Mycologia, 97(3), 2005, pp. 659–666.

Penicillium coffeae, a new endophytic species isolated from a coffee plant and its phylogenetic relationship to *P. fellutanum*, *P. thiersii* and *P. brocae* based on parsimony analysis of multilocus DNA sequences

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Abstract: *Penicillium coffeae* is described as a novel endophyte isolated from a *Coffea arabica* L. plant in Hawaii. The species is slow growing with short, vesiculate, monoverticillate conidiophores. Phylogenetic analysis using three loci shows that *P. coffeae* forms a strongly supported clade with *P. fellutanum, P. charlesii, P. chermesinum, P. indicum, P. phoeniceum* and *P. brocae*. Phenotypic ally these species are quite similar but can be distinguished. The EF-1α gene from *P. fellutanum, P. charlesii, P. chermesinum* and *P. indicum* lack introns, *P. coffeae and P. phoeniceum* have a previously unknown intron at codon 20 and *P. brocae* and *P. thiersii* isolates have a single intron at codon 26. The most parsimonious interpretation of intron changes on the strongly supported phylogenetic tree requires the gain of a novel intron at position 20 and loss of intron 26 to arrive at the current distribution of introns in this gene. This is one of only a few examples of intron gain in genes.

Key words: *Penicillium phoeniceum, Penicillium indicum, Penicillium ebenbitarianum, Penicillium atrovirens* var *nigrocastaneum,* molecular systematics

INTRODUCTION

Although coffee has been commercially planted for hundreds of years and the amount of published research on coffee production is staggering, very little is known about microorganisms present within the plant. Of paramount importance among such organisms are fungal and bacterial endophytes that live in the intercellular spaces of plants but cause no symptoms of disease. Fungal endophytes are known to produce bioactive products (Strobel 2003) and have been shown in some cases to have positive effects on the plant (Clay 1994, Bacon and White 2000, Azevedo et al 2000, Arnold et al 2003).

We have been studying the fungal endophyte diversity in coffee, and among the hundreds of fungi we have isolated there are several *Penicillium* species that we will report elsewhere (Vega et al in preparation). This finding might have practical importance in coffee production due to the wide array of metabolites produced by *Penicillium* species (Cole and Schweikert 2003, Cole et al 2003). Various *Penicillium* species have been reported as endophytes in plants (Spurr and Welty 1975, Collado et al 1999, Shaukat and Siddiqui 2001, Larran et al 2001, Cao et al 2002, Maria and Sridhar 2003, Yong et al 2003, dos Santos et al 2003) and in coffee seeds (Batista et al 2003, Reynaud et al 2003).

Four of the *Penicillium* isolates obtained in this study were highly similar to each other but were not assignable to any described species (Raper and Thom 1949, Pitt 1980, Ramirez 1982). BLASTN (Altschul et al 1997) searches of GenBank, using the internal transcribed spacer region (ITS) and large subunit (lsu) ribosomal DNA (rDNA) sequences from these four isolates, failed to reveal any closely related species. Accordingly, we computed phylogenetic trees from ITS and lsu rDNA sequences to place these isolates in the general *Penicillium* tree (Peterson 2000). These isolates are most closely related to *P. charlesii, P. fellutanum, P. phoeniceum, P. indicum, P. brocae* and the recently described species *P. thiersii*. Because they are phylogenetically distinct from known species, we describe these isolates as the new species *Penicillium coffeae*.

MATERIALS AND METHODS

The fungal cultures used are provided (Table I) and are available upon request from the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois.

Endophyte isolation.—Kona typica coffee plants (*Coffea arabica* L. cv. Guatemala) were sampled at the Hawaii Ag-

Accepted for publication 17 Feb 2005.
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TABLE I. *Penicillium* isolates used in DNA sequence analysis and phenotypic analysis

Penicillium brocae S. W. Peterson, Peréz, Vega & Infante

NRRL 31462 NRRL 31463 NRRL 31465 NRRL 31471 NRRL 31479 NRRL 31485 MEXICO. Chiapas, Cacahoatan. Isolated from coffee berry borer cuticles by Jeanneth Peréz, 2001.

NRRL 31469 NRRL 31473 MEXICO. Chiapas, Tapachula. Isolated from a coffee berry borer cuticle by Jeanneth Peréz, 2001.

NRRL 31472^{T} MEXICO. Chiapas, Tapachula. Isolated from a coffee berry borer feces by Jeanneth Peréz, 2001.

NRRL 32599 USA. Hawaii. Isolated from roots of coffee seedlings by Francisco Posada, 2003.

NRRL 35185 COLOMBIA. Chinchiná—Caldas. Isolated from coffee leaves by Fernando Vega, 2003.

NRRL 35209 USA. Hawaii. Isolated from coffee, by Fernando Vega, 2003.

