Two new *Preussia* species defined based on morphological and molecular evidence

Francisco Arenal[1], Gonzalo Platas[2] and Fernando Peláez[2*]

[1]Dpto. de Protección Vegetal, Centro Ciencias Medioambientales (CCMA-CSIC). Serrano, 115 Dpdo., E-28006 Madrid, Spain
[2]Centro de Investigación Básica. Merck Sharp & Dohme Research Laboratories. Josefa Valcárcel 38, E-28027 Madrid, Spain

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Two new *Preussia* species from plant debris and coprophilous substrata are described and illustrated based on molecular and morphological data. *Preussia africana* was isolated from Canary Islands, South Africa and Tanzania and *Preussia isabellae* from Puerto Rico. Morphologically *Preussia africana* resembled *P. australis* and *P. minimoides*. *Preussia isabellae* was related to *P. minima*. Parsimony analysis of the ITS1-5.8S-ITS2 region, the 5´end of the 28S rRNA gene and a fragment of the translation elongation factor 1α gene, supported the recognition of these fungi as new species.

Key words: *Ascomycota*, elongation factor, ITS, LSU, phylogeny, rDNA, taxonomy.

Introduction

The genus *Preussia* was erected by Fuckel (1866). This genus comprised species of bitunicate ascomycetes with non-ostiolate ascomata, containing dark brown multi-celled ascospores (4-16 cells) with germ slits, and are covered by a gelatinous sheath. The genus *Sporormiella* Ellis & Everhart differs from *Preussia* mainly by having ostiolate ascomata and by its coprophilous habitat. In contrast, *Preussia* includes species isolated from soil, wood and plant debris (Cain, 1961; Ahmed and Cain, 1972; Arx and Van der Aa, 1987). Recognition of both genera has caused confusion because of their shared morphological features (Auerswald, 1866; Munk, 1957). In recent years the two genera have been proposed or accepted as synonyms (Arx, 1973; Barr, 1987; Valldosera and Guarro, 1990; Guarro *et al*., 1997; Arenal *et al*., 2004), based on the inconsistency of the character of the presence of the ostiole, which is known to be influenced by the culture conditions, and may be present or absent even in

* Corresponding author: F. Peláez; e-mail: fernando_pelaez@merck.com, gonzalo_platas@merck.com, farenal@ccma.csic.es

ascomata from the same culture (Guarro *et al*., 1997). Furthermore, typical species of *Sporormiella,* as defined by Ahmed and Cain (1972) can be isolated from substrata other than dung, making the habitat an artificial feature as well (Guarro *et al*., 1997; Peláez *et al*., 1998). Other authors have reconsidered the concept of these two genera, and argued whether or not to treat them as synonyms (Arx and Van der Aa, 1987; Barr, 1990; Barrasa and Checa, 1991).

Although new *Preussia* and *Sporormiella* species continue to be described (Barr, 1990; Lorenzo, 1994; Guarro *et al*., 1997; Koryolova, 2000), an accumulating body of data suggests that, unless other more relevant characters are found that can be used to differentiate between the two genera, it may be more appropriate to synonymize them, therefore newly described species would be assigned to *Preussia*.

During a morphological and molecular analysis of a series of wild isolates of genus *Preussia* from different substrata and geographic regions, we found some interesting strains isolated from herbivore dung and plant materials from Canary Islands, Puerto Rico South Africa and Tanzania (Arenal, 2001). Their morphological characters were inconsistent with any described species of *Preussia* or *Sporormiella*, even though they were apparently related to a series of species morphologically akin including *P. australis* (Speg.) Arx, *P. intermedia* (Auersw.) S.I. Ahmed, *P. minimoides* (S.I. Ahmed & Cain) Valldos. & Guarro, *P. minima* (Auersw.) Arx and *P. similis* (Khan & Cain) Arenal. Molecular data derived from nuclear ribosomal DNA sequences, including the ITS1-5.8S-ITS2 region and the D1-D2 domains of the 28S rRNA gene, and a portion of the translation elongation factor 1α gene, combined with morphological data, prompted us to describe two new species for these isolates.

Materials and methods

Strains examined

All the strains were isolated by the authors following standard indirect isolation techniques. The strains from dung or leaf litter were isolated using a particle filtration method (Bills and Polishook, 1994). The strains from living plant materials were isolated using a surface sterilization method (Collado *et al*., 1996). The isolation media used have been also previously described (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 hour near-UV light/daylight for at least 14 days to induce sporulation. The cultures were preserved in the CIBE-Merck, Sharp & Dohme Culture Collection in 20% glycerol vials as 0.6 cm diam. frozen agar plugs at -80ºC.

