant species (Mapperson et al. 2014; Zaferanloo et al. 2014; Massimo et al. 2015), but the life cycle of these fungi within their host plants is still unknown. It is possible that these fungi colonize internal plant tissues, beneath the epidermal cell layers, without causing any apparent harm or symptomatic infections to their host. They may live within the intercellular spaces of the tissues of living cells.

Many endophytic species of grasses are also known as common coprophilous fungi (Sánchez-Márquez et al. 2012). Other endophytes of non-grass hosts remain viable after passing throughout the gut of herbivores (Devarajan and Suryanarayanan 2006). These observations suggest that the coprophilous stage is an alternate phase in the life cycle of some endophytic fungi, and that certain coprophilous fungi

Table 3 Differential compounds identified by LC-LRMS and LC-HRMS analyses for *Preussia* species

No.	RT (min)	$[M + H]^+$ exp.	Proposed ion	Main secondary experimental ions	Production media	Proposed formula	Proposed compound
1	3.75	209.0810	$C_{11}H_{13}O_4^+$	210.0838; 191.0698	MMK2	$C_{11}H_{12}O_4$	4(R or S)-Hydroxy-5-methylmellein
2	3.19	247.1322	$C_{15}H_{19}O_3^+$	248.1355; 495.7545	MMK2	$C_{15}H_{18}O_3$	*34 possible matches in DBs*
3	2.15	253.1790	$C_{15}H_{25}O_3^+$	292.1167; 348.1794; 492.2807	YES	$C_{15}H_{24}O_3$	*94 possible matches in DBs*
4	3.13	258.1150	$C_{15}H_{16}NO_3^+$	259.1145	MMK2	$C_{15}H_{15}NO_3$	TMC120*
5	2.30	271.0603	$C_{15}H_{11}O_5^+$	272.0634; 293.042	MMK2	$C_{15}H_{10}O_5$	7-Dihydroxy-3-(hydroxymethyl) anthraquinone*
6	5.10	280.2637	$C_{18}H_{34}NO^+$	281.2663	YES	$C_{18}H_{33}NO$	–
7	3.59	286.2009	$C_{15}H_{28}NO_4^+$	287.2038	YES	$C_{15}H_{27}NO_4$	–
8	2.44	307.0841	$C_{15}H_{15}O_7^+$	324.1078; 308.0843; 209.0705	MMK2	$C_{15}H_{14}O_7$	Preussochromone F*
9	2.77	310.2369	$C_{18}H_{32}NO_3^+$	311.2400	YES	$C_{18}H_{31}NO_3$	–
10	2.47	317.0659	$C_{16}H_{13}O_7^+$	334.0925; 285.0394; 318,059	MMK2	$C_{16}H_{12}O_7$	*16 possible matches in DBs*
11	2.87	335.0763	$C_{16}H_{15}O_8^+$	317.0657; 318.0690; 691.1268	MMK2	$C_{16}H_{14}O_8$	Microsphaerone A*
12	3.17	320.1491	$C_{17}H_{22}NO_5^+$	302,1379; 321,1521; 368,2062	MMK2	$C_{17}H_{21}NO_5$	Microsphaerone B
13	0.93	346.1284	$C_{18}H_{20}NO_6^+$	309.1325; 347,1313; 399.1544	MMK2	$C_{18}H_{19}NO_6$	SPF 32629B
14	1.73	360.2167	$C_{21}H_{30}NO_4^+$	361.2198	MMK2	$C_{21}H_{29}NO_4$	*6 possible matches in DBs*
15	5.63	449.2907	$C_{26}H_{43}O_7^+$	450.2936; 484.3273; 950.6221	MMK2	$C_{26}H_{42}O_7$	7,15-Isopimaradiene-3,19-diol; 3β-form, 19-O-α-D-ose*
16	2.73	714.3028	$C_{40}H_{10}N_7O_6^+$	715,3061; 716,3085	MMK2	$C_{40}H_{39}N_7O_6$	–

*Additional semi-preparative HPLC fractionation and LC-HRMS/MS were performed for accurate identifications of the compounds proposed

Table 4 Differential chemotypes identified for the analyzed *Preussia* species, sorted according their position in the phylogenetic tree. Species-specific compounds are highlighted in **bold**

