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Three distinctive *Preussia* (*Sporormiaceae*) from photosynthetic stems of *Ephedra trifurca* (*Ephedraceae, Gnetophyta*) in southeastern Arizona, USA

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Abstract. A survey of the diversity and community composition of culturable endophytic fungi associated with photosynthetic stems of the gnetophyte *Ephedra trifurca* (*Ephedraceae*) in southeastern Arizona, USA, yielded numerous isolates that are consistent morphologically with members of the genus *Preussia* (*Sporormiaceae*, *Pleosporales*, *Dothideomycetes*, *Ascomycota*). *Preussia* (including species until recently classified within *Sporormiella*) include dung-, soil-, and plant-inhabiting strains with considerable diversity worldwide. We used morphological evidence and phylogenetic analyses (nuclear ribosomal internal transcribed spacers and 5.8s gene, the adjacent D1-D2 region of the nuclear ribosomal large subunit, and for a subset of strains, the translation elongation factor 1-alpha, *EF1-a*) to identify distinctive members of the *Preussia intermedia* clade occurring as endophytes in *E. trifurca.* These include *P. arizonica* sp. nov., which also occurs as an endophyte in other plants of the region, and *P. elegans* sp. nov., which has been found only in *E. trifurca* to date. We also propose *Preussia mariae* sp. nov., allied phylogenetically with *Preussia lignicola* but distinguishable on the basis of morphology and *EF1-a* data. Our analyses illustrate the potential for several currently recognized species of *Preussia* to represent species complexes that should be resolved by analyses of additional loci and by further sampling of endophytes, which may provide an ecological connection among strains occurring within living plant tissues and as coprophilous or soil-inhabiting fungi. More broadly, our work expands the known geographic scope, host use, and diversity of *Preussia*, especially in arid lands. In conjunction with previous work, our study also provides the basis for hypotheses regarding secondary metabolites of the newly described species.

Key words: Arizona Upland, jointfir, *Pezizomycotina*, Sonoran Desert

Introduction

The Sonoran Desert of North America harbors the greatest botanical richness of any desert worldwide (Nabhan & Plotkin 1994). With over 2000 native plant species recorded across its area of more than 311,000 km^2, the Sonoran Desert biome reaches from subtropical-forest transition zones in Mexico to the Mojave Desert in west-central Arizona and southeastern California, USA; the Chihuahua Desert in southeastern Arizona and north-central Mexico; and the uplands of these areas, which harbor woodlands and forests (Shreve 1951). Classical characterization of vegetation in the Sonoran Desert includes seven bioregions, of which the Arizona Upland subdivision is the highest in elevation, wettest, and seasonally coldest (Shreve 1951).

Plant cover in the Arizona Upland subdivision consists largely of thornscrub, small leguminous trees, and diverse succulents (Shreve 1951). Annual rainfall ranges from 75 to 300 mm with roughly equitable input from rainy seasons in summer and winter (McGinnies 1976). The biota of the region is well-represented in the Tucson Mountains (Pima County, Arizona, USA), a small, isolated desert range in which more than 620 species of vascular plant species are recognized (Rondeau et al. 1991). The flora of that area is particularly rich in *Asteraceae*, *Fabaceae*, and *Poaceae*, as well as diverse *Cactaceae*.

Along with these diverse angiosperms, *Ephedra* spp. (*Ephedraceae*, *Gnetophyta*) also occur commonly in the Tucson Mountains. *Ephedraceae* is a monotypic family of

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gymnosperms that comprises largely leafless shrubs and small trees with photosynthetic terminal stems. *Ephedra* currently includes ~40 species that occur in arid habitats of the northern hemisphere and South America. The genus is known for its production of caffeine and ephedrine, with a long history of use by humans as a stimulant and for medicinal purposes (Dimmitt 2000; González-Juárez et al. 2020).

Six species of *Ephedra* are recognized in the Sonoran Desert bioregion as a whole (Dimmitt 2000). *Ephedra trifurca* Torr. ex S. Wats. (longleaf jointfir, Mexican tea) is distributed throughout northern Mexico and the southwestern United States, and it is particularly common in the Tucson Mountains (Rondeau et al. 1991). In that area, it co-occurs with diverse angiosperms such as *Parkinsonia microphylla* and *Vachellia constricta* (*Fabaceae*), *Larrea tridentata* (*Zygophyllaceae*), *Simmondsia chinensis* (*Simmondsiaceae*), *Ambrosia deltoidea* (*Asteraceae*), *Fouquieria splendens* (*Fouquieriaceae*), and various cacti, including *Carnegiea gigantea*, *Ferocactus wislizeni*, *Cylindropuntia acanthocarpa*, *Cochemiea grahamii*, and *Opuntia engelmannii* (*Cactaceae*).

As part of surveys to characterize endophytic fungal communities associated with distinctive plants in the Arizona Upland division of the Sonoran Desert, we isolated diverse endophytes from healthy photosynthetic stems of mature *E. trifurca* in the Tucson Mountains. Of 53 fungal isolates obtained in culture, 27 were consistent in terms of ascus and ascospore morphology with *Preussia* (*Sporormiaceae*, *Pleosporales*, *Dothideomycetes*, *Ascomycota*), previously noted to be diverse and abundant in angiosperms in the Sonoran Desert (see Massimo et al. 2015).

As currently circumscribed, *Preussia* Fuckel (1867) encompasses species that inhabit soil, wood, plant debris, and dung, as well as endophytes that occur within living plant tissues without causing disease (see Kruys & Wedin 2009). Endophytic *Preussia* associated with Mediterranean plants in Spain have been studied recently for their taxonomic richness and the considerable diversity of their secondary metabolites (Arenal et al. 2007; González-Menéndez et al. 2017). Accepted names in the genus traditionally include *Preussia* and *Sporomiella*, but *Preussia* is now preferred (for a discussion see González-Menéndez et al. 2017; for recent examples implementing *Preussia* preferentially, see Crous et al. 2020, 2021; and for a discussion of the challenges of classification into *Preussia* or *Sporomiella* based solely on morphological characters or substrate, see Kruys & Wedin 2009).

Assessments of morphological traits and phylogenetic analyses of distinctive endophytes from *E. trifurca* in Arizona led us to describe three species within *Preussia.* We contextualize our analyses of *Ephedra* endophytes with newly collected strains from angiosperms in coastal chaparral and inland montane plant communities in the western USA (Woods 2022). We focus on the *P. intermedia* clade (sensu Kruys & Wedin 2009), which is the largest monophyletic lineage in *Preussia*. Our analyses complement recent work on the genus by expanding the known ecological scope of *Preussia* and highlighting their capacity to occur endophytically in native *Gnetophyta* in North America.

Materials and methods

In September 2009, fresh and apparently healthy aboveground tissues were collected from three individuals of *Ephedra trifurca* in the Tucson Mountains, west of Tucson, Arizona, USA (32.2175°N, 111.1028°W, 910 m a.s.l.). Three haphazardly chosen stems, each with multiple small branches, were collected per individual plant.

Within 24 hours of collection, stems were washed in running tap water to remove debris, cut into 2.5 mm segments, and then surface-sterilized via sequential immersion in 95% ethanol (10 s), 0.5% sodium hypochlorite (as dilute Clorox bleach; 2 minutes), and 70% ethanol (2 minutes) (Arnold & Lutzoni 2007). Segments were placed in four sets of 12 on 2% malt extract agar (MEA) in 100 mm Petri dishes under sterile conditions (48 pieces/individual). Plates were maintained at room temperature with natural light-dark cycles. Emergent fungi were subcultured onto 2% MEA at intervals over 60 days. Overall, 27 isolates resembling *Preussia* were transferred to 2% MEA and potato dextrose agar (PDA) plates for further evaluation.

