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Penicillium species endophytic in coffee plants and ochratoxin A production

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Abstract: Tissues from *Coffea arabica*, *C. congensis*, *C. dewevrei* and *C. liberica* collected in Colombia, Hawaii and at a local plant nursery in Maryland were sampled for the presence of fungal endophytes. Surface sterilized tissues including roots, leaves, stems and various berry parts were plated on yeast-malt agar. DNA was extracted from a set of isolates visually recognized as *Penicillium*, and the internal transcribed spacer region and partial LSU-rDNA was amplified and sequenced. Comparison of DNA sequences with GenBank and unpublished sequences revealed the presence of 11 known *Penicillium* species: *P. brevicompactum*, *P. brocae*, *P. cecidicola*, *P. citrinum*, *P. coffeae*, *P. crustosum*, *P. janthinellum*, *P. olsonii*, *P. oxalicum*, *P. sclerotiorum* and *P. steckii* as well as two possibly undescribed species near *P. diversum* and *P. roseopurpureum.* Ochratoxin A was produced by only four isolates, one isolate each of *P. brevicompactum*, *P. crustosum*, *P. olsonii* and *P. oxalicum.* The role these endophytes play in the biology of the coffee plant remains enigmatic.

Key words: *Coffea arabica*, *Coffea congensis*, *Coffea dewevrei*, *Coffea liberica*, endophytes, OTA

INTRODUCTION

Endophytes are fungi or bacteria that occur inside plant tissues without causing any apparent symptoms (Wilson 1995). Several roles have been ascribed to fungal endophytes, including a role against insects (Breen 1994, Arnold and Lewis 2005). Surveys for endophytes have been conducted in dozens of plants, including many important agricultural commodities such as wheat (Larran et al 2002a), bananas (Pocasangre et al 2000, Cao et al 2002), soybeans (Larran et al 2002b) and tomatoes (Larran et al 2001), but coffee endophytes remain largely unexplored with the exception of bacterial endophytes (Vega et al 2005).

Several species of *Penicillium* were isolated as part of a large survey for fungal endophytes in coffee (Vega et al in prep). Members of this genus produce a variety of metabolites (Mantle 1987, Abramson 1997, Samson and Frisvad 2004), and contamination with one of these metabolites, ochratoxin A (OTA), can be a problem in many commodities, including coffee (Bucheli and Taniwaki 2002), due to its possible adverse effect on human health. Species of *Aspergillus* are the main OTA producers in tropical and semi-tropical areas (Abramson 1997). Among *Penicillium* species, Pitt (1987) found that only *P. verrucosum* Dierckx produced OTA, while Larsen et al (2001) reported *P. verrucosum* and *P. nordicum* Dragoni & Cantoni as OTA producers. Here we report on the identity of *Penicillium* species occurring as endophytes in coffee and on their OTA production.

MATERIALS AND METHODS

Sampling.—Apparently healthy coffee (*Coffea* spp.) tissues were collected randomly in coffee plantations in Hawaii (2003) and Colombia (2003) and from seedlings obtained at The Behnke Nurseries Co. in Beltsville, Maryland (2004). The Maryland seedlings were purchased by the Behnke Nurseries Co. from Colasanti's Wholesale in Ontario Canada, which in turn imports the seeds from Costa Rica and germinates them in North America. Tissues sampled consisted of leaves, roots, stems and various parts of the coffee berry (crown, peduncle, pulp and seeds).

Fungal isolation.—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 0.5% 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 individually placed in 5 cm diam petri dishes containing yeast-malt agar (YMA; Sigma Y-3127, Sigma-Aldrich Co., St. Louis,

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Missouri) to which 0.1% stock antibiotic solution was 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 media).

Fungal identification.—*Penicillium* strains were identified based on the DNA sequence of a ca 1200 nucleotide sequence fragment containing the ITS1, 5.8S rDNA, ITS2 and D1–D2 region of LSU rDNA (ID region, Peterson 2000, Peterson et al 2003). Briefly, DNA was isolated from mycelium harvested from agar cultures and broken mechanically with glass beads. Proteins were extracted using phenol-chloroform, nucleic acids were precipitated with ethanol, pelleted, redissolved in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and further purified by adsorption to silica in the presence of concentrated NaI (Peterson 2000). Purified DNA was stored in TE buffer at −20 C.

The ID region was amplified using primers ITS-5 (White et al 1990) and D2R (Peterson et al 2003), standard buffer (White et al 1990) and Taq polymerase (RedTaq, Sigma, St. Louis). The thermal profile was 96 C 2 min, followed by 35 cycles of 96 C 30 s, 51 C 30 s, 72 C 90 s and a final extension of 5 min at 72 C. Amplicon quality was assessed by agarose gel electrophoresis and ethidium bromide staining. Amplicons were purified using Millipore Multiscreen PCR clean-up plates (Millipore, Billerica, Massachusetts).