Strain	Taxonomy	Compounds (Table 3)																Chemotype
		1	2	**3**	4	**5**	**6**	7	8	**9**	**10**	**11**	**12**	**13**	14	**15**	**16**	
CF-277801	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	**A**
CBS 395.81	*Preussia cymatomera*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-277586	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-258370	*Preussia grandispora*	−	+	−	+	−	−	+	−	−	−	−	−	−		−	−	**B**
CF-279773	*Preussia grandispora*	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	**B**
CBS 125.66	*Preussia subticinensis*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**C**
CF-278595	*Preussia subticinensis*	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	**C**
CF-277817	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CBS 318.51	*Preussia aemulans*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-277822	*Preussia funiculata*	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	**D**
CF-282341	*Preussia* sp.	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	**E**
CF-160923 (S15)	*Preussia africana*	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	**F**
CF-279770	*Preussia africana*	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	**F**
CF-209171	*Preussia* sp.	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	**G**
CF-160907 (S1)	*Preussia intermedia*	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	**H**
CF-160910 (S3)	*Preussia intermedia*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279774	*Preussia intermedia*	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	**H**
CF-210023	*Preussia similis*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-285357	*Preussia similis*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279765	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-282334	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-282345	*Preussia lignicola*	−	−	−	+	−	−	−	−	−	−	−	+	−	+	−	−	**I**
CF-155365	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-091932	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CF-160911	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CF-285375	*Preussia australis*	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	**J**
CBS 671.77	*Preussia isomera*	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	**K**
CF-160916	*Preussia minimoides*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-285378	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	**A**
CF-160936 (S25)	*Preussia isabellae*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-095571	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CBS 524.50	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-209022	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-215748	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279768	*Preussia minima*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-160934 (S23)	*Preussia mediterranea*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None
CF-279766	*Preussia* sp.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	None

might have coevolved with grazing animals and plants (Porras-Alfaro et al. 2008).

The spores of coprophilous species are often surrounded by mucilage or have gelatinous appendices that attach easily to plant surfaces. When a plant is foraged by a herbivore, the spores travel through their digestive tract and, finally, when ending up in a new dung pile, the spores germinate and produce new fruit bodies (Kruys and Wedin 2009). An alternative hypothesis is that some of these coprophilous fungi were erroneously reported as endophytes, as their surface-sterilant resistant propagules could also occur passively on plant surfaces (Newcombe et al. 2016).

From the total number of 32 *Preussia* strains that were isolated in our study, the most frequent species was *P. lignicola*,

with eight isolates obtained from five different plant species (*Dittrichia viscosa*, *Retama sphaerocarpa*, *Viscum album*, *Erica australis*, and *Genista umbellata*) collected from all habitats sampled (Table 2). This confirmed previous results that indicated a wide distribution of this species in desert plants and its broad host range (Massimo et al. 2015). This is the first report of *P. lignicola* from the Iberian Peninsula. Another strain of *P. lignicola* was isolated from dung, as was *P. lignicola* strain CBS 264.69 from the Netherlands. Our second most frequently isolated species is *P. minima*, with four isolates. It was obtained from animal dung and plants, which may highlight its ability to alternate between endophytic and coprophilous lifestyles and explain why this species was isolated from different substrates worldwide.

The general topology of the ITS/28S phylogenetic tree was in agreement with previous studies (Kruys and Wedin 2009; Massimo et al. 2015). Eleven of the 21 *Preussia* sp. isolates from Arizona desert plants (Massimo et al. 2015) are included within the "Minima" complex.

Many known and frequent taxa represent heterogeneous species complexes, which remain to be resolved by a combination of genotype- and phenotype-derived data (Stadler 2011). Several polyphasic studies using chemotaxonomic, morphological, and molecular data have clarified the similarities between the different genera among Xylariales; for example, between *Daldinia*, *Entonaema*, and *Rhopalostroma* (Stadler et al. 2014). Although a relevant number of chemotaxonomic studies have been carried out, secondary metabolites have only been examined extensively in species of *Aspergillus*, *Penicillium*, and *Fusarium*.

Although few other studies exist that compare the secondary metabolite profiles and phylogeny (Frisvad et al. 2008), chemotaxonomic affinities only in *Alternaria* and *Ascochyta* but not other Dothideomycetes have been examined (Andersen et al. 2008; Kim et al. 2016). These recent studies highlight that this approach has the potential to provide valuable information related to ecology, and that its use in fungal biology needs to be further explored (Kim et al. 2016).

