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Penicillium brocae, a new species associated with the coffee berry borer in Chiapas, Mexico

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Abstract: *Penicillium brocae* is a new monoverticillate species isolated from coffee berry borers collected at coffee plantations in Mexico near Cacahoatán, Chiapas, and from borers reared on artificial diets at ECOSUR laboratory facilities in Tapachula, Chiapas. Phenotypically, it is in *Penicillium* series *Implicatum*, but because it does not conform to known species we have described it as new. ITS and large subunit rDNA were sequenced and compared to determine the phylogenetic position of this species. It is most closely related to *Penicillium adametzii. Penicillium brocae* has only been found in association with the coffee berry borer and is one of several fungi that grow in coffee berry borer galleries. *Penicillium brocae* may provide the exogenous sterols necessary for the coffee berry borer's development and thus be mutualistically associated with the insect.

Key Words: broca, DNA sequences, fungi, internal transcribed spacer, ribosomal DNA, Scolytidae, Trichocomaceae

INTRODUCTION

The coffee berry borer [*Hypothenemus hampei* (Ferrari); Coleoptera: Scolytidae] is the most important pest of coffee plantations over nearly the entire range of coffee cultivation (Murphy and Moore 1990), causing worldwide damages as high as US $500 million per year (P. Baker pers comm). Both adult and larval stages cause damage as they feed inside the coffee berry, reducing the quality and yield of coffee (Le Pelley 1968). The female bores into the fruit and lays her eggs inside rearing chambers. Eggs are laid at a rate of two or three per day for several weeks, resulting in 30 to 70 progeny in a single berry. After hatching, the larvae immediately start to feed on the endosperm. The larval period ranges from 9 to 20 d depending on temperature (Bergamin 1943). Fertilization of the females is by sibling mating inside the coffee berry (Brun et al 1995), so that the coffee berry borer (CBB) lives outside of the berry only for the short period when the female is searching for a new coffee berry to infest (Murphy and Moore 1990). Under field conditions, the generation time for this species is 45 d (Baker et al 1992).

As an exotic pest in Mexico, the biology and ecology of the CBB have been extensively studied (Barrera 1994 and references therein) in order to develop pest management methods based on biological control agents. However, little attention has been paid to the microorganisms associated with the CBB and its galleries. This type of study has important implications for future control of this insect. Laboratory experiments demonstrated that the CBB has low fecundity when fed on coffee berry tissues only, but optimum fecundity can be obtained by adding ergosterol to the insect diet (Morales-Ramos et al 2000). *Fusarium solani* (Martius) Saccardo growing on the coffee berry tissues produces ergosterol and *F. solani* conidia are carried on the cuticle of the beetles (Morales-Ramos et al 2000). On this basis, Morales-Ramos et al (2000) hypothesized a symbiotic relationship between the fungus and the beetle.

As part of a study aimed at understanding the basic biology of the coffee berry borer, several fungi were isolated from the gut, cuticle and feces of CBB adults obtained from coffee plantations or reared on an artificial diet in the laboratories at ECOSUR (El Collegio de la Frontera Sur). In order to determine the identity of these isolates we have used standard phenotypic identification techniques as well as DNA sequencing. Large subunit rDNA has been used to determine the

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TABLE I. Origin of *Penicillium* species isolated from coffee berry borers (*Hypothenemus hampei*). All isolates came from Chiapas State, Mexico, and were isolated by Jeanneth Pérez. GenBank accession numbers are for the ID region DNA sequences