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 of four sequential subcultures on PDA and OMA for each strain. 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 range of spore length, following the methodology described in Arenal *et al*. (2004). Measurements in descriptions are given as (minimum value-)(mean-2SD)-mean-(mean+2SD)(-maximum value), as well as the Q value and number of measurements, according to 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 Ahmed and Cain (1972) was adopted. The colour codes from Kornerup and Wanscher (1978) 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 with 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 *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 deposited in TreeBASE (M1773, M1774). The program GapCoder (Hennequin *et al*., 2003; Young and Healy, 2003) was used to improve the quality of the alignment of the EF-1α fragment. All sequences were deposited

in GenBank (Table 1). The phylogenetic relationships among the two new *Preussia* species and other closely related species, were examined using maximum parsimony method inferred by heuristic search of the aligned sequences using PAUP 4.0 b10 (Swofford, 2001). Heuristic search was performed with simple addition of sequences and TBR branch swapping, with MaxTrees set to 100. All characters were unordered and equally weighted, with gaps treated as missing data. Two phylogenetic analysis were made, one with the ITS sequences combined with the D1-D2 region of the 28S rRNA gene, and another with the EF-1α fragment alone. The confidence of the branches was measured by bootstrap analysis with 1000 bootstrap replicates using heuristic search (Felsenstein, 1985), and by decay indexes (Bremer, 1994), calculated with SEPAL v1.4 software. The trees were visualized with the application Treeview 1.5.

Results

Sequencing and phylogenetic analysis

In order to clarify the relationships of the two new species with other morphologically similar *Preussia* species, we sequenced the ITS, the 5' region of the 28S rRNA gene and a fragment of the EF-1α of all isolates of *P. africana* and *P. isabellae*. The sequences were aligned with those from a group of isolates unambiguously identified by morphology as other *Preussia* species collected from diverse geographic origins (Table 1).

Single DNA fragments of 465-485 bp for the ITS region, 584-587 bp for the D1-D2 domains of the 28S rRNA gene and 370-450 bp for the EF-1α gene, were obtained in the amplification reactions for all the *Preussia* isolates analyzed (Table 1). For the ITS/D1-D2 fragment a total of 1016 characters were aligned, of which 815 were constant, 123 were variable but parsimony-uninformative, and 78 were parsimony informative. Fifty two equally most-parsimonious trees were obtained, with identical topologies regarding all the aspects discussed below. One representative tree is shown in Fig. 1. The tree length was 282 steps, with consistency index CI = 0.830, homoplasy index HI = 0.170, retention index RI = 0.817, rescaled consistency index RC = 0.678. For the EF-1α fragment a total of 564 characters were aligned, of which 185 were constant, 153 were variable and parsimony-uninformative, and 226 were parsimony informative. Six equally parsimonious trees were obtained, with identical topologies regarding all the aspects discussed below. One representative tree is shown in Fig. 2. The tree length was 600 steps, with CI = 0.827, HI = 0.173, RI = 0.853 and RC = 0.705.

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

Strain	Species	Substrate	Origin	GenBank accession numbers		
				ITS	28S	EF-1α
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	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
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
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
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

The *Preussia* strains were grouped as monophyletic clades corresponding to each species, supported by high bootstrap values (94-100%) in both phylogenetic trees, except for *P. minimoides*. The two strains sequenced from this species appeared grouped together in a monophyletic clade with a moderate bootstrap value (87%) in the ITS/D1-D2 phylogram, but not in the EF-1α tree. All the strains of *P. africana* fell within a clade with very high bootstrap support for both regions sequenced (100% for the EF-1α and 98% for ITS/D1-D2). *Preussia isabellae*, represented by a single isolate, was consistently in a basal position to *P. minima,* which formed a well-supported monophyletic clade in both phylogenetic trees.

Taxonomy

Preussia africana Arenal, Platas & Peláez, **sp. nov.** (Figs. 3-11)

Etymology: referring to its apparent geographic distribution.

Pseudotheciis (180-)210-290 µm in diametro, sparsis vel aggregatis, immersis vel semiimersis, subglobosis vel piriformibus, atro-brunneis vel nigris, glabris et ostiolatis. *Collo* breve papilliformi vel cylindraceo, 50-60 × 20-40 µm. *Peridio* membranaceo pseudoparenchymatico, glabro, 10-15 µm, bistratoso. *Ascis* 94-110 × 15-17 µm, octosporis, cylindraceo-clavatis, superne rotundatis, inferne attenuatis, breve stipitatis, usque 13 × 4.5 µm. *Pseudoparaphysibus* 1-2 µm crassis, filiformibus, numerosis, ramosis et septatis. *Ascosporis* 32.5-44 × 4-7 µm, oblique uniseriatis vel biseriatis, cylindraceis, quattuorcellularibus, olivaceo-brunneis, transverse septatis et leviter constrictis, articulis similibus, facile sedecentibus; stria germinationis oblique usque parallela et rectis vel leviter curvatis. Vagina mucosa hyalina et angusta.

