A new endophytic species of *Preussia* (*Sporormiaceae*) inferred from morphological observations and molecular phylogenetic analysis

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A new species of *Preussia* as an endophyte of different typical Mediterranean plant species is newly described and illustrated based on molecular and morphological features. *Preussia mediterranea* resembles *P. australis*, *P. africana* and *P. similis* from a morphological point of view, but appeared closer to *P minima* and *P. isabellae* by molecular analysis. Phylogenetic analysis of the entire ITS region combined with the D1-D2 domains of the 28S rRNA gene, and a fragment of the elongation factor EF-1α gene, using Maximum Likelihood and a Bayesian Markov Chain Monte Carlo approach, generally resulted in statistically well-supported clades for all the species studied. Morphological data and molecular phylogenetic analysis supported the recognition of the new taxon.

Key words: Ascomycota, Bayesian analysis, elongation factor, ITS, LSU, maximum likelihood, phylogeny, Spain, Sporormiaceae, rDNA, taxonomy

Introduction

Mediterranean habitats are mainly constituted by different types of sclerophyllous evergreen forests. In Europe, 80% of endemic plants belong to the Mediterranean region (Fady-Welterlen, 2005; Nieto Feliner *et al*., 2005). The broad biodiversity of these thermophilous habitats is well represented in the Iberian Peninsula, and constitutes a privileged area for biodiversity studies (Greuter, 1994). Moreover, the Mediterranean Basin is one of the most important 25 hotspots identified for biodiversity conservation, containing an exceptional concentration of endemic species (Myers *et al*., 2000). Despite its importance, the Mediterranean environment has not been subjected to

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exhaustive studies concerning endophytic fungi, with only a few disperse examples in the literature (*e.g*. Fisher *et al*., 1992a; 1992b; 1994; Collado *et al*., 1996; 1999; 2002; Peláez *et al*., 1998; Girlanda *et al*., 2002; Bills *et al*., 2004; Hoyo and Gómez-Bolea, 2004; Oberwinkler *et al*., 2006). This means that a large number of new species is likely to be awaiting discovery.

During a survey screening for secondary metabolites from endophytic fungi of plants collected in Mediterranean habitats, we found several interesting strains from the same *Preussia* species, isolated from plant material collected in central and western Spain. The genus *Preussia* (*Sporormiaceae*) was erected by Fuckel (1866) and comprises species from soil, wood and plant debris (Cain, 1961; Ahmed and Cain, 1972; Arx and van der Aa, 1987; Arenal *et al*. 2004; 2005). *Sporormiella* Ellis & Everh. is a similar genus, defined originally to include exclusively coprophilous species (Cain, 1961; Ahmed & Cain, 1972). Further studies have demonstrated no difference between the two genera with respect to their habitat and other diagnostic morphological features, such as the morphology of ascomata (ostiolate *vs*. non-ostiolate). Thus, some authors consider more appropriate to treat them as synonyms (Valldosera and Guarro, 1990; Guarro *et al*., 1997; Arenal *et al*., 2004; 2005).

The morphological characteristics of these endophytic *Preussia* isolates showed evident resemblances with *P. australis* (Speg.) Arx, *P. africana* Arenal, Platas & Peláez, *P. isabellae* Arenal, Platas & Peláez, *P. similis* (Khan & Cain) Arenal, *P. intermedia* (Auersw.) S.I. Ahmed and *P. minima* (Auersw.) Arx. The molecular data derived from the ITS regions, the variable domains D1-D2 of the 28S rRNA gene, and a fragment of the elongation factor EF-1α gene, combined with morphological data, led us to erect a new *Preussia* species. The taxonomic and phylogenetic affinities of the new taxon with several closely related species are also discussed.

Materials and methods

Sampling and isolation

The collection areas were located in the center and west of Spain and are one of the most representative regions of the Mediterranean Basin. The plant species sampled are restricted to the Mediterranean area and are distinctive of different types of evergreen sclerophyllous forest formations. The strains were isolated as endophytes following standard indirect isolation techniques, using a surface sterilization method as described in Collado *et al*. (1996). The strains were grown at 22ºC and 80% relative humidity on PDA and OMA (oat meal agar) and exposed to alternate cycles of 12 h near-UV light/daylight for at least

14 days to induce sporulation. The cultures were preserved in the CIBE-Merck, Sharp & Dohme Research Laboratories and CCMA-CSIC culture collections in 20% glycerol vials as 0.6 cm diam frozen agar plugs at –80ºC. Dried cultures were deposited in the Herbarium of the Alcalá de Henares University (Spain). The geographical origin, isolation substrata and GenBank accession numbers of the isolates are listed in Table 1.

Morphological data

Microscopic features were examined after sporulation in four sequential subcultures on PDA and OMA for each strain studied. Slides were made using a Leica Wild M8 dissection scope, in water or lactophenol cotton blue, and observed under a Leitz Diaplan microscope. Photographs were made with an Olympus DP-12 microscope digital camera system, incorporated to the Leitz microscope. Twenty one measurements were made from each sporulating culture in order to define the exact range of spore length, following the methodology described in Arenal *et al*. (2004). Measurements in the description are given as (minimum value-)(mean-2SD)-mean-(mean+2SD)(-maximum value), as well as the Q value and number of measurements, following the recommendations of Heinemann and Rammeloo (1985). Ascospore measurements were made at their widest point and did not include the gelatinous sheath. The microscopic terminology of Cain (1961) and Ahmed and Cain (1972) was adopted. The color codes from Kornerup and Wanscher (1989) were used in the description of the gross morphology of the colonies.