NRRL number	ECOSUR number	Source and Genbank number
Penicillium brocae		
31462	RI-CU R36-31	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484391.
31463	RI-CU R49-46	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484392.
31465	RI-CU R6-8	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484393.
31469	DI-CU R2-2	Tapachula, ECOSUR insect rearing laboratory, isolated from cuticle 27 June 2001. AF484394.
31471	RI-CU R37-32	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484395.
31472	DI-HE R12-8	Tapachula, ECOSUR insect rearing laboratory, isolated from feces 27 June 2001. *Ex type* culture. AF484396.
31473	DI-CU R17-11	Tapachula, ECOSUR insect rearing laboratory, isolated from cuticle 27 June 2001. AF484397.
31479	RI-CU R31-28	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484398.
31485	RI-CU R7-10	Cacahoatán, Rosario Izapa, isolated from cuticle 27 April 2001. AF484399.
Penicillium citrinum Thom		
31461	RI-TD R3-4	Cacahoatán, Rosario Izapa, isolated from the gut 28 April 2001.
31468	DI-TD R27-3	Tapachula, ECOSUR insect rearing laboratory, isolated from the gut 2 July 2001. AF484400.
31475	MP-TD R1-3	Union Juarez, Montaperla, isolated from the gut 13 July 2001. AF484401.
31478	MP-CU R7-5	Union Juarez, Montaperla, isolated from the cuticle 12 July 2001. AF484402.
31481	RI-CU R6-7	Cacahoatán, Rosario Izapa, isolated from the cuticle 27 April 2001. AF484403.
31486	RI-CU R1-1	Cacahoatán, Rosario Izapa, isolated from the cuticle 27 April 2001. AF484404.
Penicillium crustosum Thom		
31466	DI-HE R7-7	Tapachula, ECOSUR, isolated from the feces 28 June 2001. AF484407.
31480	RI-TD R26-12	Cacahoatán, Rosario Izapa, isolated from the gut 28 April 2001. AF484408.
31487	DI-CU R16-4	Tapachula, ECOSUR, isolated from the cuticle 27 June 2001. AF484409.
Penicillium olsonii Bainier & Sartory		
31464	AL-TD R36-5	Cacahoatán, La Alianza, isolated from the gut 17 March 2001. AF484405.
31467	AL-CU R16-1	Cacahoatán, isolated from the cuticle 16 March 2001. AF484406.

phylogeny of many fungi including *Penicillium* (Tuthill et al 2001) and it was used here in combination with the ITS region because of the extensive database of sequences available (Peterson 2000). We encountered a number of isolates that we identified as species of *Fusarium* and *Penicillium*, but also a group of isolates that did not fit into any of the described species of *Penicillium*. Because these isolates could not be accommodated in known species, a description and a new name are provided here.

MATERIALS AND METHODS

Sampling.—Two hundred coffee berries (*Coffea arabica* L.) infested by the CBB, were collected in Rosario Izapa (N 14° 57′ 54.1″; W 92° 09′ 6.4″) in the vicinity of Cacahoatán, Chiapas. This coffee plantation is located 427 m above sea level and planted with the variety 'Oro Azteca'. The berries were dissected in the laboratory and approximately 500 coffee berry borer females were obtained.

Adult females were disinfected superficially in a solution of 5% sodium hypochlorite for 5 min and rinsed in sterile distilled water. Afterwards they were submerged in a solution of 0.05% ascorbic acid + 0.05% citric acid for 5 min. Samples from three parts of the borers, i.e., (i) cuticle (50), (ii) gut (50) and (iii) feces (25) were taken and placed in Eppendorf vials containing 100 μL of saline solution. These samples were shaken in ultrasonic cleaners at a frequency of 42 KHz for 10 s.

Ten-μL aliquots from each sample were spread on Petri dishes containing potato-dextrose-agar (PDA) acidified with lactic acid (pH 4.0–4.5) to inhibit bacterial growth. Samples were incubated at room temperature (ca 30 C) for 7 d. After that time, the colony forming units (CFU) were counted, and the microorganisms were isolated and purified for identification. The origin of the fungal isolates used in this study are listed in TABLE I.

Phenotypic analysis.—Cultures for phenotypic study were grown using the media and conditions specified by Pitt (1979). Color names used in the description are from Ridgway (1912). Microscopic examinations were performed using a Zeiss compound microscope fitted with a Kodak 420B digital camera operated at photosensitivity of ASA 100. Lighting was either Köhler, differential interference contrast, or phase contrast, and specimens for photography were teased apart and mounted in a small drop of low melting temperature agarose (0.5%, ca 40 C) supplemented with 0.5% Kodak Photoflo. The contrast and brightness of photographs were adjusted and plates of figures were put together using Photoshop 6.0.1.

