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Geosmithia morbida sp. nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in USA

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Abstract: Widespread morbidity and mortality of *Juglans nigra* has occurred in the western USA over the past decade. Tree mortality is the result of aggressive feeding by the walnut twig beetle (*Pityophthorus juglandis*) and subsequent canker development around beetle galleries caused by a filamentous ascomycete in genus *Geosmithia* (Ascomycota: Hypocreales). Thirty-seven *Geosmithia* strains collected from *J. californica*, *J. hindsii*, *J. major* and *J. nigra* in seven USA states (AZ, CA, CO, ID, OR, UT, WA) were compared with morphological and molecular methods (ITS rDNA sequences). Strains had common characteristics including yellowish conidia en masse, growth at 37 C and absence of growth on Czapek-Dox agar and belonged to a single species described here as *G. morbida*. Whereas *Geosmithia* are common saprobes associated with bark beetles attacking hardwoods and conifers worldwide, *G. morbida* is the first species documented as a plant pathogen.

Key words: insect-associated fungi, *Juglans nigra*, *Juglans* species, phytopathogenic fungi, thousand cankers disease

INTRODUCTION

Widespread branch dieback and mortality of *Juglans nigra* (black walnut) has occurred in several western states including Colorado (CO), Idaho (ID), New Mexico (NM), Oregon (OR), Utah (UT) and Washington (WA) of USA since the mid-1990s. *Juglans nigra* is not native to this region but has been widely planted as an ornamental and nut tree species. Affected trees initially exhibit yellowing and wilting of the foliage followed by progressive branch dieback. Trees are killed within 3–4 y of initial symptoms. Tree mortality is the result of aggressive feeding by the walnut twig beetle, *Pityophthorus juglandis* Blackman, (Coleoptera, Curculionidae, Scolytinae), and subsequent canker development surrounding beetle galleries in the phloem. The number of cankers formed on branches and the trunk is enormous, hence the name thousand cankers to describe the disease. A previously undescribed species of *Geosmithia* (Ascomycota: Hypocreales: Bionectriaceae) was consistently isolated from the canker margins, beetle galleries and adult beetles on *Juglans nigra*. Artificial inoculations of *Juglans nigra* demonstrated this fungus was responsible for canker development (Tisserat et al. 2009). *P. juglandis* and *G. morbida* have not been reported in the native range of *J. nigra* in eastern North America until the recent alarming report from eastern Tennessee. Here both the beetle and *Geosmithia* symbiont were found on *J. nigra* with TCD (Tennessee Department of Agriculture, media release: 5 Aug 2010).

P. juglandis first was described from *J. major* and possibly is native also to other walnut species in the southwestern United States (*J. californica* and *J. hindsii*) (Blackman 1928, Wood and Bright 1992). In 2008 and 2009 *G. morbida* was isolated from necrotic phloem surrounding *P. juglandis* galleries in *J. major* in native stands in AZ and NM, but the fungus was not causing branch dieback or mortality in this species. Both *P. juglandis* and *G. morbida* also have recently (2008) been associated with dieback of *J. hindsii* and *J. californica* in their native range in CA. However pathogenicity of *G. morbida* to these species has not yet been documented. *Geosmithia* is a genus of mitosporic filamentous fungi with a worldwide distribution containing 22 published species (Kolařík and Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008) and at least 20 more unpublished species. Certain *Geosmithia* species sporadically occur on broad range of substrates, including plant debris, cereals and in soil (Kolařík et al. 2004, Pitt and Hocking 2009), however most are exclusive associates of subcortical insects including scolytids (Coleoptera, Curculionidae, Scolytinae) and bostrichids (Coleoptera, Bostrichidae). *Geosmithia* spp. are found typically in association with phloeophagous bark beetles (Kolařík et al. 2006, 2007, 2008) but also with woodboring ambrosia beetles, where they can act as