Penicillium charlesii G. Smith

NRRL 778 ITALY. Isolated from moldy corn (*Zea mays* L.) by J. V. H. Charles in 1931. Received in 1932 from G. Smith as culture number P146.

NRRL 1887^{T} ITALY. Isolated from moldy corn (*Zea mays* L.) by J. V. H. Charles in 1931. Received in 1942 from G. Smith as culture number P146, ex type culture of *P. charlesii*.

NRRL 6208 JAPAN. Isolated from soil by Shigeo Abe, prior to 1956. Type culture of *Penicillium fellutanum* v *nigrocastaneum* Abe (nomen nudem).

NRRL 3464 JAPAN. Isolated from soil by Shigeo Abe, prior to 1956. Ex type culture of *Penicillium atrovirens* G. Smith.

Penicillium chermesinum Biourge

NRRL 2048^{NT} PANAMA. Isolated from cloth in deterioration testing by William H. Weston, prior to 1945. Ex neotype culture of *P. chermesinum*.

Penicillium coffeae S. W. Peterson, Vega, Posada & Nagai

NRRL 35363^{T} NRRL 35364 NRRL 35365 NRRL 35366 USA. Hawaii, Oahu, Aiea. Isolated from coffee peduncle KO33 by Francisco Posada, January 2003.

Penicillium fellutanum Biourge

NRRL 746^{T} USA. Massachusetts, Woods Hole Marine Biological Station. Isolated from an unrecorded substrate by Oscar W. Richards, October 1935. Ex type culture.

NRRL 3760^{T} SYRIA. Irna. Isolated from desert soil by V. Ch. Baghdadi, March 1964. Ex type culture of *Penicillium ebenbitarianum* Baghdadi.

NRRL 29654 USA. Georgia, Warm Springs. Isolated from a pyrenomycete stroma on dead hardwood in a mixed hardwood forest by D. T. Wicklow April, 2000.

Penicillium indicum Sandhu & Sandhu

NRRL 3387^{T} INDIA. New Delhi. Isolated from sputum sample by Drs. D. K. and R. S. Sandhu, 1962.

Penicillium phoeniceum van Beyma

NRRL 2070^{T} THE NETHERLANDS. Baarn. Isolated from sooty mold of palms by F. H. van Beyma, 1933. Type strain of *P. phoeniceum*.

Penicillium thiersii S. W. Peterson, Bayer & Wicklow

NRRL 28147^{T} NRRL 29162 USA. Wisconsin, New Glarus, New Glarus Woods State Park. Isolated from an old, black stroma of *Hypoxylon,* encrusting the surface of a dead maple log (*Acer saccharum* Marsh.), and collected 21 Aug. 1996 by H. D. Thiers as No. 55623.

NRRL 31609 USA. Illinois: Peoria, Galena Road. Isolated as *Penicillium* sp. from the shell of a mature, fallen walnut fruit (*Juglans* sp.) collected September 2001 by J. J. Scoby.

NRRL 32383 USA. Illinois: Peoria, Galena Road. Isolated as *Penicillium* sp. from the shell of a mature, fallen walnut fruit (*Juglans* sp.) collected October 2002 by J. J. Scoby.

riculture Research Center, Kunia Field Station, in Kunia, Hawaii (21°23.255′N, 158°2.113′W, elevation 54 feet) on 9 Jan 2003 and on various other occasions thereafter. Plant tissues were washed individually in running tap water and moved to the laminar flow hood where sections were cut with a sterile scalpel. These sections were surface-sterilized by dipping in 0.525% sodium hypochlorite for 2 min, 70% ethanol for 2 min and rinsing in sterile distilled water followed by drying on sterile filter paper (Arnold et al 2001). The edges of each sampled tissue were cut off and discarded, and subsamples of the remaining tissue measuring approximately 2 × 3 mm were placed individually in 5 cm diam Petri dishes containing yeast-malt agar (YMA; Sigma Y-3127, Sigma-Aldrich Co., St Louis, Missouri) with 0.1% stock antibiotic solution added (stock: 0.02 g each tetracycline, streptomycin and penicillin in 10 mL sterile distilled water, filter sterilized; from this 1 mL was added per liter of medium).

Phenotypic analysis.—Cultures were grown on Czapek yeast-extract agar (CYA), malt-extract agar (MEA) and glycerol-nitrate agar (G25N) under the conditions recommended by Pitt (1980). Colonies were observed with a dissecting microscope and a compound microscope (Zeiss axioskope) equipped with phase and differential interference contrast (DIC). Scanning electron microscopy was performed on samples fixed with osmium tetroxide, dehydrated with acetone, critical point dried and coated with gold-palladium (Peterson 1992). Colony color names are based on the Ridgway (1912) nomenclature. Microscopic measurements were analyzed statistically with Excel (Microsoft, Bellevue, Washington). Photographs were taken with a Kodak 420B digital camera with macrolenses and with an adaptor tube for the axioskope.

