

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*, *Spororminula*, 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 ascospores, 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 ascospores and cylindrical to cylindrical-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. systemospora* (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* (Váldosera 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, cisetin, 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-mm² 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 |
|---|-------------------------|------------------------------|--------------------|------------------------------|------------------------------|
| Asteric acid | <i>Preussia</i> sp. | Rice-based media | Solid | Endothelin binding inhibitor | Talontsi et al. (2014) |
| Auranticins A, B | <i>P. aurantiaca</i> | Rice-based media | Solid | Antifungal and antibacterial | Poch and Gloer (1991) |
| 7-Chloro-6-methoxymellein | <i>P. affinis</i> | Corn starch and molasses | Liquid | Antifungal | McGahren and Mitscher (1968) |
| Antibiotic FR 173945 | <i>P. aemulans</i> | Starch and peach powder | Liquid | Antifungal | Sato et al. (1998) |
| Antibiotic WF 15604A | <i>P. minima</i> | Starch acid and cotton flour | Liquid | Antifungal | Hatori et al. (2004) |
| Australifungin | <i>P. australis</i> | Corn-based media | Solid | Cytotoxic | Hensens et al. (1995) |
| C ₂₈ H ₄₂ N ₄ O ₈ | <i>P. minimoides</i> | Corn meal and sucrose | Liquid | Antifungal | Clapp-Shapiro et al. (1998) |
| Cissetin | <i>Preussia</i> sp. | Rice-based media | Solid | Antibacterial | Talontsi et al. (2014) |
| Cryptosporiopsin | <i>P. affinis</i> | Corn starch and molasses | Liquid | Antifungal | McGahren et al. (1969) |
| Culpin | <i>Preussia</i> sp. | Soluble starch and glucose | Liquid | Antifungal and antibacterial | Robinson et al. (1988) |
| Cyperin | <i>P. fleischhakkii</i> | Potato dextrose broth | Liquid | Phytotoxic | Weber and Gloer (1988) |
| Hyalopyrone | <i>P. teretispora</i> | Soy flour medium | Liquid | Phytotoxic | Wang et al. (1995) |
| Leptosin A, C | <i>P. typharum</i> | Cheerios breakfast cereal | Solid | Cytotoxic | Du et al. (2014) |
| Preussiadin A, B | <i>P. typharum</i> | Cheerios breakfast cereal | Solid | Cytotoxic | Du et al. (2014) |
| Microsphaeropsone A | <i>P. minima</i> | Rice-based media | Solid | Antifungal | Xiong et al. (2014) |
| Preussiafuran A, B | <i>Preussia</i> sp. | Rice-based media | Solid | Cytotoxic | Talontsi et al. (2014) |
| Preussin | <i>Preussia</i> sp. | Soluble starch and glucose | Liquid | Antifungal | Johnson et al. (1989) |
| Preussochromone A, B, C, D | <i>P. africana</i> | Rice-based media | Solid | Cytotoxic | Zhang et al. (2012) |
| Preussomerin A, E, D, G, B | <i>P. isomera</i> | Potato dextrose broth | Liquid | ras Farnesyl-protein | Weber and Gloer (1991) |
| Similin A, B | <i>P. similis</i> | Soybean and dextrose | Liquid | Antifungal | Weber et al. (1992) |
| Spiropreussione | <i>Preussia</i> sp. | Wheat bran and glucose | Liquid | Cytotoxic | Chen et al. (2009) |
| Spominarin A, B | <i>P. minimoides</i> | Rice-based media | Solid | Antifungal | Mudur et al. (2006) |