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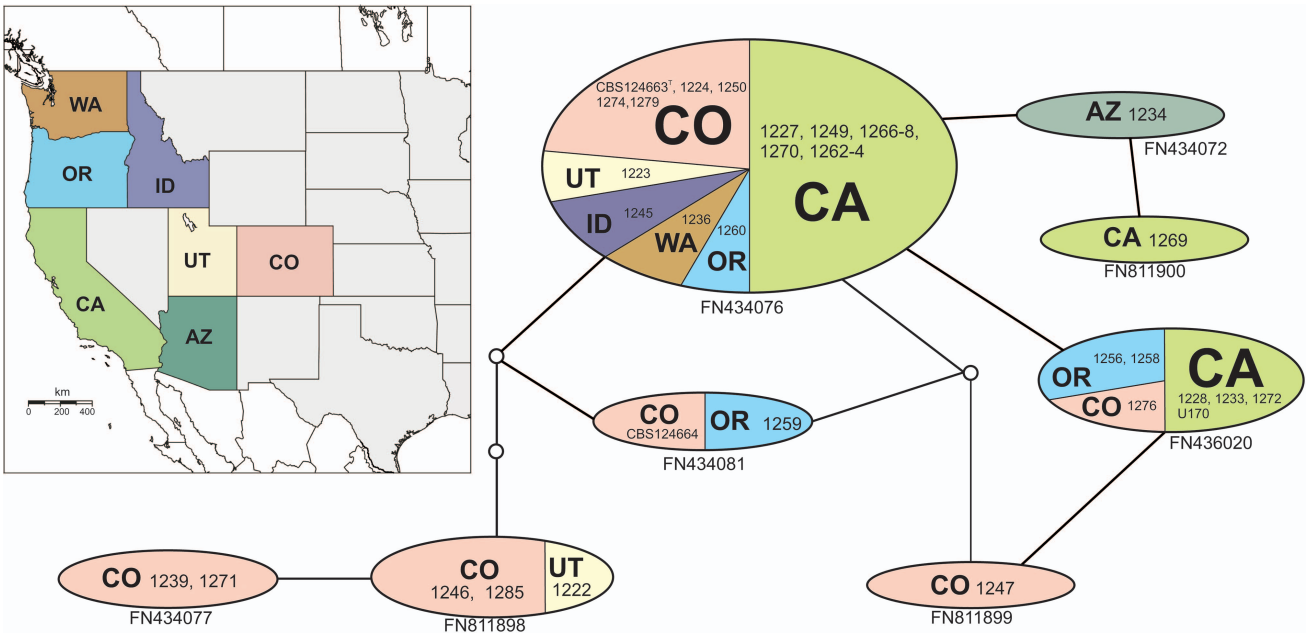

FIG. 1. Haplotype network of *G. morbida* ITS rDNA from 37 strains. The circles refer to haplotypes detected in the samples and their size is proportional to the sampled haplotype frequency. Isolates were collected in the states of of Arizona (AZ), California (CA), Colorado (CO), Idaho (ID), Oregon (OR), Utah (UT) and Washington (WA). Hosts of the isolates were: 1227, 1228, 1249, 1261–1264, 1266–1270 and 1272 from *J. californica*; 1233 and U170 from *J. hindsii*, 1234 from *J. major* and 1258–1260 from undetermined *Juglans* species or hybrids; all other isolates were recovered from *J. nigra*. The line between the haplotypes represents one base change and the small circles represent haplotypes not present in the sample. GenBank accession numbers representing each haplotype are given below the circles. (See article online for color figure.)

primary or auxiliary ambrosia fungi (Kolařík and Kirkendall 2010). While ambrosial *Geosmthia* spp. provide the main nutritional source for their vectors and represent an extreme example of nutritional mutualism, little is known about interactions of other symbiotic *Geosmithia* species or their vectors and host plants. Several attempts to evaluate phytopathogenicity of *Geosmithia* have been conducted. A *Geosmithia* sp. associated with *Cryphalus piceae* (Col., Curculionidae, Scolytinae) was non-pathogenic to *Abies alba* seedlings (Jankowiak and Kolařík 2010). Čížková (2005) reported that the *Quercus* inhabiting *G. langdonii* and *G. pallida* inhibited the growth of *Lepidium sativum* but were non-pathogenic to *Quercus* seedlings. Scala et al. (2007) found that the strain of *Geosmithia pallida* obtained from wilting *Ulmus* in Italy possessed a cerato-ulmin toxin, the protein involved in Dutch elm disease (DED). This strain was unable to cause DED symptoms on inoculated *U. glabra* trees. Nevertheless *Geosmithia* spp. co-occur regularly with phytopathogenic *Ophiostoma* species on elms and their contribution to DED complex is little explored and deserves further study.