Morphological assessment

All isolates were cultivated on PDA for morphological assessments of colony characteristics, spore morphology, and growth rates. We examined structures on three cultures of each focal strain. Colony diameter was measured at 21–23°C with ambient light-dark cycles. Sexually reproductive strains placed on PDA bore asci containing ascospores after ~3 weeks at ~22°C. Ascospore morphology was consistent with *Preussia* (see Kruys & Wedin 2009).

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from each isolate following Arnold & Lutzoni (2007). A 600–800 base pair (bp) fragment consisting of the nuclear ribosomal internal transcribed spacer and 5.8s (ITS rDNA) and the first 400–600 bp of the nuclear ribosomal large subunit (LSU rDNA) was amplified by the polymerase chain reaction (PCR) with primers ITS1F and LR3 (Arnold et al. 2009). PCR protocols followed Higgins et al. (2007). PCR products were visualized with SYBR Green on 1% agarose gels in TAE. Positive amplicons were cleaned, normalized, and sequenced bidirectionally on an AB3700i at the University of Arizona Genomics Core. Quality scoring and contig assembly by *phred* and *phrap* (Ewing & Green 1998; Ewing et al. 1998) were implemented in Mesquite (Maddison & Maddison 2009), followed by manual editing in Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). All sequence data generated for this study were submitted to GenBank (Table 1).

Sequences were submitted to BLAST searches (Altschul et al. 1990) of GenBank to estimate taxonomic

Table 1. GenBank accession numbers for sequence data generated for this study. The table includes type strains (bold), paratypes (*), and related endophytes from *Ephedra trifurca* in the Tucson Mountains, as well as newly collected endophytes marked DOW in Fig. 1. Endophytes DOW261 and DOW280 were isolated from *Baccharis sarothroides* (*Asteraceae*) at Cabrillo National Monument, California, USA. Endophytes DOW004, DOW005, and DOW044 were isolated from *Ceanothus* (*Rhamnaceae*) at Mt. Lemmon, Arizona, USA. Additional strains from *E. trifurca* that were assigned to *P. arizonica* on the basis of morphology are deposited at the RL Gilbertson Mycological Herbarium under codes DS0002, DS0003, DS0028, DS0031, DS0043, DS0046, DS0047, and DS0049. Strain DS0018 from *E. trifurca* was assigned to *P. mariae* based on morphology, and it also is archived at the Gilbertson.

Species	Isolate	ITS-LSU rDNA (*EF1-a*)
Preussia arizonica	**ARIZ-AEA-DS0001**	OP876768 (OP889250)
	DS0004	OP876769
	DS0008*	OP876770
	DS0009	OP876771
	DS0016	OP876772
	DS0017	OP876773
	DS0023	OP876774
	DS0024	OP876775
	DS0035	OP876776
	DS0044	OP876777
	DS0045	OP876778
	DS0048	OP876779
	DS0050	OP876780
Preussia elegans	**ARIZ-AEA-DS0014**	OP876781
	DS0025*	OP876782
Preussia mariae	**ARIZ-AEA-DS0040**	OP876761 (OP889251)
	DS0006	OP876763
	DS0007	OP876762
	DOW261	OP876760
Preussia sp.	DOW004	OP876764
Preussia sp.	DOW005	OP876765
Preussia sp.	DOW044	OP876766
Preussia sp.	DOW280	OP876767

placement and define taxon sampling for phylogenetic analyses. Focal strains consistently showed affinity for *Preussia* species in BLASTn searches of rRNA/ITS data restricted to type and reference material. For example, queries for isolate DS0001 retrieved *P. persica* as the top match (NR137730; Asgari & Zare 2010). As of 2021, queries for DS0014 and DS0040 retrieved *P. procaviicola* as the top match (NR173052; Crous et al. 2021). Percent identity of these matches was 97.87%, 94.27%, and 94.70%, respectively, for the compared portions of the sequences. BLASTn searches for all three strains based on LSU rDNA and restricted to type and reference material closely matched *Westerdykella dispersa* and the newly described *P. procaviicola* and *P. procaviae* (Crous et al. 2020). Some relevant taxa, such as *P. lignicola*, are not included in the type and reference list at GenBank for ITS rDNA or LSU rDNA, and thus could not be matched by our queries.

Phylogenetic analyses of ITS-LSU rDNA

To clarify the phylogenetic placement of representative strains from *E. trifurca*, we designed our taxon sampling following Kruys & Wedin (2009) and González-Menéndez et al. (2017). We focused on the *P. intermedia* clade, added sequences obtained from the Arizona strains from *E. trifurca* and recently published sequences for *P. procaviicola* and *P. procaviae* (Crous et al. 2020, 2021), and included recently isolated endophytes from coastal and montane plant communities in the region (denoted with the prefix DOW; see Table 1). We used *P. aemulans*, *P. funiculata*, *P. flanagannii*, and *P. fleischhakii* as outgroup taxa following Kruys & Wedin (2009) and González-Menéndez et al. (2017).

The data set was aligned with MUSCLE (Edgar 2004) at EMBL-EBI (www.ebi.ac.uk; Madeira et al. 2022) and adjusted by eye in Mesquite 3.70 (Maddison & Maddison 2021). The resulting alignment consisted of 68 terminals and 1026 characters, of which 185 were variable and 152 were parsimony-informative. The alignment has been made public at FigShare (DOI: 10.6084/m9.figshare.21291423).

We used the automated model selection option in PAUP 4.0a (Swofford 2003) to determine the best-fitting model of evolution (i.e., TrNef + I + G). Bayesian analyses were implemented on MrBayes 3.2.2 (Huelsenbeck & Ronquist 2001) on XSEDE via the CIPRES Science Gateway portal V3.3 (Miller et al. 2010). Analyses consisted of 3 million generations, sampling every 1000th tree, with four chains and a random starting tree. The first 30% of trees were discarded as burn-in. Maximum likelihood analyses were implemented in GARLI (Zwickl 2006) and parsimony analyses were conducted in PAUP 4.0a (Swofford 2003). Branch support was determined as Bayesian posterior probabilities and both maximum likelihood and maximum parsimony bootstrap analyses (1000 replicates each).

These analyses resolved the placement of one group of morphologically distinctive strains as a well-supported clade consisting only of endophytes from *E. trifurca* (DS0001, DS0004, DS0008, DS0009, DS0016, DS0017, DS0023, DS0024, DS0035, DS0044, DS0045, DS0048, DS0050). The placement of a second strain with distinctive morphology also was clarified, with strong support (DS0014).

A third set of strains (DS0006, DS0007, DS0040) was placed with strong support in a clade consisting of a reference strain of *P. lignicola* (CBS363.69, deposited as *Sporormia lignicola* and isolated originally from rabbit dung in the Netherlands). This clade also contained endophytic fungi that were isolated from plants in Spain and were assigned to this species on the basis of ITS-LSU rDNA analyses and morphology in a genus-wide analysis by González-Menéndez et al. (2017). An additional endophyte isolated from an angiosperm in the coastal southwestern USA also was detected in this clade (Woods 2022; Table 1). Morphological traits of the endophytes of *Ephedra* differed from the features of *P. lignicola* as described by Phillips & Plowright (1877) (see below), and the broad geographic range of the clade defined as *P. lignicola* was intriguing. Therefore, we amplified a portion of translation elongation factor 1-alpha (*EF1-a*) for selected isolates to test the prediction that the *Ephedra* endophytes in this clade are distinct from *P. lignicola*.

