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Penicillium thiersii, *Penicillium angulare* and *Penicillium decaturense*, new species isolated from wood-decay fungi in North America and their phylogenetic placement from multilocus DNA sequence analysis

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Abstract: We describe three new fungicolous species on the basis of phenotypic and phylogenetic differences from known species. *Penicillium thiersii, P. angulare* and *Penicillium decaturense* are described. *Penicillium thiersii* phenotypically is identified on the basis of several characteristics including growth rates, vesicle size and condium shape and roughening. *Penicillium angulare* is related most closely to *P. adametzioides* but differs from it by restricted growth rates and conidiophores greater than 60 μm in length. *Penicillium decaturense* is related most closely to *P. miczynskii* but differs from that species by growth rate, minimum growth temperature and pigment production on MEA. Multilocus phylogenetic analysis confirmed the genetic distinctiveness of *P. decaturense* and the closely related species *P. miczynskii, P. chrzaszczii* and *P. manginii*. *Penicillium rivolii* is a synonym of *P. waksmanii* on the basis of this analysis. Analysis of the EF-1α gene shows rapid changes of position, number and length of introns between the species, suggesting a recent evolutionary origin for the introns.

Key words: bioactivity, calmodulin, fungi, fungicolous, introns, ITS, natural products, rDNA, systematics, translation elongation factor 1-alpha

INTRODUCTION

In a survey of fungicolous fungi for novel bioactive metabolites (Wicklow et al 1998, Holler et al 2002), we isolated numerous cultures of *Penicillium* as colonists of the sporocarps of wood decay fungi (ascomycetes and basidiomycetes) that were collected for this study by Dr Harry D. Thiers, Dr Bruce W. Horn and one of us (DTW). Many of these isolates could not be identified satisfactorily using the available *Penicillium* monographs (Raper and Thom 1949, Pitt 1980, Ramirez 1982). In an attempt to identify these taxa we sequenced the ITS and ca. 650 nucleotides of the large subunit rDNA (the combined sequences are termed the ID region) for comparisons to homologous sequences obtained from ex-type *Penicillium* cultures. Some isolates were so different from known species that the ID sequence alone strongly suggested that they were new taxa. For other isolates, genes encoding translation elongation factor 1-alpha (EF-1α) and calmodulin (CAL) also were sequenced because there were few differences from known species in the ID region.

Among these cultures were three putative new species of particular interest because they are the sources of a variety of novel bioactive compounds (Li et al 2002, 2003; Zhang et al 2003). In the present study, we provide descriptions of these new *Penicillium* species.

MATERIALS AND METHODS

Isolation of cultures.—Collections of wood decay fungi, with portions of the woody substratum on which fungal sporocarps had formed were placed in individual plastic bags and stored frozen (−7 C) until they could be processed. To isolate microfungal colonists, sporocarp surfaces, including stromata (Xylariales) and basidiomata of polypores (Polyporaceae) were abraded gently using a sterilized fingernail file. The filings of stromata or polypore tissues were plated directly onto the surface of dextrose-peptone yeast-extract agar (DPYA) containing streptomycin (25 mg/L) and tetracycline (1.25 mg/L) (Papavizas and Davey 1959). Plates were incubated in the dark at 25 C for 5 d and cultures representing each colony type, showing a distinctive morphology on DPYA, were isolated as slant cultures on potato-dextrose agar (PDA, Difco). After 7–12 d incubation, the tube cultures were segregated into groups of presumptive taxa, examined for cultural purity and maintained for identification. Cultures of *Penicillium* then were subcultured onto slant cultures of Czapek's agar (CZA, Difco). After 10–14 d incubation the tube cultures were segregated further

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into presumptive taxon groupings. A maximum of eight representatives were kept for each taxon.

A culture of *Penicillium* was isolated in Sep 2001 from the shell (endocarp) of a mature fallen walnut fruit (*Juglans* sp.) collected in Peoria, Illinois, and it had DNA sequences matching one of the new *Penicillium* species and therefore was included in this study. We compiled the geographic and substratum origin of the isolates, NRRL accession numbers for the cultures and GenBank accession numbers for the ID region DNA sequences derived from them (TABLE I). These cultures are preserved permanently in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois.

Growth media and conditions and DNA isolation.—Cultures were grown on Czapek's agar with yeast extract (CYA), malt-extract agar (MEA) and 25% glycerol agar (G25N). Preparation of these media is detailed by Pitt (1980). Morphological and physiological observations were made on cultures that had been grown 7 d at 5 C, 25 C or 37 C in the dark (Pitt 1980). The color names are from Ridgway (1912) and are cited by plate number. Statistical treatment of the data was performed using Microsoft Excel.

For DNA isolation, mycelium was scraped from a 7–10 d old culture grown on an agar slant in a test tube (Peterson et al 2003). The mycelium was placed in a screw-cap tube with buffer and glass beads, and the mycelium was broken by vortex mixing. Proteins were extracted with phenol/chloroform (1 g/mL), nucleic acids were precipitated with ethanol, and the final purification of the DNA was by adsorption to a silica matrix in the presence of a chaotropic agent (GeneClean, Qbiogene, La Jolla, California) as described by the manufacturer.

The ITS-lsu rDNA fragment was amplified using published PCR techniques (White et al 1990, Peterson 2000).

Calmodulin (CAL) fragments were amplified as detailed by Peterson et al (2001) with the universal 3′ primer CF4 (5′–tttYtgcatcatRagYtggac) and a series of 5′ primers were developed and used for different taxa, CF1B (5′–gccgactcttgactgaa), CF1C (5′–gaagaacaggtctccgag), CF1D (5′–caggtctccgagtacaag). CFM (5′–gacaaggatggcgatggt) and CFMR (5′–accatcgccatccttgtc) were used as internal primers for sequencing.

Translation elongation factor 1-alpha (EF-1α) fragments were amplified using the universal 3′ primer EF6 (5′–cttStYccaRcccttgtacca) and 5′ primers EF1c (5′–tcgtcgttatcggccacgtc) and EF1d (5′–ggccacgtcgattccgg. EFM (5′–tggaaRggYcaRacNgc) and EFMr (5′–gcNgtYtgRaaYttcca) were used as internal sequencing primers.

The reaction buffer was described by White et al (1990) and the thermal profile was 96 C for 2 min followed by 42 cycles of 96 C for 30 s, 51 C for 30 s, 72 C for 90 s and a final elongation reaction of 5 min at 72 C. Amplified fragments were purified using the Millipore Multiscreen PCR system as detailed by the manufacturer (Millipore, Billerica, Massachusetts). Purified fragments were sequenced with the terminal primers used in amplification plus the internal primers noted and fluorescent dye labeled dideoxy nucleotide terminators in the Applied Biosystem Dye-deoxy sequencing kits. Sequences were read on an Applied Biosystems model 377, 3100 or 3730 DNA sequencer. The sequencing procedures are performed in accordance with the manufacturer's instructions.

DNA sequence analysis.—Sequences were rough aligned using Clustal V (Thompson et al 1994) and a text editor was used to visually optimize the Clustal V alignment. Maximum parsimony trees were calculated using PAUP* (Swofford 1998) in heuristic search with random addition order (10 replications). Bootstrap values were determined using PAUP* heuristic search in 1000 replicates. Tree diagrams were viewed using TreeView (Page 1996) and redrawn for publication using CorelDraw 9.0.

Scanning electron microscopy.—SEM samples were prepared by OsO_4 fixation overnight, dehydration in increasingly concentrated acetone, critical-point drying and sputter coating with gold/palladium (Peterson 1992). Specimens were examined and images digitally recorded using a JEOL scanning electron microscope. Photographs were taken using a Kodak 420B digital camera. Microphotographs were made using the Kodak 420 B digital camera attached to a Zeiss axioscope with phase-contrast or DIC illumination from samples teased apart and mounted in 0.5% Kodak photoflo. Photographs and SEM images were sized and fitted into composite figures using Photoshop 6.0.1.