We identified a set of morphological and molecular genetic characteristics of *Geosmithia* isolates collected from *J. nigra*, *J. major*, *J. californica* and *J. hindsii* throughout western USA that were unique from published and unnamed *Geosmithia* species in the collection of the first author. The species from *Juglans* is described here as *Geosmithia morbida* sp. nov.

MATERIALS AND METHODS

Fungal cultures and fungal morphology.—Thirty-seven fungal isolates from *Juglans* spp. throughout western USA (AZ, CA, CO, NM, ID, OR UT, WA) were isolated on one-half strength PDA (½ PDA, Difco Corp., Sparks, Maryland) containing 100 mg/L chloramphenicol and streptomycin sulfate from *Juglans* spp. exhibiting disease symptoms (Tisserat et al. 2009). These single-spore strains were characterized with ITS rDNA sequences (FIG. 1) and a subset of 12 strains representing main ITS rDNA haplotypes, and places of origin were selected for the morphological characterization (TABLE I). The ex-type and other representatives of *Geosmithia* from walnut were deposited in the Culture Collection of Fungi, Prague, Czech Republic, and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. A dried herbarium specimen of the holotype was deposited in the herbarium of the Mycological Department, National Museum in Prague (PRM). All measurements and observations were done on fungal structures grown 7–14 d on malt extract agar (MEA, malt extract Oxoid 20 g L-1, glucose 20 g L-1, peptone 1 g L-1,

TABLE I. Characterization of strains used in the description of *Geosmithia morbida*. Only characters showing intraspecies variability are presented

Source	Origin, host, collection date, collector	Diameter growth after 14 days (mm) on MEA, 25 C	Conidium size (µm)	GenBank accession number
1268	CA, *J. californica*, 2008, Seybold	30	(4.5–)5.3(–6) × 2	FN434076
1260	OR, *Juglans* sp., 2008, Pscheidt	30	(4.5–)5.3(–8) × 2	FN434075
CCF 4010 (1271, PRM 859765, PRM 859767)	CO, *J. nigra*, 2008, Utley	38	(4.3–)5.0(–5.5) × 2	FN434077
1223	UT, *J. nigra*, 2007, Funk	50–55	(4.3–)5.0(–5.5) × 2	FN434080
1276	CO, *J. nigra*, 2008, Utley	30	(4.6–)5.6(–7) × 2.3	FN436020
CCF 3880 (1234, PRM 859763-4)	AZ, *J. major*, 2008, Cranshaw	50	(4–)4.7(–5.2) × 2.2	FN434072
CBS 124663 (CCF 3881, 1217, PRM 915943, 859766)	CO, *J. nigra*, 2007, Tisserat	30–45	(4–)4.7(–6) × 2.2	FN434082
1256	OR, *J. nigra*, 2008, Pscheidt	30	(4.2–)4.9(–5.8) × 2	FN434073
1272	CA, *J. californica*, 2008, Seybold	37	(4.3–)5.0(–5.5) × 2	FN434078
U170	CA, *J. hindsii*, 2009, Graves & Kolařík	35	(4.3–)5.0(–5.5) × 2	FN434079
1259	OR, *Juglans* sp., 2008, Pscheidt	30	(4.3–)5.0(–5.5) × 2	FN434074
CBS 124664 (CCF 3879, 1218, PRM 915940-2)	CO, *J. nigra*, 2007, Tisserat	40–50	(3.9–)4.8(–5.5) × 2	FN434081

agar 20 g L-1) and incubated in the dark or in incidental light at 25 C. Other media used for colony descriptions were Czapek yeast agar (CYA), Czapek-Dox agar (CDA) (Pitt 1979) or ½ PDA. Fungal structures were mounted both in lactophenol with cotton blue and in water for better observation of the cell wall surface. Structures were examined with phase and differential interference contrast (Olympus BX-51 with digital camera) and measured with Quick Photo® software. Measurements are reported as the maximum, average and minimum values of 50 observations.