Phylogenetic analyses of *EF1-a* data

We used primers EF1-983F and EF1-2218R (Carbone & Kohn 1999; Rehner & Buckley 2005) to amplify a fragment of *EF1-a* for type isolates DS0001 and DS0040. We focused on these strains because closely related taxa were represented by this *EF1-a* fragment in public databases. Our amplification methods are described in Del Olmo-Ruiz (2012).

We obtained publicly available reference data from GenBank by retaining only *Preussia* matches in BLASTn searches with DS0001 and DS0040 sequences as queries. The resulting data set consisted of *EF1-a* data for *P. funiculata* (outgroup, see Fig. 1), *P. intermedia*, *P. africana*, *P. lignicola*, *P. minima*, and two unidentified, endophytic strains of *Preussia* from a study conducted in France (Cambon et al., unpublished: direct submission to GenBank). All species of the *P. intermedia* clade with available data were represented.

The data set was aligned with MUSCLE (Madeira et al. 2022) and adjusted by eye in Mesquite 3.70 (Maddison & Maddison 2021), with a final length of 633 characters, of which 83 were variable and 50 parsimony-informative. The data were analyzed as above and the alignment is archived at FigShare (DOI: 10.6084/m9.figshare.21291423).

Characterization of antifungal activity and endohyphal bacteria

Recent work on the diversity of secondary metabolites in *Preussia* (González-Menéndez et al. 2017) led us to test focal strains for antifungal activity *in vitro.* We then examined the strains for endohyphal bacteria, which can influence metabolite production or diversity in their host fungi (see Arendt et al. 2016).

We measured antifungal activity in assays in which two representative pathogens from southern Arizona, *Cladosporium cladosporioides* (*Cladosporiaceae*) and *Colletotrichum trifolii* (*Glomerellaceae*), were challenged by the type strain of each species. Each pairing was repeated three times on 2% MEA in 100 mm plates. Plates were incubated at 21–23°C and colony diameter of the pathogen was measured over 19–22 d. We measured the diameter of colonies of each strain in axenic growth on 2% MEA as controls.

To screen isolates for endohyphal bacteria, we used primers specific to bacteria (10F and 1507R) in PCR to amplify 16S rRNA from seven representative isolates encompassing the three species described here (for methods, see Hoffman & Arnold 2010). The presence or absence of bacteria within living hyphae was confirmed by light microscopy after LIVE/DEAD staining (Hoffman & Arnold 2010; Arendt et al. 2016).

Results

Isolates of *Preussia* were common among the endophytes obtained in culture from *E. trifurca* in the Tucson Mountains of Arizona, USA. Phylogenetic analyses based on ITS-LSU rDNA were largely congruent with the topologies reported by Kruys & Wedin (2009) and González-Menéndez et al. (2017). Morphological and phylogenetic analyses support the circumscription of three species, although further analysis of the *P. lignicola* species complex is warranted given the limited taxon sampling available for *EF1-a* analysis (Figs 1–2). The three species identified here differ significantly in growth rate *in vitro* (Table 2). All isolates inhibited representative plant pathogens *in vitro* (Table 2). Six of seven tested strains harbored viable endohyphal bacteria, as described below.

Taxonomy

Preussia arizonica D. C. Sandberg & A. E. Arnold, sp. nov. (Fig. 3)

MycoBank MB 841762

Type: USA, Arizona, Pima County, Tucson Mountains. Endophytic in healthy photosynthetic stems of *Ephedra trifurca.* Collected by Dustin C. Sandberg and A. Elizabeth Arnold in autumn of 2009. Isolated by D. C. Sandberg from surface-sterilized stem tissue as described in Massimo et al. (2015). ARIZ-AEA-DS0001 – holotype; preserved in a metabolically inactive state (lyophilized) at the Robert L. Gilbertson Mycological Herbarium, University of Arizona.

Description. Colonies on PDA reaching 80 mm diameter in 18 days at 22–23°C. Texture velvety, adpressed, and partially submerged; colony dark jade green to dark green (27E6, 27F7; Kornerup & Wanscher 1967), and growing edge white to light beige typically about 1mm wide, with an undulating surface. Mature colonies bear cottony white, irregular patches of aerial hyphae on surface in some cases, but these often become less prevalent after subculturing. Variation in intensity of green coloration observed among strains. On MEA, aerial hyphae are absent and the colony has a uniform dark green appearance (27F6), with pseudothecia lacking. On PDA, pseudothecia sparse to aggregated, semi-immersed, subglobose to globose, dark brown to black, glabrous, ostioles not evident. Peridium pseudoparenchymatous,

Table 2. Growth rates and inhibitory activity of *Preussia* spp. isolated from healthy photosynthetic stems of *Ephedra trifurca*. Different superscripts indicate significant differences (ANOVA, $F_{(2,21)} = 10.54$, $P = 0.0007$; Tukey HSD with alpha = 0.05). Inhibition values are in mm, representing the decrease in growth relative to controls. N = number of *Preussia* isolates tested.

Proposed species	Number of isolates obtained	Colony diameter on MEA, mm, after 13 d at 22–23°C: mean ± SD (N)	Inhibition of *Co. trifolii* on MEA: mean ± SD (N)	Inhibition of *Cl. cladosporioides* on MEA: mean ± SD (N)
Preussia arizonica	21	35.4 ± 4.3^{a} (19)	16.6 ± 1.1 (19)	14.5 ± 1.3 (19)
Preussia elegans	2	22.5^{b} (1)	15.5 (1)	15.0 (1)
Preussia mariae	4	26.3 ± 4.7^{b} (4)	15.9 ±1.3 (4)	13.1 ± 1.6 (4)