Sequencing reactions were performed on both strands of each amplicon using ABI Big-Dye reaction kit 3.1 (Applied Biosystems, Foster City, California); excess dye was removed by ethanol precipitation. DNA sequences were read on an ABI 3730 DNA (Applied Biosystems, Foster City, California) sequencer. Complementary strands of the DNA were aligned and corrected using Sequencher (Gene Codes Corp., Ann Arbor, Michigan). BLAST was implemented locally and a database of *Penicillium* sequences was assembled from published sequences of ex-type cultures (GenBank) and unpublished sequences of other ex-type isolates (Peterson 2000, unpubl). If an unknown sequence was a perfect match to the sequence from an ex-type culture, the unknown was assigned to that species. Sequences were also compared to all GenBank accessions using BLAST. Sequences of the isolates reported here are deposited in GenBank and accession numbers are listed (TABLE I). Fungal isolates are accessioned in the ARS Culture Collection (NRRL) Peoria, Illinois. Some isolates also were examined for phenotypic characters and keyed using Pitt et al (1990) or Ramirez (1982).

Ochratoxin A detection.—All the isolates listed (TABLE I) were tested for OTA presence. OTA was extracted and analyzed using the methodology of Bragulat et al (2001). Three agar plugs (0.5 mm diam × 0.5 mm length) were removed from the fungal cultures growing on YMA for three months and added to an HPLC (Shimadzu LC-AT, Columbia, Maryland) autosampler vial (33 mm × 10 mm) of known weight and weighed again. The plugs were extracted in 0.8 ml methanol : formic acid (25 : 1) for 1 h at 25 C. The samples were filtered through 13 mm nylon membrane discs (Osmonics Laboratory Products, Minnetonka, Minnesota) and OTA separated by isocratic C18 reverse phase HPLC with a mobile phase of acetonitrile : water : acetic acid (57 : 41 : 2) at 0.8 mL/min. OTA was quantified using a fluorescence detector (Shimadzu RF-10A, Columbia, Maryland) (excitation 330 nm and emission 460 nm). The minimum detectable amount was 0.01 ng. Three sample replicates were analyzed for each isolate. An OTA (Sigma-Aldrich Co., St. Louis, Missouri) standard curve was prepared for quantification and *Aspergillus ochraceus* Wilhelm was used as a positive control (kindly provided by J. White, Rutgers University). *Aspergillus westerdijkiae* Frisvad & Samson, a species known to produce ochratoxins and which has been isolated from surface-disinfected green coffee beans in India (Frisvad et al 2004) also was used as a positive control. The *A. westerdijkiae* strains were isolated from a coffee berry borer parasitoid (*Prorops nasuta* Waterston; Hymenoptera: Bethylidae) and from a coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae), both collected at the National Coffee Research Center (CENICAFÉ) in Chinchiná, Caldas, Colombia (Peterson et al 2005a).

RESULTS

Comparison of ID region sequences with GenBank and unpublished sequences revealed the presence of 13 *Penicillium* species endophytic in *Coffea arabica*: *P.* sp. (near *P. diversum* Raper & Fennell), *P. brevicompactum* Dierckx, *P. brocae* Peterson et al, *P. cecidicola* Seifert et al, *P. citrinum* Thom, *P. coffeae* Peterson et al, *P. crustosum* Thom, *P. janthinellum* Biourge, *P. olsonii* Bainier & Sartory, *P. oxalicum* Currie & Thom, *P. sclerotiorum* Beyma, *P.* sp. (near *P. roseopurpureum* Dierckx), and *P. steckii* Zalessky (TABLE I). *P. olsonii* was also identified in *C. liberica* Bull ex Hiern, *C. dewevrei* DeWild & Durand, and *C. congensis* from the Kona Experimental Station in Kona, Hawaii (TABLE I). The sequences of six isolates identified as *P. brevicompactum* differed from the sequence of the ex-type isolate at 0, 1 or 2 base positions with total similarity of 1138, 1139 or 1140 matches out of 1140 bases. Each of these sequences had been found previously to be representative of the species in genealogical concordance phylogenetic species recognition (GCPSR) study (Peterson 2004). Sequences from the 13 isolates identified as *P. olsonii* were identical with the sequence from an ex-type isolate (1140 bases) and were representative of variation found in the species (Peterson 2004). Three isolates possessed sequences identical to the ID region sequence (1110 bases) of *P. citrinum* ex-type and are identified as such. The ITS sequence of NRRL 35178 (591 bases) was identical to the ITS sequence from an ex-type culture of *P. crustosum* (FRR 1669; FRR Culture Collection, Food Science Australia, PO Box 52, North Ryde, NSW 1670 Australia) and was

Table I. Origin, identification and GenBank numbers of the endophyte species isolated in this study