DNA sequencing and phylogenetic analysis

DNA extraction and PCR amplification procedures were performed as described by Bills *et al*. (1999). The ITS1-5.8S-ITS2 region was amplified using primers ITS1F and ITS4 (White *et al*., 1990), whereas the D1-D2 domains of the 28S rRNA gene was amplified using primers LR0R and LR16 (Bunyard *et al*., 1994). For the fragment of EF-1α, that includes one intron, we used the primers EF1-728F and EF1-986R described by Carbone and Kohn (1999). All the resulting PCR products were purified using GFX™ PCR Gel Band Purification Kit (Amersham Pharmacia Biotech Inc, USA), before sequencing. The amplified products were sequenced using an ABI PRISM™ Dye Terminator Cycle Sequencing Kit (Perkin Elmer). The samples were sequenced in both directions as described for the ITS region (Sánchez-Ballesteros *et al*., 2000), for the D1-D2 domains of the 28S rRNA gene (Acero

Table 1. *Preussia* isolates examined, their substrata and geographical origin.

Strain code	Species	Substrate	Origin	GenBank accession numbers		
				ITS	28S	EF-1α
CBS 28767	*P. aemulans*	Greenhouse soil	Wageningen, Netherlands	DQ468017	DQ468037	DQ468001
S12	*P. africana*	Goat dung	Iringa, Tanzania	AY510420	AY510384	AY510405
S14	*P. africana*	Zebra dung	Kwazulu-Natal, South Africa	AY510417	AY510382	AY510403
S15	*P. africana*	Zebra dung	Kwazulu-Natal, South Africa	AY510421	AY510385	AY510404
S17	*P. africana*	*Viburnum tinus* leaves	Tenerife, Canary Islands, Spain	AY510418	AY510383	AY510402
S5	*P. australis*	Gazelle dung	Cape Point, South Africa	AY510411	AY510376	AY510399
S6	*P. australis*	Gazelle dung	Luderitz, Namibia	AY510412	AY510377	AY510401
S7	*P. australis*	Zebra dung	Hester Malan Reserve, South Africa	AY510413	AY510378	AY510400
CBS 36149	*P. fleischhakii*	Man's nail	Groningen, Netherlands	DQ468018	DQ468038	NA
CBS 56563	*P. fleischhakii*	Wheat field soil	Kiel-Kitzeberg, Germany	DQ468019	DQ468039	DQ468004
S1	*P. intermedia*	Elk dung	Arizona, USA	AY510415	AY510380	AY510398
S3	*P. intermedia*	Goat dung	Cefalonia, Greece	AY510414	AY510379	AY510396
S4	*P. intermedia*	Goat dung	Cefalonia, Greece	AY510416	AY510381	AY510397
UAMH7460	*P. intermedia**	*Populus tremuloides*	Hinton BC, Canada	DQ468020	DQ468040	NA
S22	*P. mediterranea*	*Quercus ilex* leaves	Guadalajara, Spain	DQ468021	DQ468041	DQ468009
S23	*P. mediterranea*	*Cistus albidus* leaves	Monfragüe, Cáceres, Spain	DQ468022	DQ468042	DQ468010
S30	*P. mediterranea*	*Quercus suber* leaves	Sierra Villuercas, Cáceres, Spain	DQ468023	DQ468043	DQ468011
S31	*P. mediterranea*	*Alnus glutinosa* leaves	Sierra Villuercas, Cáceres, Spain	DQ468024	DQ468044	DQ468012
S34	*P. mediterranea*	*Daphne gnidium* leaves	Sierra Villuercas, Cáceres, Spain	DQ468025	DQ468045	DQ468013

Table 1 continued. *Preussia* isolates examined, their substrata and geographical origin.

Strain code	Species	Substrate	Origin	GenBank accession numbers		
				ITS	28S	EF-1α
S13	*P. minima*	Gazelle dung	Hobatere, Namibia	AY510426	AY510391	AY510410
S21	*P. minima*	Rhinoceros dung	Kwazulu-Natal, South Africa	AY510425	AY510390	AY510408
S26	*P. minima*	Leaf litter	South Dakota, USA	AY510427	AY510392	AY510409
CBS 52450	*P. minima*	Goat dung	Santa Rosa, Panama	DQ468026	DQ468046	DQ468003
WQ64	*P. minima*	Unknown	Spain	DQ468027	DQ468047	NA
S10	*P. minimoides*	Pig dung	Chaco, Argentina	AY510423	AY510388	AY510406
S18	*P. minimoides*	*Prunus lusitanica* leaves	Tenerife, Canary Islands	AY510422	AY510387	AY510394
S25	*P. isabellae*	Leaf litter	Puerto Rico	AY510424	AY510389	AY510407
S19	*P. similis*	Dung	Arizona, USA	AY510419	AY510386	AY510395
CBS 80473	*P. similis**	Saline dessert soil	Kuwait	DQ468028	DQ468048	DQ468006
WQ63	*Westerdykella sp.*	Unknown	Spain	DQ468029	DQ468049	NA
CBS 15667	*W. dispersa*	Soil	Nigeria	DQ468016	DQ468036	DQ468000
CBS 71271	*W. dispersa*	Greenhouse soil	Naaldwijk, Netherlands	DQ468031	DQ468051	DQ468005
CBS 50875	*W. dispersa*	Salt-marsh soil	Metsamor, Armenia,	DQ468030	DQ468050	DQ468002