RESULTS

DNA sequences (ITS and lsu-rDNA) from the putative new species were compared to those in GenBank and to a number of additional sequences derived from ex type cultures of *Penicillium* species (S.W. Peterson unpubl) using BLAST (Altschul et al 1997) in a local implementation. The sequences from NRRL 28147, NRRL 28162 and NRRL 31609 were identical and differed at about 5% of the nucleotide positions from the most closely related *Penicillium* species. Sequences from NRRL 28140 and NRRL 28157 were identical and differed from the sequence of *P. adametzioides* at about 1% of the nucleotide positions. Sequences of NRRL 28119, NRRL 28152 and NRRL 28160 were identical and differed from that of NRRL 1077, the ex-type culture of *P. miczynskii* at less than about 1% of nucleotide positions. A parsimony tree was calculated that included *Penicillium* species in GenBank plus some unpublished sequences (more than 160 taxa not shown here). Strict consensus of the 25 000 trees generated in that analysis showed that NRRL 28157 always occurred on the branch with *P. adametzioides,* that NRRL 28160 always branched next to *P. miczynskii,* and that NRRL 28147 branched deeply in the tree and these data provided no consensus on its location relative to other species.

A subset of 48 ID region sequences, including representatives of each *Penicillium* phylogenetic group (Peterson 2000), were analyzed using the maximum parsimony criterion (FIG. 1). While many branches

TABLE I. Origin of fungi used in this study

Species	NRRL number	Geographic origin and substratum of isolates	GenBank accession numbers for ID region
Penicillium thiersii	28147	UNITED STATES, WISCONSIN, from a *Hypoxylon* stroma. Ex type.	AY313611
Penicillium thiersii	28162	UNITED STATES. WISCONSIN, from a *Hypoxylon* stroma.	AF125936
Penicillium thiersii	31609	UNITED STATES. ILLINOIS, from a walnut shell (*Juglans* sp.).	AY313612
Penicillium angulare	28157	UNITED STATES. NEW MEXICO, from an old polypore. Ex type.	AF125937
Penicillium angulare	28140	UNITED STATES. NEW MEXICO, from an old polypore.	AY313613
Penicillium decaturense	28152	UNITED STATES, ILLINOIS, from an old resupinate fungus. Ex type.	AF125946
Penicillium decaturense	28119	UNITED STATES. From a wood decaying fungus	AY313614
Penicillium decaturense	28160	UNITED STATES. ILLINOIS, from *Ischnoderma* sp.	AY313615
Penicillium decaturense	29675	UNITED STATES. GEORGIA, from *Trichaptum biformis*.	AY313616
Penicillium decaturense	29708	UNITED STATES. GEORGIA, from a basidiomycete	AY313617
Penicillium decaturense	29807	UNITED STATES. FLORIDA, from a pyrenomycete stroma.	AY313618
Penicillium decaturense	29828	UNITED STATES. FLORIDA, from *Trichaptum biformis*.	AY313620
Penicillium decaturense	29840	UNITED STATES. FLORIDA, from a polypore fruiting body.	AY313619
Penicillium miczynskii Zaleski	1077	POLAND. From forest soil. Ex type.	AF033416
Penicillium miczynskii	28096	UNITED STATES. NEW MEXICO, from a dead polypore.	AY443453
Penicillium waksmanii Zaleski	777	POLAND. From forest soil. Ex type	AF033417
Penicillium waksmanii	28095	UNITED STATES. NEW MEXICO, from a dead polypore.	AY443452
Penicillium sp.	29686	UNITED STATES. GEORGIA, from an old *Trametes* sp. fruit body.	AY443459
Penicillium sp.	29736	UNITED STATES. GEORGIA, from an old *Collybia* sp. fruit body.	AY443461
Penicillium aculeatum Raper & Fennell	2129	UNITED STATES. FLORIDA, from type C bisquits. Ex type.	AF033397
Penicillium adametzioides Abe Ex G. Smith	3405	JAPAN. From soil. Ex type.	AF033403
Penicillium atramentosum Thom	795	FRANCE. From camembert cheese. Ex type.	AF033483
Penicillium bilaiae Chalabuda	3391	UKRAINE. From soil. Ex type.	AF033402
Penicillium brocae Peterson, Perez, Vega & Infante	31472	MEXICO. CHIAPAS, from insect frass. Ex type.	AF484396
Penicillium canescens Sopp	910	ENGLAND. From soil. Ex neotype.	AF033493
Penicillium capsulatum Raper & Fennell	2056	PANAMA. From a camera lens. Ex type.	AF033429
Penicillium chrzaszczii Zaleski	903	POLAND. From forest soil. Ex type.	AY313621
Penicillium citrinum Thom	1841	From G. Smith, London as P 6, Biourge's type of *P. aurifluum*. Ex type.	AF033422
Penicillium corylophilum Dierksx	802	From Biourge. Ex neotype.	AF033450
Penicillium cyaneum (Bainier & Sartory) Biourge	775	FRANCE. From Bainier collection. Ex type.	AF033427
Penicillium decumbens C. Thom	741	UNITED STATES. FLORIDA. From Biourge. Ex type.	AF033453
Penicillium herquei Bainier & Sartory	1040	FRANCE. From leaf of *Agauria pyrifolia*. Ex type.	AF033405
Penicillium jensenii Zaleski	909	POLAND. From forest soil. Ex type.	AY443470
Penicillium kojigenum Abe Ex G. Smith	3442	SCOTLAND. From soil. Ex type.	AF033489
Penicillium lividum Westling	754	SCOTLAND. From soil. Ex neotype.	AF033406
Penicillium manginii Duche & R. Heim	2134	From soil by Duche. Ex type.	AY443469

TABLE I. Continued

Species	NRRL number	Geographic origin and substratum of isolates	GenBank accession numbers for ID region
Penicillium melinii C. Thom	2041	UNITED STATES. From soil. Ex neotype.	AF033449
Penicillium paxilli Bainier	2008	PANAMA. From photographic film. Ex neotype.	AF033426
Penicillium quercetorum Baghdadi	3758	SYRIA. From Soil. Ex type.	AY443471
Penicillium raperi G. Smith	2674	UNITED KINGDOM. From cultivated soil. Ex type.	AF033433
Penicillium restrictum Gilman & Abbott	1748	HONDURAS. From soil. Ex type.	AF033457
Penicillium rivolii Zaleski	906	POLAND. From forest soil. Ex type.	AF033419
Penicillium rolfsii C. Thom	1078	UNITED STATES. FLORIDA, From pineapple. Ex type.	AF033439
Penicillium sclerotiorum J.F.H. Beyma	2074	INDONESIA. From air. Ex type.	AF033404
Penicillium soppii Zaleski	2023	POLAND. From forest soil. Ex type.	AF033488
Penicillium spinulosum Thom	1750	GERMANY. From culture contaminant. Ex type.	AF033410
Penicillium thomii Maire	2077	UNITED STATES. From a pine-cone. Ex neotype.	AF034448
Penicillium vinaceum Gilman & Abbott	739	UNITED STATES. UTAH, from soil. Ex type.	AF033461

were supported in more than 90% of the bootstrap samples, the more basal branches of the tree had only weak statistical support. Additional sequences from CAL and EF-1α were obtained for the species most closely related to *P. miczynskii* to compare with the ID region tree.

The PCR amplified CAL fragment included amino acid codons 9–148 (total protein consists of 149 amino acids). Introns were inserted after codon 20, at codon 26 between nucleotides 1 and 2, at codon 68 between nucleotides 1 and 2, and at codon 139 between nucleotides 1 and 2, positions identical to those found in the CAL gene of *A. oryzae*. Intron sequences were aligned easily for the ingroup with small length differences, and while some similarity to outgroup was observed, length differences were more pronounced between ingroup and outgroup (up to 50% length difference). The protein coding region was aligned with no length differences. Nucleic acid sequences were used to predict amino acid sequences of the proteins. The ingroup amino acid sequences were identical, but the proteins predicted for *P. jensenii* and *P. rolfsii* showed two amino acid differences from the ingroup.