| Sporostatin | <i>Preussia</i> sp. | Glucose and peptone broth | Liquid | Cytotoxic | Kinoshita et al. (1997) |
| Sporovexin A, B, C | <i>P. vexans</i> | Potato dextrose broth | Liquid | Antifungal and antibacterial | Soman et al. (1999) |
| Terezine A, B, C, D | <i>P. teretispora</i> | Soy flour medium | Liquid | Antifungal and antibacterial | Wang et al. (1995) |
| Zaragozic acid B | <i>P. intermedia</i> | 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 | |
| <i>Forliomyces uniseptata</i> | MFLUCC 15-0765 (ex-type) | <i>Spartium junceum</i> | Spain | KU721772 | KU721762 | Phukhamsakda et al. (2016) |
| <i>Preussia aemulans</i> | CBS 287.67 | Soil | The Netherlands | DQ468017 | DQ468037 | Arenal et al. (2007) |
| <i>Preussia aemulans</i> | CBS 318.81 | Soil | The Netherlands | KX710218 | – | – |
| <i>Preussia africana</i> | S17 (holotype) | <i>Viburnum tinus</i> leaf | Tenerife, Spain | AY510418 | AY510383 | Arenal et al. (2005) |
| <i>Preussia africana</i> | CF-279770 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710221 | – | – |
| <i>Preussia africana</i> | CF-098213 | <i>Erica araganeensis</i> | Spain, Lugo | KX710222 | – | – |
| <i>Preussia africana</i> | S14 | Zebra dung | South Africa | AY510417 | AY510382 | Arenal et al. (2005) |
| <i>Preussia africana</i> | DN136 | <i>Ramalina calicaris</i> | China | JQ031265 | – | Zhang et al. (2012) |
| <i>Preussia africana</i> | S15 | Zebra dung | South Africa | AY510421 | AY510385 | Arenal et al. (2005) |
| <i>Preussia africana</i> | S12 | Goat dung | Tanzania | AY510420 | AY510384 | Arenal et al. (2005) |
| <i>Preussia cymatomera</i> | CBS 396.81 | <i>Juniperus communis</i> | Switzerland | KX710252 | – | – |
| <i>Preussia flanaganii</i> | CBS 112.73 (isotype) | Sandy soil | Mexico | AY943061 | – | – |
| <i>Preussia fleischhakkii</i> | CBS 565.63 | Soil | Germany | GQ203761 | GQ203721 | Kruys and Wedin (2009) |
| <i>Preussia fleischhakkii</i> | CBS 361.49 | Man's nail | The Netherlands | DQ468018 | DQ468038 | Arenal et al. (2007) |
| <i>Preussia funiculata</i> | UPS:Huhndorf et al. 2577 (F) | Porcupine dung | USA | GQ203762 | GQ203722 | Kruys and Wedin (2009) |
| <i>Preussia isomera</i> | CBS 415.82 | – | Venezuela | KX710243 | – | – |
| <i>Preussia isomera</i> | NBRC 30581 | Soil | Nepal | NBRC 03058101 | – | – |
| <i>Preussia isomera</i> | CBS 388.78 | Cow dung | Venezuela | GQ203763 | GQ203723 | Kruys and Wedin (2009) |
| <i>Preussia isomera</i> | CBS 671.77 | – | Japan | KX710241 | – | – |
| <i>Preussia mediterranea</i> | S23 (holotype) | <i>Cistus albidus</i> leaf | Spain, Caceres | DQ468022 | DQ468042 | Arenal et al. (2007) |
| <i>Preussia mediterranea</i> | S30 | <i>Alnus glutinosa</i> leaf | Spain, Caceres | DQ468023 | DQ468043 | Arenal et al. (2007) |
| <i>Preussia mediterranea</i> | S34 | <i>Daphne gnidium</i> leaf | Spain, Caceres | DQ468025 | DQ468045 | Arenal et al. (2007) |
| <i>Preussia mediterranea</i> | S31 | <i>Quercus suber</i> leaf | Spain, Caceres | DQ468024 | DQ468044 | Arenal et al. (2007) |
| <i>Preussia mediterranea</i> | S22 | <i>Quercus ilex</i> leaf | Spain, Guadalajara | DQ468021 | DQ468041 | Arenal et al. (2007) |