*CBS80473 appears originally identified as *S. intermedia*, and UAMH7460 as *S. similis*.
NA: Not available

et al., 2004), and for the EF-1α (Carbone and Kohn, 1999). DNA sequences were visually aligned with the multiple sequence alignment editor GeneDoc 2.5 and datasets deposited in TreeBASE (SN2857). Due to the existence of some ambiguously aligned regions in the EF-1α fragment, the application GapCoder (Hennequin *et al*., 2003; Young and Healy, 2003) was used to recode the ambiguous regions without violating positional homology, to improve the quality of the alignment. All the sequences were deposited in GenBank (Table 1).

Two separate phylogenetic analyses were made, one with the sequences of the entire ITS1-5.8S-ITS2 region combined with the D1-D2 domains of the 28S rRNA gene, and the other with the sequences of the EF-1α gene fragment.

The phylogenetic relationships among the new *Preussia* species and other closely related species were estimated by two different methods of analysis, the Bayesian Monte Carlo Markov Chains approach (MCMC) as implemented in the program MrBayes v3.0b4 (http://www.morphbank.ebc.uu.se/mrbayes; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), and the Maximum Likelihood method (ML) using PAUP* v4.0b10 (Swofford, 2002).

For the Bayesian MCMC analysis, Monte Carlo Markov Chains of trees were constructed and used to approximate the *a posteriori* probability of monophyletic groups. Four incrementally heated simultaneous MCMC were run over 2 M generations using the general time-reversible model (GTR) of DNA substitution. The model assumed a portion of invariable sites, with gamma-distributed substitution rates for the remaining sites and random starting trees. The nucleotide substitution models were GTR+G+I for the ITS-D1-D2 region and GTR+G for the EF-1α fragment. Trees were sampled every 100 generations, resulting in an overall sampling of 20,000 trees. This analysis was repeated twice to test the reproducibility of the results (Huelsenbeck *et al*., 2002). The convergence of the Markov chains was checked with the Tracer software, v1.0 (Rambaut and Drummond, 2003). In order to obtain estimates for the *a posteriori* probabilities, the initial 1000 trees were excluded for the posterior analysis. From those 19,000 trees sampled, and after the process reached stationarity, a 50% majority-rule consensus was computed to obtain an estimate of the *a posteriori* probabilities.

For ML analysis, the DNA nucleotide substitution model was defined by calculating comparison of the best likelihood for 56 models using hierarchical Likelihood Ratio Tests (hLRTs) (Huelsenbeck and Crandall, 1997) with the program MODELTEST v3.7 (Posada and Crandall, 1998). For the entire ITS-D1-D2 region, MODELTEST analysis selected the GTR+G+I model of nucleotide substitution, assuming the shape parameter of the gamma-distributed substitution rates (a = 0.51) 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. For the EF-1α fragment, MODELTEST analysis selected the GTR+G model of nucleotide substitution assuming the shape parameter of the gamma-distributed substitution rates (a=1.2113), and an estimation of nucleotide frequencies as (A = 0.39290, C = 0.27070, G = 0.15860, T = 0.17780). In both cases no molecular clock was assumed and initial runs were conducted with a starting tree obtained via Neighbor-Joining (NJ) method and Tree-Bisection-Reconnection (TBR) branch-swapping algorithm. A bootstrap analysis with 1000 replicates was run to determine the confidence of the branching points (Felsenstein, 1985).

Results

Molecular phylogenetic analysis

Amplification and sequencing of the ITS and D1-D2 regions was positive for all the strains selected. For the EF-1α fragment there were four strains that had to be excluded from the analysis due to failed amplification after repeated attempts (see Table 1).

The size of DNA fragments was of 464-486 bp for the ITS region, and of 458 bp for the D1-D2 domains. For the ITS-D1-D2 fragment a total of 1021 characters were finally aligned, of which 772 were constant. The PCR product size for the EF-1α gene fragment ranged from 249 to 333 bp. The aligned EF-1α sequences resulted in a matrix of 432 characters, but after being codified by the GapCoder software, a final data matrix of 622 characters was processed, of which 127 were constant.

From the Bayesian MCMC analysis of the ITS-D1-D2 fragment a single tree having a total tree length of 465 steps was obtained (Fig. 1). Bayesian analysis of the EF-1α dataset resulted in a tree of 1335 steps (Fig. 2). The results of the ML analysis of both sequence alignments (ITS-D1-D2 and EF-1α) were essentially consistent with those obtained by MCMC (not shown). Likewise, maximum parsimony analysis resulted in phylogenetic trees with very similar topology (not shown).