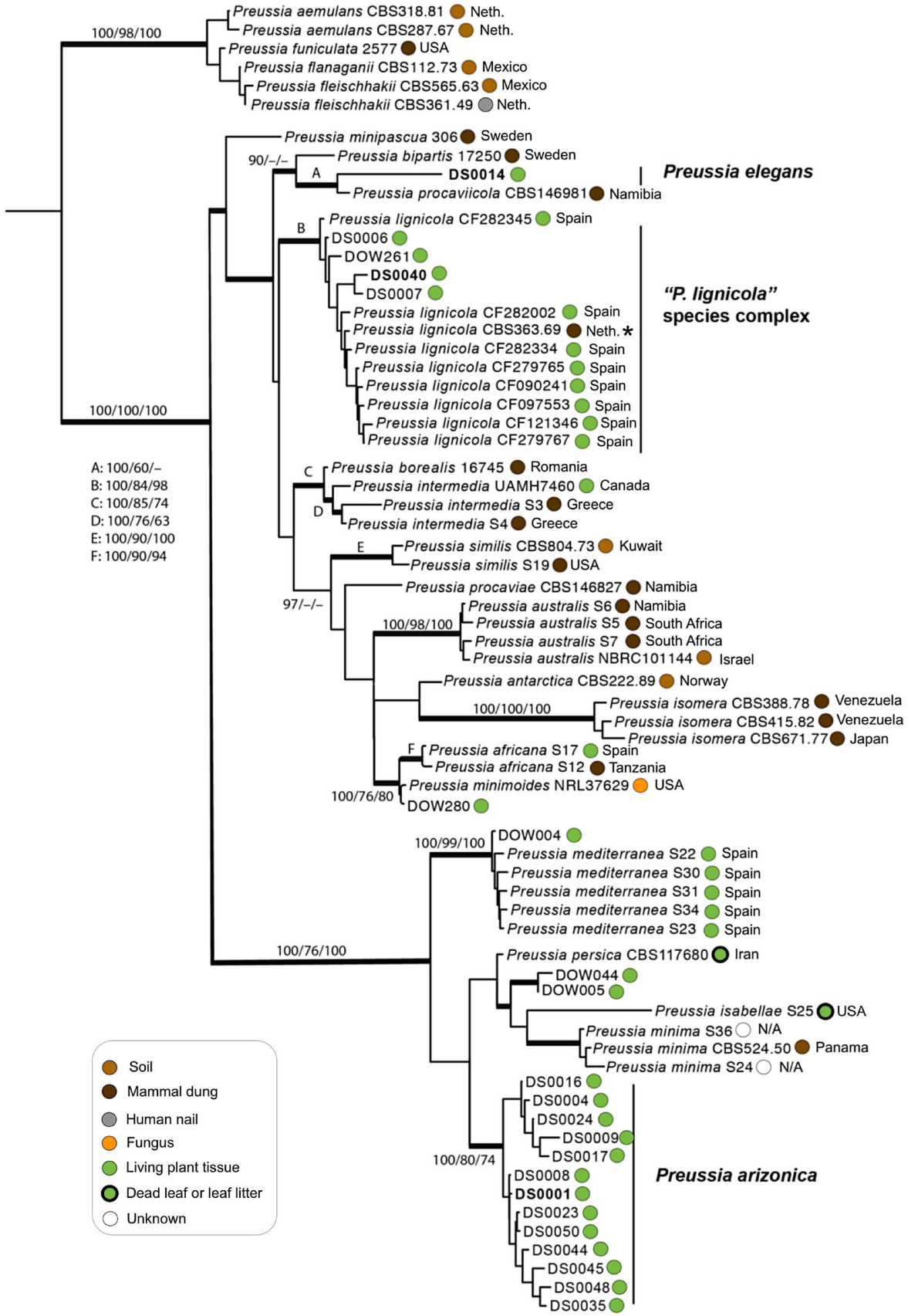

Figure 1. Phylogenetic relationships of the *Preussia intermedia* clade as inferred via Bayesian analyses of ITS-LSU rDNA. Support values are Bayesian posterior probability (≥ 90) / maximum likelihood (ML) bootstrap (≥ 60) / maximum parsimony (MP) bootstrap (≥ 60), with thickened branches indicating that branches were represented in at least two analysis sets (Bayesian as well as ML and/or MP). Circles indicate host- or substrate use as observed here (DS and DOW strains) or as reported by Kruys and Wedin (2009), González-Menéndez et al. (2017), or records in GenBank or CBS-KNAW (https://wi.knaw.nl/page/Collection). Geographic origin data were obtained from the same sources. Separately published ITS rDNA and partial LSU rDNA sequences for two species were concatenated prior to analysis: for *P. procaviae*, NR171769.1 and NG074498.1 from CBS146827, respectively, and for *P. procaviicola*, NR173052.1 and NG076741.1 from CBS146981, respectively (see Crous et al. 2020, 2021). Neth = the Netherlands. The asterisk highlights that *P. lignicola* is not represented in GenBank by sequence data from the type specimen.

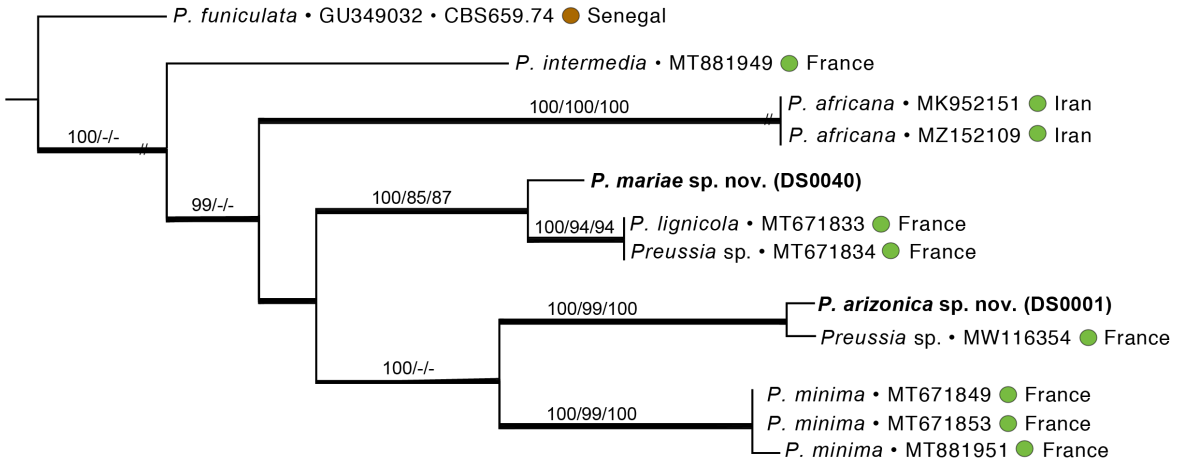

Figure 2. Phylogenetic relationships of the *Preussia intermedia* clade with *P. funiculata* as outgroup, as inferred via Bayesian analyses of *EF1-a*. We focused on clarifying the positions of DS0040 (*P. mariae*) and DS0001 (*P. arizonica*), as DS0014 (*P. elegans*) was placed with high support in the ITS-LSU rDNA analysis (Fig. 1). Support values are Bayesian posterior probability (≥ 90) / maximum likelihood (ML) bootstrap (≥ 60) / maximum parsimony (MP) bootstrap (≥ 60), with thickened branches indicating that branches were represented in at least two analysis sets (Bayesian as well as ML and/or MP). Circles indicate host- or substrate use as observed here (DS strains) or as reported by Kruys and Wedin (2009), González-Menéndez et al. (2017), or examination of records in GenBank or CBS-KNAW (https://wi.knaw.nl/page/Collection). Geographic origin data were obtained from the same sources. Two sequences are publicly available as representatives of *P. lignicola*, but the sequence representing CBS264.69 is >8.4% divergent from all other *Preussia* species in GenBank, and thus was not included here. When it was used in a repeated version of this analysis, there was no change in our inference with regard exclusion of *P. mariae* from *P. lignicola* (data not shown).

membranous, glabrose, and thick. Ascomata borne on brown, septate, flexuous hyphae. Asci octosporous, bitunicate, cylindrical to clavate, elongate and often curved; generally rounded and gradually to abruptly tapering into a short stipe; (98, minimum) 100–180 (185, maximum) µm × (16)18–22(24) µm. Pseudoparaphyses filiform, septate, and longer than the asci, mixed with them, and bifurcate. Ascospores four-celled, uniseriate or biseriate, cylindrical, elongate, hyaline to olivaceous (4E4) when young and maturing to dark brown (6E4); transversely septate, with constrictions at septae broad and shallow; (18)18–24(25) µm × (4)4–8(9) µm. Middle cells of the ascospores are of equal length and broader than terminal cells, with rounded apices that make them appear more spherical than terminal cells; germ slit diagonal, oblique or parallel and straight to sinuous. Gelatinous sheath hyaline and narrow. Anamorph: unknown.

Etymology. Refers to the US state in which it was collected. It is the home state of the majority of the authors of this paper and home to the university in which all authors earned or are earning their undergraduate or graduate degrees.