DNA isolation, amplification and sequencing.—Cultures were grown either on agar slants in tubes or on 4.5 cm diam Petri dishes containing MEA. After 7–10 d mycelium was scraped from the colonies, placed in a disposable tube with acid-washed glass beads and buffer, followed by vortex mixing (60 s) to break the cell walls. Proteins were extracted with phenol : chloroform (1:1); the aqueous phase was isolated by centrifugation for 5 min at 2000 *g*, and nucleic acid were precipitated by addition of 1.3 volume 95% ethanol. Nucleic acids were dissolved in TE buffer and adsorbed to silica particles in the presence of concentrated NaI (GeneClean, Qbiogene Inc., Carlsbad, California) and eluted in TE buffer. DNA solutions were stored at −20 C.

The ITS and partial large subunit rDNA (ID region) was amplified with primers ITS-5 (White et al 1990) and D2R in the protocol of Peterson et al (2004). The calmodulin gene (CAL) was amplified with primer CF1d and CF4, and translation elongation factor 1-α (EF 1-α) was amplified with primers EF1b and EF6 in the procedures of Peterson et al (2004).

Amplified gene fragments were purified with 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 internal primers (Peterson et al 2004) and fluorescent dye labeled dideoxy nucleotide terminators in the Applied Biosystems Dye-deoxy sequencing kits. Sequences were read on an Applied Biosystems model 377, 3100 or 3730 DNA sequencer (Applied Biosystems Inc., Foster City, California). Sequencing procedures were performed in accordance with the manufacturer's instructions.

Phylogenetic analysis.—DNA sequences were aligned with Clustal W (Thompson et al 1994) followed by visual corrections with a text editor. Modeltest 3.06 (Posada and Crandall 1998) was used to determine the evolutionary model that best fit the data. Trees were calculated with PAUP* 4.0 β10 (Swofford 2003) with maximum parsimony or maximum likelihood criterion, random addition order and TBR branch swapping. Bootstrap values were calculated using heuristic search and 1000 samples. The congruence of alternative trees was determined with the Kishino-Hasagawa test in PAUP*. Trees were viewed with TreeView (Page 1996) and formatted for publication with CorelDraw 9.

RESULTS

Several species of *Penicillium* and other genera were isolated as endophytes of coffee. The details of those isolates will be published elsewhere (Vega et al in preparation). The new species was isolated from only one peduncle in coffee plant K033 at the Kunia Field Station. Four separate isolates were obtained, but it seems likely that these isolates are clonally related. The DNA sequences from the four isolates were identical, further suggesting that they are clonal.

The ID region sequences range was 1129–1152 nt in length. The aligned dataset of 1156 nt included 1008 constant and 103 parsimony informative characters. Heuristic search of the dataset produced 10 equally parsimonious trees of 159 steps with CI = 0.8113 and RC = 0.7780. The best model for this data was GTR + I + Γ, with I = 0.7023 and the shape parameter alpha = 0.6682. The sequences are deposited in GenBank with accession numbers AF033399, AF125936, AF484391–AF484399 and AY742692–AY742708.

Calmodulin sequence lengths were 677–704 nt with an aligned length of 737 nt. All length differences in CAL were due to indels in the introns. All isolates possessed intron sequences at codon 20 (phase 0), and at codons 26, 68 and 139 (all phase 1). Introns at phase 0 are inserted between codons, phase 1 between bases 1 and 2 of a codon and phase 2 between bases 2 and 3. The amino acid sequences predicted from the coding regions of species in this study were identical although those DNA sequences differed. Parsimony analysis of the coding region was performed with the conditions specified above. The data set included 307 constant and 66 parsimony informative positions and gave two equally parsimonious trees of 113 steps with CI = 0.7434 and RC = 0.7030. The best model for this data was GTR + I + Γ with I = 0.6749 and the shape parameter = 0.1643. The sequences are deposited in GenBank with accession numbers AY741726–AY741754.

Elongation factor-1α sequences were aligned in a data set of length 742 nt, with the individual sequences ranging from 583–684 nt. Isolates of *P. coffeae* and *P. phoeniceum* each possessed an intron inserted at codon 20 (phase 1), *P. thiersii* and *P. brocae* isolates contained a single intron at codon 26 (phase 1) and *P. charlesii, P. indicum, P. chermesinum* and *P. fellutanum* isolates contained no intron sequences in the region sequenced. The intron at codon 20 has not been reported previously. Amino acid sequences predicted from the coding region DNA reveal EF-1α proteins with amino acid differences at amino acid positions 77, 79, 155, 157, 179, 196 and 198. Most amino acid differences are between the outgroup species