| <i>Preussia minimoides</i> | MEXU 26355 | <i>Hintonia latiflora</i> | Mexico | KF557658 | KF557659 | Leyte-Lugo et al. (2013) |
| <i>Preussia minimoides</i> | NRRL 37629 | <i>Trametes hirsutum</i> | Hawaii | GU183123 | – | Mudur et al. (2006) |
| <i>Preussia minimoides</i> | S10 | Pig dung | Argentina | AY510423 | AY510388 | Arenal et al. (2005) |
| <i>Preussia minimoides</i> | S18 | <i>Prunus lusitanica</i> | Canary island | AY510422 | AY510387 | Arenal et al. (2005) |
| <i>Preussia persica</i> | CBS 117680 | Barley dead leaf | Iran | GQ292750 | GQ292752 | Asgari and Zare (2010) |
| <i>Preussia polymorpha</i> | CBS 117679 (holotype) | Barley dead leaf | Iran | GQ292749 | GQ292751 | Asgari and Zare (2010) |
| <i>Preussia</i> sp. | ELV3.2 | <i>Eustrephus latifolius</i> | Australia | JN418773 | KF269206 | Mapperson et al. (2014) |
| <i>Preussia</i> sp. | ELV3.11 | <i>Eustrephus latifolius</i> | Australia | JN418774 | KF269205 | Mapperson et al. (2014) |
| <i>Preussia</i> sp. | CF-095571 | <i>Pinus pinaster</i> | Spain, Lerida | KX710247 | – | – |
| <i>Preussia</i> sp. | CF-155367 | – | – | – | – | – |
| <i>Preussia</i> sp. | WO-009 | Soil | Spain | KX710239 | – | – |
| <i>Preussia</i> sp. | CF-209171 | Kudu | South Africa | KX710223 | – | – |
| <i>Preussia</i> sp. | CF-277787 | Soil | Spain, Granada | KX710262 | – | – |
| <i>Preussia</i> sp. | CF-277801 | Soil | Spain, Granada | KX710260 | – | – |
| <i>Preussia</i> sp. | CF-277817 | Soil | Spain, Granada | KX710251 | – | – |
| <i>Preussia</i> sp. | CF-277822 | Soil | Spain, Granada | KX710220 | – | – |
| <i>Preussia</i> sp. | CF-277849 | Soil | Spain, Granada | KX710263 | – | – |
| <i>Preussia</i> sp. | CF-277856 | Soil | Spain, Granada | KX710253 | – | – |
| <i>Preussia</i> sp. | CF-277965 | Soil | Spain, Granada | KX710261 | – | – |
| <i>Preussia</i> sp. | CF-279766 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710250 | – | – |
| <i>Preussia</i> sp. | CF-279773 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710256 | – | – |
| <i>Preussia</i> sp. | CF-282341 | <i>Ditrichia viscosa</i> | Spain, Granada | KU295582 | – | González-Menéndez et al. (2016) |
| <i>Preussia</i> sp. | CF-285370 | <i>Anabasis articulata</i> | Spain, Almeria | KX710254 | – | – |
| <i>Preussia</i> sp. | CF-285378 | <i>Salsola oppositifolia</i> | Spain, Almeria | KX710249 | – | – |
| <i>Preussia</i> sp. | CF-285772 | <i>Phragmites australis</i> | Spain, Granada | KX710255 | – | – |
| <i>Preussia</i> sp. | SNP008 | <i>Larrea tridentata</i> | USA | KP335214 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP034 | <i>Simmondsia chinensis</i> | USA | KP335239 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP057 | <i>Parkinsonia microphylla</i> | USA | KP335262 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP156 | <i>Phoradendron californicum</i> | USA | KP335359 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP164 | <i>Larrea tridentata</i> | USA | KP335365 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP208 | <i>Larrea tridentata</i> | USA | KP335402 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP220 | <i>Larrea tridentata</i> | USA | KP335411 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP223 | <i>Larrea tridentata</i> | USA | KP335414 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP232 | <i>Larrea tridentata</i> | USA | KP335422 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP235 | <i>Parkinsonia microphylla</i> | USA | KP335425 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP252 | <i>Simmondsia chinensis</i> | USA | KP335440 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP301 | <i>Parkinsonia microphylla</i> | USA | KP335487 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP309 | <i>Simmondsia chinensis</i> | USA | KP335495 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP334 | <i>Larrea tridentata</i> | USA | KP335519 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP392 | <i>Larrea tridentata</i> | USA | KP335569 | – | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP408 | <i>Larrea tridentata</i> | USA | KP335584 | – | Massimo et al. (2015) |

Table 2 (continued)

| Species | Strain code ^a | Substrate | Origin | GenBank accession numbers ^b | | Reference |
|---------------------------------------|--------------------------|-----------------------------------|---------------------------|--|----------|--|
| | | | | ITS | 28S | |
| | | <i>Phoradendron californicum</i> | | | | |
| <i>Preussia</i> sp. | SNP419 | <i>Simmondsia chinensis</i> | USA | KP335595 | | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP420 | <i>Simmondsia chinensis</i> | USA | KP335596 | | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP437 | <i>Simmondsia chinensis</i> | USA | KP335613 | | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP458 | <i>Parkinsonia microphylla</i> | USA | KP335634 | | Massimo et al. (2015) |
| <i>Preussia</i> sp. | SNP459 | <i>Larrea tridentata</i> | USA | KP335638 | | Massimo et al. (2015) |
| <i>Preussia subticinensis</i> | CBS 443.9 | – | France | KX710258 | | – |
| <i>Preussia subticinensis</i> | CBS 125.66 | – | Germany | KX710259 | | – |
| <i>Preussia subticinensis</i> | CF-278595 | Soil | Spain, Granada | KX710257 | | – |
| <i>Preussia terricola</i> | CBS 317.65 | <i>Musa sapientum</i> | Honduras | GQ203765 | GQ203725 | Kruys and Wedin (2009) |
| <i>Preussia terricola</i> | CBS 527.84 | Elephant dung | Tanzania | GQ203764 | GQ203724 | Kruys and Wedin (2009) |
| <i>Preussia typharum</i> | CBS 107.69 | Deer dung | Japan | GQ203766 | GQ203726 | Kruys and Wedin (2009) |
| <i>Preussia typharum</i> | 718249 | Degraded organic matter | USA | JX143871 | | – |
| <i>Preussia typharum</i> | NBRC 32847 | Unidentified plant | Iraq | NBRC 03284701 | | – |
| <i>Preussia typharum</i> | CF-085890 | Microbial mats | Spain, Tarragona | KX710219 | | – |
| <i>Preussia vulgaris</i> | UPS:Strid 18884 | Hare dung | Sweden | GQ203767 | GQ203727 | Kruys and Wedin (2009) |
| <i>Sparticola junci</i> | MFLUCC 15-0030 (ex-type) | <i>Spartium junceum</i> | Spain | KU721775 | KU721765 | Phukhamsakda et al. (2016) |
| <i>Sparticola forliciesenica</i> | MFLUCC 14-1097 (ex-type) | <i>Spartium junceum</i> | Spain | KU721773 | KU721763 | Phukhamsakda et al. (2016) |
| <i>Sporormiella affinis</i> | UPS:Lundqvist 17739-j | Rabbit dung | Denmark | GQ203770 | GQ203730 | Kruys and Wedin (2009) |