The amplified fragment of EF-1α included amino acid codons 15–215 of the 460 AA protein. Introns were inferred with insertion points, at codon 26 between nucleotides 1 and 2; after codon 28; after codon 45; after codon 56; at codon 92 between nucleotides 1 and 2; after codon 132, and after codon 133. Intron lengths varied from 56 to 108 at the different positions, with length differences of 20–30% where more than one species possessed the intron (TABLE II). EF-1α from *A. oryzae* has introns at codons 45 and 92 only. The protein coding region was aligned with no length differences, and nucleic acid sequences were used to predict amino acid sequences of the proteins. There were amino acid changes at 11 positions, with most changes occurring in outgroup species relative to ingroup. Intron sequences were used in BLAST searches of the GenBank nucleic acid databases, but no significant homology was found to reverse transcriptases or any other genes.

Heuristic search of the CAL data produced more than 100 equally parsimonious trees of length 607. The strict consensus of those trees is indicated by bold lines in the tree diagram (FIG. 2). Heuristic search of the EF-1α data produced more than 100 equally parsimonious trees and the strict consensus of those trees is indicated by bold lines (FIG. 3). Bootstrap values are on the tree branches.

TAXONOMY

Penicillium thiersii SW Peterson, EM Bayer & DT Wicklow, sp. nov. FIGS. 4, 7–12

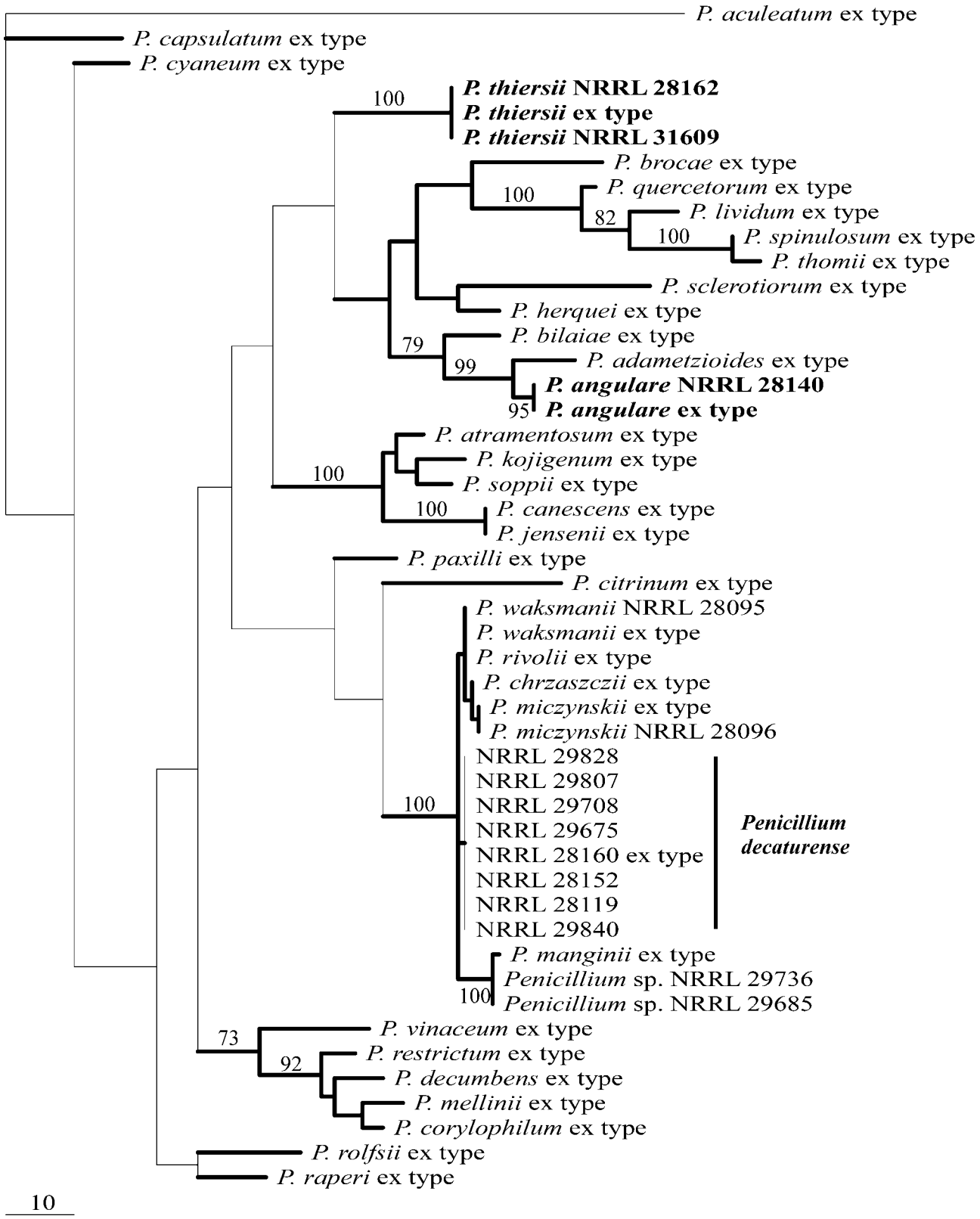


FIG. 1. Phylogram calculated using ID region data and parsimony criterion in PAUP*. Dataset composed of 1180 characters, of which 126 were excluded from analysis because of indels, 806 were constant, 82 were parsimony noninformative and 166 were parsimony informative. The search produced 20 equally parsimonious trees of 579 steps, with CI = 0.5475 and RC = 0.4324. The bootstrap values are placed on the branches of one of the maximum parsimony trees. *Penicillium angulare* is most closely related to *P. adametzioides* and *P. bilaiae,* the relationship of *Penicillium thiersii* to other *Penicillium* species is

TABLE II. Location of introns in EF-1α. Numbering of introns was explained in text. Intron 56 is shared by the ingroup but not outgroup species, intron 133 is shared by the *P. waksmanii* group. Introns 26, 45, and 92 are unique to the outgroup species

Species	26(½)	28+	45+	56+	92(½)	132+	133+
A. oryzae	—	−	+	−	+	−	—
P. rolfsii	+	—	+	—	+	—	—
P. jensenii	−	−	+	−	+	−	—
P. manginii	—	+	—	+	—	—	—
Penicillium sp.	—	+	—	+	—	—	—
P. waksmanii	—	—	—	+	—	—	+
P. rivolii	—	—	—	+	—	—	+
P. chrzaszczii	—	—	—	+	—	—	+
P. miczynskii	—	—	—	+	—	+	—
P. decaturense	—	—	—	+	—	—	—
P. sclerotiorum	+	—	—	—	—	—	—

Coloniae velutinosae, radialiter sulcatae, barium-flavae in CYA; velutinosae, glauco-griseae in MEA; color faciei aversae primulino-flavus ad ochraceus in CYA, olivaceo-flavus in MEA, moderatim bene crescentes. Conidiophora monoverticillata, vesiculata, 250–500 × 3–4 μm, vesicula 7–10 μm cum verticillis phialidum ampulliformium 8 ad 12, 9–12 × 2.0–3.0 μm, producentia conidia levia et elliptica 3–4 × 2.0–2.5 μm.