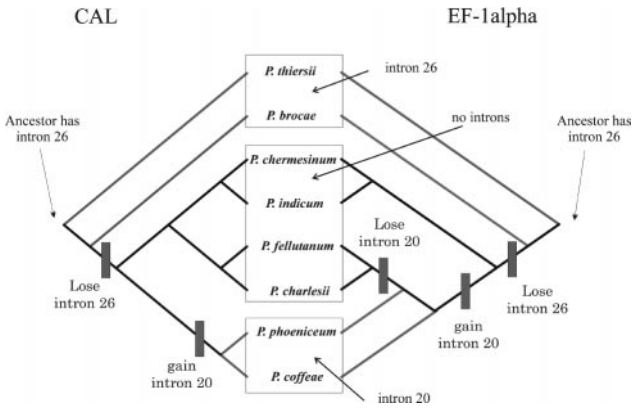


FIG. 1. Single most parsimonious trees obtained from the calmodulin (CAL, left) and elongation factor-1α (EF-1α, right) datasets. The trees are noncongruent, but statistical analysis does not favor one topology over the other. Hypothesized gains and losses of introns that would explain the current distribution of EF-1α introns are labeled on the trees. The calmodulin based tree is favored because it requires the smallest number of intron gains and losses. TreeBase No. SN2019.

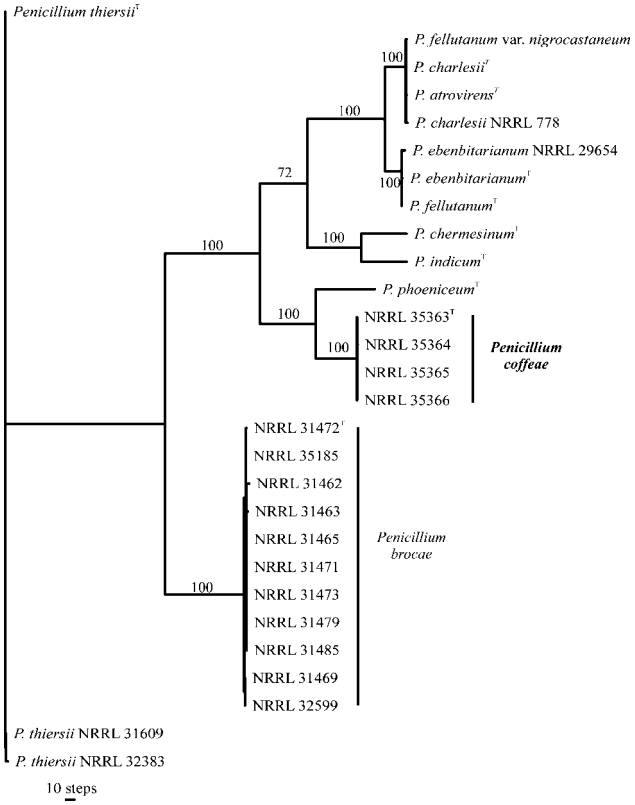


FIG. 2. Single most parsimonious tree based on concatenated data from the ITS and partial large subunit rDNA (ID region), calmodulin gene (CAL) and elongation factor-1α loci (EF1-α). Bootstrap sample percentages supporting branches are placed on internodes. *Penicillium atrovirens* and *P. fellutanum* var *nigrocastaneum* are synonyms of *P. charlesii,* and *P. ebenbitarianum* is a synonym of *P. fellutanum. Penicillium coffeae* is most closely related to *Penicillium phoeniceum.* Ex type or ex neotype cultures are denoted by a superscript T or NT. TreeBase No. SN2019.

and the ingroup. Introns were excluded from analysis and the data set included 598 constant and 57 parsimony informative characters. Heuristic search of the data produced two equally parsimonious trees of 97 steps with CI = 0.6598 and RC = 0.6110. The best model for these data was the GTR + I + Γ with I = 0.7122 and the shape parameter = 0.4780. The sequences are deposited in GenBank with accession numbers AY741755–AY741783.