DNA extraction.—Cultures were grown for 7–10 d on malt extract agar (MEA) slopes in tubes. The mycelium and the upper 2–3 mm of agar were excised from the culture tube, placed in a 15 mL tube with 1 g glass bead (0.5 mm diam), 3 mL extraction buffer (Raeder and Broda 1985) and 3 mL of chloroform-phenol (1:1 v:w). Mycelium was broken by vortex mixing of the sample and glass beads for 45–60 s. Proteins were extracted by gentle rocking of the phenol-chloroform-buffer emulsion for 20 min, and DNA was recovered with the aqueous layer after phase-separation centrifugation at ca 2000 *g* for 5 min. DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 6.0) and 1.3 volumes of 95% ethanol. Precipitate was pelleted by centrifugation for 5 min at ca 2000 *g*. The pellet was redissolved in 200 μL TE/10 buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0), and DNA was adsorbed to a silica matrix in the presence of concentrated NaI (GeneClean, Qbiogene, Carlsbad, California), rinsed and eluted into 250 μL TE/10. Purified DNA was stored at −20 C.

Post-isolation DNA procedures.—Amplification of the ITS1–5.8S rDNA-ITS2-LSU rDNA (ID region, ca 1200 nt) was performed as previously described (Peterson 2000). Sequencing reactions were performed using Applied Biosystems, Inc. (ABI) BigDye version 2.0. Excess dye was removed by ethanol precipitation and DNA sequences were read on an ABI 3100 DNA sequencer, all according to manufacturer's instructions.

Phylogenetic analysis.—DNA sequences were aligned with other *Penicillium* spp. sequences obtained from GenBank, using CLUSTALW (Thompson et al 1994). Alignments were further refined using a text editor. Phylogenetic analyses of the aligned sequences were performed using maximum parsimony and maximum likelihood as implemented in PAUP* ver. 4 beta 8 (Swofford 1998). PAUP* trees were viewed in TREEVIEW (Page 1996) and final versions were formatted in CorelDraw ver. 9.0. Alignments were deposited in TreeBASE, accession number M1289.

RESULTS

Penicillium brocae SW Peterson, Pérez, Vega et Infante, sp. nov.

Coloniae crescentes in agaro CYA post septem dies 17–22 mm diam, convexae, sulcatae, valide glauco-caeruleo-virides ad caeruleo-glauco-virides (id est caeruelum 'Medici'), conidiogenesis fortis, superficiem velutinosam cum incremento lanoso per mediam partem efficiens, effluvium clarum adest plerumque cum pigmento electro-flavo et dissolubili, sclerotia ver ascomata absunt, color partis aversae pallide ad fusce aureo-flavus (id est flavum 'primuline' ad 'naphthalene'). Incrementum nullum ad 5 C vel 37 C. Conidiophora plerumque ex hyphis basalibus, levia, brevia, non ramosa, 2–3–(4) μm × (10)–40–150 μm tumore apicali 4–6–(10) μm terminata, phialides 4–8–(10) in verticillis 2–3 μm × (6)–8–10–(12) μm, conidia spherica, tenuiter asperata, 2.5–3.5–(4.5) μm diam formantia.

Colonies grown for 7 d on Czapek's yeast autolysate agar (CYA) (FIG. 1 left) at 25 C attain 17–22 mm diam, are slightly convex, 2–3 mm high centrally, with 4–8 low sulcations, deep grayish blue-green (R-XLVIII) centrally changing along the radius to near Medici blue (R-XLVIII) and white peripherally, consisting of a tough basal felt of hyphae, sunken ca 1 mm into the agar, sporulating heavily and producing a velutinous surface except the central area that has a lanose white overgrowth, clear exudate moderate to abundant present centrally, soluble pigment amber yellow (R-XVI) usually present and visible 10 mm or more from the colony edge, no sclerotia or ascomata, reverse color primuline yellow (R-XVI) centrally changing to amber yellow and finally naphthalene yellow at the edge.