Colonies (FIG. 4) grown 7 d on CYA at 25 C 37–42 mm diam, consisting of a closely woven felt of hyphae, thin (ca. 1 mm), velutinous, radially sulcate, pinard yellow (R-IV) to barium yellow (R-XVI), colony margin on the surface, a 1–2 mm peripheral white band of hyphae, moderate amounts of clear exudate present, no soluble pigments produced, sporulation moderate, colony reverse primuline yellow (R-XVI) marginally to yellow ocher (R-XV) centrally. Conidiophores scattered through the colony 250–500 × 3–4 μm, arising from basal hyphae, and slightly roughened near the apex terminating in a 7–10 μm diam vesicle (X = 7.61 n = 15, FIGS. 7–9). Penicillus (FIGS. 7–10) monoverticillate bearing 8–12 ampulliform, 9–12 × 2.0–3.0 μm phialides (X = 11 ± 1.6 × 2.9 ± 0.3 n = 18, FIG. 10). Conidia (FIGS. 11, 12) are ellipsoidal, 3–4 × 2.0–2.5 μm (X = 3.6 ± 0.4 × 2.5 ± 0.3 n = 18), smooth in light microscopy (FIG. 11).

Colonies grown 7 d on MEA at 25 C 37–40 mm diam, velutinous to lanose, colored dark glaucous gray (R-XLVIII), marginal ring white 3–4 mm, moderate to heavy sporulation, exudate and soluble pigments absent, colony reverse olive yellow (R-XXX) to yellowish citrine (R-XVI). Conidiophores, phialides, conidia and other microscopic features as described from CYA.

No growth or germination of conidia on CYA at either 5 C or 37 C. On G25N, colonies 14–15 mm diam after 7 d at 25 C.

Etymology. Named in honor of Professor Harry D. Thiers.

HOLOTYPE. BPI 842269 here designated. Dried colony of NRRL 28147 grown 7 d at 25 C on MEA and CYA.

Cultures examined.—UNITED STATES. ILLINOIS: Peoria, Galena Road, ca. 40°44′N, 89°35′W, isolated from the shell of a mature, fallen walnut fruit (*Juglans* sp.) collected Sep 2001, *J.J. Scoby* (Culture NRRL 31609). WISCONSIN: New Glarus, New Glarus Woods State Park, ca. 42°48′N, 89°38′W, isolated as "Myc-500 *Penicillium* sp." from an old, black stroma of *Hypoxylon* encrusting the surface of a dead maple log (*Acer saccharum* Marsh.) collected 21 Aug 1996, *H.D.Thiers No. 55623* (Culture NRRL 28147 ex type); culture NRRL 28162, a second isolate from the same stroma as above.

Commentary.—*P. thiersii* colonies grown on CYA tend to develop sporulation tardily, and this might be the cause of the bright yellow colony color. After 14 d growth, colonies of NRRL 28147 display moderately heavy sporulation in the marginal 30% of the colonies in bluish-green.

On the basis of long vesiculate stipes and rugose conidial walls, *P. thiersii* fits into ser. *Glabra* (Pitt 1980). Green conidia, in mass, and ellipsoidal, ru-

←

not established by the current dataset. *Penicillium decaturense* forms a strongly supported clade with *P. miczynskii*. Treebase accession number M2076-8.

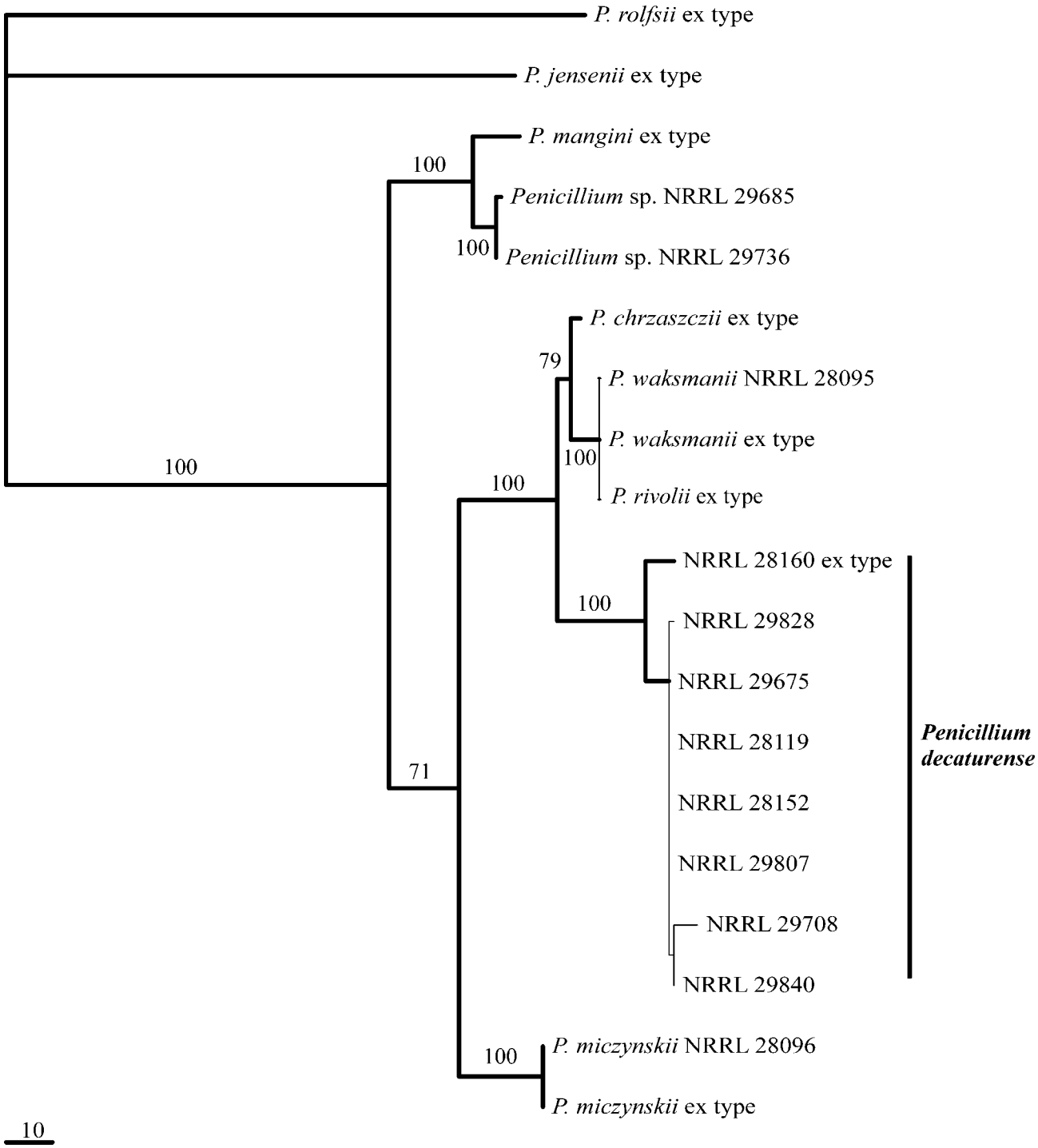


FIG. 2. One of more than 100 equally most parsimonious trees of 451 steps derived from the calmodulin dataset. The data included 136 phylogenetically informative sites. The consistency index was 0.8936, and the recalculated consistency index was 0.7947. Bootstrap percentages above 70 are indicated on the tree. Treebase M2076-8. GenBank No. AY443472–AY443490.

gose conidia suggest *P. thomii* (Pitt 1980). Arguing against inclusion of these isolates in *P. thomii* are the CYA growth rates (*P. thomii* 40–60 mm, *P. thiersii* 38–42 mm), G25N growth rates (*P. thomii* 20–24 mm, *P. thiersii* 14–16 mm), vesicle size (*P. thomii* 4–6 μm, *P. thiersii* 7–10 μm diam) and the failure of *P. thiersii* to form the pinkish sclerotia commonly found in *P. thomii* isolates.