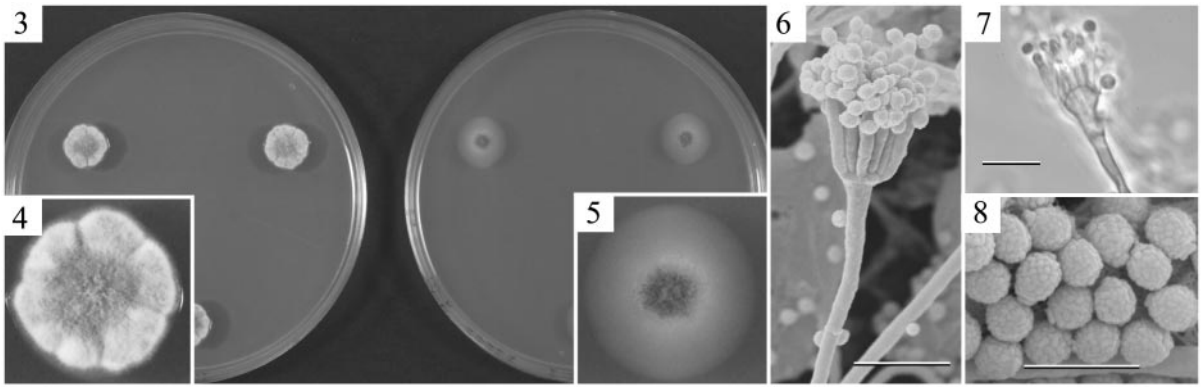
Reduced datasets containing only the ex type isolates were constructed and evaluated with PAUP*. ID data produced three most parsimonious trees; CAL data produced a single most parsimonious tree; and EF-1α data produced a single most parsimonious tree. Noncongruence of the trees was statistically insignificant as assessed with the Kishino-Hasegawa test and the GTR + I + Γ model. The most parsimonious trees from EF-1α and CAL are presented (FIG. 1), with predicted losses and gains of introns that would explain the current distribution of introns among the species. Tree CAL is the most parsimonious requiring a single loss of intron 26 and a single gain of intron 20. Because the incongruence was statistically insignificant, the data were concatenated into a single dataset. Its analysis produced the single tree (FIG. 2) that has the same configuration as tree CAL (FIG. 1).

TAXONOMY

Penicillium coffeae S. W. Peterson, Vega, Posada & Nagai, sp. nov. FIGS. 3–8

Coloniae parvae, post septem dies 11–17 mm diam in CYA, vel 11–13 mm diam in MEA; coloniae in CYA radialiter sulcatae, griseae ad caeruleo-virides pendentes ex productione conidiorum; coloniae in MEA planae, fusco-glaucae, incrementum submersum 4–5 mm largius quam incrementum in superficie. Conidiophora oriuntur a hyphis basalibus, monoverticillata, levia, 50–200 × 2.0–2.5 um cum vesiculo terminali 3–8 um diam; cellulae conidiogenosae ampulliformes, 8.5–12 × 2.0–2.5 um, in vorticillis 6–10 vel plures continentibus, producentes conidia levia et spherica 2.5–3.5 um diam.

Colonies after 7 d on CYA 11–17 mm diam (FIGS. 3, 4), radially sulcate, lanose, lacking exudate, color variable with state of sporulation from grayish-olive to Artemisia or Gnaphalium green, margin scalloped, reverse is light drab to light grayish, soluble pigments and sclerotia are absent. Colonies on MEA after 7 d 11–13 mm diam (FIGS. 3, 5), plane, velutinous, lacking exudate, celandine green, with heavy sporulation, submerged growth accounting for two-thirds of colony diameter, reverse colonial buff to ivory yellow,



FIGS. 3–8. *Penicillium coffeae* NRRL 35363. 3. Colonies grown 7 d on CYA (left) and MEA (right). 4. Close-up view of a CYA grown colony showing sulcation and scalloped margin. 5. Close-up view of an MEA-grown colony showing the small aerial portion of the colony and the larger submerged portion of the colony. 6. SEM of a mature conidiophore showing the smooth stalk and crowded whorl of phialides. 7. Light micrograph (DIC) showing the inflated vesicle typical of conidiophores in this species. 8. SEM showing faintly roughened spherical conidia. Bar in FIGS. 6, 7 = 10 μm; FIG. 8 = 5 μm.

soluble pigments and sclerotia are absent. Conidiophores (FIGS. 6, 7) similar on both media arising from basal hyphae, monoverticillate, smooth 50–200 × 2.0–2.5 μm with a terminal vesicle 3–8 μm diam, conidiogenous cells ampuliform 8.5–12 × 2.0–2.5 μm in whorls of 6–10 or more producing smooth spherical conidia 2.5–3.5 μm diam (FIG. 8).

Etymology. Epithet is based on the host plant.

HOLOTYPE: BPI863480 here designated, consists of colonies of NRRL 35363 grown 7 d on CYA and MEA agars, affixed to a slide mailer and dried.

Penicillium coffeae is most readily recognized by the monoverticillate, vesiculate conidiophores, small colony diameter and the large proportion of submerged colony growth on MEA. In this last character it differs from the other species considered here, and this appears to be a good character for distinguishing the species.

Cultures of *P. charlesii* and *P. fellutanum* were examined with the diagnostic media of Pitt (1980). Most characters recorded (TABLE II) were not sufficiently distinct to differentiate between these two species, a situation that led Pitt (1980) to place the names in synonymy. However we found that conidia of *P. charlesii* were elliptical, averaging 3.1 × 2.7 μm, and finely roughened while the conidia of *P. fellutanum* isolates were nearly spherical, 2.7 × 2.5, μm and smooth. *Penicillium indicum* could be distinguished on the basis of greater colony diameter on CYA and MEA at 25 C and heavy clear exudate on CYA (TABLE II). *Penicillium chermesinum* made relatively large diameter colonies on CYA and MEA but produced slightly smaller, more elliptical conidia than *P. indicum*. *Penicillium phoeniceum* produced small colonies, as did *P. fellutanum, P. charlesii* and *P. coffeae* but could be distinguished from *P. charlesii* and *P. fellutanum* by being strictly monoverticillate, producing slightly larger phialides and spherical 3.1–3.2 μm diam conidia. *Penicillium coffeae* produced colonies composed mostly of submerged growth on MEA compared to the other species whose submerged and surface growths were of nearly equal diameter. CYA colonies tended to be dull grayish blue and produced smooth, spherical conidia of 2.6 μm diam.