Notes. *Preussia arizonica* was the most common species isolated from *E. trifurca* in the Tucson Mountains of Arizona. On both PDA and 2% MEA (Table 2), it was the fastest-growing of the three species considered here. We have observed apparently conspecific endophytes from tissues of various angiosperms in the same mountain range, including healthy stems of *Simmondsia chinensis* (*Simmondsiaceae*), *Parkinsonia microphylla* (*Fabaceae*), and *Phoradendron californicum* (*Viscaceae*), and healthy leaves of *Larrea tridentata* (*Zygophyllaceae*) (Massimo et al. 2015). Thus the species appears to be common locally, with a wide host range. Surveys across the region including over 6000 isolates in desert and montane settings failed to detect the species in higher-elevation woodlands and forests, suggesting that it may be a distinctive species of the desert biome (see Huang et al. 2018). It also was not detected in surveys of coastal plants wherein other *Preussia* species were detected (Woods 2022). *Preussia arizonica* was reconstructed as sister to the clade containing *P. persica*, *P. isabellae*, and *P. minima*, as well as various DOW strains (DOW044, DOW005), albeit without strong support.

We detected living endohyphal bacteria in living hyphae of all four representative isolates of this species that we examined (the holotype, DS0001, as well as isolates DS0009, DS0043, and DS0046, the latter two of which were identified as *P. arizonica* based on morphology). The bacteria represent diverse *Alphaproteobacteria* and were closely related to endohyphal bacteria from other endophytes in the *Dothideomycetes* (including *Alternaria*, *Dothidea*, and *Phoma*) that were isolated previously from leaves of gymnosperm trees in the region (Hoffman & Arnold 2010).

Material examined. USA, Arizona, Pima Co., Tucson Mountains: 21 isolates from living stems of *E. trifurca.*

Vouchers and data deposition. In addition to the holotype, living and lyophilized vouchers of the paratype DS0008 are deposited in the publicly accessible Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ; https://www.gilbertsonherbarium.net/resources.html; accession numbers match isolate numbers), with data available at MyCoPortal.org and MycoBank. Based on phylogenetic analysis (*; see Fig. 1) and morphological assessment, the following strains were assigned to *P. arizonica*, in addition to the type and paratype: DS0002, DS0003, DS0004*, DS0009*, DS0016*, DS0017*, DS0023*, DS0024*, DS0028, DS0031, DS0035*, DS0043, DS0044*, DS0045*, DS0046, DS0047, DS0048*, DS0049, and DS0050*. ITS-LSU rDNA sequences from the strains marked with (*) are deposited in GenBank (Table 1), and living specimens of all strains are deposited at ARIZ.

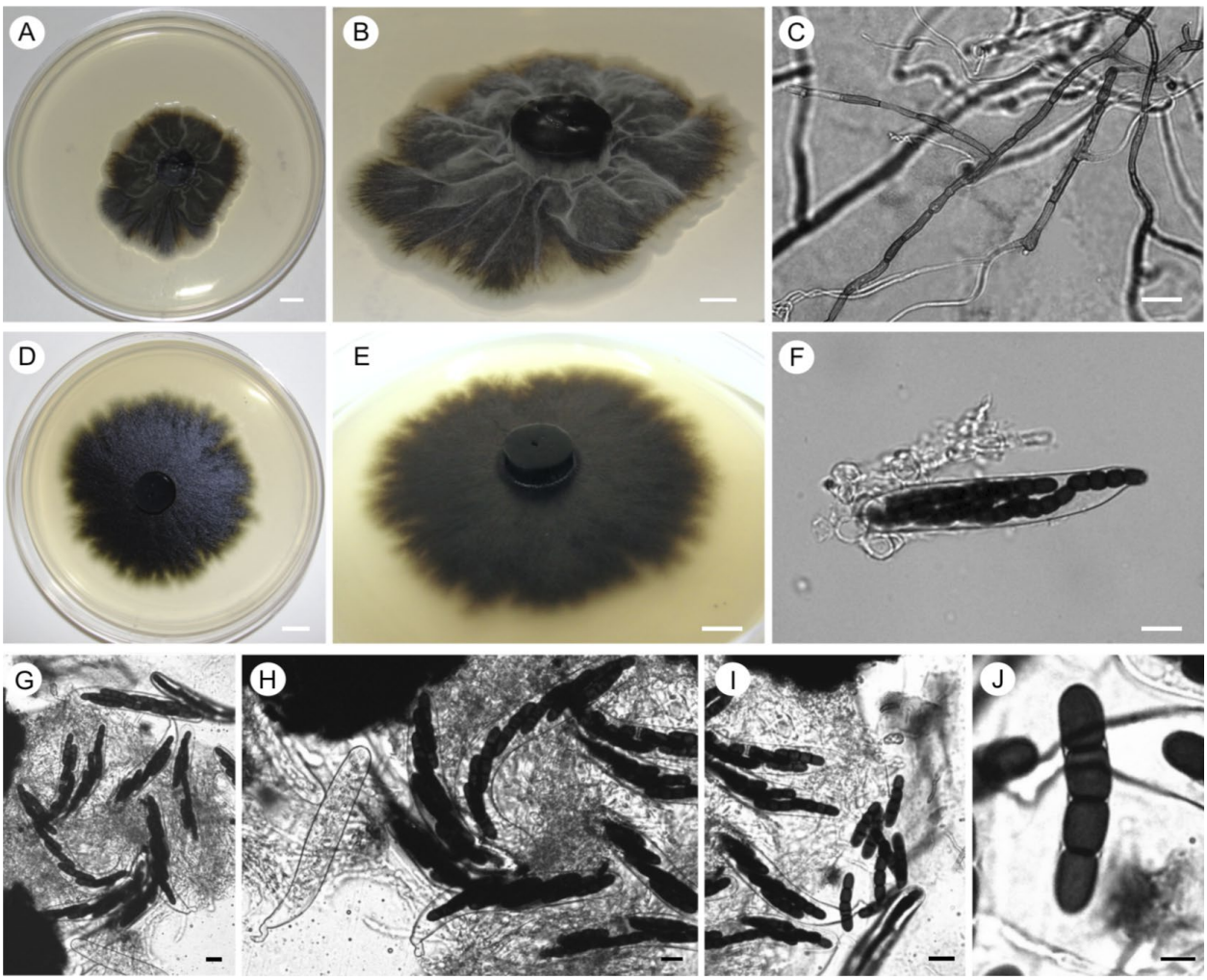

Figure 3. *Preussia arizonica* (type, ARIZ-AEA-DS0001). A – culture on PDA; B – culture in detail on PDA; C – hyphae; D – culture on MEA; E – culture in detail on MEA; F–J – asci and ascospores. Scales: A, B, D, E = 1 cm; C, F–I = 10 µm; J = 5 µm.

Preussia elegans D. C. Sandberg & A. E. Arnold, sp. nov. (Fig. 4)

MycoBank MB 841763

Type: USA, Arizona, Pima County, Tucson Mountains. Endophytic in healthy photosynthetic stems of *Ephedra trifurca.* Collected by Dustin C. Sandberg and A. Elizabeth Arnold in autumn 2009. Isolated by D. C. Sandberg from surface-sterilized stem tissue as in Massimo et al. (2015). ARIZ-AEA-DS0014 – holotype; preserved in a metabolically inactive state (lyophilized) at the Robert L. Gilbertson Mycological Herbarium, University of Arizona.