Temperature studies.—Isolates CBS 124663 and CBS 124664 were grown on sterile wheat seeds 10 d at 21 C in a manner described by Tisserat et al. (1989). A 3 mm diam sterile cork borer was used to remove an agar plug from the center of 80 mm diam Petri plates containing ½ PDA, and a colonized wheat berry of one of the *Geosmithia* isolates was inserted. Three plates of each isolate were incubated in the dark at 13, 21, 25, 30 or 41 C, and the maximum diameter of fungal growth was recorded daily. The experiment was repeated with similar results. The growth of *Geosmithia* isolates on MEA and CYA at selected temperatures were determined by measuring diameter growth at 7 and 14 d after placement of agar containing mycelium in the center of the plate.

DNA analysis.—Single-spore isolates were grown in yeast extract broth 5–7 d on a rotary shaker and the mycelium collected. Genomic DNA was isolated from the mycelium with Easy DNA Kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. A 560 bp nuclear rDNA fragment containing the internal transcribed spacers (ITS1 and ITS2) and 5.8S subunit was amplified with universal primers ITS1 and ITS4 according to White et al. (1990). PCR products were purified with Pure Link PCR purification kit (Invitrogen Carlsbad, California) and sequenced directly with ABI 3130xL genetic analyzer to process sequencing samples prepared with ABI BigDye® Terminator 3.1 sequencing chemistry. A genotype network of the strains from *Juglans* was constructed with statistical parsimony in TCS 1.21 (Clement et al. 2000). Sequences were aligned to representatives of published *Geosmithia* spp. in MUSCLE (Edgar 2004). Maximum likelihood (ML) analyses were performed in PHYML (Guindon et al. 2005) with default settings, and Bayesian analyses were performed with MrBayes 3.1 (Ronquist and Huelsenbeck 2003). The latter was based on the substitution models determined with MrModeltest 2.2 (Nylander 2004), metropolis-coupled Markov chain Monte Carlo search algorithm with 1 000 000 generations and calculation of Bayesian posterior probabilities after discarding a burn-in of 500 generations. Analyses with minimum evolution (ME) method and LogDet distance algorithm were performed in MEGA 4 (Kumar et al. 2004). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Support for branches in ML and NJ trees were estimated with 1000 bootstrap replicates. *Acremonium alternatum* Link, ancestrally related to *Geosmithia* (Rossman et al. 2001), was chosen as outgroup. Sequences were submitted to GenBank (see FIG. 1 and TABLE I for Accession numbers) and sequence alignment was deposited in the TreeBase (www.treebase.org/) database under submission code SN4939.

RESULTS

We detected eight rDNA ITS haplotypes from 37 *Geosmithia* isolates collected throughout western USA (FIG. 1). The aligned ITS rDNA haplotypes of the *Juglans G. morbida* isolates showed six variable positions (five of them in the poly-C region) in the ITS1 region. Distribution of haplotypes was not correlated with the geographic site or the *Juglans* species from which isolates were collected. Multiple