Penicillium species	Isolated from:	Location:	NRRL #	GenBank
P. brevicompactum	Seed from ripe berry	USA. Hawaii. Foster Arboretum N 21°18.959′, W 157°51.444′, January, 2003	32574	DQ123636
	Seed from ripe berry[1]	USA. Hawaii. Foster Arboretum N 21°18.959′, W 157°51.444′, January, 2003	32579	DQ123637
	Seed from ripe berry	USA. Hawaii, Kona. Holualoa Kona Coffee Co. N 19°32.020′, W 155°55.746′, January, 2003	32580	DQ123638
	Seed from ripe berry	USA. Hawaii, Kona. Howard Yamasaki farm N 19°25.976′, W 155°52.902′, January, 2003	32582	DQ123639
	Leaves from seedlings	USA. Hawaii, Kunia. Kunia Field Station, N 21°23.255′, W 158°02.113′, March 2003	32600	DQ123640
	Leaves	COLOMBIA. Caldas, Chinchiná (CENICAFÉ) N 5°00′, W 75°36′, July 2003	35184	DQ123641
P. brocae	Roots of seedlings	USA. Hawaii, Kunia. Kunia Field Station N 21°23.255′, W 158°02.113′, March 2003	32599	DQ123642
	Leaves	COLOMBIA. Caldas, Chinchiná (CENICAFÉ) N 5°00′, W 75°36′, July 2003	35185	DQ123643
P. cecidicola	Stem	USA. Maryland, Beltsville. Behnke's Nurseries N 39°02.424′, W 076°54.347′, November 2003	35466	DQ123648
P. citrinum	Peduncle	USA. Hawaii, Kunia. Kunia Field Station N 21°23.255′, W 158°02.113′, March 2004	35434	DQ123644
	Roots, leaves of seedlings	USA. Maryland, Beltsville, Behnke's Nurseries N 39°02.424′, W 076°54.347′, January 2004	35448–35449	DQ123645 DQ123646
P. coffeae[2]	Peduncle	USA. Hawaii, Kunia. Kunia Field Station N 21°23.255′, W 158°02.113′, January 2003	35363	AY742704
P. crustosum	Seed from ripe berry[3]	MEXICO. Chiapas, Cacaohoatán, Rancho El Paraíso N 15°00′.27.6″, W 92°09′51.2″, Feburary 2003	35178	DQ123647
near *P. diversum*	Crown	USA. Hawaii. Kunia Field Station N 21°23.255′, W 158°02.113′, January, 2003	35186	DQ123635
P. janthinellum	Roots	USA. Maryland, Beltsville. Behnke's Nurseries N 39°02.424′, W 076°54.347′, January 2004	35451	DQ123649

TABLE I. Continued

Penicillium species	Isolated from:	Location:	NRRL #	GenBank
P. olsonii	Seed from ripe berry	USA. Hawaii, Kona. Dragon's Lair Coffee Farm N 19°25.536′, W 155°52.829′, January 2003	32581	DQ123650
	Coffee berry pulp	USA. Hawaii, Kona. Dragon's Lair Coffee Farm N 19°25.536′, W 155°52.829′, January 2003	32577	DQ123651
	Peduncle[4]	USA. Hawaii, Kona. Dragon's Lair Coffee Farm N 19°25.536′, W 155°52.829′, January 2003	35166	DQ123652
	Seed from ripe berry	USA. Hawaii, Kunia. Kunia Field Station N 21°23.255′, W 158°02.113′, January 2003	35167	DQ123653
	Peduncle	USA. Hawaii, Kona. Greenwell Farms N 19°30.673′, W 155°55.308′, January 2003	35168	DQ123654
	Peduncle	USA. Hawaii, Kauai. Kauai Coffee Co. N 21°53′53″, W 159°33′30″, January 2003	35169	DQ123655
	Crown	USA. Hawaii, Oahu, Haleiwa. Waimea Arboretum N 21°37.834′, W 158°02.877′, January 2003	35171	DQ123656
	Peduncle (*C. liberica*)	USA. Hawaii, Kona. Kona Experimental Station N 19°32.048′, W 155°55.494′, January 2003	35174	DQ123657
	Peduncle	USA. Hawaii. Foster Arboretum N 21°18.959′, W 157°51.444′, January 2003	n/a	DQ123658
	Leaves (*C. dewevrei*)	USA. Hawaii, Kona. Kona Experimental Station N 19°32.048′, W 155°55.494′, January 2003	n/a	DQ123659
	Peduncle (*C. congensis*)	USA. Hawaii, Kona. Kona Experimental Station N 19°32.048′, W 155°55.494′, January 2003	n/a	DQ123660
	Peduncle	USA. Hawaii, Kona. Kona Experimental Station N 19°32.048′, W 155°55.494′, January 2003	n/a	DQ123661
	Peduncle	USA. Hawaii, Kona. Lehuula Farms N 19°32.020′, W 155°55.746′, January 2003	n/a	DQ123662
P. oxalicum	Leaves[5]	COLOMBIA. Caldas, Chinchiná,CENICAFÉ N 5°00′, W 75°36′, July 2003	35183	DQ123663

