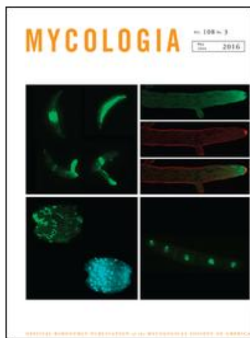

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Gibberella xylarioides (anamorph: *Fusarium xylarioides*), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the *G. fujikuroi* species complex

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Abstract: Tracheomycosis or coffee wilt has emerged as a major disease of robusta coffee in Uganda in the past 10 years. Coffee wilt historically has been associated with *Fusarium xylarioides* Steyaert (teleomorph *Gibberella xylarioides* Heim and Sacc.), a species that has been classified as a member of *Fusarium* section *Lateritium.* We investigated the molecular phylogenetics of fusarial coffee wilt isolates by generating partial DNA sequences from two protein coding regions, translation elongation factor 1-α and beta-tubulin, in 36 isolates previously identified as *F. xylarioides* and related fusaria from coffee and other woody hosts, as well as from 12 isolates associated with a current coffee wilt outbreak in Uganda. These isolates fell into two morphologically and phylogenetically distinct groups. The first group was found to represent previously unidentified members of the *Gibberella fujikuroi* species complex (GFC), a clade that replaces the artificial *Fusarium* section *Liseola.* This group of isolates fit the original description of *F. xylarioides,* thus connecting it to the GFC. The second group, which was diverse in its morphology and DNA sequences, comprised four distinct lineages related to *Fusarium lateritium.* Our finding of unrelated species associated with coffee wilt disease has important implications regarding its epidemiology, etiology and control.

Key words: coffee wilt, *Fusarium lateritium, Gibberella fujikuroi,* section *Liseola*

INTRODUCTION

Fusarium xylarioides Steyaert is the etiological agent most often associated with coffee wilt disease, a vascular wilt or tracheomycosis that has seriously affected coffee production in Africa. Nelson et al (1983) listed this species as ''insufficiently documented,'' and stated that isolates from diseased coffee plants from Ethiopia were similar to a ''female'' strain described by Booth (1971), but most fusariologists (Booth 1971, Gerlach and Nirenberg 1982, Gordon 1952) recognized the species and placed it in *Fusarium* section *Lateritium.* However, because section *Lateritium* was found to be nonmonophyletic in this study, we have elected to use ''*Lateritium* clade'' in reference to those taxa that appear to be monophyletic in this study. So-called ''male'' and ''female'' isolates described by Booth (1971) were thought to exhibit dimorphism in conidial and colony characters, with ''male'' isolates producing thin, elongated, 5–7-septate sporodochial conidia (often referred to as macroconidia) typical of *Lateritium* clade fusaria, and ''female'' isolates producing shorter, highly curved, 0–3-septate conidia. In diseased trees, the sexual stage *Gibberella xylarioides* Heim and Sacc. forms readily in the cracks of stem bark from the collar region of dying trees. Booth (1971) noted that *G. xylarioides* perithecia form in culture when opposing mating-types are brought together, thus inferring that the fungus is heterothallic. The existence of ''male'' and ''female'' strains was questioned by Gerlach and Nirenberg (1982), whose description of *F. xylarioides* included only the ''female'' conidial morphology, as did the original description of this species (Steyaert 1948).

Members of the *Lateritium* clade have been associated with diseases of coffee. *F. stilboides* Wollenweber causes bark and fruit rots of citrus and coffee (Gerlach and Nirenberg 1982). This species was considered a synonym of *F. lateritium* by a number of authors (Bilai 1955, Nelson et al 1983, Snyder and Hansen 1945). *F. lateritium* var. *longum* also has been

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associated with rot diseases of citrus and coffee. This taxon was considered a synonym of *F. stilboides* by some authors (Bilai 1955, Booth 1971) and of *F. lateritium* by others (Gordon 1952, Snyder and Hansen 1945, Subramanian 1971), while Gerlach and Nirenberg (1982) maintained it as a unique taxon. Producing long, thin sporodochial conidia that are often five or more septate, these species possess a morphology more typical of *F. lateritium* and its presumed relatives than *F. xylarioides* as described by Steyaert (1948) and Gerlach and Nirenberg (1982). Gerlach (1978) proposed that ''male'' isolates were mutants or variants of *F. stilboides*.