Description. Colonies on PDA reaching 80mm diameter in 22 days at 22–23°C. Texture cottony, adpressed, and partially submerged, with entire colony dark green to (28E8) to light greenish white (30A2), at times with a fine white margin of aerial hyphae. On MEA, aerial hyphae absent to rare, with hyphae submersed or growing thinly across the surface, uniformly dark green (28E8) to dark greenish-brown (27F4), and pseudothecia absent. On PDA, pseudothecia sparse to aggregated, semi-immersed, subglobose to globose, dark brown (6E4) to black, glabrous, ostioles not evident. Peridium pseudoparenchymatous, membranous, glabrose, and thick. Ascomata borne on brown, septate, flexuous hyphae. Asci octosporous, bitunicate, cylindrical to clavate, generally rounded and abruptly tapering into a short stipe; (85)90–160(165) µm × (9)12–20(22) µm. Pseudoparaphyses filiform, septate, and longer than the asci, mixed with them, and bifurcate. Ascospores four-celled, uniseriate or biseriate, cylindrical, hyaline to olivaceous when young (4E4) and maturing to dark brown (6E4); transversely septate, with constrictions at septae broad and shallow; (10)12–16(16) µm × (3)3–6(7) µm. Middle cells of ascospores are of equal length and broader than terminal cells, with rounded apices; germ slit diagonal, oblique or parallel and straight to sinuous. Gelatinous sheath hyaline and narrow. Anamorph: unknown.

Etymology. Refers to the long, elegant asci of this species.

Notes. This species was encountered twice in our surveys of *E. trifurca* endophytes. The two isolates were obtained from different branches of the same plant and had identical ITS-LSU rDNA and *EF1-a* sequences. For this reason, we included only one isolate in phylogenetic analyses (DS0014). On PDA and on 2% MEA (Table 2), this was the most slowly growing species of the three considered here. This species has not been detected in large surveys of endophytes in other plants in the region, nor is it closely related to known strains from other regions of

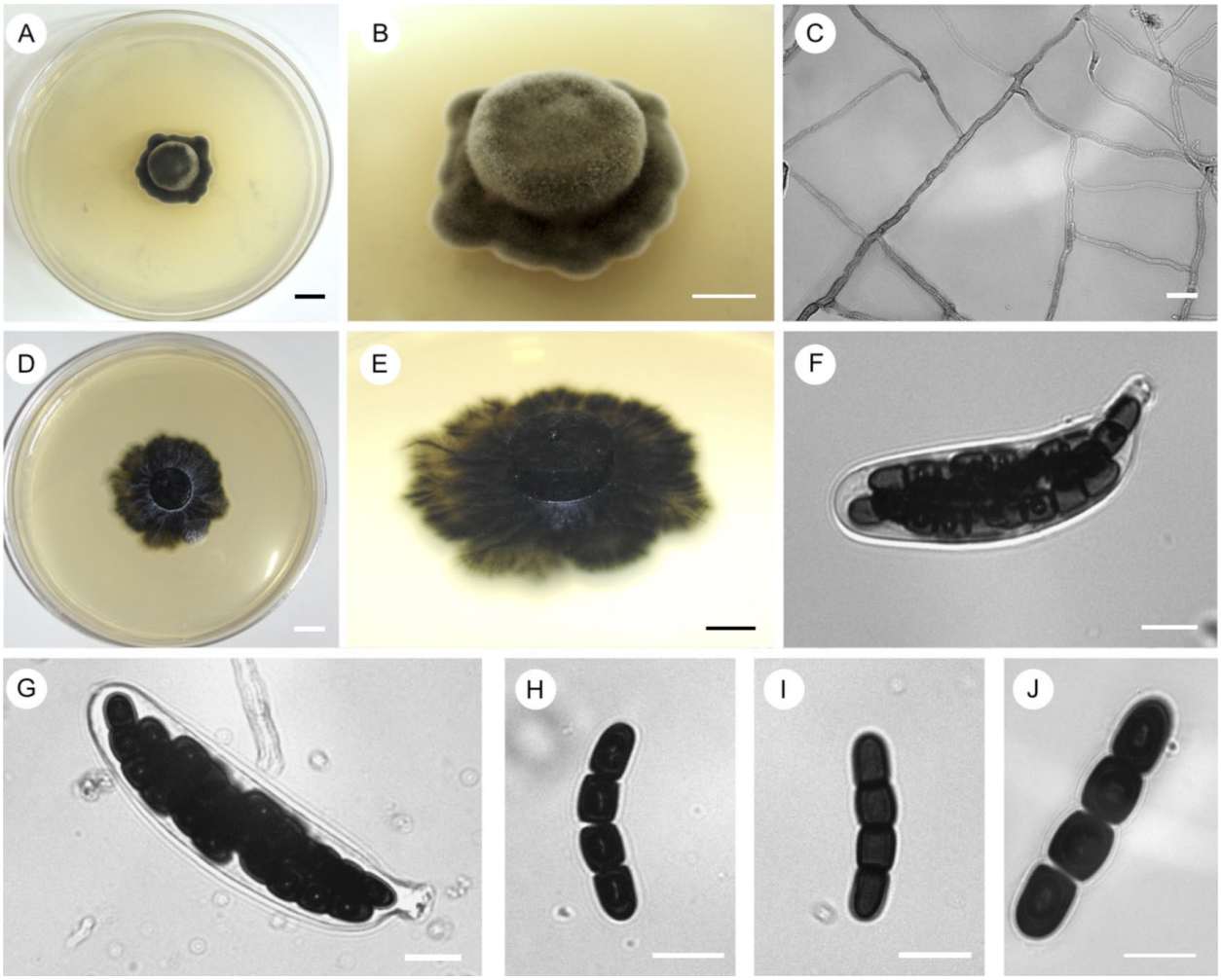

Figure 4. *Preussia elegans* (type, ARIZ-AEA-DS0014). A – culture on PDA; B – culture in detail on PDA; C – hyphae; D – culture on MEA; E – culture in detail on MEA; F–J – asci and ascospores. Scales: A, B, D, E = 1 cm; C, F–I = 10 µm; J = 5 µm.

North America. In Bayesian analyses of ITS-LSU rDNA, it was placed with weak support as sister to *P. procaviicola*, isolated from dung in Namibia (Crous et al. 2021). *Preussia procaviicola* has globose to pyriform ascomata with asci and ascospores that are highly variable in length, with generally wider asci and larger ascospores relative to *P. elegans* (see Crous et al. 2021). We did not detect living endohyphal bacteria in living hyphae of the type strain.

Material examined. USA, Arizona, Pima County, Tucson Mountains: two isolates from living stems of *E. trifurca.*

Vouchers and data deposition. Living and lyophilized vouchers of the paratype (DS0025) are deposited in the publicly accessible Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ; accession number matches isolate number; see above for holotype information), with data available at MyCoPortal.org and MycoBank. Sequence data for the type and paratype are deposited in GenBank (Table 1).

Preussia mariae D. C. Sandberg & A. E. Arnold, sp. nov. (Fig. 5)

MycoBank MB 841764

Type: USA, Arizona, Pima County, Tucson Mountains. Endophytic in healthy photosynthetic stems of *Ephedra trifurca.* Collected by Dustin C. Sandberg and A. Elizabeth Arnold in autumn 2009. Isolated by D. C. Sandberg from surface-sterilized stem tissue as in Massimo et al. (2015). ARIZ-AEA-DS0040 – holotype; preserved in a metabolically inactive state (lyophilized) at the Robert L. Gilbertson Mycological Herbarium, University of Arizona.