The phylogenetic tree obtained from the analysis of the ITS-D1-D2 region (Fig. 1) grouped the *Preussia* species studied as monophyletic clades. All these clades were supported by high posterior probabilities values (80-100%), except for the clade containing the strains of *P. minima*, which showed a relatively lower level of statistical support (75%). All the *Preussia* strains formed a single cluster, although without statistical support. The *Westerdykella* strains were grouped in a clade rooted at the base of the tree. *Preussia fleishakii* and *P. aemulans* grouped in a well supported branch that was basal to a main clade with statistical support (86%) containing the remaining *Preussia* species studied. This main clade contained several statistically well supported branches (84-100%). *Preussia grandispora* clustered with *P. pilosella*; while the new species *P. mediterranea* grouped with *P. isabellae* and *P. minima*. *Preussia similis* clustered with *P. intermedia*; and *P. australis* with *P. africana*. The latter four species were grouped in a higher level clade with good support (96%).

The topology of the tree based on the ML analysis of the ITS-D1-D2 region was essentially identical (not shown). There was just a slight difference, in the grouping of *P. fleishakii* and *P. aemulans* together with the

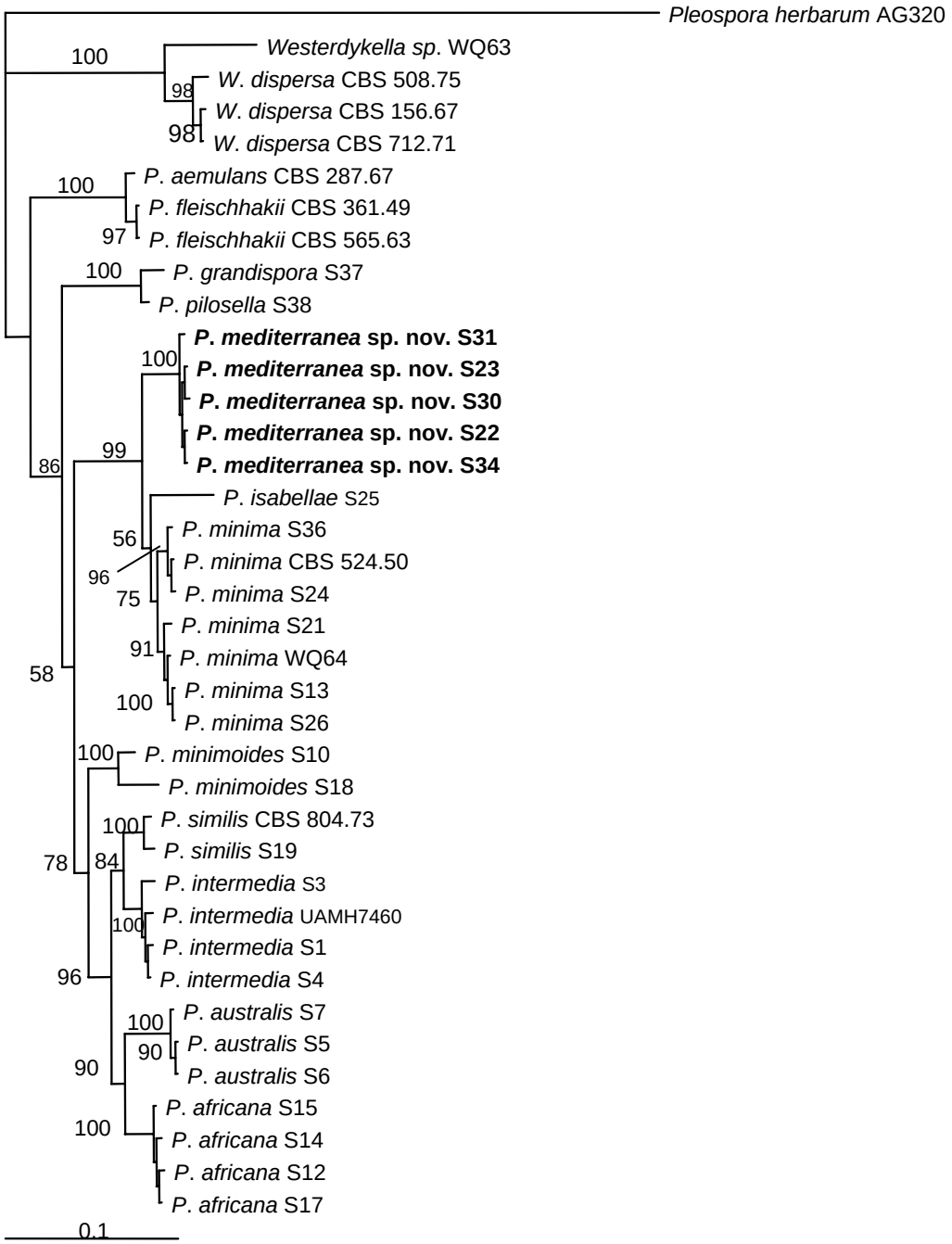


Fig. 1. Phylogenetic tree generated by Bayesian-MCMC analysis using the ITS-D1-D2 sequences, with *Pleospora herbarum* AG320 as outgroup. The MCMC posterior probabilities are indicated above or at the branches. The scale bar indicates the nucleotide substitution.