| <i>Sporormiella alloiomeria</i> | UPS:Lundqvist 21345-p | Goat dung | Norway | GQ203771 | GQ203731 | Kruys and Wedin (2009) |
| <i>Sporormiella antarctica</i> | CBS 222.89 | Soil | Norway | KX710224 | | – |
| <i>Sporormiella australis</i> | S5 | Gazelle dung | South Africa | AY510411 | AY510376 | Arenal et al. (2005) |
| <i>Sporormiella australis</i> | CF-285375 | <i>Launaea arborescens</i> | Spain, Almeria | KU295583 | | González-Menéndez et al. (2016) |
| <i>Sporormiella australis</i> | NBRC 101144 | Soil | Israel | NBRC 11731401 | | – |
| <i>Sporormiella australis</i> | S7 | Zebra dung | South Africa | AY510413 | AY510378 | Arenal et al. (2005) |
| <i>Sporormiella australis</i> | CF-091932 | <i>Tamarix canariensis</i> | Spain, Almeria | KX710240 | | – |
| <i>Sporormiella australis</i> | UPS:Lundqvist 20884-a | Rabbit dung | France | GQ203773 | GQ203732 | Kruys and Wedin (2009) |
| <i>Sporormiella australis</i> | S6 | Gazelle dung | Namibia | AY510412 | AY510377 | Arenal et al. (2005) |
| <i>Sporormiella bipartis</i> | UPS:Lundqvist 17250-a | <i>Lagopus muta</i> dung | Sweden | GQ203774 | GQ203733 | Kruys and Wedin (2009) |
| <i>Sporormiella borealis</i> | UPS:Lundqvist 16745-c | Horse dung | Romania | GQ203775 | GQ203734 | Kruys and Wedin (2009) |
| <i>Sporormiella dakotensis</i> | UPS:Thulin 2570-g | Cow dung | Ethiopia | GQ203776 | GQ203735 | Kruys and Wedin (2009) |
| <i>Sporormiella dubia</i> | UPS:Strid 19562-G | Horse dung | Iceland | GQ203777 | GQ203736 | Kruys and Wedin (2009) |
| <i>Sporormiella grandispora</i> | S37 | <i>Phragmites communis</i> | Madrid, Spain | DQ468032 | DQ468052 | Arenal et al. (2007) |
| <i>Sporormiella heptamera</i> | UPS:Lundqvist 3090-b | Horse dung | Sweden | GQ203778 | GQ203737 | Kruys and Wedin (2009) |
| <i>Sporormiella intermedia</i> | UPS:Kruys 304 | Cow dung | Sweden | GQ203779 | GQ203738 | Kruys and Wedin (2009) |
| <i>Sporormiella intermedia</i> | S1 | Elk dung | USA | AY510415 | AY510380 | Arenal et al. (2005) |
| <i>Sporormiella intermedia</i> | CF-208569 WQ-056 | Dung | Spain | KX710225 | | – |
| <i>Sporormiella intermedia</i> | CF-279774 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710226 | | – |
| <i>Sporormiella intermedia</i> | CF-209155 | <i>Lithodora diffusa</i> | Portugal, Algarve | KX710227 | | – |
| <i>Sporormiella intermedia</i> | S4 | Goat dung | Greece | AY510416 | AY510381 | Arenal et al. (2005) |
| <i>Sporormiella intermedia</i> | S3 | Goat dung | Greece | AY510414 | AY510379 | Arenal et al. (2005) |
| <i>Sporormiella intermedia</i> | UAMH 7460 | <i>Populus tremuloides</i> | Canada | DQ468020 | DQ468040 | Arenal et al. (2007) |
| <i>Sporormiella irregularis</i> | UPS:Lundqvist 16568-f | Cow dung | Hungary | GQ203780 | GQ203739 | Kruys and Wedin (2009) |
| <i>Sporormiella isabellae</i> | S25 (holotype) | Leaf litter | Puerto Rico | AY510424 | AY510389 | Arenal et al. (2005) |
| <i>Sporormiella leporina</i> | UPS:Richardson MJR93/04 | Spruce grouse dung | Canada | GQ203782 | GQ203741 | Kruys and Wedin (2009) |
| <i>Sporormiella leporina</i> | UPS:Lundqvist 19873-a | Hare dung | Sweden | GQ203781 | GQ203740 | Kruys and Wedin (2009) |