TABLE I. Continued

Penicillium species	Isolated from:	Location:	NRRL #	GenBank
near *P. roseopurpureum*	Seed from ripe berry	USA. Hawaii, Kunia. Kunia Field Station N 21°23.255′, W 158°02.113′, January 2003	32575	DQ123664
P. sclerotiorum	Crown	USA. Hawaii, Kauai. Kauai Coffee Co. N 21°53′53″, W 159°33′30″, January 2003	32583	DQ127231
P. steckii	Pulp from berry	USA. Hawaii, Oahu, Manoa. Lyon Arboretum N 21°20.035′, W 157°48.228′, January 2003	35367	DQ123665
	Roots	USA. Maryland, Beltsville. Behnke's Nurseries N 39°02.424′, W 076°54.347′, November 2003	35463	DQ123666

[1] Detectable ochratoxin A level of 0.037 ppb.
[2] See Peterson et al, 2005b.
[3] Detectable ochratoxin A level of 0.074 ppb.
[4] Detectable ochratoxin A level of 0.025 ppb.
[5] Detectable ochratoxin A level of 0.037 ppb.

identified on that basis. ID region sequence of NRRL 35183 (1147 bases) was identical with the ID region sequence of an ex-type culture of *P. oxalicum.* The ID region sequence (1133 bases) of NRRL 35283 differed from the sequence of a *P. sclerotiorum* ex-type culture at one base position. Isolates NRRL 35367 and NRRL 35463 (1152 and 1151 bases respectively) differed from the sequence of *P. steckii* ex-type (1151 bases) at one or two base positions. The ID regions sequence of NRRL 35451 (1144 bases) differed from the sequence of an ex-type isolate (1145 bases) of *P. janthinellum* at a single base position. The *Penicillium* species isolate NRRL 35466 has an ID region sequence that differed from that of an ex-type isolate of *P. cecidicola* at a single nucleotide position. Isolate NRRL 35466 was grown in culture and fit well the phenotypic description of the species (Seifert et al 2004). The only departure from expectation was the failure of our culture to produce synnemata in culture. Seifert et al (2004) noted that their isolates produced fewer synnemata in culture than they did on natural substrates (wasp galls). We identify this isolate as *P. cecidicola.* NRRL 35186 has an ID region sequence that differs at three base positions from the sequence of *P. diversum* (ex-type GB DQ308553). Phenotypically, NRRL 35186 produced a thin surface growth on agar that was composed of symmetrical biverticillate penicilli with 3–4 spreading metulae (15–18 × 2.5–3 μm) with whorls of 3–5 phialides (ampuliform, 7–9 × 2–2.5 μm) and smooth ellipsoidal conidia 2–2.5 × 1.5–2.0 μm. Conidia typically occurred in adherent chains of 15–25 conidia. Colonies were dark green in the conidial area and uncolored in reverse. No exudate, soluble pigments, sclerotia or ascomata were observed. The penicilli contain fewer metulae and the whorls of phialides are less dense, but otherwise the morphology fits well with *P. diversum.* The ID region sequence of NRRL 32575 most closely resembles the sequence from *P. roseopurpureum* ex-type, but differs from that species at 20 base positions. Microscopically, the culture produces monoverticillate and furcate penicilli. Unlike *P. velutinum* and *P. charlesii* that possess divaricate penicilli that resemble furcate penicilli, NRRL 32575 contains monoverticillate, divaricate and truly biverticillate appearing penicilli. *Penicillium roseopurpureum* is strictly monoverticillate. NRRL 32575 appears to be an undescribed species of *Penicillium.*

OTA was detected in only four of the isolates listed (TABLE I). Two of these, *P. brevicompactum* and *P. crustosum,* were isolated from seeds in ripe berries and produced 0.037 ppb and 0.074 ppb, respectively (TABLE I). *P. olsonii* isolated from a peduncle produced 0.025 ppb, and *P. oxalicum* from leaves produced 0.037 ppb (TABLE I). The type of medium used has been shown to have an effect on metabolite production in other fungal endophytes, e.g., *Taxomyces andreanae* and *Penicillium raistrickii* Smith (Stierle and Stierle 2000). This suggests that the