DISCUSSION

Penicillium coffeae was discovered from nature as an endophyte of a single coffee plant in Hawaii. The four isolates in this study are probably clonal. Thus more isolates are needed to assess the phenotypic and genotypic diversity of the species. Other *Penicillium* species recovered from coffee plants as endophytes include the brevianamide-producing species *P. brevicompactum* (Vega et al in preparation). Brevianamides have insecticidal properties (Patterson et al 1987) that could be beneficial if formed by the endophytic fungus. *Penicillium brocae* also has been isolated as a coffee plant endophyte (Vega et al in preparation). While it makes a suite of novel antitumor metabolites, the brocaenols (Bugni et al 2003) antiinsect activity has not been assessed. *Penicillium chermesinum* is the only member of this clade with published secondary metabolite studies (Cole and Schweikert 2003). Agurell (1964) reported the production of costaclavine by *P. chermesinum,* an alkaloid previously found in ergot (a disease caused by *Claviceps purpurea*). Ergot alkaloids often are viewed as functional antiherbivory compounds (Clay 1988). Chemical studies of the metabolites of *P. coffeae* would help clarify the relationship of the fungus and the coffee plant. The rarity of *P. coffeae* isolations

TABLE II. Select phenotypic characters from the ingroup species

Species	Diam on CYA	Diam on MEA	Diam on G25N	Conidiophore	Conidial size	Conidium shape
P. coffeae	11–17 (12.8 ± 2.0)	11–13 (12.3 ± 0.6)	11–13 (12.3 ± 0.8)	Monoverticillate	2–4 (2.6 ± 0.6) × 2–3.5 (2.6 ± 0.6)	spherical
P. charlesii	11–17 (14.8 ± 1.9)	14–17 (14.8 ± 1.2)	2–9 (6.4 ± 3.6)	Monoverticillate /Furcate	2–4 (3.1 ± 0.6) × 1.5–4 (2.7 ± 0.6)	elliptical
P. fellutanum	11–20 (15.2 ± 3.2)	11–16 (14.2 ± 2.1)	7–9 (7.9 ± 0.8)	Monoverticillate /Furcate	2–3.5 (2.7 ± 0.4) × 1.5–3.5 (2.5 ± 0.4)	spherical
P. phoeniceum	15–16 (15.3 ± 0.6)	14	8–9 (8.7 ± 0.6)	Monoverticillate	2.5–3.5 (3.2 ± 0.3) × 2.5–3.5 (3.1 ± 0.4)	spherical
P. chermesinum	26–27 (26.7 ± 0.6)	23	10–11 (10.7 ± 0.6)	Monoverticillate	1.5–2.5 (2.2 ± 0.3) × 1.5–2.5 (1.9 ± 0.3)	spherical–elliptical
P. indicum	31–32 (31.7 ± 0.6)	29–30 (29.3 ± 0.6)	12–13 (12.3 ± 0.6)	Monoverticillate	2–3 (2.2 ± 0.3) × 1.5–2.5 (2.1 ± 0.2)	spherical–elliptical

might have to do with its slow growth and thus the probability of its being overgrown by other species. On the other hand, Santamaría and Bayman (2005) observed that dominant endophytes of coffee in some areas are often of minor importance or not found in other areas. Sampling some of the native trees and herbaceous plants in Hawaii for this species, as well as other coffee farms, would provide valuable additional information about host plants and the fungal distribution.

Penicillium fellutanum and *P. charlesii* are separated on the basis of subtle phenotypic characters, but more observations using different media and growth conditions might reveal additional and more distinctive characters. Pitt et al (2000) treated *P. charlesii* as a synonym of *P. fellutanum*. These sibling species are isolated genetically and have diagnosable characters, and we recognize them as species. Ramirez (1982) considered *P. fellutanum* to be strictly monoverticillate, while *P. charlesii* contained furcate and monoverticillate conidiophores. We have observed both monoverticillate and furcate conidiophores in isolates of each species and have been unable to separate the species on the basis of conidiophore complexity. The isolate of *P. atrovirens* and the isolate of *P. fellutanum* var. *nigrocastaneum* are indistinguishable from *P. charlesii,* and they are synonyms of *P. charlesii*. The ex type isolate of *P. ebenbitarianum* is on the same terminal branch as *P. fellutanum* and is a synonym of that species. These synonymies are in agreement with Pitt et al (2000) except that we accept *P. charlesii* as a distinct species.