| <i>Sporormiella lignicola</i> | CF-282334 | <i>Ditrichia viscosa</i> | Spain, Granada | KX710231 | | – |
| <i>Sporormiella lignicola</i> | CBS 363.69 | Rabbit dung | The Netherlands | GQ203783 | DQ384098 | Kruys and Wedin (2009) |
| <i>Sporormiella lignicola</i> | CF-282002 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710232 | | – |
| <i>Sporormiella lignicola</i> | CF-282345 | <i>Ditrichia viscosa</i> | Spain, Granada | KX710233 | | – |
| <i>Sporormiella lignicola</i> | CF-279765 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710234 | | – |
| <i>Sporormiella lignicola</i> | CF-097553 | <i>Viscum album</i> | Spain, Guadalajara | KX710235 | | – |
| <i>Sporormiella lignicola</i> | CF-121346 | <i>Erica australis</i> | Spain, Caceres | KX710236 | | – |
| <i>Sporormiella lignicola</i> | CF-279767 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710237 | | – |
| <i>Sporormiella lignicola</i> | CF-214984 WQ-124 | Dung | Spain | KX710238 | | – |
| <i>Sporormiella lignicola</i> | CF-090241 | <i>Genista umbellata</i> | Spain, Almeria | KX710230 | | – |
| <i>Sporormiella longisporopsis</i> | UPS:Lundqvist 16551-g | Rabbit dung | Hungary | GQ203784 | GQ203742 | Kruys and Wedin (2009) |
| <i>Sporormiella megalospora</i> | UPS:Kruys 305 | Cow dung | Sweden | GQ203785 | GQ203743 | Kruys and Wedin (2009) |
| <i>Sporormiella minima</i> | S13 | Gazelle dung | Namibia | AY510426 | AY510391 | Arenal et al. (2005) |
| <i>Sporormiella minima</i> | CF-160935 | Elephant dung | India | KX710243 | | – |
| <i>Sporormiella minima</i> | CF-066028 | Vegetation | Dominican Republic | KX710244 | | – |
| <i>Sporormiella minima</i> | CF-279768 | <i>Retama sphaerocarpa</i> | Spain, Granada | KX710245 | | – |
| <i>Sporormiella minima</i> | CF-215748 WQ-140 | Dung | Spain | KX710246 | | – |
| <i>Sporormiella minima</i> | NBRC 32842 | Garden soil | India | NBRC 03284201 | | – |
| <i>Sporormiella minima</i> | CBS 524.50 | Goat dung | Panama | DQ468026 | DQ468046 | Arenal et al. (2007) |
| <i>Sporormiella minima</i> | 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 | |
| <i>Sporormiella minima</i> | NBRC 8595 | Soil | – | NBRC -00859501 | | – |
| <i>Sporormiella minima</i> | S26 | Leaf litter | USA | AY510427 | AY510392 | Arenal et al. (2005) |
| <i>Sporormiella minima</i> | S21 | Rhinoceros dung | South Africa | AY510425 | AY510390 | Arenal et al. (2005) |
| <i>Sporormiella minima</i> | CF-209022 WQ-064 | Dung | Spain | KX710248 | – | – |
| <i>Sporormiella minipascua</i> | UPS:Kruys 306 | Cow dung | Sweden | GQ203787 | GQ203745 | Kruys and Wedin (2009) |
| <i>Sporormiella muskokensis</i> | NBRC 8539 | Soil | – | NBRC 00853901 | | – |
| <i>Sporormiella octomera</i> | UPS:Huhndorf et al. 2579 | Porcupine dung | USA | GQ203788 | GQ203746 | Kruys and Wedin (2009) |
| <i>Sporormiella pilosella</i> | S38 | <i>Quercus ilex</i> twigs | Guadalajara Spain | DQ468033 | DQ468053 | Arenal et al. (2007) |
| <i>Sporormiella pulchella</i> | UPS:MJR67/01. #216605 | Rabbit dung | USA | GQ203789 | GQ203747 | Kruys and Wedin (2009) |
| <i>Sporormiella septenaria</i> | UPS:Espigores 00036 | Sheep dung | Argentina | GQ203790 | GQ203748 | Kruys and Wedin (2009) |