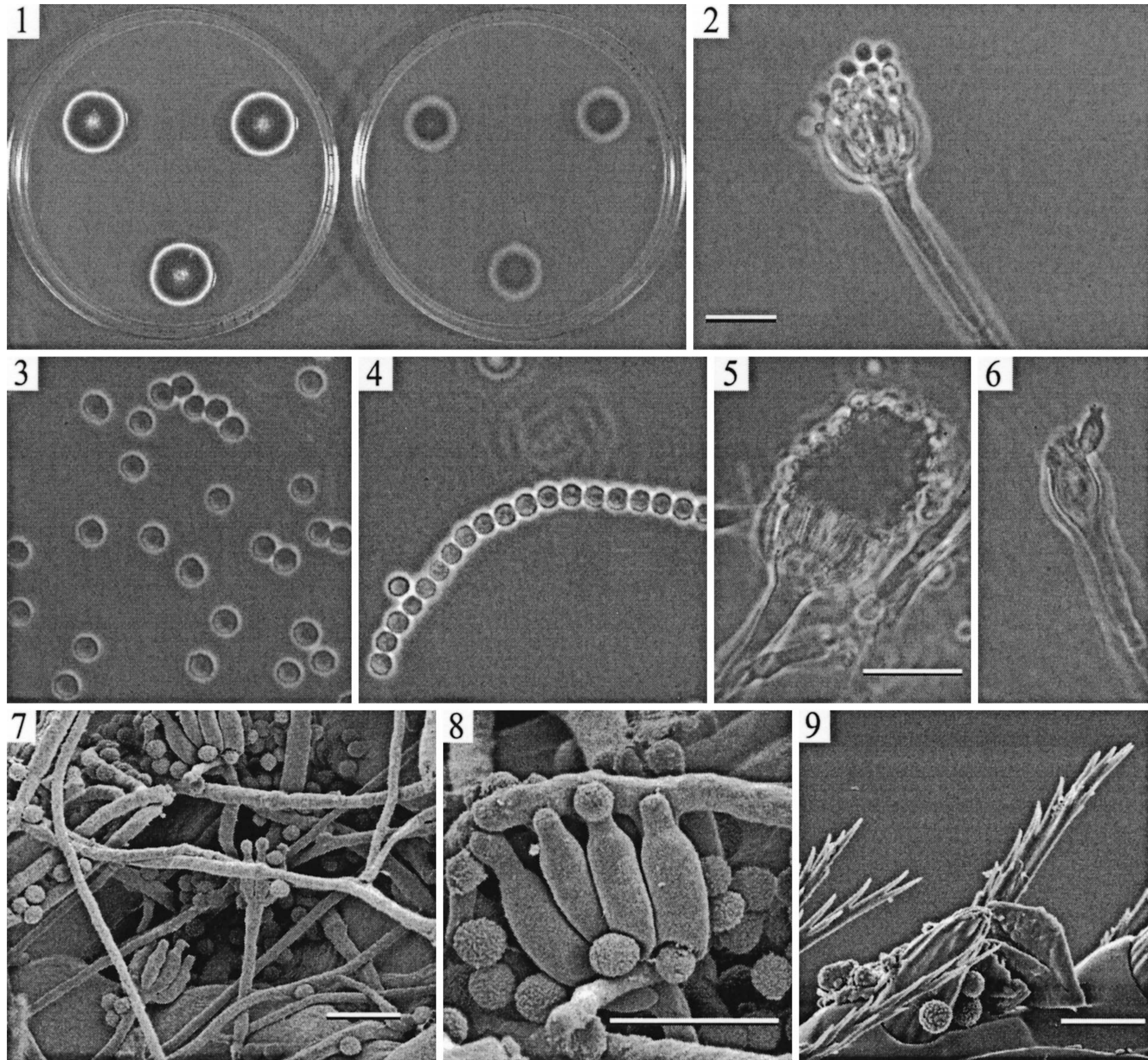
Colonies grown for 7 d on MEA (FIG. 1 right) at 25 C attain a diam of 14–20 mm, are plane, velutinous, heavily sporulating, Artemisia green (R-XLVII) to deep bluish glaucous (R-XLII) centrally with a growth of white at the peripheral margin, with sparse surface and subsurface growth extending ca 2 mm from the visible white periphery, reverse color near Massicot yellow (R-XVI) with undertones of tea green, no exudate, soluble pigments, sclerotia, or ascomata.

When incubated at 5 C or at 37 C, none of the isolates grew nor did the conidia germinate. On G25N medium, colony diam after 7 d at 25 C ranged from (4)–7–9 mm and the appearance of the colonies was very similar to the appearance of colonies grown at 25 C on CYA.

Conidiophores (FIGS. 2, 5, 6, 7) arising mostly from basal hyphae, smooth, short, unbranched 2–3–(4) μm [$\bar{x}$ = 2.8, SD = 0.5] × (10)–40–150 μm [$\bar{x}$ = 66, SD = 30.6] terminated by an apical swelling 4–6–(10) μm [$\bar{x}$ = 5.8, SD = 1.5], bearing whorls of 4–8–(10) phialides 2–3 μm [$\bar{x}$ = 2.75, SD = 0.4] × (6)–8–10–(12) μm [$\bar{x}$ = 8.96, SD = 1.5] producing spheroidal, slightly roughened conidia 2.5–3.5–(4.5) μm [$\bar{x}$ = 2.91, SD = 0.34] diam (FIGS. 3, 4, 8).