The *Gibberella fujikuroi* species complex (GFC) is a monophyletic and diverse group of approximately 50 phylogenetic species, many of which remain unnamed (O'Donnell et al 1998a, O'Donnell et al 2000). This complex is divided into three subclades, often referred to as African, American and Asian, based on the putative geographic origin of most of the species within them. The GFC approximates *Fusarium* section *Liseola* as defined by a number of authors, except it contains a number of taxa that produce chlamydospores. However, as defined morphologically, section *Liseola* is paraphyletic because it excluded chlamydospore-producing taxa. The GFC includes a number of diverse plant pathogens, including fusaria causing ear rot of corn (*F. verticillioides, F. proliferatum, F. subglutinans*), pitch canker of pine (*F. circinatum*), mango malformation (*F. mangiferae, F. sterilihyphosum* (Britz et al 2002)) and the gibberellin-induced bakanae disease of rice (*F. fujikuroi*), as well as numerous species that produce mycotoxins such as fumonisins and monilformin (Marasas et al 2001). A number of species recently have been recognized as members of the African clade of the GFC based on phylogenetic evidence (O'Donnell et al 1998a), including *F. udum,* which was placed in section *Elegans* by some authors (Wollenweber and Reinking 1935, Subramanian 1971, Gerlach and Nirenberg 1982) and in section *Lateritium* by others (Booth 1971). *F. udum* causes vascular wilt diseases of woody hosts, particularly *Cajanus cajan* (pigeon pea). It produces conidia with strongly curved or hooked apical cells, along with longer, up to 5-septate conidia, from densely and irregularly branched conidiophores. The apparently heterothallic sexual stage of *F. udum, G. indica* B Rai & RS Upadhyay has been observed on the roots and collars of wilted pigeon pea plants (Rai and Upadhyay 1982). In addition, *F. denticulatum,* originally identified as *F. lateritium* from sweet potato, also is nested within the African clade of the GFC. In his description of *G. xylarioides* Heim noted similarities between the *F. xylarioides* anamorph and the strongly falciform conidia of '*F. moniliforme* var. *anthophilum*' (now recognized as *F. anthophilum*) as illustrated by Wollenweber and Reinking (1935) and thus concluded that *F. xylarioides* belonged in Section *Liseola* (Heim 1950). This observation received little recognition perhaps in part due to the described production of chlamydospores by *F. xylarioides,* which would preclude its inclusion in section *Liseola*. In fact a number of diverse fusaria are known to produce highly curved conidia (e.g., *F. inflexum, F. avenaceum* var. *volutum,* insect-associated species such as *F. larvarum* and members of the GFC such as *F. succisae* and *F. udum*), and later descriptions of *F. anthophilum* do not depict the highly curved conidia illustrated in Wollenweber's and Reinking's (1935) monograph (Gerlach and Nirenberg 1982, Nelson et al 1983).

Two endemic species of coffee are cultivated in eastern Africa (Simpson and Ogorzaly 2001): *Coffea canephora* Pierre (robusta) and *C. arabica* L. (arabica), with robusta accounting for up to 90% of production in Uganda. Before the appearance of coffee wilt in 1993 only minor diseases were associated with robusta in Uganda (Hakiza and Mwebesa 1997). Coffee wilt can attack all stages of growth, from seedlings to mature plants, and infected plants show 100% mortality. Symptoms include wilting, defoliation and blue-black streaks in the wood and under the bark (Flood 1996, Guillemat 1946, Pochet 1988, Waller and Brayford 1990). On a multistemmed coffee plant, the external symptoms occur sequentially until all stems/branches are killed. Coffee berries on affected plants ripen prematurely and dry up but remain attached to the branches. Brown sunken lesions at the end of the stalk bearing berries also may be observed. Although pruned plants or stumps may sprout new suckers that appear healthy, the plants do not recover.