| <i>Sporormiella similis</i> | CF-285357 | <i>Asparagus horridus</i> | Spain, Almeria | KX710228 | – | – |
| <i>Sporormiella similis</i> | S19 | Undetermined dung | USA | AY510419 | AY510386 | Arenal et al. (2005) |
| <i>Sporormiella similis</i> | CBS 804.73 | Saline dessert soil | Kuwait | DQ468028 | DQ468048 | Kruys and Wedin (2009) |
| <i>Sporormiella similis</i> | CF-210023 WQ-023 | Dung | Spain | KX710229 | – | – |
| <i>Sporormiella vexans</i> | UPS:23.VIII.1995, Andersson | Moose dung | Sweden | GQ203793 | GQ203751 | Kruys and Wedin (2009) |
| <i>Spororminula tenerifae</i> | CBS 354.86 | Rabbit dung | Tenerife | GQ203794 | GQ203752 | Kruys and Wedin (2009) |
| <i>Verruculina enalia</i> | CF-090068 | Soil | Singapore | KX710217 | – | – |
| <i>Westerdykella dispersa</i> | CBS 297.56 | <i>Phlox drummondii</i> | USA | GQ203797 | GQ203753 | Kruys and Wedin (2009) |
| <i>Westerdykella ornata</i> | CBS 379.55 (holotype) | Mangrove mud | Mozambique | AY943045 | GU301880 | Schoch et al. (2009) |
| <i>Westerdykella purpurea</i> | 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 rDNA; ITS internal transcribed spacer regions of the rDNA and intervening 5.8S rDNA

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/phyloids/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. alloiomeria*, *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. thypharum*, *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, biserially 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, asteric 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, curvicolliide A, 11-deacetoxywortmannin, equisetin, funicone, globosuxanthone A, 2-(2-hydroxy-5-

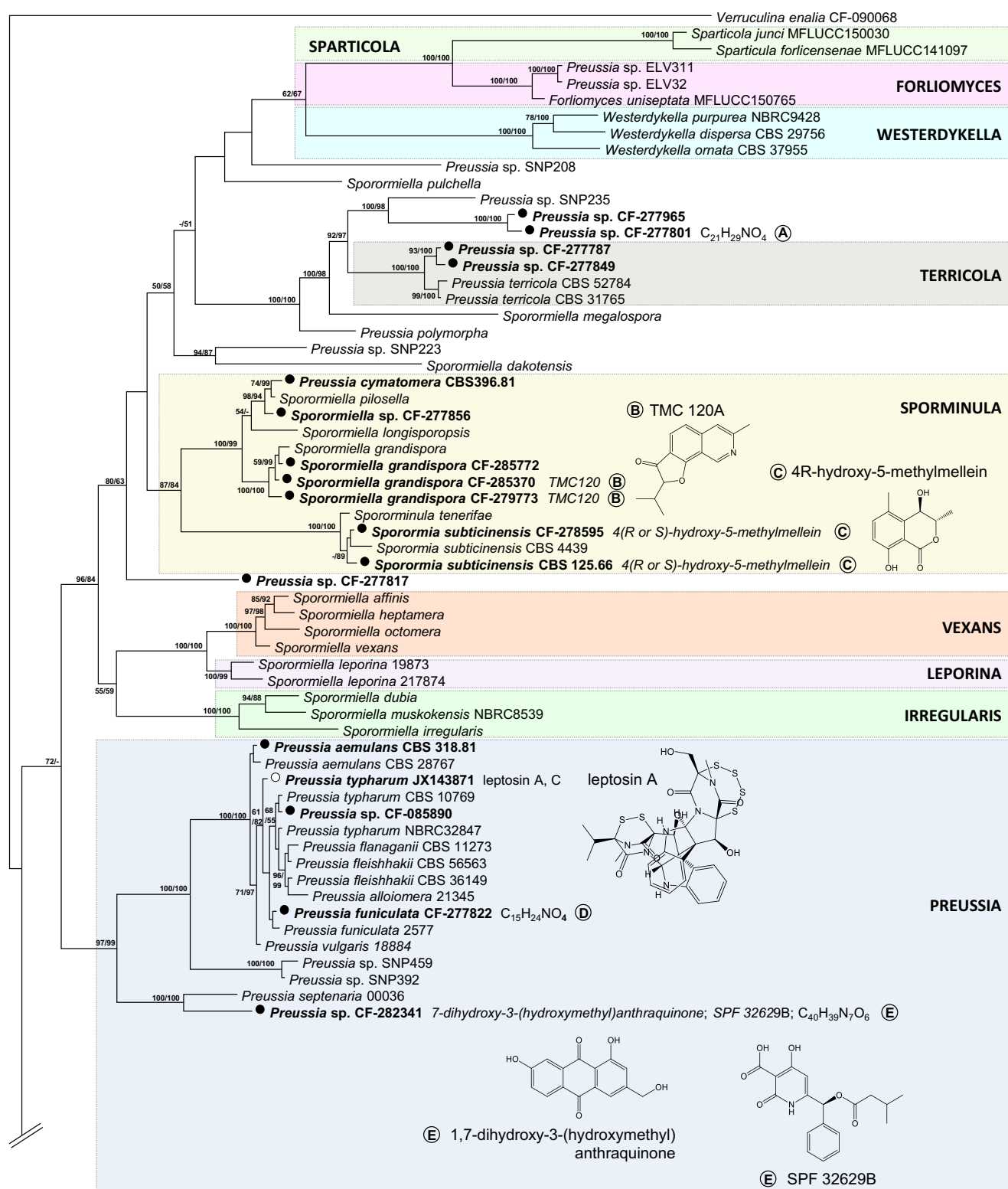


Fig. 1 Consensus tree from Bayesian phylogeny inferences based on ITS/28S sequences of selected *Preussia* species and related genera. The symbol ● identifies strains from the Iberian Peninsula isolated in this study and the symbol ○ indicates producers of compounds described in the literature. The most 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

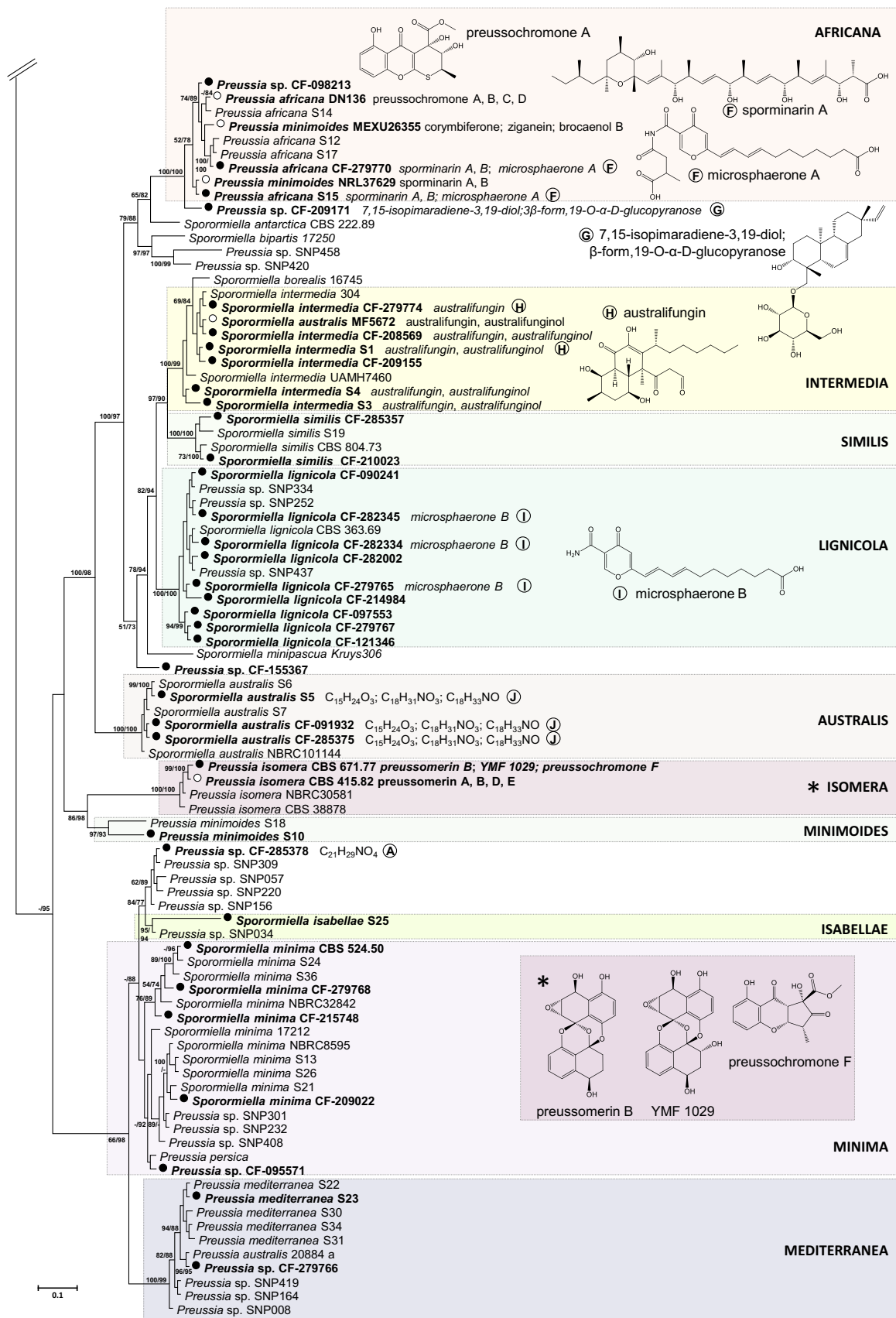
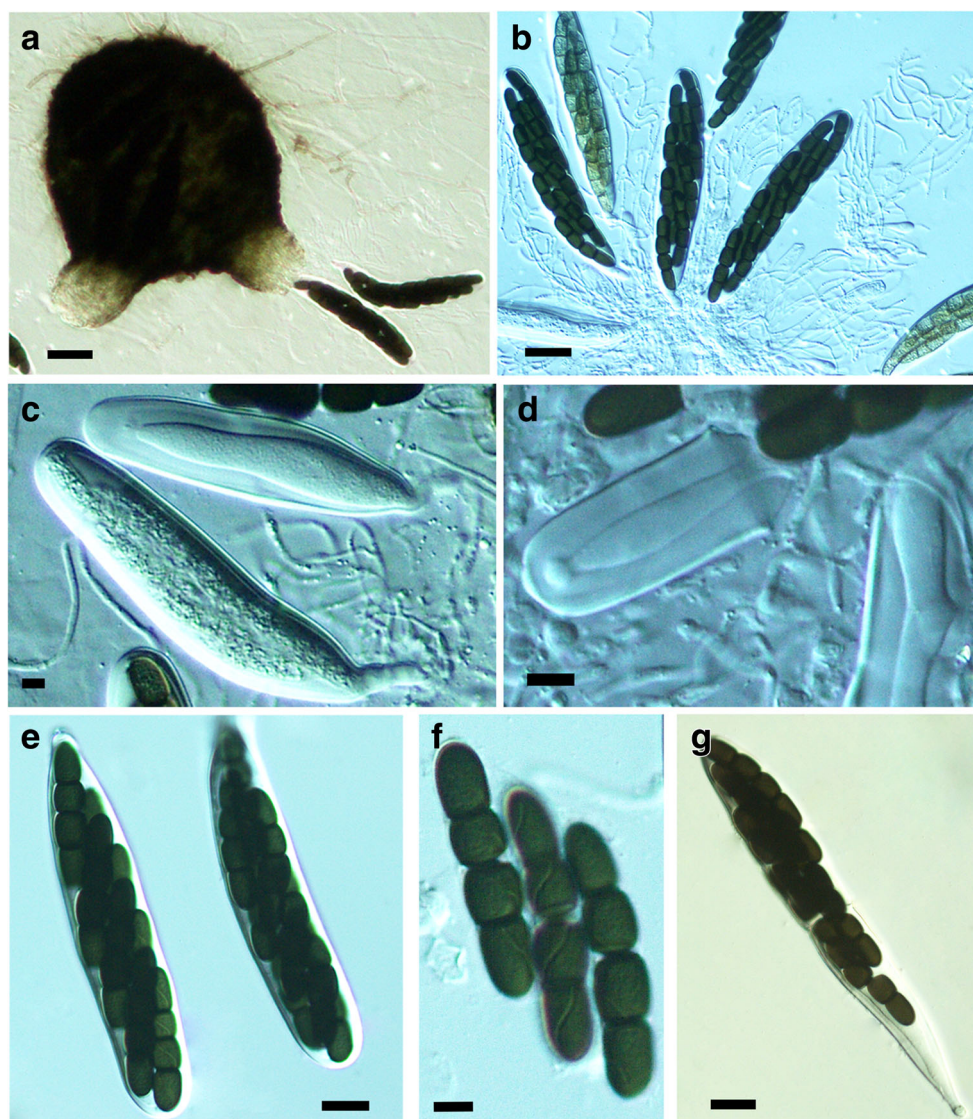


Fig. 1 (continued)

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 C₁₅H₁₈O₃, TMC120, and C₁₅H₂₇NO₄ (chemotype B). The “Subticinensis” clade presented differentially 4-hydroxy-5-methylmellein (chemotype C). The “Africana” clade showed C₁₅H₁₈O₃, C₁₆H₁₂O₇, and microsphaerone A (chemotype F). All members of the “Lignicola” clade were characterized as producers of TMC120, microsphaerone B, and C₂₁H₂₉NO₄ (chemotype I). The “Australis” clade (chemotype J) included C₁₅H₂₄O₃, C₁₈H₃₃NO, and C₁₈H₃₁NO₃ 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 ₇ O ₆ ⁺ | 715,3061; 716,3085 | MMK2 | C ₄₀ H ₃₉ N ₇ O 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 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | A |
| CBS 395.81 | <i>Preussia cymatomera</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-277586 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-258370 | <i>Preussia grandispora</i> | - | + | - | + | - | - | + | - | - | - | - | - | - | - | - | - | B |
| CF-279773 | <i>Preussia grandispora</i> | - | + | - | + | - | - | + | - | - | - | - | - | - | - | - | - | B |