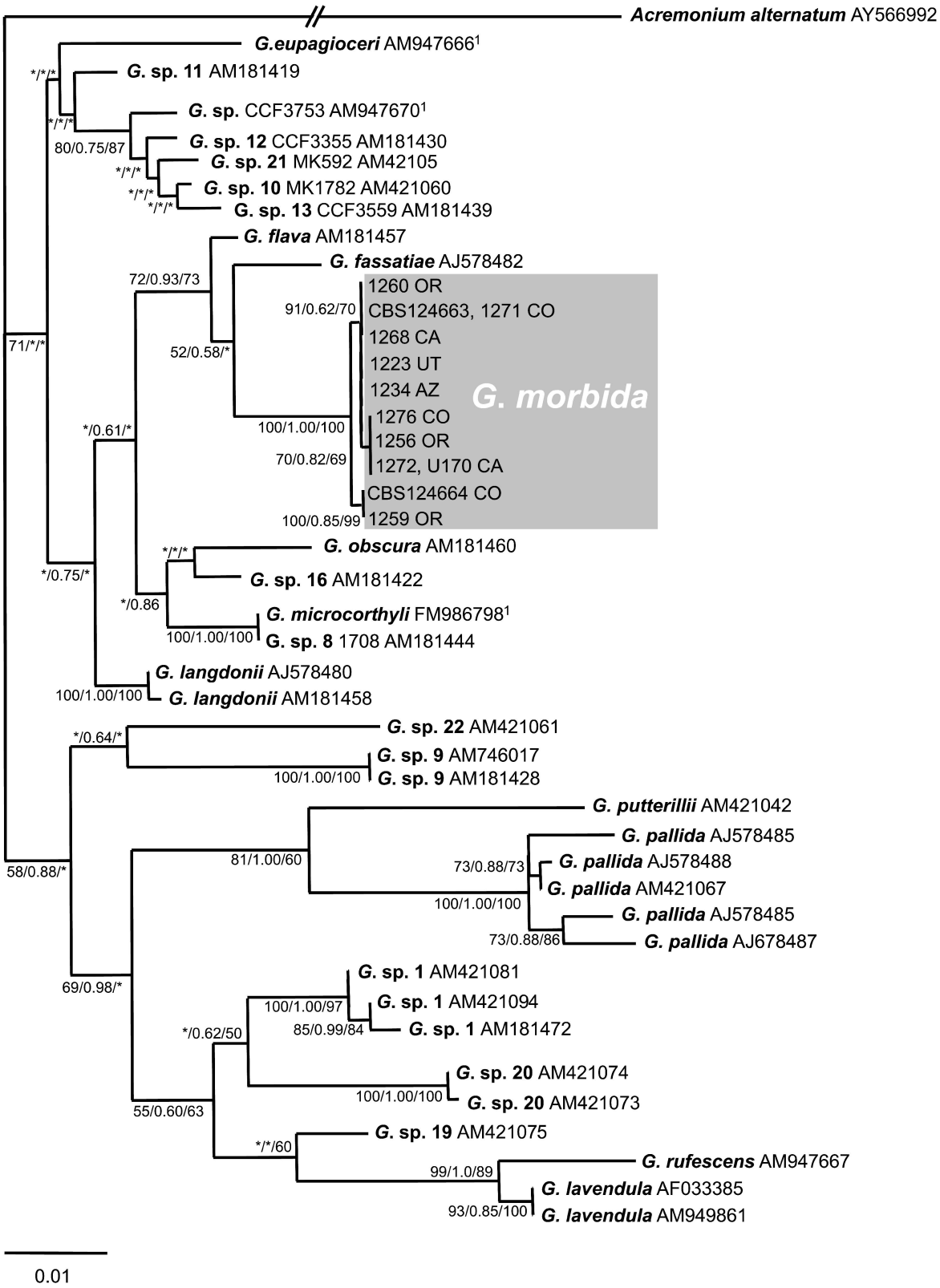

FIG. 2. A phylogeny of *Geosmithia* based on ITS rDNA sequence data representing all published species and *G. morbida*. The best tree resulting from heuristic maximum likelihood analysis in PHYML is presented. Numbers next to the internal nodes are maximum likelihood bootstrap, Bayesian MCMC posterior probabilities and minimum evolution bootstrap. Branch leading to the outgroup sequences is one-fourth actual length. The species numbering is from Kolařík et al. (2007, 2008).

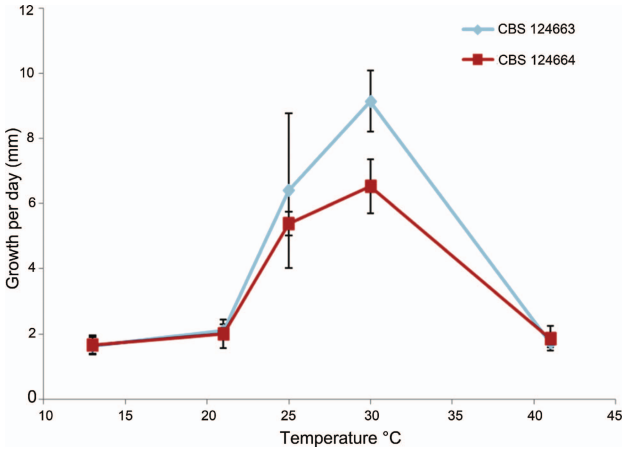

FIG. 3. Average daily diameter growth of *G. morbida* isolates CBS 124663 and CBS 124664 on ½ PDA at various temperatures. Bars represent standard errors.