Description. Colonies on PDA reaching 80 mm diameter in 20 days at 22–23°C. Texture cottony to glabrous, adpressed, and partially submerged, with center of colony dark green (30F7) to grey (4B2) and growing edge white to light beige, often with tan to buff (4B3) to ash grey (1B2) undertones and rings of pseudothecia surrounding an undulate, velvety center. Mature colonies frequently but not always bear cottony white, irregular patches of aerial hyphae on the colony surface. Variation in intensity of greenish coloration was observed among strains of this species on PDA. On MEA, aerial hyphae typically absent, with hyphae appearing brown (6D7) to greenish brown (29F5) and at times sectoring in different shades; pseudothecia rare. On PDA, pseudothecia sparse to aggregated, semi-immersed, subglobose to globose, dark brown (6E4) to black, glabrous, ostioles not evident. Peridium pseudoparenchymatous, membranous, glabrose, and thick. Ascomata borne on brown, septate, flexuous hyphae. Asci octosporous, bitunicate, cylindrical to clavate, very elongated, gradually to abruptly tapering into a short stipe; variable in length, typically (157)160–200(203) µm

× (12)14–16(17) µm at maturity. Pseudoparaphyses filiform, septate, and longer than the asci, mixed with them, and bifurcate. Ascospores four-celled, uniseriate or biseriate, cylindrical, hyaline to olivaceous (4E4) when young and maturing to dark brown (6E4); transversely septate, with constrictions at septae broad and shallow; (18)20–40(40) µm × (5)6–10(11) µm. Middle cells are of equal length and are only very slightly broader than, or of equal width relative to, terminal cells, with rounded apices; germ slit diagonal, oblique or parallel and straight to sinuous. Gelatinous sheath hyaline and narrow. Anamorph: unknown.

Etymology. Refers to the lead author's mother, who has supported all his endeavors in science and has inspired him throughout his life.

Notes. The comprehensive analysis by González-Menéndez et al. (2017) highlights several well-supported clades within "*P. lignicola*". We anticipated that this clade could represent a species complex worthy of careful examination with additional loci beyond ITS-LSU rDNA. Indeed, we found that *Preussia mariae* could not be distinguished from *P. lignicola* on the basis of ITS-LSU rDNA data alone (Fig. 1). However, analyses of the coding region of *EF1-a* (Fig. 2) suggest, albeit with limited taxon sampling, that it is related to but distinct from *P. lignicola*.

We explored this inference more fully by considering morphological information. The original description notes that *P. lignicola* has asci measuring 200 µm in length and 27 µm in width (Phillips & Plowright 1877). The asci of the isolates considered here are typically shorter in length and markedly narrower in width. The isolates considered here also are narrower and typically shorter than those of the strains classified as *P. lignicola* by González-Menéndez et al. (2017) (e.g., CF-27965). Pseudothecia of *P. mariae* are more often globose than subglobose, whereas those of *P. lignicola* are described as subglobose exclusively (Phillips & Plowright 1877). Ascospores of *P. lignicola* were described by Phillips & Plowright (1877) as 60 µm long and 14 µm in diameter; those observed here were notably smaller. The ascospores we observed also were smaller than those reported for *P. lignicola* by González-Menéndez et al. (2017). Phillips & Plowright (1877) recorded *P. lignicola* from rotten

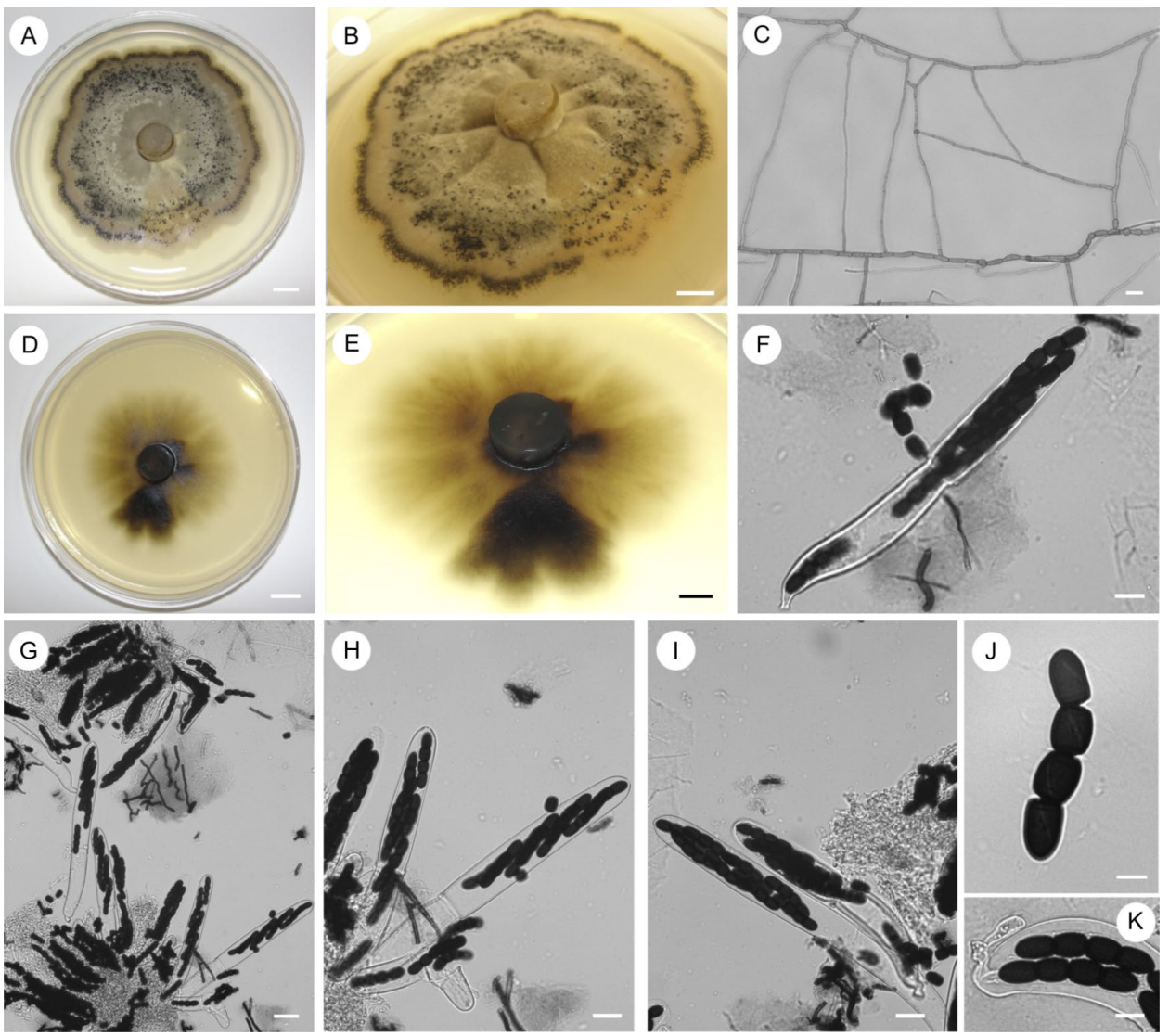

Figure 5. *Preussia mariae* (type, ARIZ-AEA-DS0040). A – culture on PDA; B – culture in detail on PDA; C – hyphae; D – culture on MEA; E – culture in detail on MEA; F–I – asci and ascospores; J, K – ascospores. Scales: A, B, D, E = 1 cm; C, F–I = 10 µm; J, K = 5 µm.

wood of ash in the United Kingdom, contrasting with the endophytic occurrence of *P. mariae*; however, many clades in the *P. intermedia* group have wide geographic- and host ranges (Kruys & Wedin 2009; see also Fig. 1). Based on the confluence of morphological evidence and available data from *EF1-a*, we consider *P. mariae* distinct from *P. lignicola*, and suggest that the European isolates considered by González-Menéndez et al. (2017), and the strain for which an *EF1-a* sequence is available (from France, Cambon et al., GenBank submission) likely represent *P. lignicola* sensu Phillips & Plowright 1877.