| CBS 125.66 | <i>Preussia subticinensis</i> | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C |
| CF-278595 | <i>Preussia subticinensis</i> | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C |
| CF-277817 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CBS 318.51 | <i>Preussia aemulans</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-277822 | <i>Preussia funiculata</i> | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | D |
| CF-282341 | <i>Preussia</i> sp. | - | - | - | - | + | - | - | - | - | - | - | + | - | - | + | - | E |
| CF-160923 (S15) | <i>Preussia africana</i> | - | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | F |
| CF-279770 | <i>Preussia africana</i> | - | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | F |
| CF-209171 | <i>Preussia</i> sp. | - | + | - | - | - | - | - | - | - | - | - | - | - | + | - | - | G |
| CF-160907 (S1) | <i>Preussia intermedia</i> | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | H |
| CF-160910 (S3) | <i>Preussia intermedia</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-279774 | <i>Preussia intermedia</i> | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | H |
| CF-210023 | <i>Preussia similis</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-285357 | <i>Preussia similis</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-279765 | <i>Preussia lignicola</i> | - | - | - | + | - | - | - | - | - | - | + | - | + | - | - | - | I |
| CF-282334 | <i>Preussia lignicola</i> | - | - | - | + | - | - | - | - | - | - | + | - | + | - | - | - | I |
| CF-282345 | <i>Preussia lignicola</i> | - | - | - | + | - | - | - | - | - | - | + | - | + | - | - | - | I |
| CF-155365 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-091932 | <i>Preussia australis</i> | - | - | + | - | - | + | - | - | + | - | - | - | - | - | - | - | J |
| CF-160911 | <i>Preussia australis</i> | - | - | + | - | - | + | - | - | + | - | - | - | - | - | - | - | J |
| CF-285375 | <i>Preussia australis</i> | - | - | + | - | - | + | - | - | + | - | - | - | - | - | - | - | J |
| CBS 671.77 | <i>Preussia isomera</i> | - | - | - | - | - | - | + | - | - | - | - | - | + | - | - | - | K |
| CF-160916 | <i>Preussia minimoides</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-285378 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | A |
| CF-160936 (S25) | <i>Preussia isabellae</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-095571 | <i>Preussia</i> sp. | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CBS 524.50 | <i>Preussia minima</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-209022 | <i>Preussia minima</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-215748 | <i>Preussia minima</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-279768 | <i>Preussia minima</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-160934 (S23) | <i>Preussia mediterranea</i> | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | None |
| CF-279766 | <i>Preussia</i> 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 Botryosphaerales, 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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