Typus: In foliis *Viburni tini* subsp. *rigidi*. Tenerife, Canary Islands, Spain, 2 Apr. 1995, leg. A. Santos. Cultura sicca (**holotypus**) in Herbarium AH (AH32767)

Colonies on PDA attaining 70-75 mm diam. in 14 days at 23ºC. Texture cottony to floccose showing frequently white to light cream sectors (10YR 7/4), adpressed and occasionally submerged, light brown (10YR 7/3) to black. *Ascomata* scattered or aggregated, superficial or partially immersed in culture media when young. *Pseudothecia* (180-)210-290 µm diam., globose, subglobose to pyriform, smooth, ostiolate, brown to dark brown (10YR 5/3, 4/3); neck small, 50-60 × 20-40 µm, papilliform to cylindrical; glabrous, but with short ornamental hyphae measuring 10-20 × 2.5-4.5 µm, sometimes present at the base of the ascomata. *Peridium* dark brown (10YR 5/3, 4/3), pseudoparenchymatous in surface view, membranaceous, coriaceous and 10-15 µm thick. *Asci* 94-110 × 15-17 µm eight-spored, cylindrical-clavate, stipitate, nonamyloid, broadly rounded above, gradually to abruptly tapering into a short and robust stipe up to 13 × 4.5 µm. *Pseudoparaphyses* 1-2 µm diam., filiform, septate, interspersed with asci. *Ascospores* (32.5-)32.9-37.3-41.6(-44) × 4-5.3-7 µm, Q = 4.6-7.3-9.9(-10.4) (n = 168), biseriate, cylindrical, hyaline to olivaceous when young, becoming olivaceous brown to dark brown (10YR 6/3) when mature; four-celled, transversely septate, cells separable at the central septa, constrictions at septa broad and shallow, middle cells of equal length and broader than terminal cells, with rounded apices; germ slit oblique to parallel and straight to slightly sinuous; gelatinous sheath hyaline and narrow.

Anamorph: unknown.

Habitat: On living plant material and herbivore dung.

Known distribution: Canary Islands (Spain), Tanzania and South Africa.

Material examined: SPAIN, Canary Islands (Tenerife), Llano de los Viejos, on living leaves of *Viburnum tinus* subsp. *rigidum*, 2 April 1995, col. A. Santos, S17 (AH32767; **holotype here designated)**; South Africa, Kwazulu-Natal, zebra dung, 22 January 1995, col. M.J. Wingfield, S14 (AH32768); South Africa, Kwazulu-Natal, zebra dung, 23 January 1995,

col. M.J. Wingfield, S15 (AH32769); Tanzania, Iringa, goat dung, 26 June 1992, col. D. Moyer, S12 (AH32770).

Preussia isabellae Arenal, Platas & Peláez, **sp. nov.** (Figs. 12-20)

Etymology: dedicated to Isabel Soto.

Pseudotheciis (90-)100-130(140) µm in diametro, sparsis vel aggregatis, immersis usque semiimersis, subglobosis vel piriformibus, atro-brunneis vel nigris, glabris et ostiolatis. *Peridio* pseudoparenchymatico et membranaceo, 12-15 µm crasso, glabro, bistratoso. *Ascis* 94-110 × 17-20.5 µm, octosporis, cylindraceo-clavatis, superne late rotundatis, inferne in stipitem attenuatis. *Pseudoparaphysibus* 2 µm crassis, filiformibus, numerosis, ramosis et septatis. *Ascosporis* 29-42 × 4-6.5 µm, oblique uniseriatis vel biseriatis, cylindraceis, quattuorcellularibus, olivaceo-brunneis et brunneis, transverse septatis et leviter constrictis, articulis prope similibus, cellulis maturis facile sedecentibus; stria germinationis parallela, abrupte curvata ad centrum. Vagina mucosa hyalina et angusta.

Typus: In ligno plantae. Puerto Rico, 12 Jan 1996, leg. J. Guarro. Cultura sicca (**holotypus**) in Herbarium AH (AH32771).

Colonies on PDA attaining 75-80 mm diam. in 14 days at 23ºC. Texture cottony, adpressed, brown to black (10YR 5/3) with white patches. *Ascomata* scattered or in small groups, superficial or partially immersed when young in culture media surface. *Pseudothecia* subglobose to pyriform, (90-)100-130(140) µm in diam., dark brown to black (10YR 4/3), smooth and membranaceous; glabrous, but with short ornamental hyphae, 7-10 × 2.5-4 µm, sometimes present at the base of the ascomata. *Peridium* pseudoparenchymatous in surface view, membranaceous, glabrous, peridial cells 12-15 µm in diam. *Asci* 94-110 × 17-20.5 µm, eight-spored, cylindrical-clavate, stipitate, nonamyloid, broadly rounded above, gradually to abruptly tapering into a short stipe. *Pseudoparaphyses* 2 µm in diam., filiform, abundant, branched and septate. *Ascospores* (29-)30.7-35.8-40.9(-42) × 4-5.3-6.5 µm, Q = 5.2-6.8-8.9(-9.7) (n = 168), uniseriate to biseriate, cylindrical, olivaceous brown to dark brown (10YR 6/3) when mature; four-celled, transversely septate, cells easily separable, constrictions at septa broad and shallow, cells nearly equal in size, terminal cells with rounded apices; germ slit parallel with a strong to sinuous curvature at the middle; gelatinous sheath hyaline and narrow.

Anamorph: unknown.

Habitat: On unidentified plant debris.

Known distribution: Puerto Rico.

Material examined: PUERTO RICO, on woody plant debris, 12 January 1996, col. J. Guarro, S25 (AH32771; **holotype here designated**).