We found additional strains of *P. mariae* occurring endophytically in a coastal angiosperm in California (DOW261, Table 1, Fig. 1) and, in subsequent sampling for other purposes, a senesced leaf of a liliaceous host in a garden setting in Tucson, AZ (data not shown). We also detected members of this group in previous work on angiosperm endophytes in the Tucson Mountains (Massimo et al. 2015), as illustrated by the placement of strains SNP252, SNP334, and SNP437 in the *P. lignicola* clade by González-Menéndez et al. (2017). We observed endohyphal bacteria in both of the isolates we examined from this species (the paratype, DS0007, and morphologically similar isolate DS0018), to be characterized in future work.

Material examined. USA, Arizona, Pima Co., Tucson Mountains: four isolates from living stems of *E. trifurca.*

Vouchers and data deposition. Living and lyophilized vouchers of the paratypes (DS0006, DS0007) are deposited in the publicly accessible Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ; accession numbers match isolate numbers; see above for holotype information), with data available at MyCoPortal.org and MycoBank. Sequence data for the type and paratypes, and for the related strain DOW261, are deposited in GenBank (Table 1). Isolate DS0018 was identified as a member of this species on the basis of morphology.

Discussion

The Sonoran Desert is of interest for the discovery of endophyte biodiversity because of its high plant richness and its coupling of relatively copious annual rainfall with seasonal aridity and extreme temperatures. A survey of endophytes associated with diverse woody plants in the Tucson Mountain area by Massimo et al. (2015) revealed many endophyte taxa but focused exclusively on angiosperm hosts. Several studies have documented novel secondary metabolites from individual endophytic fungi isolated from plants in the region (e.g., Bashyal et al. 2005; Kithsiri Wijeratne et al. 2006; Zhan et al. 2007), including *Chaetomium globosum*, *Chaetomium chiversii*, and *Fusarium oxysporum* from *Ephedra fasciculata* in central Arizona. More generally, endophytes of *Ephedraceae* have been examined primarily in Europe (e.g., *E. nebrodensis*; Pelaez et al. 1998) and have not been characterized in detail in the deserts of North America. By examining endophytes of a locally abundant species of *Ephedra*, we recovered a phylogenetically diverse array of fungi, among which isolates of three putative species of *Preussia* presented sexual structures *in vitro* on PDA. The prevalence of *Preussia* spp. in *E. trifurca* from the Tucson Mountains, and the morphological and phylogenetic distinctiveness of these strains, led us to propose three new species.

Preussia is rich in endophytes, as showcased by González-Menéndez et al. (2017) and other studies focusing on arid and seasonally arid lands (e.g., Massimo et al. 2015; González-Menéndez et al. 2018; Huang et al. 2018; Woods 2022). ITS-LSU rDNA data were sufficient to distinguish two of the endophyte species from *E. trifurca* from known species, but we found that the *P. lignicola* group requires additional loci to diagnose species boundaries. Data from *EF1-a* were helpful but limited in scope because of sparse taxon sampling in public databases, arguing for a careful revision of this clade with multi-locus and/or genomic data. Even so, *EF1-a* can contribute to ongoing efforts to disentangle relationships within *Preussia* and related *Sporormiaceae*, for which the mitochondrial small subunit (mtSSU) and β-tubulin also have shown considerable promise (see Kruys & Wedin 2009).

Although endophytic fungi are widely acknowledged as a rich source of unexplored fungal diversity, studies in only a few regions of the world have yielded new species descriptions in the past two decades (e.g., for *Preussia*: Arenal et al. 2007; Kruys & Wedin 2009; Asgari & Zare 2010; Mapperson et al. 2014). In part this reflects the tendency of some endophytic fungi to not produce reproductive structures in culture, as well as an increased reliance on high-throughput sampling methods. The *Preussia* strains isolated here and in our previous work (Massimo et al. 2015; Woods 2022) grow well in culture and produce sexual structures on PDA. Had we worked only with MEA we would not have observed such structures, highlighting a need to cultivate apparently vegetative cultures on multiple media to improve taxonomic identification, and diagnose the novelty, of endophytes from diverse plants.

In working with endophytic *Preussia* for this and related studies, we have noticed that the strains often are not resilient to extended storage in sterile water and can grow less vigorously or lose viability over long periods of time. In some cases, strains are overtaken by bacteria upon regrowth from living vouchers, suggesting that some of the endohyphal bacteria detected here may have a negative effect during culture storage. One solution is to isolate and cultivate *Preussia* strains on media containing antibiotics (e.g., Hoffman & Arnold 2010). However, it is increasingly clear that endohyphal bacteria can influence fungal phenotypes, including the expression of secondary metabolites (Araldi-Brondolo et al. 2017). Thus, care would need to be taken to clarify the roles of endohyphal bacteria in contributing directly, or indirectly, to the remarkable chemodiversity of secondary metabolites documented recently across the genus (see González-Menéndez et al. 2017). More generally, phylogenetic placement of *P. arizonica*, *P. elegans*, and *P. mariae* in or near lineages characterized in chemotaxonomy analyses by González-Menéndez et al. (2017) suggests that the species described here may be constitutive

producers of important secondary metabolites. Further exploration can clarify whether the major metabolites described by González-Menéndez et al. (2017) drive the antifungal activity observed here.

The topology of our ITS-LSU rDNA tree is generally consistent with the inferences of Kruys & Wedin (2009) and González-Menéndez et al. (2017). The intermixing of closely related taxa from multiple continents (e.g., in the *P. lignicola* species complex; see also the *P. mediterranea* clade, which includes a DOW strain from montane *Ceanothus* in Arizona; Fig. 1, Table 1) suggests an interesting biogeographic history for the genus. Our analyses also reveal intermixing of fungi with distinctive ecological modes in many clades, consistent with previous studies suggesting that endophytism is a highly labile trophic mode (Arnold et al. 2009). The occurrence of these three *Preussia* species in a gnetophyte expands the taxonomic breadth for host associations in the genus. In turn, the observation of three distinctive species in only a small sample from *Ephedra* a single area suggests that further research may discover additional *Preussia* species in the long-lived photosynthetic stems of this intriguing and ethnobotanically important genus.

We conjectured previously that endophytes of desert plants may have host-generalist strategies, sheltering *in planta* from the thermal and radiation extremes common outside of plant tissues (Massimo et al. 2015). Host generalism can be a trait of a given isolate, a species as a whole, or a clade such as the *P. lignicola* species complex. We found that *P. arizonica* has an apparently broad host range, whereas *P. elegans* has been detected only in *E. trifurca* despite extensive sampling of other hosts and substrates in the region (see Huang et al. 2018). *Preussia mariae* has been identified from *E. trifurca* only, but as part of the *P. lignicola* species complex it is in a clade containing isolates from an angiosperm in the southwestern US as well as diverse plants from Europe (see González-Menéndez et al. 2017 and Fig. 1). Further exploration at the full genome level, as well as multi-locus sequencing, can help clarify the host use of each species and identify the cryptic species that may occur within species complexes.

Our analyses included recently isolated strains of *Preussia* from angiosperms in a montane forest and coastal region in the western USA (Woods 2022). These isolates also represent considerable phylogenetic diversity, including strains that are well-supported as sister to *P. minimoides*, occur within the *P. lignicola / P. mariae* species complex, and may represent a distinctive species as yet unidentified but potentially affiliated phylogenetically with *P. isabellae* and *P. minima* (Fig. 1). We anticipate that arid and seasonally arid areas of the western USA may harbor additional diversity in *Preussia* worth of exploration (Massimo et al. 2015), as in Spain (see Arenal et al. 2007; González-Menéndez et al. 2017). More broadly, our study expands the records of endophytism in *Preussia*, enriches the known diversity of the genus, and highlights a potential for additional diversity in the arid and semi-arid lands of southwestern North America.

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