The evaluation of the different chemotypes present in the studied *Preussia* isolates revealed 16 compounds that can be used to distinguish *Preussia* species. We proposed component identities for eight of the 16 compounds. Four presented several possible compounds for each molecular formula identified and the other four could not be identified in the databases, suggesting that they may correspond to undescribed compounds (Table 3). Eleven of them were uniquely formed by certain species and they could be used to resolve groups of closely related species. This is the case for microsphaerone A formed in *P. africana* and microsphaerone B formed in *P. lignicola* (CF-279765) while no such compounds were encountered in other closely related *Preussia* sp.

The first fungal strain described to produce microsphaerone A and B was the mitosporic fungus *Microsphaeropsis* sp. (Wang et al. 2002). *Preussia subticinensis* also produced a specific ochratoxin derivative (Cole et al. 2003), previously described in a strain of *Microsphaeropsis* sp. as 4(R/S)-hydroxy-5-methyl-mellein (Höller et al. 1999). Young *Microsphaeropsis* pycnidia may be easily mistaken for a *Phoma* species (Boerema et al. 2004), with still colorless conidia when immature. This raises the question whether the strain was misidentified as a *Preussia* anamorph. A recent publication from *P. minima* reported the isolation of three novel linear pyran–furan fused furochromones, sporormielleins A–C, and three biogenetically related compounds, sporormiellones A and B, and microsphaeropsone A (Xiong et al. 2014). Microsphaeropsone A is a secondary metabolite intermediate generated by the sporormielleins AC production pathway, confirming our hypothesis that these metabolites are present in another species of *Preussia* (Xiong et al. 2014). On the other hand, the comparative analysis of the presence or absence of several specific *m*/*z* ions (chemotypes) for each *Preussia* strain proved to be also useful for discriminating species that did not present species-specific compounds, as in *P. grandispora* (chemotype B) or *P. funiculata* (chemotype D) (Fig. 1, Tables 3 and 4).

Regarding the bioactive secondary metabolites dereplicated in the extracts, several mellein (ochracein) derivatives were also found in three *Preussia* strains (CF-282341, CF-277856, and CBS 125.66). These precursors of ochratoxins (Harris and Mantle 2001) were originally discovered in *Aspergillus ochraceus* and then in different taxa of the Botryosphaeriales, Pleosporales, and Xylariales (Rukachaisirikul et al. 2013; Stadler 2011). Preusserin (Johnson et al. 1989) is produced by *A. ochraceus* (Schwartz et al. 1988) and several species of *Preussia*. The analyzed strains of *P. africana* produced sporminarin A and B and strains of *P. similis* contained brefeldin A and 11-deacetoxywortmannin. The compounds cytochalasin, globosuxanthone A, or brevianamide F were produced by some strains included in the clades "Australis", "Intermedia", and "Minima".

Limitations to the detection of already known active compounds in these species can be explained by a differential production under the specific fermentation conditions used in this study (MMK2 and YES). Most of the discussed molecules had been previously reported from rice- or corn-based solid media cultures (Hensens et al. 1995; Mudur et al. 2006; Zhang et al. 2012; Xiong et al. 2014) (Table 1). It is well known that culture media compositions affect the production of fungal secondary metabolites. Microorganisms growing on a solid medium are in various physiological conditions, which may stimulate the expression of different biosynthetic gene clusters (de la Cruz et al. 2012). To confirm this hypothesis, we studied the production of australifungin and

australifunginol by adopting the same solid media and conditions used by Mandala et al. (1995) and using the original australifungin producer strain (MF5672). Australifungin was detected in five and australifunginol in four species of the "Intermedia" clade (Fig. 1). This experiment confirms that specific conditions and taxon-specific optimizations are required for triggering the production of certain compounds.

Conclusions

Preussia lignicola, a species reported for the first time from the Iberian Peninsula, was encountered in five of the 14 different plant species analyzed. Another 19 *Preussia* species were identified from the phylogenetic and morphological analyses, of which three either formed phoma- or chrysosporium-like anamorphs, while four did not sporulate in culture.

Eleven of the 16 identified secondary metabolites produced by the *Preussia* isolates can be chemotaxonomically used to distinguish six species. In addition, phylogenetic analysis identified 11 different chemotypes among 22 of the species studied, supporting that secondary metabolites characterization is a useful tool for taxonomic descriptions. More culturing conditions should be added to further identify other chemotypes to distinguish the rest of the *Preussia* species.

This analysis also identified four putative new secondary metabolites with no matches in the natural products databases of known compounds, suggesting that the potential of *Preussia* species for the discovery of new natural products is untapped.

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