Holotype. BPI 841763 is a dried culture of NRRL 31472, isolated from feces of a coffee berry borer reared on an artificial diet at ECOSUR, Tapachula, Chiapas, Mexico, 28 June 2001 by Jeanneth Pérez.

Etymology. La broca del café is the Spanish name of the coffee berry borer.



FIGS. 1–9. *Penicillium brocae*. 1. NRRL 31472 grown 7 d at 25 C on CYA (right) whose central white overgrowth, white marginal area and velutinous appearance are characteristic, and MEA (left) whose plane velutinous growth is typical of several *Penicillium* species. 2. Smooth walled conidiophore with knobbed apex bearing a whorl of phialides. 3. Slightly roughened conidia. 4. Conidia in a long chain. 5. Conidiophores with parallel chains of hydrophobic conidia trapping a large air bubble, the knobby apex is visible. 6. Unusually shaped apical swelling on a conidiophore. This was not common, but seen on a regular basis in some isolates. 7, 8. SEM of *P. brocae* growing on gallery walls of a coffee berry infested by the coffee berry borer. 9. Conidia resembling those of *P. brocae* lodged at the base of setae and asperites of the coffee berry borer. Magnification bars equal 10 μm, use scale in Fig. 2 for Figs. 3, 4, and 6.

Colony color in reverse varied only slightly among the nine isolates of *P. brocae*, ranging from a pale yellow to a pale golden yellow on CYA. Sulcation of the colonies was well developed in seven of the isolates consisting of 4–7 long, deep, radial furrows, but two of the isolates had shallow or short radial furrows. Exudate was consistently present on the colonies grown on CYA, but varied by isolate from heavy to slight. Soluble pigment was pale yellow and present in seven of the isolates with two isolates showing no soluble pigment at seven days' growth. Isolate NRRL 31479 differed from the other isolates in producing lanose colonies, and it produced few or no conidia on CYA. Stalk length was fairly consistent with average lengths in the 50–90 μm range and extremes were 10 and 200 μm. Vesicle size was fairly consistent near 4–6 μm, but a small proportion of the vesicles in some isolates were unusually elongated

(FIG. 6). The monoverticillate penicillus structure was very consistent in all isolates, with only rare branching of the conidiophores.

Penicillium brocae was isolated eight times from the cuticle of CBB adults and once from the feces of the adults that were collected from coffee plantations in Cacahoatán or reared on artificial diets in laboratories at ECOSUR, Tapachula, Chiapas. In addition to *P. brocae*, six isolates of *P. citrinum*, three isolates of *P. crustosum*, two isolates of *P. olsonii* (TABLE I) and an unidentified biverticillate *Penicillium* species were obtained.

Gallery walls of infested coffee berries examined by SEM revealed the presence of *Penicillium brocae* (FIGS. 7, 8) as well as terverticillate *Penicillium* species (not shown). Some coffee berry borers were also examined by SEM (FIG. 9). Conidia indistinguishable from those of *P. brocae* were found near the bases of the setae and asperites.

Phenotypically, *Penicillium brocae* fits in subgenus *Aspergilloides* ser. *Implicata* (Pitt 1979) on the basis of the monoverticillate conidiophores with terminal vesicles and restricted growth, but the combination of characters present does not fit any of the species in this series (Pitt 1979). In a synoptic examination of the monoverticillate species, *P. brocae* resembles *Penicillium fellutanum* Biourge on the basis of slowly growing colonies, comprised of a low dense hyphal felt, a central hyaline lanose overgrowth on CYA, and the production of an amber yellow soluble pigment. However, *P. fellutanum* produces larger (12–16 mm) G25N colonies than *P. brocae* (7–9 mm); conidiophores of *P. fellutanum* arise from aerial hyphae while those of *P. brocae* are from basal hyphae; the phialides and conidia of *P. fellutanum* are smaller than those of *P. brocae* and the conidia of *P. fellutanum* are ellipsoidal versus spheroidal in *P. brocae*.