Discussion

Employing the morphological characters in the keys of Cain (1961) and Ahmed and Cain (1972), the two new species are related to a series of *Preussia*

species characterized by the absence of hairs in the pseudothecia and neck, 8-spored cylindrical asci abruptly contracting below into a short stipe, and four-celled ascospores with a length ranging from 37 to 60 µm. The species within this group can be distinguished based on spore size, morphology and disposition of the germ slit.

Preussia africana has a spore length range (32.5-44 µm) overlapping between *P. minimoides* (28-36 µm) and *P. australis* (38-46 µm). However, the germ slit in *P. africana* is oblique to parallel and straight to slightly sinuous, while it is strongly oblique to diagonal in *P. australis*, and sigmoid or sinuous in *P. minimoides* (Ahmed and Cain, 1972). Other characters, such as cells not easily separable, or the terminal spore cells longer and narrower toward the end, do not fit exactly with the morphological characteristics of *P. australis* and *P. minimoides* (Ahmed and Cain, 1972). However, these three species share other features, such as the morphology of the asci, broadly rounded, frequently broader near the middle and abruptly constricted in a short stipe, as well as the presence of a narrow and hyaline gelatinous ascospore sheath. The overlapping size range makes it possible to misidentify strains of *P. africana*, ascribing them to either *P. australis* or *P. minimoides*, unless a significant number of ascospores are measured and the morphology and disposition of the germ slit are carefully examined (Arenal *et al*., 2004). *Preussia africana* seems to have both coprophilous and non-coprophilous habitat; three of the four isolates found were recovered from dung of herbivore species, but one strain was isolated as an endophyte from surface-sterilized living leaves of *Viburnum tinus* (Peláez *et al*., 1998). Likewise, other *Preussia* and *Sporormiella* species have been often reported as endophytes (e.g. Guarro *et al*., 1997; Peláez *et al*., 1998).

Another new species, *P. isabellae*, is morphologically most comparable to *P. minima*. The two species differ only slightly in spore length range (28-34 µm in *P. minima* vs. 29-42 µm in *P. isabellae*). However, the shape of the germ slit in *P. isabellae*, although presenting a curvature in the middle, is not kink-like as described in *P. minima*, characterized by a strong wider curvature (Ahmed and Cain, 1972). Other morphological ascospore characters, such as easily separable cells at septa and the hyaline narrow gelatinous sheath, are shared by the two species. Again, both species could be confused unless enough ascospores are measured to determine a reliable size range, as described by Arenal *et al*. (2004). Actually, it would be difficult to justify *P. isabellae* as a new species solely based on morphology, given the overlapping with *P. minima*. However, molecular phylogenetic analyses clearly support *P. isabellae* as a new species, as discussed below. The single strain of *P. isabellae*