We used molecular phylogenetics to investigate the identity of fusaria associated with coffee wilt. Toward this end we determined partial DNA sequences from two protein coding regions, translation elongation factor 1-α (tef) and beta tubulin (*benA*), from 36 putative *Lateritium* clade fusaria previously identified as *F. xylarioides, F. stilboides* or *F. lateritium,* associated with coffee or other woody hosts, and analyzed them phylogenetically.

MATERIALS AND METHODS

Sample collection and pathogen isolation.—Isolates analyzed in this study are listed (TABLE I). For the Ugandan isolates, stem and/or branch tissue showing typical coffee wilt symptoms were collected from farmers' fields in nine districts where robusta is a major cash crop. Isolation of the pathogen was initiated within 7 d of sample collection. Samples

TABLE I. Isolates analyzed in this study

Accession number	Received or originally identified as	Host/substrate	Geographic origin	Current identification
FRC[a] L-55 = NRRL 13622[a] = ATCC[a] 60188	*F. lateritium*	elm with canker dieback	LOUISIANA, USA	*Lateritium* Clade III
L-69	*F. stilboides*	*Coffea arabica* berries	ZIMBABWE	*Lateritium* Clade I
L-81 = Sydney[a] F9064	*F. lateritium/stilboides*	orange twig	NEW CALEDONIA	*Lateritium* Clade IIA
L-82 = Sydney F9065	*F. lateritium/stilboides*	orange twig	NEW CALEDONIA	*Lateritium* Clade IIB
L-83	*F. lateritium/stilboides*	coffee berry	NEW GUINEA	*Lateritium* Clade I
L-84	*F. lateritium/stilboides*	coffee berry	NEW GUINEA	*Lateritium* Clade I
L-86	*F. lateritium/stilboides*	coffee berry	NEW GUINEA	*Lateritium* Clade I
L-87	*F. lateritium/stilboides*	coffee berry	NEW CALEDONIA	*Lateritium* Clade I
L-95 = MRC[a] 1845[b]	*F. xylarioides* 'male'	*Coffea arabica*	ETHIOPIA	*Lateritium* Clade I
L-96 = MRC 1853[b]	*F. xylarioides* 'female'	*Coffea arabica*	ETHIOPIA	*F. xylarioides*
L-101 = BBA[a] 62455	*F. xylarioides*	*Coffea canephora* vascular bundle	GUINEA	*Lateritium* Clade I
L-102 = BBA 62721 = CBS[a] 749.79 = NRRL 25804	*F. xylarioides*	*Coffea canephora*	GUINEA	*F. xylarioides*
L-107 = MRC 1926	*F. lateritium/stilboides*	coffee	ZIMBABWE	*Lateritium* Clade I
L-110 = Sydney F9084	*F. lateritium*	coffee twig	PAPUA NEW GUINEA	*Lateritium* Clade III
L-112 = Sydney F9086	*F. lateritium*	coffee twig	PAPUA NEW GUINEA	*Lateritium* Clade III
L-120	*F. lateritium*	coffee	not known	*Lateritium* Clade IIB
L-125	*F. lateritium*	*Coffea arabica*	ETHIOPIA	*F. xylarioides*
L-126	*F. xylarioides*	*Coffea arabica*	ETHIOPIA	*F. xylarioides*
L-127	*F. xylarioides*	*Coffea arabica*	ETHIOPIA	*F. xylarioides*
L-128	*F. xylarioides*	*Coffea arabica*	ETHIOPIA	*F. xylarioides*
L-200	*F. lateritium/stilboides*	soil	PHILIPPINES	*Lateritium* Clade IIA
L-375	*F. lateritium*	dry coffee berry	BRAZIL	*Lateritium* Clade I
L-376	*F. lateritium*	coffee seed	BRAZIL	*Lateritium* Clade I
L-388	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-389	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-390	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-391	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-392	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-393	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-394	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-395	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-396	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-397	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-399	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-400	*F. xylarioides*	*Coffea canephora*	UGANDA	*F. xylarioides*
L-402	*F. stilboides*	coffee bark	MALAWI	*Lateritium* Clade I
L-405 = NRRL 25485 = CBS 746.79 = BBA 63887	*F. stilboides* var. *stilboides*	citrus	NEW ZEALAND	*Lateritium* Clade IIA
CBS 258.52 = NRRL 25486	*Gibberella xylarioides* ex-type	*Coffea* sp.—trunk	IVORY COAST	*F. xylarioides*