On the basis of no sclerotium production, velutinous colonies and ellipsoidal conidia, *P. thiersii* fits

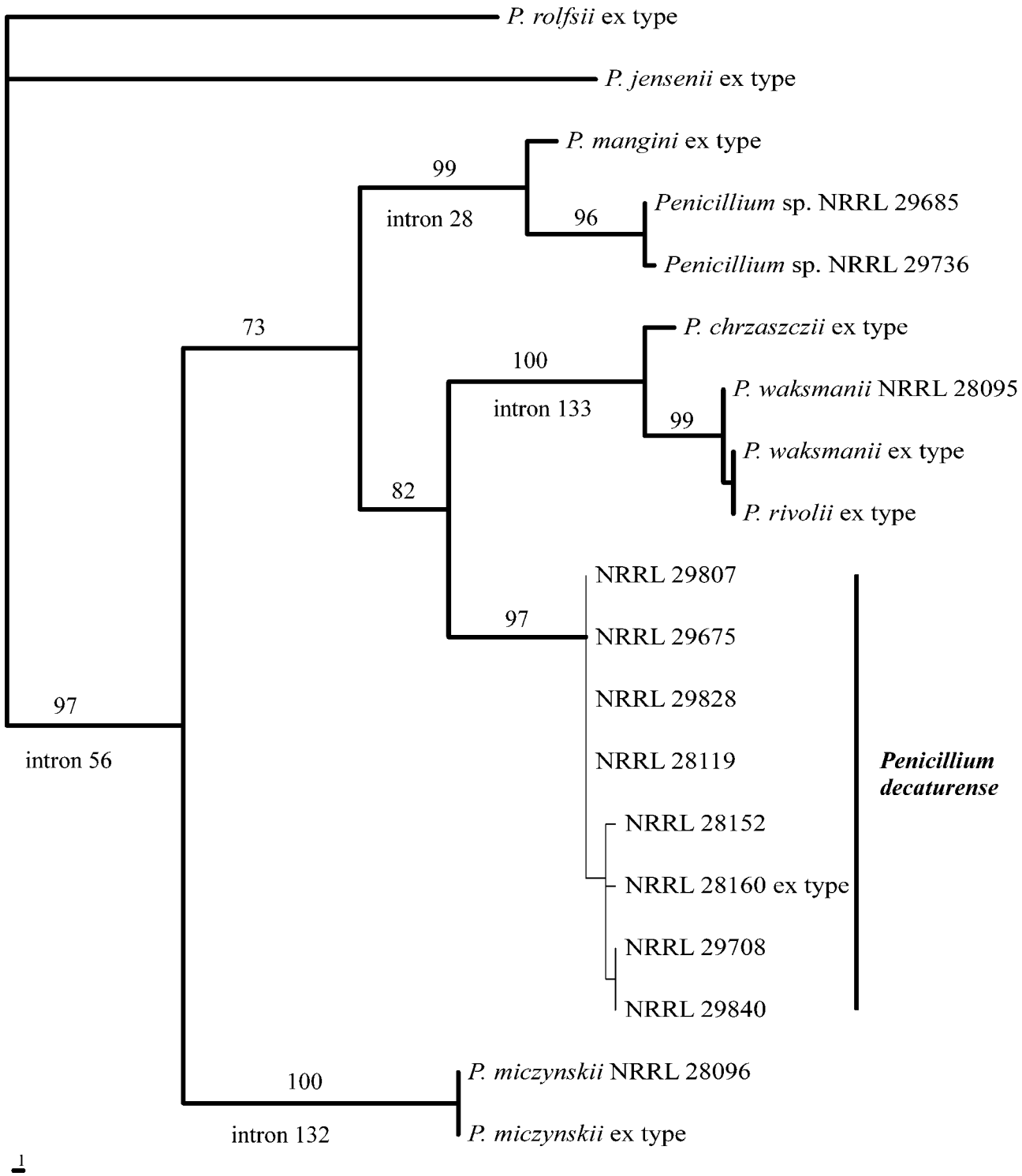
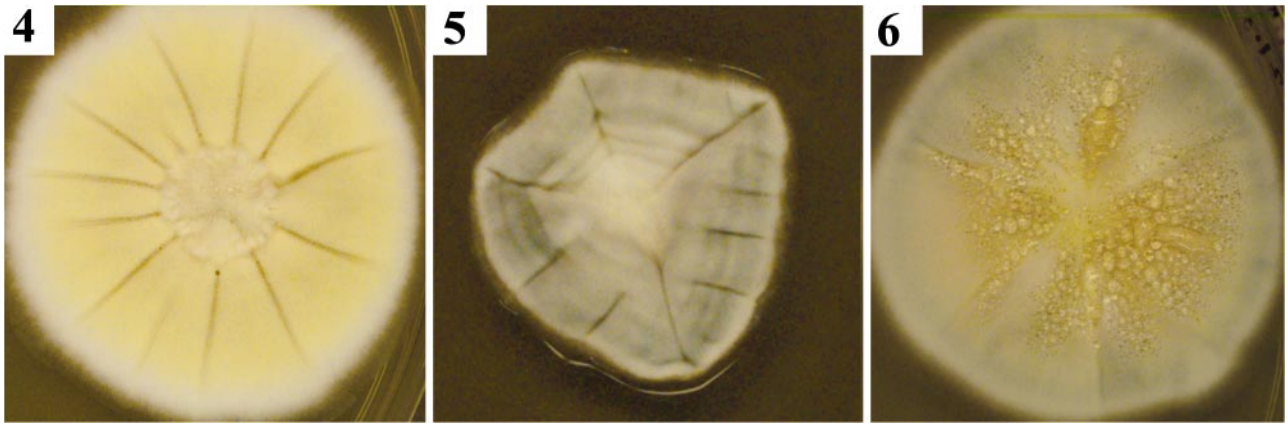
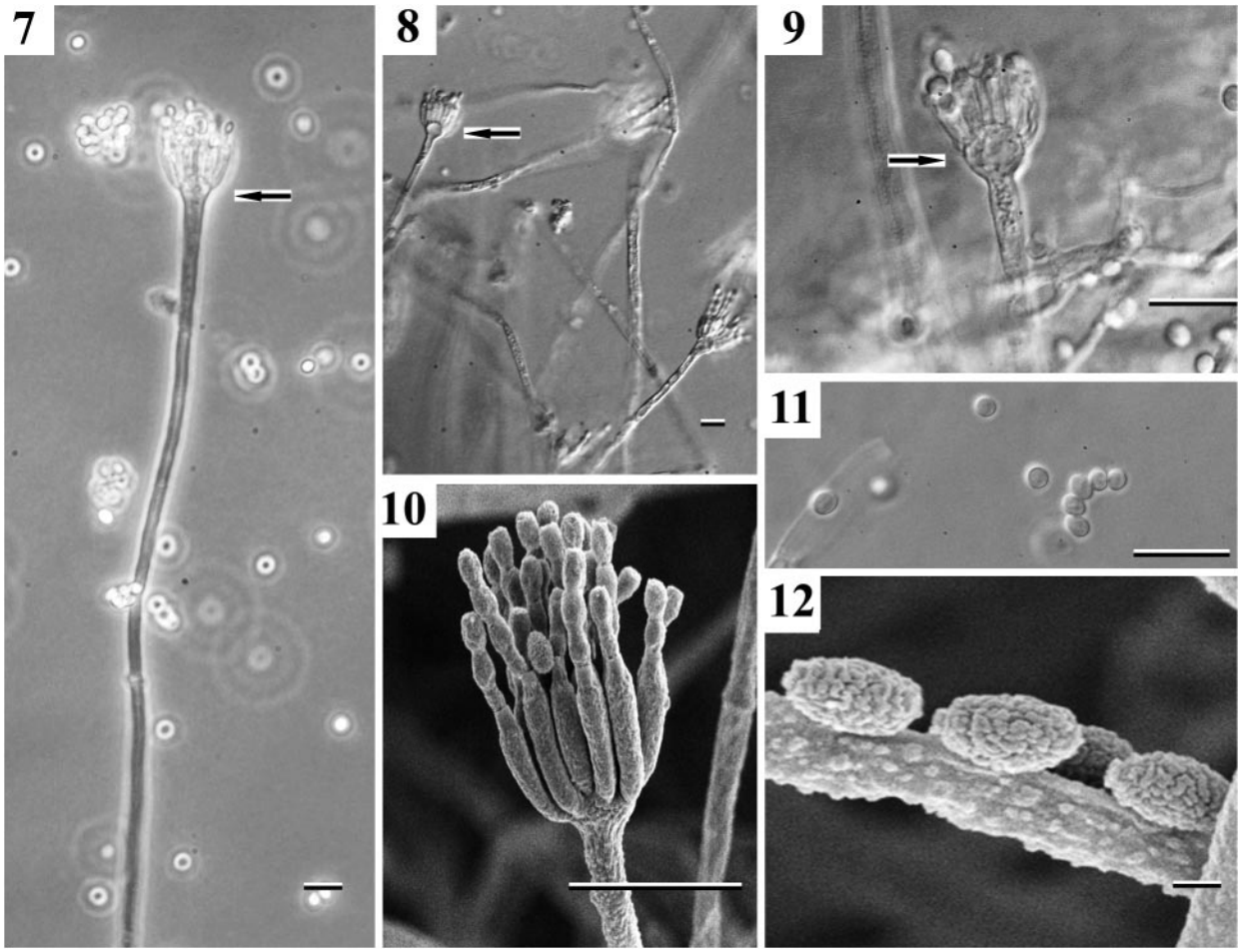