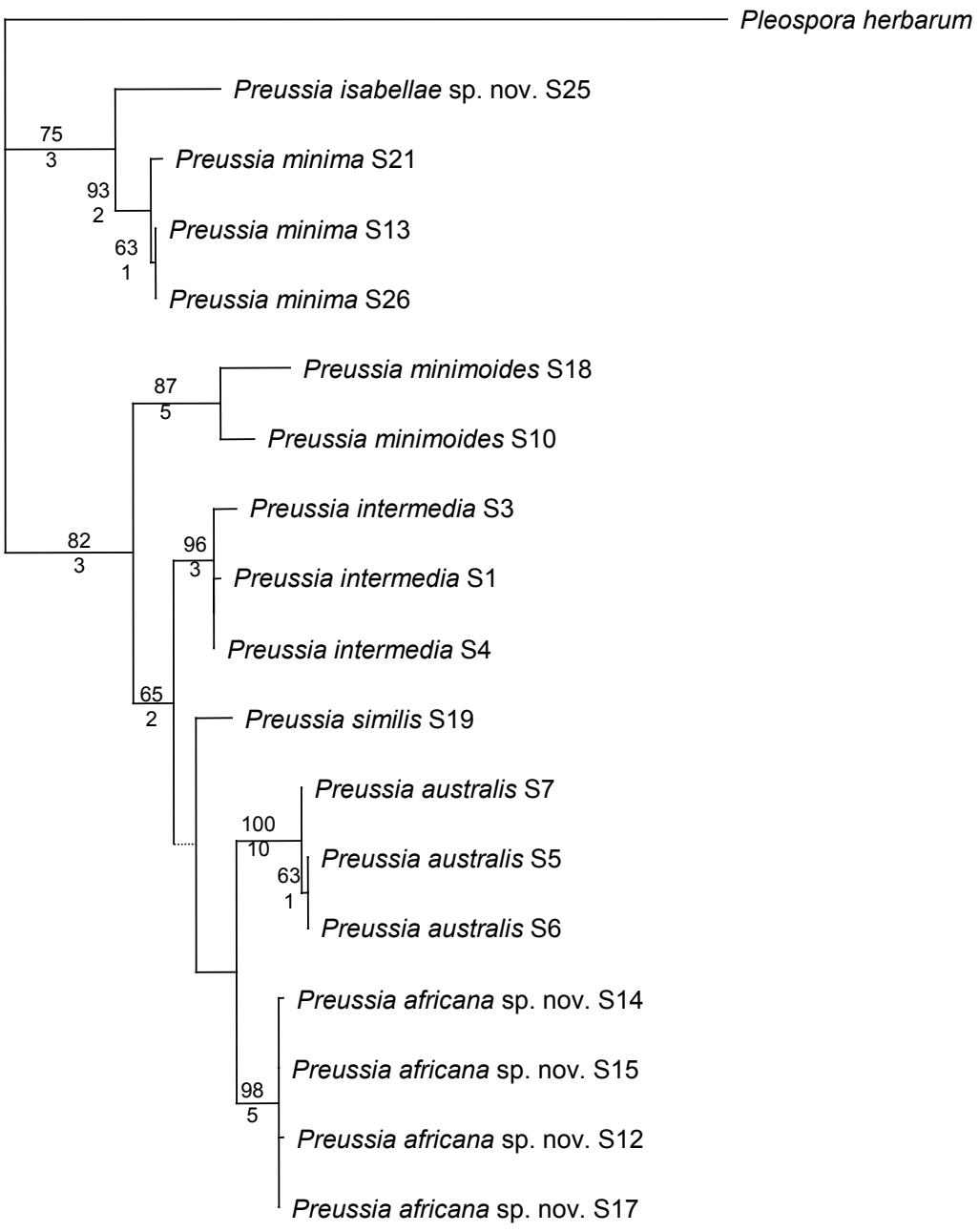


Fig. 1. Phylogram representing one of the 52 most parsimonious trees derived from the ITS/D1-D2 region. All the branches are conserved in the strict consensus tree, except the branch containing *P. similis*, *P. australis* and *P. africana* (indicated by a dotted line), which collapses. Bootstrap support values (when more than 50%) and decay index values are shown at the internodes. *Pleospora herbarum* (*Dothideales*) (AF071345 and U43476) is used as outgroup taxon to root the tree.

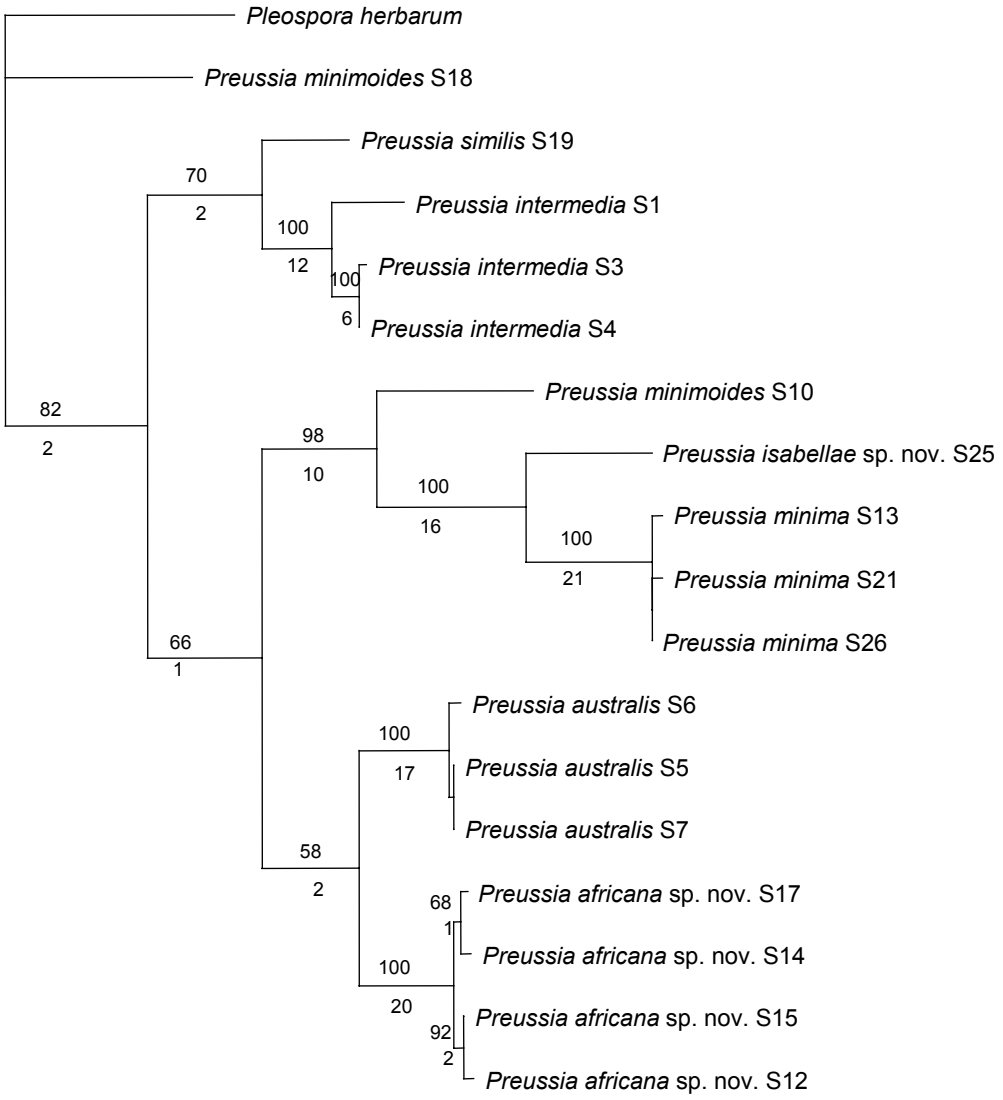
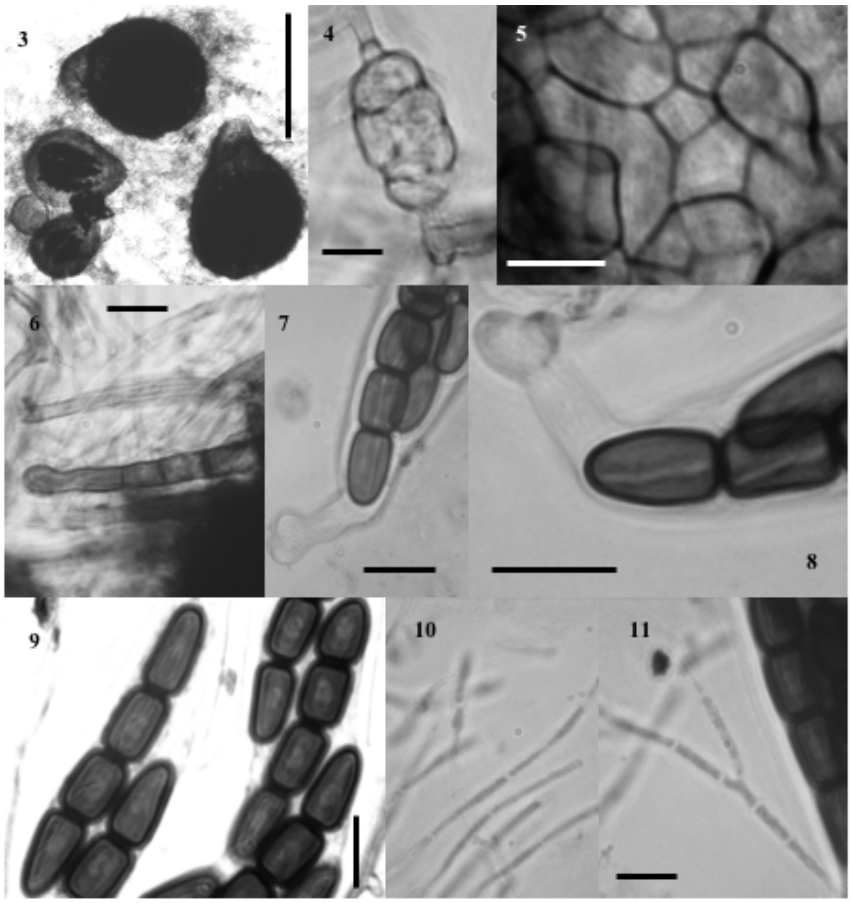