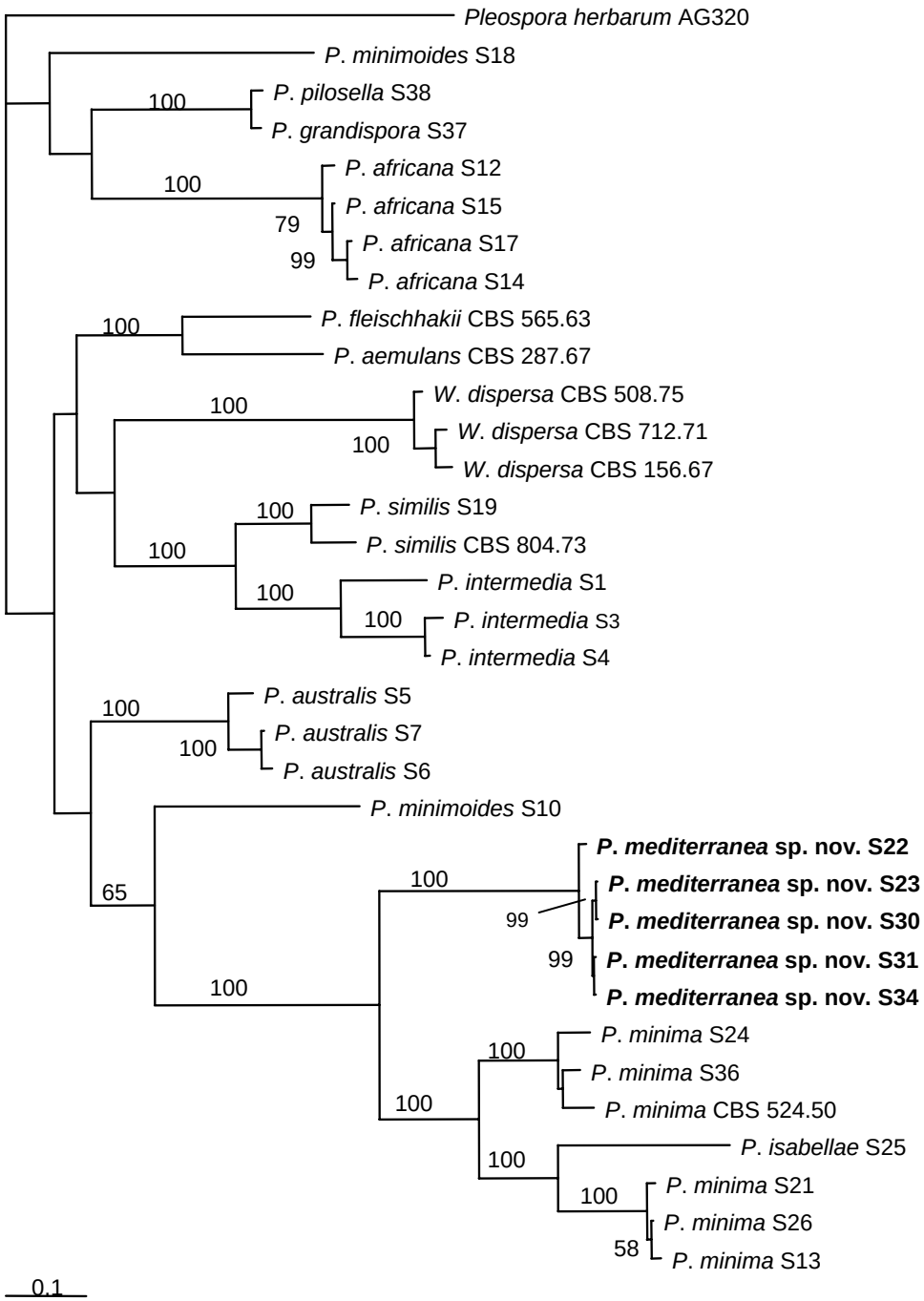


Fig. 2. Phylogenetic tree generated by Bayesian-MCMC analysis using the EF-1α sequences, with *Pleospora herbarum* AG320 as outgroup. The MCMC posterior probabilities are indicated above or at the branches. The scale bar indicates the nucleotide substitution.

Westerdykella strains at the base of the tree, but this relationship did not receive statistical support. All the internal branches of the main clade were identical, in most of the cases bootstrap support values were consistent with the posterior probability values obtained in the Bayesian analysis.

The Bayesian phylogenetic analysis based on EF-1α sequences resulted in a tree conserving most of the monophyletic groups described above, but somewhat less congruent with morphological criteria than the one based on ITS-D1-D2 sequences (Fig. 2). The main differences were that the two strains of *P. minimoides* appeared separated, although placed in clades without statistical support, and the strains of *P. minima* and *P. isabellae* appeared intermingled. Also, although the strains of *Westerdykella* were grouped in a monophyletic clade, this was placed together with other *Preussia* species, instead of rooted at the base of the tree. Still, *P. mediterranea* appeared grouped with *P. minima* and *P. isabellae* in a well supported branch; and *P. similis* was placed as a sister group of *P. intermedia*. However, *P. australis* and *P. africana* did not appear grouped together.

In any case, all the strains of *P. mediterranea* fell within a single monophyletic clade in the phylogenetic analysis run with the two sets of sequences, and by all the methods of analysis used, with maximum posterior probabilities and bootstrap support.