Penicillium brocae also resembles *P. citreonigrum* Dierckx, but is distinguished from the latter species, which has olivaceous-gray colony color on CYA and vegetative hyphae that are typically yellow versus the hyaline hyphae of *P. brocae*. Growth on G25N is 7–9 mm for *P. brocae* and conidia are 2.5–3.5 μm diam, while cultures of *P. citreonigrum* typically attain 11–14 mm diam on G25N and conidia are 1.8–2.5 μm diam.

Penicillium brocae also resembles *P. charlesii* G. Sm., but the latter species forms CYA colonies made of a low, dense hyphal felt, and growth rate is moderate to slow, colonies are sulcate, and an amber soluble pigment is produced (Raper and Thom 1949, Ramirez 1982). However, *P. brocae* has spheroidal conidia 2.5–3.5–(4.5) μm formed on unbranched conidiophores, while *P. charlesii* has ellipsoidal conidia 2.5–3.0 × 3.0–4.0 μm produced from branched conidiophores. Also, while the growth of *P. brocae* is slow on all media, *P. charlesii* grows rapidly on MEA.

Phylogenetic analysis.—Of the 1182 base positions in the ID region alignment, 126 characters were eliminated because of insertion/deletion events, 783 characters were constant, 64 variable characters were parsimony-uninformative and 209 characters were parsimony-informative. Heuristic search found 114 equally most parsimonious trees of 783 steps. The tree statistics were consistency index (CI) = 0.4764, homoplasy index (HI) = 0.5236, CI excluding uninformative characters = 0.4193, HI excluding uninformative characters = 0.5807, retention index (RI) = 0.7029 and rescaled consistency index (RC) = 0.3348.

In maximum parsimony analysis, *P. brocae* is most closely related to *P. adametzii* Zalessky, *P. herquei* Bainier & Sartory, *P. adametzioides* S. Abe ex G. Sm., *P. bilaiae* Chalab., and *E. hirayamae* D. B. Scott & Stolk (FIG. 10), but the relatively low bootstrap value (65%) on this clade makes the placement of *P. brocae* tentative. The strict consensus tree branches of the 114 equally most parsimonious trees are bold (FIG. 10) and show that *P. brocae* remains in the same clade and in the same relative position to *P. adametzii* and the other *Penicillium* species on that branch. A constraint tree under the parsimony model, forcing *P. brocae* to form a clade with *P. charlesii* and *P. fellutanum* or *P. citreonigrum* was rejected with strong statistical support ($P = 0.004$) using the Kishino-Hasegawa test.

DISCUSSION

Penicillium brocae is phylogenetically distinct from all other *Penicillium* species and is not closely related to *P. charlesii* or *P. fellutanum* that it resembles most. The strains assigned to the species have identical DNA sequences in the region examined and the distance between *P. brocae* and other *Penicillium* species is far greater than the genetic distance between most species. The DNA sequence data show that *P. brocae* is phylogenetically distinct from all other *Penicillium* species, and we have provided a description of the phenotypic differences that make this species distinct.

Penicillium brocae and several other *Penicillium* and *Fusarium* species have been isolated from the cuticle of adult coffee berry borers, and also from feces and guts. Morales-Ramos et al (2000) only isolated *Fusarium solani* from the cuticle of coffee berry borers, whether collected in Allada, Benin, or in Chiapas. The most readily apparent explanation lies in the different surface sterilization techniques, but other unknown factors may also apply.

Ambrosia beetles (Coleoptera: Scolytidae) are known to have mutualistic associations with fungi that