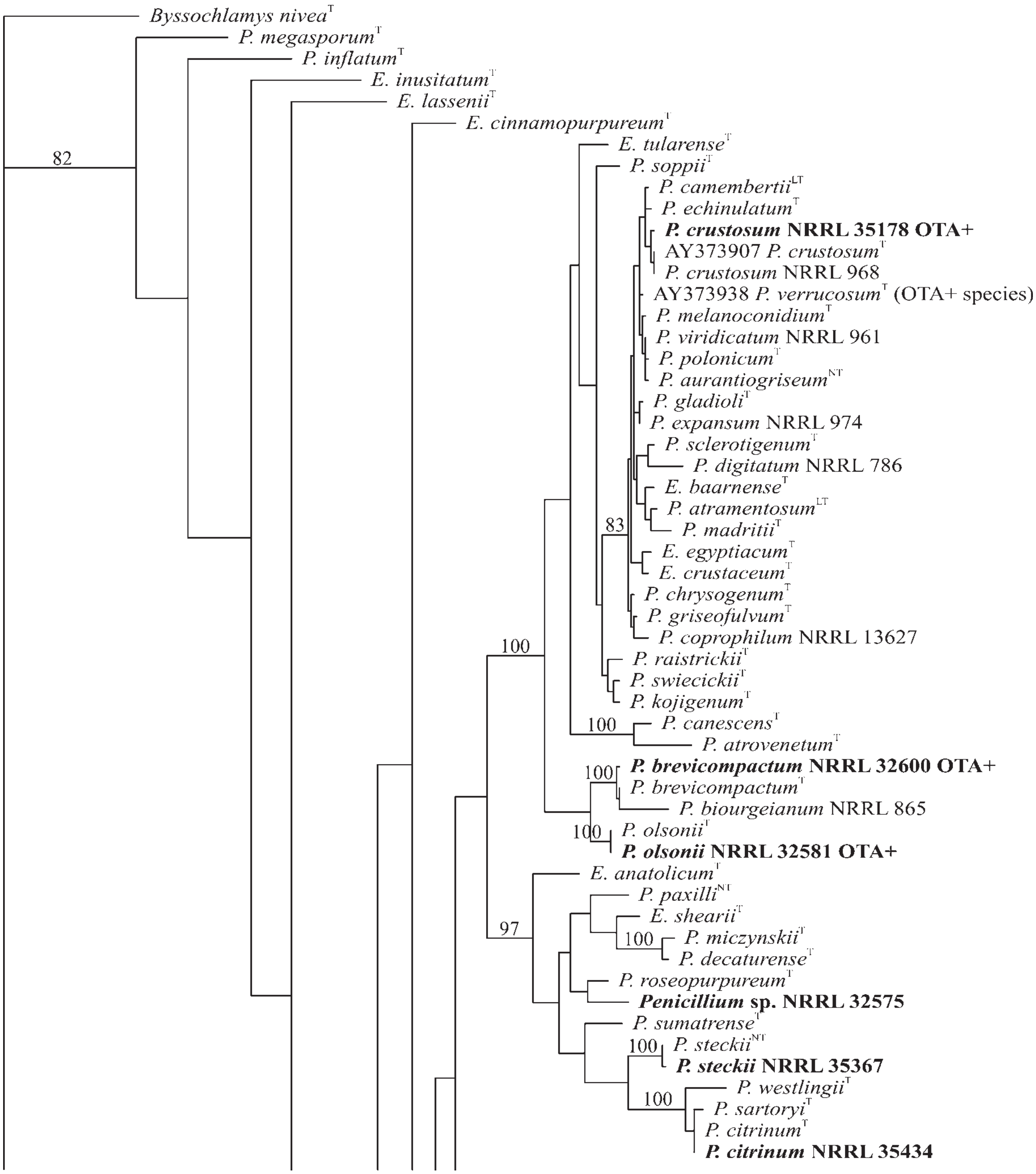

FIG. 1. Phylogenetic tree calculated from ITS and LSU rDNA sequences using PAUP* and parsimony criterion. Over 500 equally parsimonious trees were found. Numbers above internodes are bootstrap values calculated in 1000 samples. Isolates in bold type are endophytic species including only one isolate per species. OTA producing isolates are noted in the figure. Except for the *P. oxalicum* isolate, all OTA producing isolates are in the clade containing subgenus *Penicillium*. Tree statistics: length = 1827, CI = 0.3415, RC = 0.2544. The superscripts T, LT and NT stand for Type, LectoType and NeoType isolate, respectively.

amount of OTA production by the four *Penicillium* strains from coffee might be different in other media, as is the case for OTA levels in *A. westerdijkiae* isolates tested in three different media (Vega et al unpublished) and various OTA-producing *Aspergillus* species tested in two different media (Tsubouchi et al 1985). *A. ochraceus* (positive control) produced 5000–25 000 ppb OTA depending upon the medium tested (data not shown). The second positive control, *A. westerdijkiae* isolated from *P. nasuta*, produced 974 ppb and *A. westerdijkiae* isolated from *H. hampei* produced 2706 ppb.