haplotypes were present in each state and tree species, whereas identical haplotypes often were found in different states and species. A subset of isolates exhibiting the various ITS rDNA haplotype sequences were compared to sequences of species and unnamed isolates of *Geosmithia* from other hosts (FIG. 2). This manually adjusted ITS alignment contained 47 *Geosmithia* sequences each containing 582 nucleotid bases (including alignment gaps). Of these 430 were conserved, 143 were variable and 85 were parsimony informative. ME, ML and MP analyses showed agreement in topology of highly supported nodes only (≥ 60, 0.7). Sequences of *Geosmithia* from *Juglans* formed well supported clade that was distinct from all other *Geosmithia* spp. Strains from *Juglans* together with *G. fassatiae* and *G. flava* formed a well supported phylogenetic group characterized by white to yellow colonies and association with bark beetles living on hardwoods. Strains from *Juglans* represent an easily morphologically recognizable and homogenous group, distinct from other species. Variability in growth rate and conidial and phialide sizes were noted but these differences were not correlated with specific haplotypes identified by ITS sequences (TABLE I).

Growth on ½ PDA was faster than on MEA and was optimal at 31 C with limited growth at 41 C (FIG. 3). In a subsequent study we did not detect any growth of isolates CBS 124663 and CBS 124664 on ½ PDA at 48 C (data not shown). However when the colonized wheat seeds exposed to 48 C were transferred to fresh ½ PDA and returned to 31 C mycelial growth resumed normally.

TAXONOMY

Morphological characteristics and DNA sequence analyses of the *Juglans* isolates indicated they represent a single and undescribed species, which is described here.

Geosmithia morbida M. Kolařík, E. Freeland, C. Utley, & N. Tisserat sp. nov. FIG. 4
MycoBank MB518713

Coloniae in agaro matli (MEA) post septem diebus in 25 C diam 18–40 mm attingentes; post septem diebus in 37 C diam 15–25 cm cresecentes; in agaro Czapekii (CDA) conidia non germinantia; pars aversa coloniae culturarum MEA velutina; conidiogenesis flava. Conidiophora penicillata, verruculosa, biverticillata usque pentaverticillata; stipes 20–250 µm longi; penicilli 30–60 µm longi; conidia cylindrica, (4.0–)4.5–6(–8) × (1.5–)2(–2.5) µm; portata in columnis usque 200 mm longis.

Etymology: refers to its pathogenicity to *Juglans nigra*.

Specimens examined: USA. Colorado: Boulder, 40°00′41.45″N, 105°16′07.57″W, 1620 m. Isolate from an adult of *Pityophthorus juglandis*, VII-2007, N. Tisserat, (HOLOTYPE PRM 915940, ISOTYPES PRM 915941-2, culture ex-type CCF 3879, CBS 124664). (For additional material examined see TABLE I.)

Habitat: galleries of *Pityophthorus juglandis* and adjacent phloem.

Distribution: western USA from California to Colorado.

Teleomorph: unknown.

Colonies on MEA attaining 18–40 mm diam in 7d, 30–55 in 14 d at 25 C; 15–25 in 7 d and 30–40 in 14 d at 37 C. On CYA at 25 C attaining 30–45 diam in 7 d, 55–65 diam in 14 d. No growth observed on CDA. Colony on MEA highly lobate, low and plane; mycelium hyaline; substrate mycelium dense, monilioid, often with numerous yeast-like and inflated globose cells (5–10 µm, originating from conidia) and conidia produced in the medium (substrate conidia) forming together a tough mass resulting in a slimy appearance of young colonies; yeast colonies also may originate from conidial suspensions placed on MEA; conidiogenesis moderate, ocher yellow; exudate absent; soluble pigment yellowish to ocher; reverse yellowish to ocher. Colony at 37 C differing by a regular margin, presence of sparse aerial mycelium and cream sporulation. Colony on CYA with regular or slightly lobate margin, plane and low, surface consisting of sterile substrate mycelium or forming floccose areas with abundant sporulation in the central area and highly floccose and less sporulating marginal area; conidiogenesis low to moderate, cream; exudate absent; soluble pigment ocher to light brown; reverse light brown to dark brown. Conidiophores roughened to distinctly verrucose, penicillate; stipe 20–200 × 2.5–3 µm, base (peg foot) consisting of curved and atypically branched cells