Fig. 2. Phylogram representing one of the six most parsimonious trees derived from the EF-1α fragment sequence. All the branches are conserved in the strict consensus tree. Bootstrap support values (when more than 50%) and decay index values are shown at the internodes. *Pleospora herbarum* (*Dothideales*) (AY510393) is used as outgroup taxon to root the tree.

was recovered from leaf litter using a particle filtration method (Bills and Polishook, 1994).

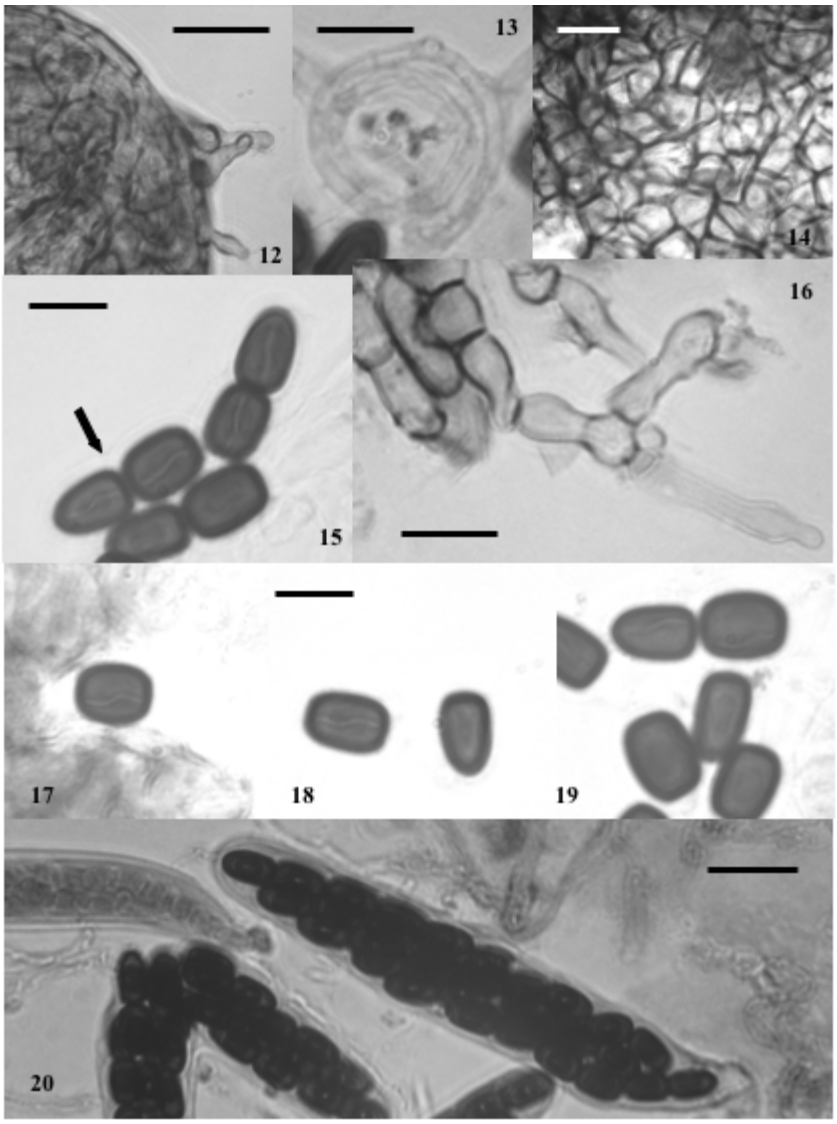
From a molecular phylogenetic point of view, the trees derived from the alignment of the sequences of the EF-1α and ITS/D1-D2 from the isolated strains and other related *Preussia* species, supported the recognition of the two new species and their separation from the allied species mentioned above (Figs. 1 and 2).