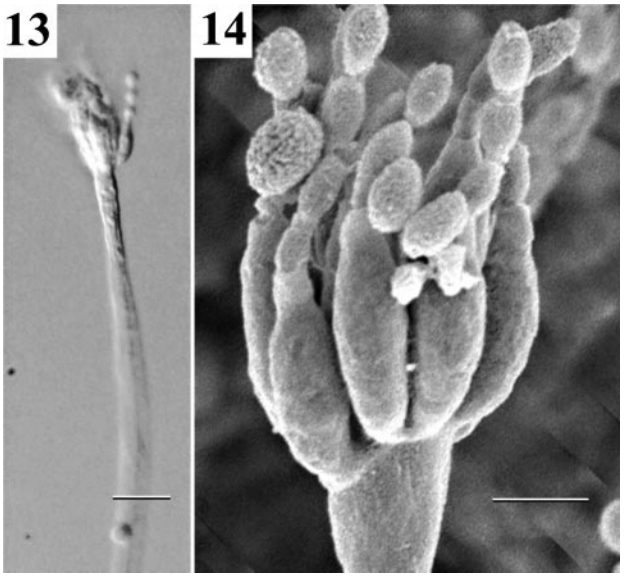
FIG. 3. One of more than 100 equally most parsimonious trees of 267 steps from the EF-1α dataset. The data included 106 phylogenetically informative sites. The consistency index was 0.8839, and the recalculated consistency index was 0.7919. Bootstrap percentages above 70 are indicated on the tree. The presence of introns is indicated, with all isolates to the right of the intron listing possessing the particular intron. Intron 56 is common to the ingroup while other introns are unique to more terminal clades. Treebase M2076-8. GenBank No. AY443450–AY443468.



FIGS. 4–6. *Penicillium* species grown on CYA 7 d. 4. *P. thiersii* NRRL 28147 showing the radial sulcations and bright yellow colony. 5. *P. angulare* NRRL 28157 showing the angular outline and sulcate centrally sunken colony. 6. *P. decaturense* NRRL 28152 showing radial sulcation, light grayish blue conidial coloration and clear to yellow exudate on the colony.



FIGS. 7–12. *Penicillium thiersii* NRRL 28147. 7–9, light micrographs using phase contrast (FIG. 7) or DIC. In each figure, the large bulbous vesicle subtending the conidiogenous cells is indicated by arrows. 10. SEM photograph of penicillus showing long narrow phialides and elongate conidia. 11. Light micrograph using DIC contrast showing conidia in aqueous mounting medium. 12. SEM view of conidia. Shrinkage of the samples during drying might have exaggerated the elliptical shape of the conidia and the ornamentation, which are not as pronounced in FIG. 11. Scale: bar = 10 μm in FIGS. 7–11, bar = 1 μm in FIG. 12.



FIGS. 13, 14. *Penicillium angulare* NRRL 28157. 13. Light micrograph of a penicillus using DIC. The conidiophore terminates with no noticeable vesicle. 14. SEM view of penicillus showing the smooth non-vesiculate stipe, smooth ampuliform conidiogenous cells (phialides) and slightly roughened elliptical conidia. Scale: bar = 10 μm in FIG. 13, bar = 5 μm in FIG. 14.

into the *P. lividum* series (Ramirez 1982) and of the species in the series, most closely resembles *P. lividum*. However, *P. lividum* colonies are lanose and blue-green while those of *P. thiersii* are barium yellow and velutinous; *P. lividum* produces yellow soluble pigments, *P. thiersii* does not; *P. lividum* produces smaller vesicles (5–6 μm) than *P. thiersii*; the phialides are larger in *P. lividum* 11–16 × 2.5–4 μm versus 9–12 × 2–3 μm; and conidia of *P. lividum* are roughened, 4–5 × 3–4 μm while those of *P. thiersii* are ellipsoidal 3–4 × 2.0 × 2.5 μm and smooth in light microscopy.

Analysis shows that *P. thiersii* phylogenetically is distinct from any of the species that it phenotypically resembles.

Penicillium angulare SW Peterson, EM Bayer & DT Wicklow, sp. nov. FIGS. 5, 13, 14

Coloniae celandinae, velutinosae et sulcatae, polygonales et in media parte depressae in CYA, gnaphalium-griseae et velutinosae in MEA, color faciei aversae baryta-flavus in CYA, Neapolitano-flavus in MEA, incrementum lentum. Conidiophora monoverticillata, non vesiculata, levia, 100–150 × 3–4 μm cum verticillis phialidum ampulliformium 6 ad 10, 8–10 × 2–3 μm, producentia conidia levia et elliptica ad 3.5 × 2.5 μm.

Colonies (FIG. 5) grown 7 d on CYA at 25 C 13–18 mm diam, velutinous and sulcate, not distinctly colored, white with light celandine areas (R-XLVII), irregular to polygonal in shape and sunken centrally (FIG. 5), margin on the agar surface, small amounts of clear exudate present, soluble pigments absent, sporulation light, colony reverse baryta yellow (R-IV). Conidiophore smooth, nonvesiculate, measuring 100–150 × 3–4 μm, penicillus (FIG. 13) monoverticillate arising from basal hyphae, with phialides 8–10 × 2–3 μm (X = 8.5 ± 0.6 × 2.6 ± 0.3 n = 15), arranged in whorls of 6–10 bearing smooth ellipsoidal conidia up to 3.5 × 2.5 μm (X = 3.2 ± 0.4 × 2.4 ± 0.4 n = 13, FIG. 14).

Colonies grown on malt agar at 25 C for 7 d 9–15 mm diam, velutinous with heavy sporulation, gnaphalium gray (R-XLVII) with a 1 mm white margin on the agar surface. Exudate and soluble pigments absent, colony reverse Naples yellow (R-XVI). Microscopic features as described from CYA.

No growth or at most micro colony formation on CYA plates at 5 C. At 37 C, no growth or germination. Colonies at 25 C on G25N agar 10–12 mm diam.

Etymology. angulare refers to the angular colony appearance when grown on CYA.

HOLOTYPE. BPI 842268 here designated. Dried colony of NRRL 28157 grown 7 d at 25 C on MEA and CYA.