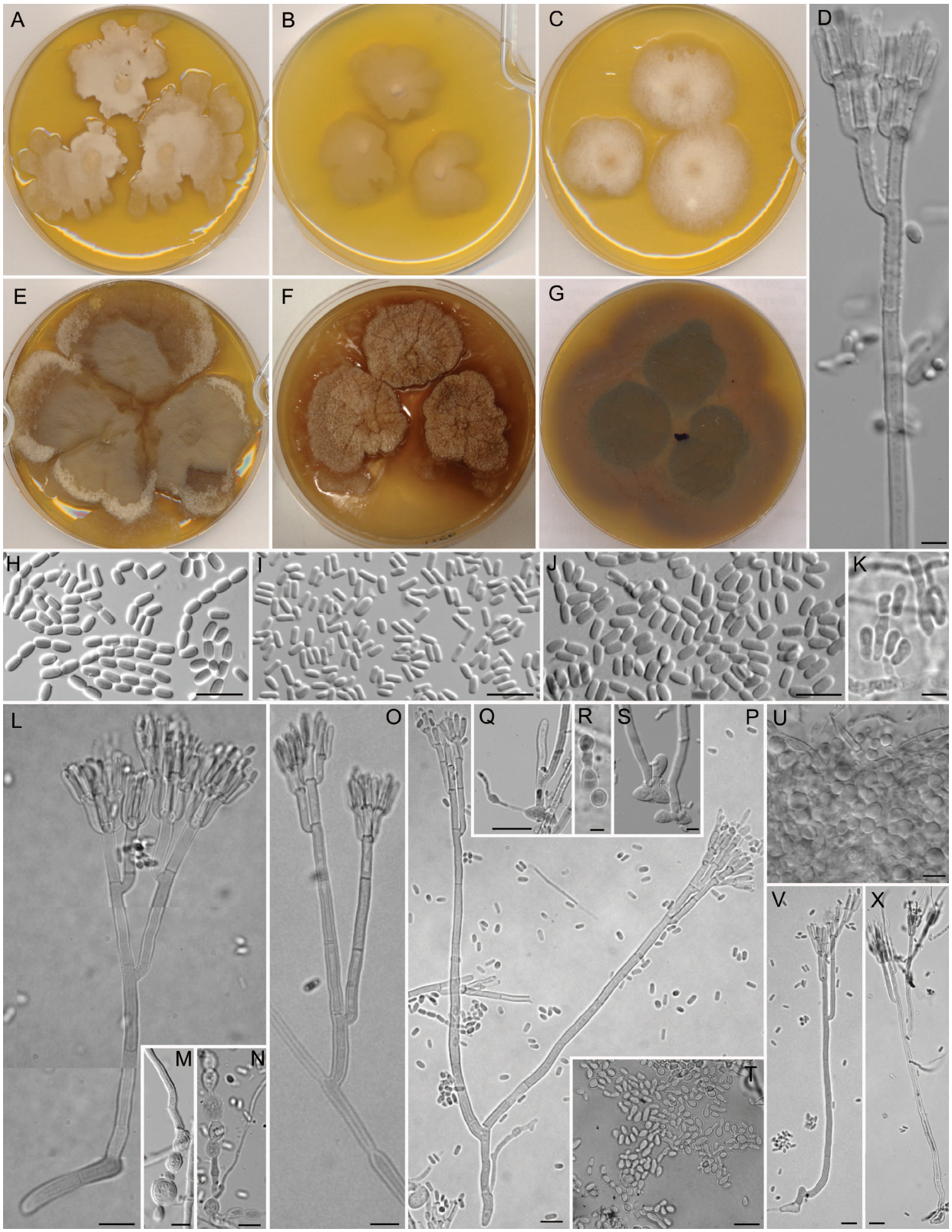

FIG. 4. *Geosmithia morbida*. Two-week-old colonies grown on MEA (A–C) and CYA (E, G) (at 25 C unless otherwise noted): A. CBS 124664. B. CBS 124663. C. CBS 124664 (37 C). E. CBS 124664. F. 1256. G. 1234. Conidiophores: D. 1221. L. 1260. O. 1268. P.1271. U. 1271. V. 1268, phialides are bearing hyphae instead of conidia. Conidia: H. CBS 124663. I. 1276. J. CBS 124664. Substrate conidia: K. 1260. Conidophore bases: M, Q. 1217. S. 1276. Monillioid mycelium and budding and inflated cells forming the basis of the colony: N. 1221. R. CBS 124664. Yeast stage: T. CBS 124663. Bars: D, K, R, S = 5 µm; H–J, L–Q, T–V = 10 µm.