Figs. 3-11. *Preussia africana*. **3**. Young and mature ostiolate ascomata. **4**. Ascoma initials. **5**. Part of the peridial wall, surface view. **6**. Ascomata hyphae. **7**, **8**. Detail of the stipe of the asci and ascospores with germ slit. **9**. Ascospores showing the morphology of the germ slit. **10**, **11**. Paraphyses. Bars: 1 = 250 µm; 2, 4 = 5 µm; 3, 5, 6, 7 = 10 µm; 8, 9 = 8 µm.

Preussia africana appeared as a sister group of *P. australis* in both phylogenetic trees, although the branch containing both species was not supported by bootstrap analysis in the tree based on ITS/D1-D2 sequences (Fig. 1), and showed only marginal support (58%) in the tree based on EF-1α sequences (Fig. 2).

The molecular phylogenetic analysis also supported the separation of *P. isabellae* from the strains belonging to *P. minima*, although the two species are closely related. *Preussia isabellae* appeared consistently as basal to the *P. minima* clade in both genomic regions analyzed, in a branch supported by 100% and 75% bootstrap in EF-1α tree (Fig. 2) and ITS/D1-D2 tree (Fig. 1) respectively.



Figs. 12-20. *Preussia isabellae*. **12**. Ascomata. **13**. Ascoma initials. **14**. Peridial wall, surface view. **15**. Ascospore. The arrow shows the gelatinous sheath. **16**. Peridial ornamentation. **17-19**. Ascospores showing the germ slit. **20**. Asci. Bars: 10, 11 = 5 µm; 12, 13, 15-17 = 10 µm; 14 = 2 µm; 18 = 20 µm.

Our phylogenetic analysis also clarified the taxonomic relationships of another interesting species included in the study, *P. similis*. Molecular data supported its segregation from *P. intermedia* and *P. australis*, to which it is morphologically related. Actually, *P. similis* more closely resembles *P. intermedia* than *P. australis*, based on the size of asci and ascospores (Khan

and Cain, 1979). These relationships were, at least, partially confirmed by our molecular data. In the phylogenetic tree derived from the EF-1α sequences (Fig. 2), the strain of *P. similis* appeared in a basal position with respect to the clade containing the *P. intermedia* strains, both species grouped in the same branch (70% bootstrap support). On the other hand, in the phylogeny inferred from the ITS/D1-D2 region (Fig. 1), *P. similis* appeared grouped with *P. australis*, *P. intermedia* and the new species *P. africana* within a clade, although with low bootstrap support (65%).

In conclusion, we have described two new *Preussia* species based on morphology and molecular evidence. Morphological characters of *P. africana* suggest it to be an intermediate taxon between *P. australis* and *P. minimoides*, whereas *P. isabellae* resembles *P. minima*. The taxa included in this work constitute a series of *Preussia* species that may often overlap in their morphological diagnostic features, being necessary to apply some caution before erecting new *Preussia* species based solely on morphology. However, the molecular phylogenetic analysis based on the genomic regions selected, justifies the existence of well-defined biological species and reveals their inferred relationships within genus *Preussia*.

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References

Acero, F.J., González, V., Sánchez-Ballesteros, J., Rubio, V., Checa, J., Bills, G.F., Salazar, O., Platas, G. and Peláez, F. (2004). Molecular phylogenetic studies on the Diatrypaceae based on rDNA-ITS sequences. Mycologia 96: 249-259.

Ahmed, S.I. and Cain, R.F. (1972). Revision of the genera *Sporormia* and *Sporormiella*. Canadian Journal of Botany 50: 419-477.

Arenal, F. (2001). *Comparación de técnicas genotípicas y fenotípicas para el análisis de la diversidad en hongos*, *aplicadas a Epicoccum nigrum Link y varias especies del género Sporormiella Ell. & Ev*. Ph.D. thesis. Universidad Autónoma de Madrid, Spain.