Taxonomy

Based on morphological observations and results of molecular data and phylogenetic analysis, we erect the new species as follows:

Preussia mediterranea Arenal, Platas & Peláez, **sp. nov.** (Figs 3-12)

Etymology: referring to its apparent biogeographic distribution.

Pseudothecia 150-235 µm in diametro, sparsa vel aggregata, semiimmersa, subglobosa ad globosa, atro-brunnea ad nigra, glabra, non ostiolata vel ostiolata. *Peridium* membranaceum pseudoparenchymaticum, 10-12 µm, bistratosum et glabrum. *Ascomata* hyphis brunneis, sepatatis et flexuosis, cellulis usque 5-15 µm longis et 2-2.5 µm in diametro ornamentata. *Asci* 99-120 × 11-16 µm, octospori, cylindraceo-clavati, superne late rotundati, inferne attenuati, stipitati, usque ad 10 × 5 µm. *Pseudoparaphyses* 2 µm, crassae, filiformes, numerosae, ramosae et septatae. *Ascosporae* 32-48 (50) × 6.4-8 (10.3) µm, oblique uniseriatae vel biseriatae, cylindraceae, quattuorcellulares, olivaceo-brunneae, transverse septatae et leviter constrictae, articulis prope similibus, cellulis maturis faciliter separabilibus, striis germinationis diagonalibus, obliquis vel parallelis in longitudine et rectis vel leviter curvatis. *Vagina mucosa* hyalina et angusta 2-4 µm lata.

Typus: In foliis *Cisti albidi*. Monfragüe, Cáceres, Hispania, Nov. 1996, leg. F. Arenal. Cultura sicca (**holotypus**) in herbario AH (AH32774).

Colonies on PDA media attaining 80 mm diameter in 14 d at 23ºC. Texture cottony, adpressed and partially submerged, light brown to black

(10YR 7/3) with cream to white (10YR 7/4) patches. *Ascomata* scattered to aggregated, developed superficially or partially immersed in culture media when young. *Pseudothecia* 150-235 µm diam, globose to spherical, smooth, almost glabrous, usually not ostiolate, light brown to dark brown (10YR 4/3). *Peridium* brown (10YR 5/3) pseudoparenchymatous in surface view, membranaceous, coriaceous and 10-12 µm thick. *Ascomata ornamentation* consisting on septate and flexuose hyphae, 5-15 × 2-2.5 µm. *Asci* 99-120 × 11-16 µm, eight spored, cylindrical to clavate, broadly rounded above and gradually to abruptly tapering into a robust stipe of 10 × 5 µm. *Pseudoparaphyses* 10-15 µm, filiform, septate and longer than the asci, mixed with them and bifurcate. *Ascospores* (32-)34.1-40.9-47.8(-50) × 6.44-8.37-10.3 µm, Q = (3.38-)3.51-4.94-6.36(-7.14), (n = 172), four-celled, cells easily separable at the central septum, uniseriate or biseriate, cylindrical, hyaline to olivaceous (10YR 5/3) when young and finally becoming olivaceous brown to dark brown (10YR 4/3) when mature; transversely septate, constrictions at septa broad and shallow, middle cells of equal length and broader than terminal cells, provided with rounded apices; germ slit diagonal, oblique or parallel and straight to sinuous; gelatinous sheath hyaline and narrow, less than 4 µm wide.

Anamorph: unknown.

Habitat: On living plant material.