(FIG. 4Q, S, V) or of single or several inflated globose cells (FIG. 4M); penicillus 30–60 µm, terverticillate or quaterverticillate, rarely more branched, symmetric or asymmetric, rami (1. branch) 15–35 × 2–3 µm, metulae (last branch) 9–11 × 2–2.5 µm, phialides 8–15 × 2–2.5 µm, 3–6 per cluster; conidia narrowly cylindrical to ellipsoid (4.0–)4.5–6(–8) × (1.5–)2(–2.5) µm, in persistent chains up to 200 µm long, conidial chains tangled, not parallel and forming a compact crust; synanamorph with conidial heads on acremonium-like conidiophores present (substrate conidia according to Kolařík et al. 2004), cylindrical to ellipsoidal with truncate base, 8–15 × 2–4 µm.

Intraspecific variability. The above description is a consensus of all strains. Intraspecific variability included differences in conidium and phialide size and shape and colony growth rate (FIG 2, TABLE I). Conidial sizes (arithmetic mean) varied from 5 × 2 µm (with phialides 8–10 × 2 µm) in CBS 124664 to 5.6 × 2.35 µm (with phialides 9.5–15 × 2.5 µm) in 1276 (FIG. 4I). Size of conidia also varied within a single strain (e.g. in strain 1260 chains of conidia 5.0 µm or 6.5 µm diam were observed.

Differential characters. The species exhibits three remarkable differential characters. It is unable to grow on CDA that is otherwise characteristic for *Geosmithia* associated with bark beetles infesting trees from family Pinaceae (unpubl) but is unique among *Geosmithia* from hardwoods. The presence of growth at 37 C and thermotolerance is an unusual character typically found only in red-spored *G. lavendula* and its relative *Geosmithia* sp. 19 (Kolarik et al. 2007). The base of the condiophores often are atypically branched or monilioid.

G. morbida is similar in colony color and micromorphology to *G. flava* and the unnamed *Geosmithia* sp. 13 (Kolařík et al. 2008) but is easily distinguished from these species based on ITS rDNA data. These species occur in the same geographic region as *G. morbida* but have different hosts. *Geosmithia* sp. 13 has similar yellowish colonies on MEA with a lobate margin and identical arrangement of conidial chains but differs by its slower growth (MEA, 20–25 mm, 14 d at 25 C). Colonies of *G. flava* tend to have regular margins and abundant sporulation with parallel conidial chains forming a deep and compact crust.

DISCUSSION

G. morbida is presented here as a genetically variable but morphologically and ecologically homogenous species clearly separated from other species. This is the first report of a phytopathogenic species in this genus and in the Bionectriaceae. Members of this family typically are fungicolous, myxomyceticolous, coprophilous or saprotrophic on plant material (Schroers 2000).

G. morbida has been isolated only from *P. juglandis* or from necrotic phloem associated with *P. juglandis* feeding on *Juglans* species in western USA. *G. morbida* was not isolated from 33 species of subcortical insects associated with 40 plant species representing all main tree hosts and *Geosmithia* insect vectors in the same area (CA, CO) during a 2009 survey (unpubl data). The host range of *G. morbida* thus is limited to *P. juglandis* (or to other nonstudied bark and wood boring insects associated with *Juglans*).

Variability in the ITS rDNA sequence in *Geosmithia* is species dependent and cannot be used as the sole criterion for species identification. An extreme example of minute variability in rDNA between species is *G. microcorthyli* and *Geosmithia* sp. 8. These species can be clearly distinguished based on morphology, host range, distribution, β-tubulin or TEF-1α sequences, but they have identical ITS rDNA sequences (Kolařík and Kirkendall 2010). In contrast a comprehensive analysis of *G. lavendula* populations from Eurasia and Africa revealed seven haplotypes and 4.3% variation in ITS rDNA sequence (unpubl). Similarly *G. morbida* has 1% variability in rDNA and at least eight haplotypes. These haplotypes are not correlated with phenotypic characters, geographic origin of the isolates or their plant hosts. The apparent complex genetic structure of *Geosmithia* suggests that its presence in western USA was unlikely the result of recent introduction and that fungus and beetle might have been established for some time outside the range reported by Wood and Bright (1992). More detailed population genetics study supported by multilocus data and detailed sampling should elucidate the evolutionary history of this fungus.

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