The phylogenetic placements of *P. indicum* and *P. phoeniceum* had not been established previously with DNA sequence data, but our data show (FIG. 2) that they form a strongly supported clade along with *P. coffeae, P. fellutanum, P. chermesinum* and *P. charlesii*. The overall phenotypic similarity of these species is striking (TABLE II). Pitt et al (2000) consider *P. indicum* to be a synonym of *P. chermesinum*. These two species are siblings, but the three genetic loci used here show that they are distinct species. There are phenotypic distinctions (TABLE II) that can be used to distinguish them. Phenotypic similarity among closely related species in well defined lineages was observed in the *P. miczynskii* clade (Peterson et al 2004) and the *P. brevicompactum* clade (Peterson 2004).

Pitt et al (2000) list *P. phoeniceum* as the anamorphic state of *Eupenicillium cinnamopurpureum*. A comparison of ID region DNA sequences from the type isolates of each species reveals a three base-length difference and a 94% similarity in Clustal W alignment. In an alignment of the EF-1α genes, *E. cinnamopurpureum* (NRRL 3326, ex type culture) has

introns at codon 26 (phase 1) and codon 45 (phase 0) versus the total lack of introns in the *P. phoeniceum* gene and the overall similarity of the coding region sequences is 70%. The phenotypic similarity of *P. phoeniceum* and the anamorphic state of *E. cinnamopurpureum* result from convergent evolution, and these two species are distinct. Peterson and Sigler (2002) found a similar situation for *P. pullum* (syn. = *P. fuscum*), which on phenotypic grounds once was considered to be the anamorph of *Eupenicillium pinetorum* (Stolk and Samson 1983), but molecular genetic studies showed it to be distinct. Convergent evolution has made it nearly impossible to assign anamorph-teleomorph connections purely on the basis of phenotype.

There is incongruence of the trees generated with CAL and EF-1α datasets. However, when the datasets were tested against the two tree topologies, neither topology was significantly better than the other for either dataset. The stochastic nature of nucleotide substitution makes it likely that the rate of change will be nonlinear over relatively short time spans and we believe this is the cause of the incongruence. This hypothesis could be tested by sequencing additional genes that have a higher intrinsic rate of change, and that could hypothetically provide linearity over a smaller time span.

Novel intron gains in protein coding DNA was a controversial topic until very recently (Logsdon 2004), and there is little certainty about the mechanisms of intron loss and gain. For this reason we have chosen the most parsimonious explanation for the present day distribution of the introns in the EF-1α gene (FIG. 1) as also was done in the *P. miczynskii* clade (Peterson et al 2004). In fact the concatenated dataset analysis also strongly supports the topology derived from the CAL sequences, which is the most parsimonious phylogeny in terms of intron loss and gain. Although intron sequences were searched against GenBank with BLAST to look for possible functions of the DNA, none were found. Lack of function is consistent with the presence or absence of introns in the examined species. Four of the species have no EF-1α introns, two species have the intron at codon 26, and two of the species have a previously unknown intron at codon 20. If there were any function assignable to the introns, greater conservation of those introns would be expected. The lack of introns in some species does not appear to reduce fitness in the species and thus supports the idea of nonfunctionality.

ACKNOWLEDGMENTS

SWP acknowledges the skillful technical assistance of Jennifer J Scoby in all phases of the systematics study and the SEM operator Arthur J Thomson. FEV thanks Ann Sidor for field assistance in Hawaii and for technical work in the laboratory. MH Hertwig-Jaksch translated the diagnosis into Latin. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

LITERATURE CITED

Agurell SL. 1964. Costaclavine from *Penicillium chermesinum*. Experientia 20:25–26.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

Arnold AE, Maynard Z, Gilbert GS. 2001. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycol Res 105:1502–1507.

———, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100: 15649–15654.

Azevedo JL, Maccheroni Jr A, Pereira JO, de Araujo WL. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electronic J Biotechnol 3:40–65.

Bacon CW, White Jr JF. 2000. Microbial Endophyte. New York: Marcel Dekker Inc. 487 p.

Batista LR, Chalfoun SM, Prado G, Freitas Schwan R, Wheals AE. 2003. Toxigenic fungi associated with processed (green) coffee beans (*Coffea arabica* L.). Int J Food Microbiol 85:293–300.

Bugni TS, Bernan VS, Greenstein M, Janso JE, Maiese WM, Mayne CL, Ireland CM. 2003. Brocaenols A–C: novel polyketides from a marine-derived *Penicillium brocae*. J Org Chem 68:2014–2017.

Cao LX, You JL, Zhou SN. 2002. Endophytic fungi from *Musa acuminata* leaves and roots in South China. World J Microbiol Biotechnol 18:169–171.