Cultures examined.—UNITED STATES. NEW MEXICO: Red River, Mount Wheeler Road, ca. 105°30′W, 36°35′N, isolated as "Myc-545 *Penicillium sp.*" from an old polypore found on a dead conifer stump collected 5 Sep 1996, *H.D. Thiers No. 55690* (Culture NRRL 28157, ex type); Mount Wheeler Road, ca. 105°30′W, 36°35′N, isolated as "Myc- 424 *Penicillium sp.*" from an old polypore found on a dead conifer stump collected 5 Sep 1996, *H.D. Thiers No. 55686* (Culture NRRL 28140).

Commentary.—*Penicillium angulare* is a species of sect. *Exilicaulis* (Pitt 1980) but differs from all species of the section in its slow growth (<25 mm) and long (>60 μm) condiophores.

Following Ramirez (1982) this isolate can be identified only as *P. hispanicum* Ramirez et al, because of the colony growth rate and monoverticillate penicillus. However, there are several differences between *P. angulare* and *P. hispanicum*. *Penicillium angulare* produces no soluble pigments, while *P. hispanicum* produces yellow-orange pigments that diffuse through the agar plate, and the colonies of *P. hispanicum* have an irregular margin, while the colonies of *P. angulare* have a smooth margin. *Penicillium angulare* produces conidiophores 100–150 × 3–4 μm with no noticeable vesicle at the apex, while conidiophores of *P. hispanicum* are up to 100 × 2–2.5 μm and terminate in an apical vesicle up to 7 μm diam. Conidia of *P. angulare* are ellipsoidal 3.5 × 2.5 μm

while the conidia of *P. hispanicum* are globose to subglobose 3–4 μm diam.

In phylogenetic analysis based on the ID sequence region (FIG. 1), *P. angulare* is most closely related to *P. adametzioides* Abe ex G. Smith but differs from that species in several ways. When grown on CYA, *P. adametzioides* colonies attain ca. 50 mm diam in 14 d, produce yellow soluble pigments and reverse color is orange, while *P. angulare* colonies attain 9–15 mm diam on CYA in 7 d, produce no soluble pigments and colony reverse color is bright yellow. Stipes of *P. adametzioides* rarely exceed 25 μm length and produce a 4.5 μm diam vesicle, while *P. angulare* stipes are 100–150 μm long and nonvesiculate. Phialides of *P. adametzioides* are 10–12 × 2.8–4.0 μm and produce smooth ellipsoidal, conidia 3–4.8 × 2.5–4.0 μm while *P. angulare* produces phialides 8–10 × 2–3 μm and smooth, ellipsoidal conida up to 3.5 × 2.5 μm. These species as well as *P. bilaiae, P. herquei, P. sclerotiorum* and *P. adametzii* occur on a strongly supported branch that has been referred to as group 3 (Peterson 2000). The very high bootstrap values strongly support the distinction of *P. angulare* from *P. adametzioides* and other species.

Penicillium decaturense SW Peterson, EM Bayer & DT Wicklow, sp. nov. FIGS. 6, 15–19

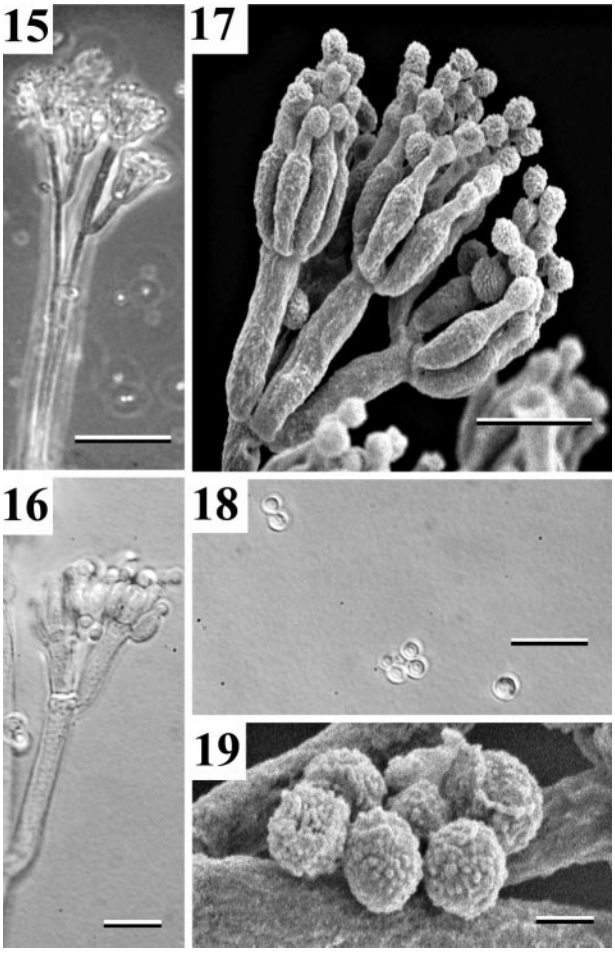
Coloniae velutinosae, sulcatae, glauco-griseae, producentes sudorem flavum in CYA, velutinosae, artemisia-virides ad saxeo-olivaceae in MEA, color faciei aversae primulino-flavus ad malum Armeniacum-luteus in CYA, pallide ad profunde uva-viridis in MEA. Conidiophora furcata, ad 150 × 2.5–3.0 μm, metulae 10–15(–20) × 2.5–3.0 μm producentes phialides ampulliformes 6–10.0 × 2.5 μm cum conidiis sphericis et levibus 2.0–2.5(–3.0) μm diam.

Colonies (FIG. 6) grown at 25 C for 7 d on CYA 31–37 mm diam, velutinous, sulcate, white and deep glaucous gray (R-XLVIII) to dawn gray (R-LII) with yellow to reddish shades centrally coming from exudate, margin on the agar surface, exudate yellow, soluble pigments absent, sporulation heavy, reverse primuline yellow (R-XVI) to apricot buff (XIV).

Colonies grown on malt agar 7 d at 25 C 26–27 mm diam, artemisia green to slate-olive (R-XLVII) margin 3 mm wide white, on the agar surface, exudate and soluble pigments absent, sporulation heavy, reverse pale to deep grape green (R-XLI), influenced by the surface color.

No growth or germination of conidia on CYA at 5 C or 37 C. On G25N at 25 C, colonies 15–17 mm diam in 7 d.

Conidiophores (FIGS. 15–17) up to 150 μm in length, smooth and arising from basal hyphae, diameter below metulae 2.5–3.0 μm, penicillus furcate (FIG. 15–17) with metulae 10–15(–20) × 2.5–4 μm



FIGS. 15–19. *Penicillium decaturense* NRRL 28152. 15, 16. Light micrographs of the penicillus structure using phase contrast or DIC illustrating the furcate penicillus. 17. SEM of a penicillus. 18. Conidia in aqueous mounting fluid showing smooth walled subglobose spores. 19. SEM view of conidia showing the subglobose to elliptical shape and lightly roughened outer wall. Scale: bar = 20 μm in FIG. 15, bar = 10 μm in FIGS. 16–18, bar = 1 μm in FIG. 19.

(X = 12 ± 2 × 3 ± 0.2 n = 90) and ampulliform phialides 6–10 × 2.5 μm (X = 7.6 ± 0.7 × 2.1 ± 0.2 n = 113). Conidia (FIGS. 18, 19) globose, smooth, 2.0–2.5(–3.0) μm diam (X = 2.13 ± 0.2 n = 115).

Etymology. The species name refers to Decatur, Illinois, collection site of the holotype.

HOLOTYPE. BPI 842267, here designated. Dried colony of NRRL 28152, grown 7 d on CYA and MEA.