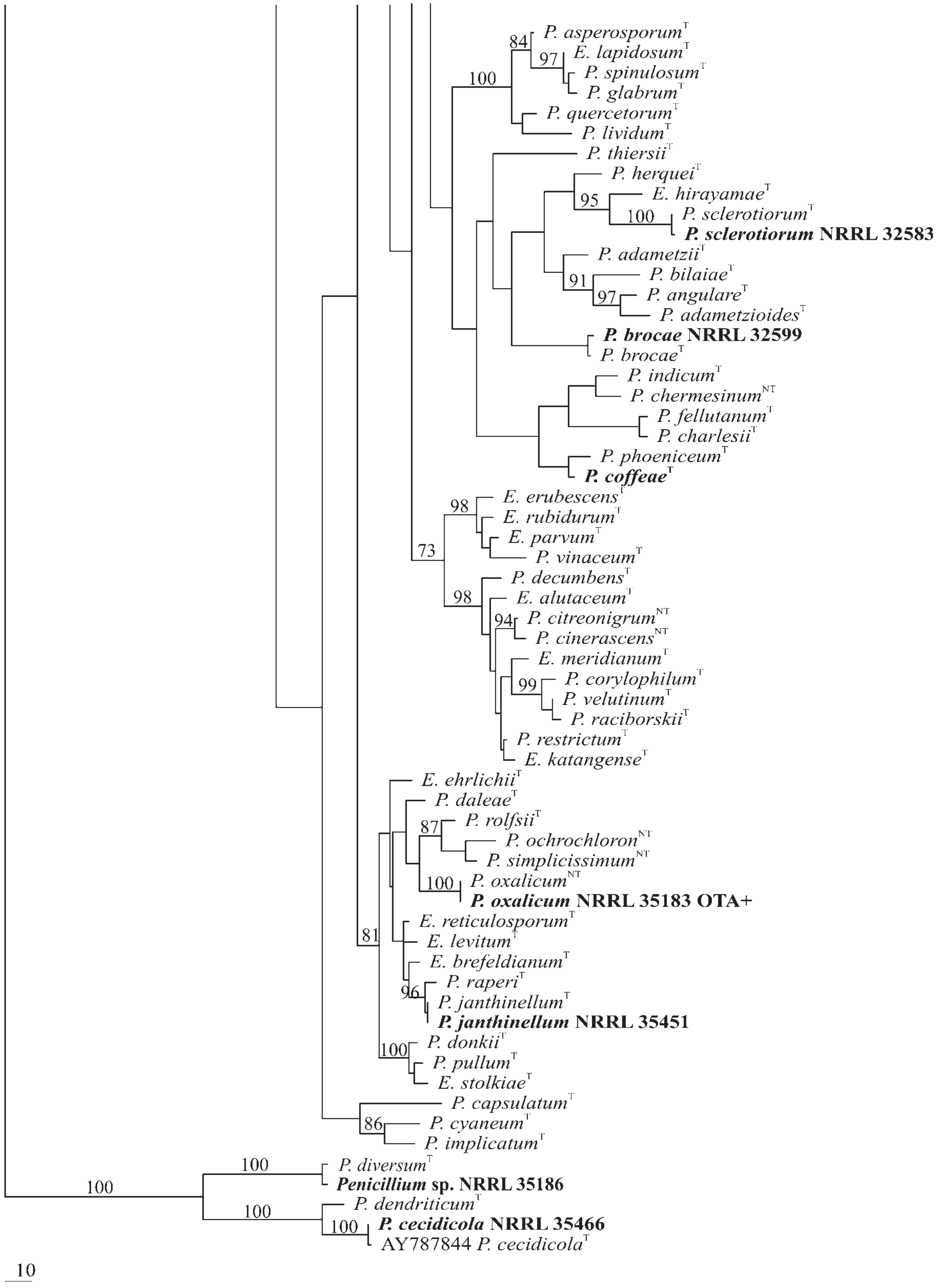

FIG. 1. Continued

DISCUSSION

Our study contrasts with previous reports of *Penicillium* endophytes in that it uses molecular data to identify 13 *Penicillium* species. The use of molecular data, particularly the ITS region, to identify environmental fungal isolates is a well established technique (Bruns and Shefferson 2004) that correlates well with other techniques used for species identification (Winton et al 2003). We have utilized the ID region that contains ITS and partial large subunit rDNA because ITS region sequences vary significantly in length between species making them difficult to align for use in phylogenetic studies. LSU rDNA sequences are less variable in length making them much easier to use in phylogenetic studies, and they also provide species-specific DNA sequences. Sequence similarity searches were performed using only ITS or using the entire ID fragment.