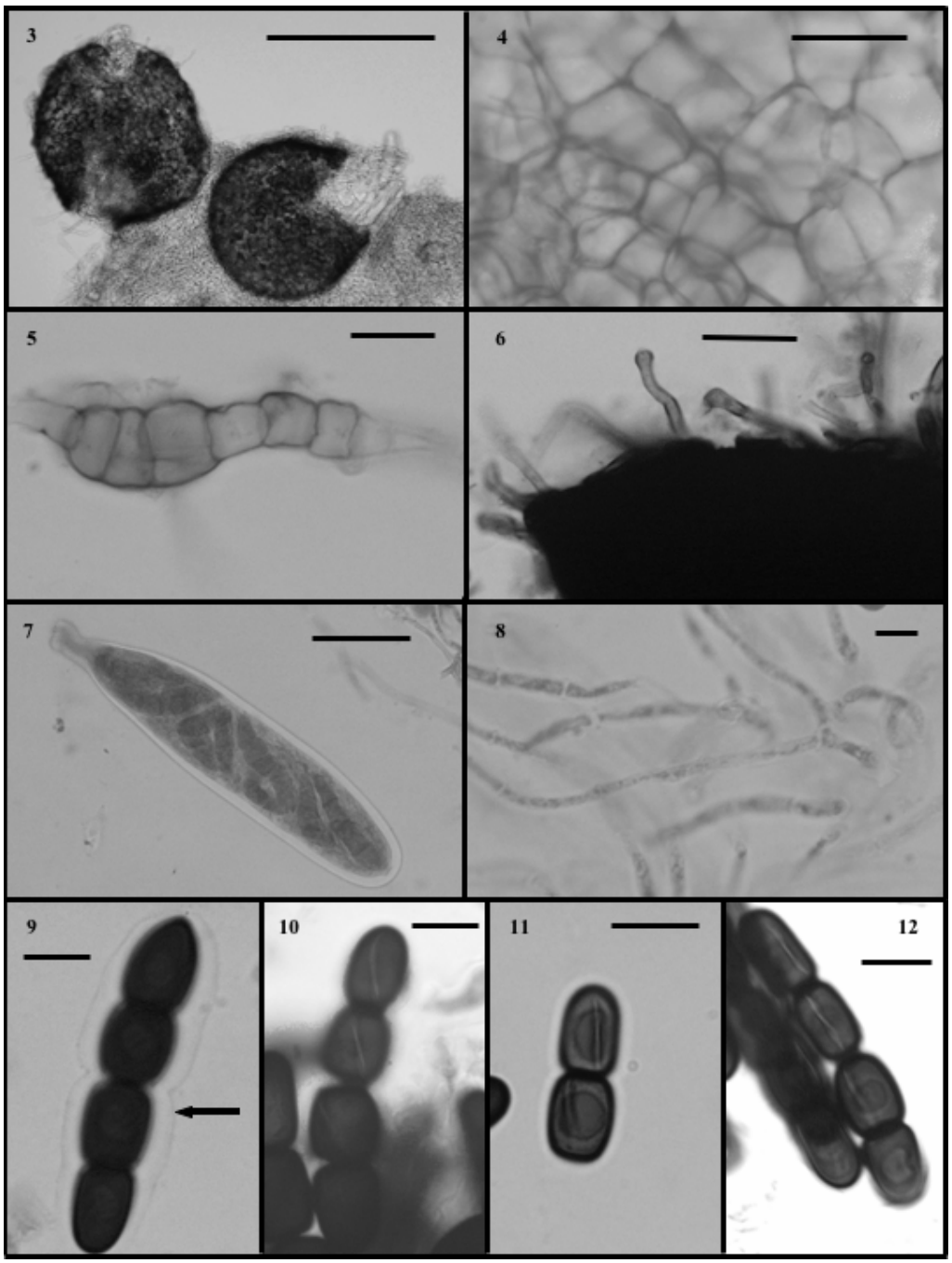
Known distribution: Monfragüe (Cáceres, Spain); Sierra de las Villuercas (Cáceres, Spain); La Matilla (Guadalajara, Spain).

Material examined: SPAIN, Monfragüe (Cáceres), on living leaves of *Cistus albidus*, November 1996, col. F. Arenal, S23 (AH32774; holotype here designated); Sierra de las Villuercas (Cáceres), on twigs and leaves of *Quercus suber*, November 1997, col. F. Arenal, S30; Sierra de las Villuercas (Cáceres), on living leaves of *Alnus glutinosa*, October 1997, col. F. Arenal, S31; Sierra de las Villuercas (Cáceres), on living leaves of *Daphne gnidium*, October 1997, col. F. Arenal, S34 and La Matilla (Guadalajara), on living leaves of *Quercus ilex* subsp. *ballota*, November 1996, col. F. Arenal, S22 (AH33911).

The rest of the strains were also identified following the methodology described in Arenal *et al*. (2004, 2005). Two strains coming from culture collections (CBS80473, and UAMH7460) were morphologically identified as different species as originally labeled (Table 1). The new identification was fully consistent with the molecular results and the phylogenetic analysis developed.

Discussion

The new described species, *Preussia mediterranea*, is morphologically similar to a group of species (*P. australis*, *P. africana*, *P. isabellae*, *P. similis*, *P. minima* and *P. intermedia*), characterized by the absence of hairs in the



Figs 3-12. *Preussia mediterranea*. **3**. Mature non-ostiolate ascomata. **4**. Part of the peridial wall in surface view. **5**. Ascoma initials. **6**. Ascomata hyphae ornamentation. **7**. Detail of the stipe and shape of the asci. **8**. Branched paraphyses. **9-12**. Ascospores showing the gelatinous sheath, and the variable morphology and orientation of the germ slit. Bars: 5 = 200 µm, 6 = 10 µm, 7 = 5 µm, 8 = 8 µm, 9 = 20 µm, 10 = 2 µm, 11-14 = 10 µm.

pseudothecia, asci broadly rounded near the apex and abruptly constricted in a short stipe, and four-celled ascospores ranging in length from 28 to 60 µm; but they can be distinguished mainly by their range of the spore length and width, the morphology and orientation of the germ slit and the thickness of the gelatinous sheath.

Preussia mediterranea is particularly similar to *P. australis*, *P. africana* and *P. similis*. The new species has a spore length range of 32-50 µm, overlapping with measurements of those three species. The germ slit in *P. mediterranea* is oblique to parallel and straight to slightly sinuous like in *P. africana*, but the spores can be slightly longer and wider (32-50 × 6.4-10.3 µm in *P. mediterranea* vs. 32.5-44 × 4-7 µm in *P. africana*). It differs from *P. australis* because the germ slit is strongly oblique to diagonal in the latter and in the more extended size range of the new taxon (32-50 × 6.4-10.3 µm *vs*. 38-46 × 7-8 µm in *P. australis*). Also, *P. mediterranea* differs slightly from *P. australis* in the narrow gelatinous sheath (2-4 µm), which is broader in the latter species. With respect to *P. similis*, *P. mediterranea* differs in the features of the germ slit, which is strongly oblique and somewhat "S" shaped in *P. similis* and in the spore size range, including smaller spores in the new species (32-50 × 6.4-10.3 µm vs. 42-52 × 9-10 µm in *P. similis*). Also, the ascospores from *P. similis* show a broader gelatinous sheath (5-8 µm).