Clay K. 1988. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology 69:10–16.

———. 1994. The potential role of endophytes in ecosystems. In: Bacon CW, White Jr JF, eds. Biotechnology of Endophytic Fungi of Grasses. Boca Raton, Florida: CRC Press Inc. p 73–86.

Cole RJ, Schweikert BJ. 2003. Handbook of Secondary Fungal Metabolites. Vols. I and II. New York: Academic Press.

———, Jarvis BB, Schweikert BJ. 2003. Handbook of Secondary Fungal Metabolites. Vol. III. New York: Academic Press.

Collado J, Platas G, González I, Peláez F. 1999. Geographical and seasonal influences on the distribution of fungal endophytes in *Quercus ilex*. New Phytol 144:525–532.

dos Santos RMG, Rodrigues-Fo E, Rocha WC, Teixeira MFS. 2003. Endophytic fungi from *Melia azeradach*. World J Microbiol Biotechnol 19:767–770.

Larran S, Monaco C, Alippi HE. 2001. Endophytic fungi in

leaves of *Lycopersicon esculentum* Mill. World J Microbiol Biotechnol 17:181–184.

Logsdon JM. 2004. Worm genomes hold the smoking guns of intron gain. Proc Natl Acad Sci USA 101:11195–11196.

Maria GL, Sridhar KR. 2003. Endophytic fungal assemblage of two halophytes from west coast mangrove habitats, India. Czech Mycol 55:241–251.

Page RDM. 1996. TreeView: an application to display phylogenetic trees on personal computers. Computer Application in Bioscience 12:357–358.

Patterson RRM, Simmonds MSJ, Blaney WM. 1987. Mycopesticidal effects of characterized extracts of *Penicillium* isolates and purified secondary metabolites (including mycotoxins) on *Drosophila melanogaster* and *Spodoptora littoralis*. J Invertebr Path 50:124–133.

Peterson SW. 1992. *Neosartorya pseudofischeri* sp. nov. and its relationship to other species in *Aspergillus* section *Fumigati*. Mycol Res 96:547–554.

———. 2000. Phylogenetic analysis of *Penicillium* based on ITS and LSU-rDNA sequences. In: Samson RA, Pitt JI, eds. Classification of *Penicillium* and *Aspergillus*: integration of modern taxonomic methods. Reading, UK: Harwood Publishers. p 163–178.

———. 2004. Multilocus DNA sequence analysis shows that *Penicillium biourgeianum* is a distinct species closely related to *P. brevicompactum* and *P. olsonii*. Mycol Res 108:434–440.

———, Bayer EM, Wicklow DT. 2004. *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. Mycologia (In press).

———, Sigler L. 2002. Four new *Penicillium* species having *Thysanophora*-like melanized conidiophores. Mycol Res 106:1109–1118.

Pitt JI. 1980. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York: Academic Press. 634 p.

———, Samson RA, Frisvad JC. 2000. List of accepted species and their synonyms in the family Trichocomaceae. In: Samson RA, Pitt JI, eds. Classification of *Penicillium* and *Aspergillus*: integration of modern taxonomic methods. Reading, UK: Harwood Publishers. p 9–50.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

Ramirez C. 1982. Manual and Atlas of the Penicillia. New York: Elsevier Biomedical Press. 874 p.

Raper KC, Thom C. 1949. A manual of the Penicillia. Baltimore, Maryland: Williams & Wilkins Co. 875 p.

Reynaud DT, Pimentel IC, Homechim M, Kania CE, Dykstra C. 2003. Fungos isolados dos grãos do café (*Coffea arabica* L.—Rubiaceae) variedade Mundo Novo. Estudios de Biología, Curitiba 25:49–54.

Ridgway R. 1912. Color Standards and Color Nomenclature. Washington, D.C.: Published by the author. 43 p., 53 plates.

Santamaría J, Bayman P. 2004. Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). Microb Ecol (In press).

Shaukat SS, Siddiqui IA. 2001. *Lantana camara* in the soil changes the fungal community structure and reduces impact of *Meloidogyne javanica* on mungbean. Phytopath Mediterranea 40:245–252.

Spurr Jr HW, Welty RE. 1975. Characterization of endophytic fungi in healthy leaves of *Nicotiana* spp. Phytopathol 65:417–422.

Stolk AC, Samson RA. 1983. The ascomycete genus *Eupenicillium* and related *Penicillium* anamorphs. Studies Mycol 23:1–149.

Strobel GA. 2003. Endophytes as sources of bioactive products. Microbe Infect 5:535–544.

Swofford DL. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal V: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

White TJ, Bruns TD, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. In: Innes MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to the methods and applications. New York: Academic Press. p 315–322.

Yong Z, Guo L-A, Wu W-F. 2003. Identification of *Taxus cuspidata* Sieb. et Zucc. endophytic fungi—new species, species known and their metabolite. J Forest Res 14: 290–294.