The accuracy and usefulness of any sequence based identification system relies on data quality, the thoroughness of taxon sampling and the species concepts applied. In *Penicillium*, the *Eupenicillium* clade has been well sampled (Peterson 2000) using ex-type isolates of most taxa and including sequences from the ITS and partial LSU rDNA region used in this study. Additional studies in *Penicillium* (Peterson 2004, Peterson et al 2004, 2005b) have used the GCPSR method (Taylor et al 2000) to assess species boundaries and found very low levels of intraspecific ID region variation for the species studied. These studies suggest that ID region DNA sequence identifications will correlate well with the more intricate GCPSR studies that clearly identify species boundaries. *Penicillium* taxonomy using the phenotypic approach can be quite difficult even for those with experience in the genus (Pitt et al 1990) and DNA sequence based identification methods provide an alternative that could be of value to specialists and nonspecialists.

The few studies reporting species identification for endophytic penicillia were performed using classical taxonomy methods. Both phenotypic and molecular identification methods found some of the same species living as endophytes. For example, dos Santos et al (2003) isolated seven *Penicillium* species (*P. citrinum*, *P. herquei* Bainier & Sartory, *P. janthinellum*, *P. rubrum* Stoll, *P. rugulosum* Thom, *P. simplicissimum* [Oudemans] Thom and *P. implicatum* Biorgue) from roots, stems, leaves and fruits of *Melia azedarach* L. while Yong et al (2003) reported *P. citrinum* as an endophyte in the bark of *Taxus cuspidata* and Singh et al (2003) reported *P. chrysogenum* Thom as an endophyte in the leaves of an unidentified plant in Peru. Evans et al (2003) isolated *P. aculeatum* Raper & Fennell, *P. glabrum* (Wehmer) Westling and *P.* sp.1 as endophytes in stems of the cacao relative *Theobroma gileri* in Ecuador. A myconodule-forming endophytic species, *P. nodositatum* Valla, has been reported in roots of *Alnus incana* (Valla et al 1989, Sequerra et al 1995); this species was originally named based on taxonomic differences with other members in the genus *Penicillium*, and was later confirmed to belong to a homogeneous taxon (i.e., *P. nodositatum*) based on molecular data (Sequerra et al 1997).

The remaining studies on *Penicillium* as an endophyte do not report a specific epithet. For example, ''*Penicillium* sp.'' has been reported as an endophyte in leaves of *Nicotiana* spp. (Spurr and Welty 1975), *Lycopersicon esculentum* Mill (Larran et al 2001), *Triticum aestivum* L. (Larran et al 2002a), *Pasania edulis* Makino (Hata et al 2002), *Glycine max* (L.) Merr. (Larran et al 2002b), *Acanthus ilicifolius* L. (Maria and Sridhar 2003), *Melia azedarach* L. (dos Santos et al 2003), and *Plumeria rubra* L. (Suryanarayanan and Thennarasan 2004); from the stalk of the grasses *Cynodon nlemfuensis* Vanderyst and *Paspalum fasciculatum* Willd. (Danielsen and Funck Jensen 1999); in *Vigna radiata* (L.) roots (Shaukat and Siddiqui 2001); in leaves and roots of *Musa acuminata* Colla (Indomal.) (Cao et al 2002); in leaves, petioles, rhizomes and roots of *Acrostichum aureum* L. (Maria and Sridhar 2003); in roots of *Pseudotsuga menziesii* (Mirb.) Franco and *Pinus ponderosa* Douglas ex Lawson & C. Lawson (Hoff et al 2004); and in the bark, stem and leaves of five medicinal plants from India (Raviraja 2005).

Concerning reports of *Penicillium* as a seed endophyte, two *Penicillium* species (*P. brevicompactum* and *P. canadense* Smith) have been reported in surface-sterilized seeds of *Pinus roxburghii* Sargent (Mittal and Sharma 1982). There are also some *Penicillium* reports in green coffee beans (Mislivec et al 1983, Batista et al 2003, Reynaud et al 2003), but these have to be taken with caution. Green coffee beans is the name commonly used for the coffee seed, which is the final product after coffee has been harvested, washed and dried and which is subsequently roasted before grinding. *Penicillium* sp. was reported by Reynaud et al (2003) in surface sterilized green seeds of *Coffea arabica* collected in Brazil while Mislivec et al (1983) reported the presence of *Penicillium* sp., *P. frequentans* (= *P. glabrum*), *P. citrinum*, *P. brevicompactum*, *P. cyclopium* Westling and *P. expansum* Link in green coffee beans from 31 coffee-producing countries, but the percent infection decreased dramatically in surface sterilized beans. These results indicate that although the fungi were found in surface sterilized beans, it is likely that these were not endophytic, otherwise there would not be any difference in percent infection in nonsurface sterilized and surface sterilized beans. A similar reduction in infection rates of eight *Penicillium* species (*P. aurantiogriseum* Dierckx, *P. brevicompactum*, *P. citrinum*, *P. corylophilum Dierckx*, *P. chrysogenum*, *P. expansum*, *P. glabrum* and *P. solitum* Westling) was reported by Batista et al (2003) after surface sterilizing green beans from Brazil in 1% sodium hypochlorite for 2 min. None of the *Penicillium* species reported by Batista et al (2003) was found to produce detectable levels of OTA in yeast-extract sucrose medium in contrast to our detection of OTA in extremely low levels in four of the isolates (TABLE I). This contrasts with Pitt (1987) who states that only *P. verrucosum* is an OTA producer and with Larsen et al (2001) who found OTA production only in *P. verrucosum* and *P. nordicum*. Our results and those of Batista et al (2003) document endophytic *Penicillium* spp. in coffee plants. However, the species found to produce OTA in this study are not likely to pose a risk to human health because the amount produced is minuscule (less than 1 ppb). The European Union (2005) has established maximum OTA levels of 5 ppb in roasted and ground coffee and 10 ppb in soluble coffee. It is still important to consider that OTA production in all endophytic *Penicillium* isolates from coffee might change if different media or natural substrates are tested. Except for the *P. oxalicum* isolate, all OTA producing isolates in this study are in the clade containing subgenus *Penicillium* (FIG. 1).