[a] Culture collection abbreviations: FRC = Fusarium Research Center, University Park, PA, USA; NRRL = National Center for Agricultural Utilization Research, Peoria, IL, USA; ATCC = American Type Culture Collection, Manasas, VA, USA; Sydney = Fusarium Research Laboratory, Sydney, NSW, AUSTRALIA; MRC = Medical Research Council *Fusarium* Collection, Tygerberg, SOUTH AFRICA; CBS = Centraalbureau voor Schimmelcultures, Utrecht, NETHERLANDS; BBA = Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem, GERMANY

[b] L-95 (=MRC 1845) and L-96 (=MRC 1853) were derived as single conidia from a single culture originally identified as *F. xylarioides* from coffee, Ethiopia.

were cut into 15–20 cm lengths and placed in envelopes for transport to the laboratory. Samples (20 mm) from the margins of infected tissue were split lengthwise, surface sterilized in 2% (v/v) Jik (3.5 % sodium hypochlorite; Reckitt Benckiser East Africa Ltd., Nairobi, Kenya) for 3 min, rinsed three times in sterile distilled water, placed onto 2% tap water agar and incubated at 25 ± 2 C under fluorescent light 4 d. Cultures were purified by transferring 1 cm^2 plugs from the growing edges to potato-dextrose agar (PDA) and then purified by single-spore isolation. Other isolates were obtained from the Fusarium Research Center (FRC) Culture Collection at Pennsylvania State University.

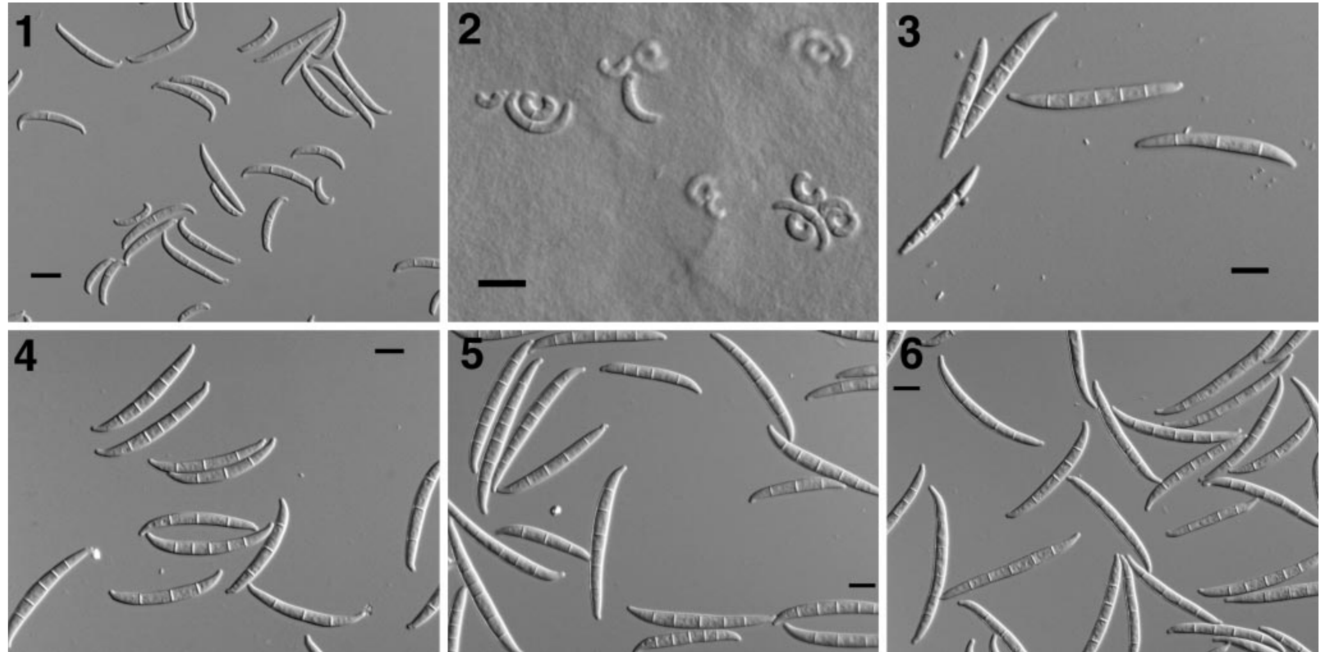
Culturing and maintenance of isolates.—The accession number, geographic area of sample collection and the source/host/substrate for isolates used in this study are listed (TABLE I). Isolates include those recently collected from an outbreak of coffee wilt on robusta in Uganda, as well as the ex-type culture of *G. xylarioides* (CBS 258.52 = NRRL 25486) and other isolates in the FRC collection that previously were identified as *F. lateritium, F. stilboides* or *F. xylarioides* from coffee, citrus and in one case soil. All cultures were derived from single conidia and were stored on synthetic low-nutrient agar (SNA) (Nirenberg 1976) slants at 4 C or lyophilized as described by Nelson et al (1983). Isolates from Uganda were deposited in the FRC collection under accession numbers L-388–397, L-399 and L-400. To study the morphological characteristics of the Ugandan isolates, they were grown on potato-dextrose agar slants (PDA; Difco Laboratories, Detroit, Michigan) and SNA and carnation leaf agar (CLA) plates (Fisher et al 1982), under black and white fluorescent lights with a 12 h photoperiod.