As shown above, the differences within this group of species are relatively subtle. Besides, all these characters must be considered carefully because of their significant variability. To reach valid conclusions it is necessary to carefully measure and study a significant number of ascospores, as previously discussed (Arenal *et al*., 2004; 2005).

The new species is described exclusively from a group of endophytic isolates recovered from plant material collected at different geographic locations in Spain. Several *Preussia* and *Sporormiella* species have been reported as fungal endophytes (Guarro *et al*., 1997; Peláez *et al*., 1998; Arenal *et al*., 2004; 2005). The recently described *P. africana*, originally isolated from living leaves of *Viburnum tinus* subsp. *rigidum* in the Canary Islands, has been subsequently isolated also from grapevine plant material in South Africa (U. Damm, pers. com.). We suspect that the endophytic habit, considered relatively unusual for this genus, may actually be the rule rather than the exception, or at least as common as the coprophilous life style.

From a molecular phylogenetic point of view, the trees derived from the MCMC and ML analysis of the sequences of the ITS-D1-D2 and EF-1α gene of the taxa studied, unequivocally supported the recognition of *P. mediterranea* as a new species. The five strains of the new species constituted a single monophyletic clade, with maximum posterior probability support and bootstrap

value for both regions sequenced. Though morphologically *P. mediterranea* resembles *P. africana*, *P. australis* and *P. similis*, the new species clustered together with *P. minima* and the recently described *P. isabellae* (Arenal *et al*., 2005) in all the phylogenetic trees, as a monophyletic cluster with good statistical support (75-100%). Morphologically *Preussia isabellae* resembles *P. minima* (Arenal *et al*., 2005), and phylogenetically appeared more closely related to *P. minima* than to *P. mediterranea*. *Preussia isabellae* appeared as a sister taxon to the clade containing all the analyzed strains of *P. minima* (Fig. 1). These results corroborate our previous data that supported the separation of *P. isabellae* from *P. minima* (Arenal *et al*., 2005). Phylogenies based on EF-1α however, failed to resolve the taxonomic placement of these two species (Fig. 2).

Interestingly, *P. similis* and *P. intermedia* grouped together in a single clade with moderate to high statistical support (84-100%) in all the trees, except for the ML analysis of the ITS-D1-D2 region, with a value lower than 50% (not shown). These results confirm the strong relationship between *P. similis* with *P. intermedia* based on the size of asci and ascospores, and previously suggested from a molecular perspective (Arenal *et al*., 2005).

The analysis of the ITS-D1-D2 sequences grouped *P. australis* together with the recently described *P. africana* (Arenal *et al*., 2005). This group showed high support in the Bayesian analysis, suggesting a strong phylogenetic affinity between these two species. However, this cluster lacked bootstrap support in the ML analysis, and these two species appeared phylogenetically distant in the analysis derived from EF-1α sequences. These results are consistent with previous findings (Arenal *et al*. 2005), and again they apparently suggest a higher correlation of the ITS-D1-D2 than of EF-1α based phylogeny with the morphological diagnostic features for this group of species.

In addition, the molecular analysis showed *P. aemulans* (Rehm) Arx and *P. fleischhakii* (Auersw.) Cain as phylogenetically closely related species and grouped them in a clade with maximum statistical support in all the phylogenetic trees. However, these two species are morphologically different in ascospore and ascus size and shape. *Preussia aemulans* has been described as a quite variable species (Arx, 1973); whereas *P. fleischhakii* is similar to *P. funiculata*, a taxon not included in our work (Cain, 1961). Further studies should include additional specimens from all of these species to clarify the phylogenetic relationships among these taxa. Likewise, the strains belonging to genus *Westerdykella* were grouped in a monophyletic clade with reliable statistical support. In the trees based on ITS-D1-D2 sequences this clade was rooted at the base of the clade containing the *Preussia* species, as expected (Fig. 1). However, in the EF-1α analysis the *Westerdykella* clade appeared as

an internal clade within genus *Preussia* (Fig. 2). This suggests the strong phylogenetic affinities between both genera previously suggested by Arx (1973).

Another important finding in this study is the apparent phylogenetic affinity between *P. grandispora* and *P. pilosella*, observed in all the trees. However, these two species are morphologically very different. *Preussia pilosella* presents conspicuously hairy perithecia, asci with a long stipe and terminal cells of the ascospores ovoid to conical in shape, characters all of them absent in *P. grandispora* (Ahmed and Cain, 1972).

In conclusion, we have described a new *Preussia* species based on morphological and molecular phylogenetic evidence. Morphological data suggest that *P. mediterranea* is related to *P. australis*, *P. africana* and *P. similis*, but the molecular phylogenetic analysis places the new species as a sister group of *P. minima* and *P. isabellae*. Phylogenies derived from sequence analyses of the ITS-D1-D2 regions are congruent to currently available morphological data whereas those from EF-1α are still obscure and do not always reflect proper morphological arrangements.

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