Of the thirteen *Penicillium* species we isolated as coffee endophytes, seven (*P. brevicompactum*, *P. crustosum*, *P.* sp. near *diversum*, *P. olsonii*, *P.* sp. near *roseopurpureum*, *P. sclerotiorum* and *P. steckii*) were associated with the coffee berry. A study by Pérez et al (2003) reported four *Penicillium* species (*P. brocae*, *P. citrinum*, *P. crustosum* and *P. olsonii*) associated with the coffee berry borer in Mexico. As adults, these insects bore a hole in the coffee berry and deposit their eggs, with larvae feeding on the endosperm, thus the insect and berry are intimately associated. Even though the coffee berry borer is not present in Hawaii or Puerto Rico, our results and those of Pérez et al (2003) raise the question of whether the insect might be inoculating the berry with fungi carried on its body or alternatively whether berries containing endophytes contaminate the insects as they feed on the berry. *Penicillium cecidicola* was isolated originally from cynipid wasp galls on oak trees in western USA (Seifert et al 2004), and the wasp was hypothesized to be a transmission vector of the fungus. In this study *P. cecidicola* was isolated from a coffee plant stem, showing that the relationship to wasp galls is not as specific as originally thought. It would be instructive to learn whether this species can be isolated from oak tissues other than the wasp galls.

It is noteworthy that the *A. westerdijkiae* isolates used as positive controls for OTA production were isolated from a coffee berry borer parasitoid (Peterson et al 2005a) and from the coffee berry borer itself. This suggests that both the parasitoid and the coffee berry borer could serve hypothetically as vehicles for the transmission of this fungus from the parasitoid to the insect and from the insect to the coffee berry. If the fungus were to establish itself in the berry and seed, it could be a source for OTA which could eventually be consumed by humans. Frisvad et al (2004) have reported on an ochratoxin producing strain of A. *westerdijkiae* from surface-disinfected green coffee beans from India. If *A. westerdijkiae* were to become endophytic in coffee seeds then it could potentially be present in seedlings emerging from those seeds, which could pose a risk of contamination in subsequent harvests from those plants. This area deserves further study.

Does the plant receive benefits for serving as a host for *Penicillium*? It is possible that due to the high number of metabolites produced by members of this genus (Mantle 1987; Frisvad and Samson 1991; Abramson 1997; dos Santos and Rodrigues-Fo 2002, 2003; Stierle and Stierle 2000; Singh et al 2003; Cole and Schweikert 2003; Cole et al 2003), the fungi might be protecting the plant against other fungi or insects, but this remains to be tested. Because OTA has anti-insect properties (Paterson et al 1987, Wicklow et al 1996), any association between the coffee berry borer and OTA producing penicillia would seem unsupportable in the long term. *Penicillium* species produce several other anti-insectan metabolites such as brevianamides, chaetoglobosins, cyclopenol, cyclopiazonic acid, E-64, griseofulvin, isoepoxydons, kojic acid, macrophorins, mycophenolic acid, okaramines, paraherquamides, patulin, penitrem A, rubratoxins, rugulosin, terphenyls, verruculogen, viomellein and xanthones (Dowd 2002). Documented production of particular fungal metabolites in planta would be necessary to show an anti-insectan or anti-herbivory value for the host plant.

The fact that no *Penicillium* species are reported as pathogens of *Coffea* spp. implies that these endophytes are not latent pathogens and suggests either commensal or mutualistic relationships. Potential benefits to *Penicillium* or other endophytes include: ''(1) greater access to nutrients; (2) protection from desiccation; (3) protection from surface-feeding insects; and (4) protection from parasitic fungi and the competition of other microbes'' (White et al 2000).

We have used molecular methods to identify 13 *Penicillium* species occurring as endophytes in coffee.

Four of these isolates were positive for OTA production in vitro, although at very low levels. The role that endophytic *Penicillium* species play in coffee plants remains unknown.

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