Arenal, F., Platas, G. and Peláez, F. (2004). Variability of spore length in some species of the genus *Preussia* (*Sporormiella*). Mycotaxon 89: 137-151.

Arx, J.A. von (1973). Ostiolate and nonostiolate pyrenomycetes. Proceedings Koninklijke Nederlandse Akademie van Wetenschappen, Series C 76: 289-296.

Arx, J.A. von and Van der Aa, H.A. (1987). *Sporormula tenerifae* gen. et sp. nov. Transactions of the British Mycological Society 89: 117-120.

Auerswald, B. (1866). In Rabenhorst. Fungi europaei exsiccatti No. 921. Hedwigia 5: 189.

Barr, M.E. (1987). *Prodomus to class Loculoascomycetes*. Published by the Author, Amherst, MA, USA.

Barr, M.E. (1990). Melanommatales (Loculoascomycetes). North American Flora II, part 13: 1-129.

Barrasa, J.M. and Checa, J. (1991). Dothideales del parque natural de Monfragüe (Cáceres). I. Boletín de la Sociedad Micológica de Madrid 15: 91-102.

Bills, G.F., Platas, G., Peláez, F. and Masurekar, P. (1999). Reclassification of a pneumocandin-producing anamorph, *Glarea lozoyensis* gen. et sp. nov., previously identified as *Zalerion arboricola*. Mycological Research 103: 179-192.

Bills, G.F. and Polishook, J.D. (1994). Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. Mycologia 86: 187-198.

Bremer, K. (1994). Branch support and tree stability. Cladistics 10: 295-304.

Bunyard, B.A., Nicholson, M.S. and Royse, D.J. (1994). A systematic assessment of *Morchella* using RFLP analysis of the 28S ribosomal RNA gene. Mycologia 86: 762-772.

Cain, R.F. (1961). Studies of coprophilous ascomycetes. VII. *Preussia*. Canadian Journal of Botany 39: 1633-1666.

Carbone, I. and Kohn, L.M. (1999). A method for designing primer sets for speciation studies in filamentous Ascomycetes. Mycologia 91: 553-556.

Collado, J., Platas, G. and Peláez, F. (1996). Fungal endophytes in leaves, twigs and bark of *Quercus ilex* from Central Spain. Nova Hedwigia 63: 347-360.

Felsenstein, J. (1985). Confidence intervals on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Fuckel, L. (1866). *Fungi rhenani*, Suppl. Fasc. 3, 1750.

Guarro, J., Abdullah, S.K., Gené, J. and Al-Saadoon, A.H. (1997). A new species of *Preussia* from submerged plant debris. Mycological Research 101: 305-308.

Heinemann, P. and Rammeloo, J. (1985). De la mesure des spores et de son expression. Agarica 6: 366-380.

Hennequin, S., Ebihara, A., Ito, M., Iwatsuki, K. and Dubuisson, J.Y. (2003). Molecular systematics of the fern genus *Hymenophyllum* s.l. (Hymenophyllaceae) based on chloroplastic coding and noncoding regions. Molecular Phylogenetics and Evolution 27: 283-301.

Khan, R.S. and Cain, R.F. (1979). The genera *Sporormiella* and *Sporormia* in East Africa. Canadian Journal of Botany 57: 1174-1186.

Kornerup, A. and Wanscher, J.H. (1978). *Methuen Handbook of Colour*. Methuen, UK.

Korolyova, O.V. (2000). New ascomycete species *Sporormiella tomilinii* Korolyova. Mycology and Phytopatology 34: 11-13.

Lorenzo, L.E. (1994). A new hairy species of *Sporormiella*. Mycological Research 98: 10-12.

Munk, A. (1957). Danish Pyrenomycetes. Dansk Botanisk Arkiv 17: 1-491.

Peláez, F., Collado, J., Arenal, F., Basilio, A., Cabello, M.A., Díez, M.T., García, J.B., González, del Val A., González, V., Gorrochategui, J., Hernández, P., Martín, I., Platas, G. and Vicente, F. (1998). Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycological Research 102: 755-761.

Sánchez-Ballesteros, J., González, V., Salazar, O., Acero, J., Portal, M.A., Julián, M., Rubio, V., Bills, G.F., Polishook, J.D., Platas, G., Mochales, S. and Peláez, F. (2000). Phylogenetic study of *Hypoxylon* and related genera based on ribosomal ITS sequences. Mycologia 92: 964-977.

Swofford, D.L. (2001). PAUP*, *Phylogenetic Analysis using Parsimony* (* and Other Methods). Ver. 4. Sinauer Associates, Sunderland, USA.

Valldosera, M. and Guarro, J. (1990). Estudios sobre hongos coprófilos aislados en España. XV. El género *Preussia* (*Sporormiella*). Boletín de la Sociedad Micologica de Madrid 14: 81-94.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols*: *A Guide to Methods and Amplifications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, USA: 315-322.

Young, N.D. and Healy, J. (2003). GapCode automates the use of indel characters in phylogenetic analysis. BMC Bioinformatics 4: 6.

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