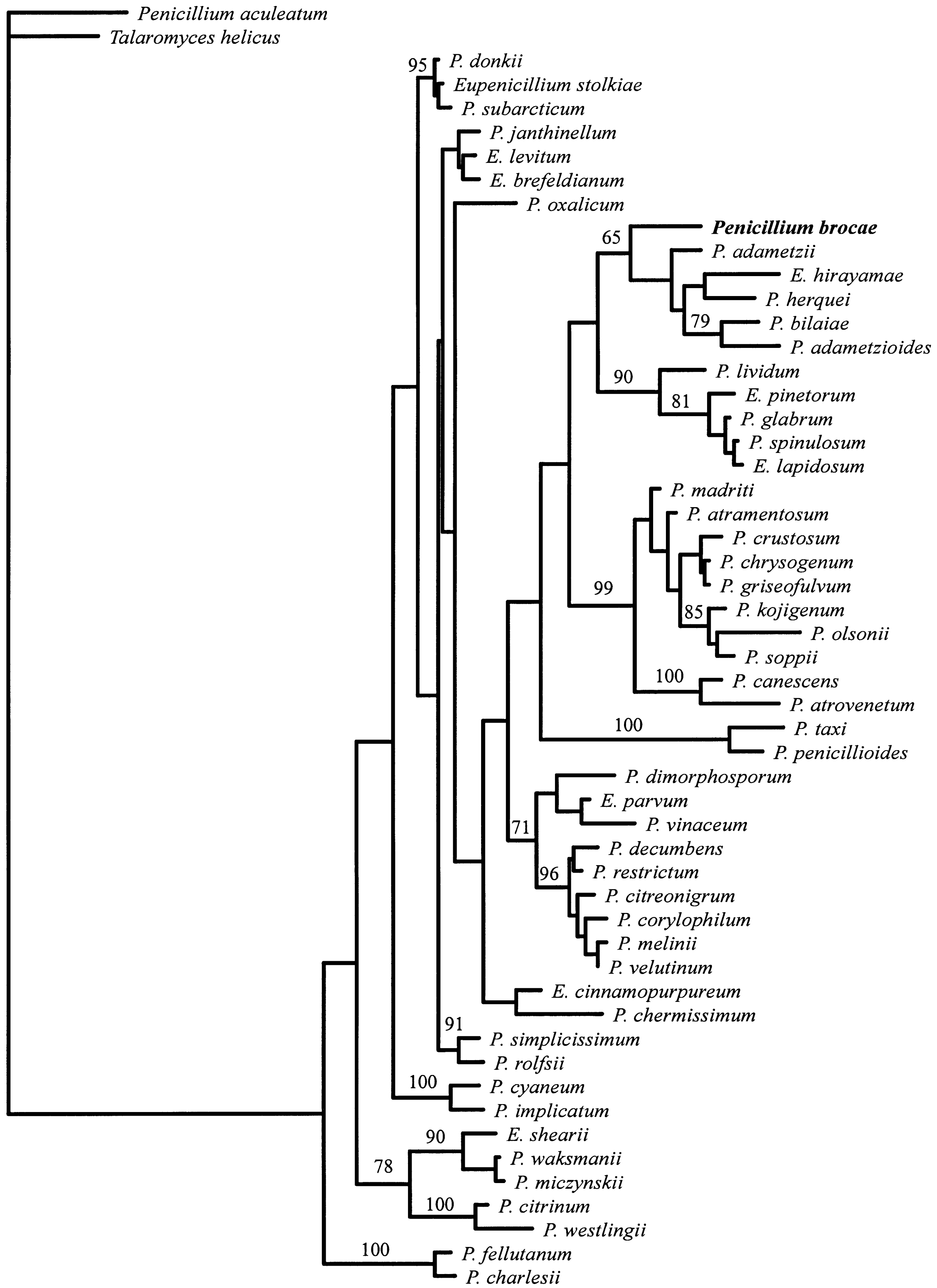


FIG. 10. Gene tree based on aligned ID region sequences derived using maximum parsimony in an heuristic search. 114 equally most parsimonious trees were found in the search. Numbers above the internodes are bootstraps percentages (based on 1000 bootstrap samples) and values below 65 are not shown. Bar in the figure represents 10 steps in the tree.

they carry on specialized structures termed mycangia. Once in the plant, the ambrosia fungi grow on galleries made by the beetles, and the insects graze on the fungal hyphae (Batra 1966). Coffee berry borers do not appear to have mycangia, but the asperites and setae on the pronotum have functional similarity to mycangia (Morales-Ramos et al 2000). Ergosterol, which is produced by the fungal growth in the beetle galleries, was a necessary factor for the growth and fecundity of the coffee berry borer (Morales-Ramos et al 2000). Insects feeding their entire life on single food sources, such as the CBB do, are often associated with symbiotic microorganisms that supplement the insects restricted diet with critical nutrients (Bean-Beard et al 2002). Because we have isolated many different species of fungi from the adult beetles and have observed *P. brocae* and other *Penicillium* species in the galleries of coffee berries, and because ergosterol is the principal sterol produced by fungi (Hawksworth et al 1995), it is possible that the symbiosis of the coffee berry borer is not exclusively with *Fusarium* species. *Penicillium brocae* also may be a symbiont that can sustain the growth and development of the coffee berry borer by providing needed sterols.

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