Cultures examined.—UNITED STATES. Locality unknown, isolated 1997 from a wood-decaying fungus collected 1996, *H.D. Thiers* (Culture NRRL 28119). ILLINOIS: Decatur, Ramsey Lake State Park, ca. 39°10′N, 89°10′W, isolated 5 Jul 1997 from an old resupinate fungus collected 12 Aug 1996, *H.D. Thiers* (Culture NRRL 28152, ex type); Peoria, North Picture Ridge Road, ca 40°47′N, 89°35′W, isolated 7 Jul

1997 from an old basidiomata of *Ischnoderma* sp. found on a dead hardwood log, 5 Sep 1996, *H.D. Thiers* (Culture NRRL 28160). FLORIDA: Crawfordsville, Wakulla Springs State Park, dry cypress swamp ca. 30°10′N, 84°22′W, isolated 20 Jun 2000 from *Trichaptum biformis* on a dead hardwood branch collected 1 May 2000, *D.T. Wicklow* (Culture NRRL 29828); Blountstown, Torreya State Park mixed hardwood-cypress-pine forest ca 30°26′N, 85°3′W, isolated 28 Jun 2000 from a polypore found on a dead pine branch collected 2 May 2000, *D.T. Wicklow* (Culture NRRL 29840). GEORGIA: Albany, Chehaw Park, mixed hardwood-pine forest ca 31°34′N, 84°10′W, isolated 4 Jun 2000 from *Trichaptum biformis* found on a dead hardwood branch collected 29 Apr 2000, *B. W. Horn* (Culture NRRL 29675); Adel, Reed Bingham State Park, hardwood swamp area ca. 31°8′N, 83°25′W, isolate 14 Jun 2000 from a basidiomycete on dead hardwood collected 29 Apr 2000, *B.W. Horn* (Culture NRRL 29708). FLORIDA: Hickory Mounds, near Ecofina River and SR 98, Sabal palm swamp ca 30°8′N, 83°40′W, isolated 19 June 2000 from a pyrenomycete stroma on dead hardwood collected 1 May 2000 (*D.T. Wicklow*).

Commentary.—Colonies on CYA may show a more lanose than velutinous texture in some isolates, and the number of sulcations varies from 3 to 8 by isolate, but they are deep and distinct. Colony color is affected by the texture but is always in light bluish shades when sporulating. Colonies developing more slowly display a white to yellowish white color until conidia develop. Exudate may be present or absent in different isolates and when present may be clear to light yellow.

According to Pitt (1980) this species may be identified as *P. madriti* or as *P. miczynskii* but it is most similar to *P. miczynskii*. While *P. miczynskii* is listed (Pitt 1980) as conidiating only sparsely on CYA and MEA, *P. decaturense* sporulates heavily on both of these media. *P. decaturense* colony growth on CYA is quite similar to that of *P. miczynskii,* as stated by Pitt (1980). However, on MEA, soluble pigments are absent in *P. decaturense* as is the yellow-orange colony reverse color of *P. miczynskii*. In addition, while cultures of *P. miczynskii* always show some growth at 5 C, *P. decaturense* shows no growth or conidium germination.

Phylogenetically, *P. decaturense* occurs on a branch with *P. miczynskii, P. rivolii, P. waksmanii,* part of group 1 (Peterson 2000). The branch is supported in 100% of the bootstrap samples. *Penicillium soppii,* sometimes regarded as a synonym of *P. miczynskii* (e.g., Pitt 1980) appears in the parsimony tree in a different branch from *P. miczynskii* (FIG. 1) which is consistent with other analyses (Christensen et al 1999). In a GenBank search, this species varies at a single base position from a putative new species '*P. luridum*' which appears in GenBank as a deposit from Tuthill and Frisvad.

DISCUSSION

Phylogenetic analysis of DNA sequence data has shown that *Penicillium* is divided into two very different clades. One clade includes species of *Eupenicillium* along with most of the species placed in subgenera *Aspergillioides, Furcatum* and *Penicillium*. The second clade contains species from *Penicillium* subg. *Biverticillium* and species with *Talaromyces* teleomorphs (Berbee and Taylor 1992, Lobuglio et al 1994, Berbee et al 1995, Ogawa and Sugiyama 2000). Peterson (1993) showed that species assigned to subg. *Penicillium* appear to be monophyletic, but Peterson et al (1999) showed that species from subgenera *Aspergillioides* and *Furcatum* are often sister taxa and no larger grouping of monoverticillate or furcate species can be defined phylogenetically. The furcate and monoverticillate penicillus are useful in taxonomy and identification of isolates, but the taxonomy of these subgenera does not reflect the phylogeny. Peterson (2000) identified seven subclades of the *Eupenicillium* lineage, most including both monoverticillate and furcate species. Some *Penicillium* species did not fall into any of these clades (e.g., *Penicillium fellutanum* and *P. charlesii*). These species branch together, but their relationship to any other *Eupenicillium* species was not resolved. *Penicillium thiersii* also is not part of any of the defined *Penicillium* subclades (Peterson 2000). Finding novel structural classes of compounds in *P. thiersii* (Li et al 2002, Zhang et al 2003) suggests that the species is distinct from previously described species, as we infer from the DNA sequence analysis. While two isolates of *P. thiersii* were obtained from the same decaying stromata of *Hypoxylon* sp. collected in southern Wisconsin, a third isolate was recorded from a walnut shell in central Illinois (TABLE I). Examination of *Penicillium* isolates from hundreds of sporocarps of wood-decay fungi collected in Georgia, Florida, Illinois, Wisconsin and New Mexico has revealed no additional cultures of *P. thiersii*.

Penicillium angulare is known only from collections of two old polypores found on decaying tree stumps within a mixed aspen-spruce forest in the Sangre de Cristo mountains of north-central New Mexico near Red River (TABLE I). No additional isolates of *P. angulare* were recorded from numerous collections of wood-decay fungi that we have examined.

Penicillium decaturense has been isolated as a colo-

nist of fungal sporocarps including pyrenomycete stromata and the basidiomata of polypores (e.g., *Trichaptum biformis* and *Ischnoderma* sp., etc.) collected in Illinois, Georgia and Florida (TABLE I). *Penicillium decaturense* is related closely to *P. miczynskii,* but the multilocus analysis shows that these two species are genetically distinct.

Penicillium waksmanii (subgenus *Furcatum,* series *Fellutana*) and *P. miczynskii* (subgenus *Furcatum,* series *Citrina*) are distinct but related species, as suggested by Peterson (2000), despite the wide taxonomic distinctions of the two (Pitt 1980). *Penicillium manginii* is a genetically and phenotypically (Christensen et al 1999) valid species, although other authorities have placed it and *P. miczynskii* in synonymy (Raper and Thom 1949, Pitt 1980). *Penicillium chrzaszcii* is seen on a distinct branch close to *P. miczynskii* at all of the loci examined and has been distinguished phenotypically (Christensen et al 1999). Peterson (2000) suggested *Penicillium rivolii* might be a synonym of *P. waksmanii* on the basis of identical ID region sequences. Multilocus analysis has confirmed synonymy to *P. waksmanii*. Raper and Thom (1949) considered it to be a synonym of *P. janthinellum.*

Introns are believed to be of ancient origin and the positions and number of introns tend to be strongly conserved (Baldauf and Doolittle 1997). This was the case for the calmodulin gene, where each of the *Penicillium* species possessed the same number and location of introns as those found in *Aspergillus oryzae*. In EF-1α, *P. jensenii* had the same number of introns at the same positions (TABLE II) as *A. oryzae* but each of the other species either lacked these two introns or had additional introns at other positions. Wendland and Kothe (1997) discovered intron differences for the EF-1α genes of a Zygomycete and a homobasidiomycete species, but the large variation of intron numbers and positions found in these *Penicillium* species is unusual. The intron positions are overlaid on a phylogenetic tree (FIG. 3), and the introns are specific to particular terminal clades. This suggests that these introns arose recently in the evolutionary history of *Penicillium* and lack the consistency of position and number found in other genes and other organisms (Doolittle 1978). The mechanism of intron insertion and deletion in the EF-1α gene is unknown but does not appear to be related to autonomous mobile genetic elements (Moran et al 1999) because Genbank searches showed no intron homology to reverse transcriptases or any other functional genes.

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