DNA extraction, PCR and nucleotide sequencing.—For DNA extraction, mycelium was transferred from PDA to 250 mL Erlenmeyer flasks containing potato-dextrose broth (Difco Laboratories, Detroit, Michigan). After 5 d growth at 25 ± 2 C without shaking, mycelium was harvested and DNA was extracted from the mycelium using a DNeasy Plant Minikit (Qiagen, Valencia, California). Polymerase chain reaction (PCR) amplification of the beta-tubulin gene region and sequencing of the 1000 bp amplicon was performed using primers bena-T1 and bena-T22 (O'Donnell and Cigelnik 1997). An approximately 690 bp portion of the translation elongation factor 1-α gene also was amplified and sequenced using primers ef1 and ef2 (O'Donnell et al 1998b). PCR for both primer sets was performed in a PTC-100 Programmable thermocycler (MJ Research Inc., Waltham, Massachusetts) using these conditions: 1× PCR buffer, 2 mM MgCl, 0.2 mM dNTP, 0.2 μM of each primer, 0.01 U Taq polymerase and 50 ng template DNA. These PCR parameters were used: 2 min at 94 C, followed by 35 cycles of 1 min at 94 C, 1 min at 53 C and 1 min at 72 C, followed by 5 min at 72 C. DNA sequencing was performed in both directions, using the same primers as used in PCR, except that the reverse primer bena-T2 was used instead of bena-T22 (O'Donnell and Cigelnik 1997). Sequences were generated using an Applied Biosystems Prism BigDye sequencing kit according to manufacturer's instructions, except that 8 μL volumes were used, and analyzed on an Applied Biosystems 3730 DNA Analyzer (PE Applied Biosystems, Foster City, California). Sequences were edited manually and deposited in GenBank under accession numbers AY707101–AY707173.

DNA sequence and phylogenetic analysis.—BLAST (Altschul et al 1990) was used to perform similarity searches comparing fusarial coffee wilt sequences with those in the GenBank database and local sequence databases, as described in Geiser et al (2004). Based on BLAST results, sequences were aligned by eye into files containing DNA sequences representing the phylogenetic breadth of the GFC (O'Donnell et al 2000), or to a new alignment of *Lateritium* clade sequences. Where identical sequences were found among isolates, a single representative was included in the phylogenetic analysis, and then its haplotype mates were added back to the inferred phylogenetic tree at the same position. Alignments were deposited in TreeBASE under accession number SN 1981. Maximum likelihood (ML) analyses were performed using the PAUP* phylogenetics package (beta versions 4.0b9–11:(Swofford 2003)). ModelTest version 3.0.6 (Posada and Crandall 1998) was used in conjunction with PAUP* to determine appropriate evolutionary models for ML analysis, which identified these models for the five different datasets analyzed phylogenetically: *Lateritum* clade tef (TrN model (Tamura and Nei 1993) with gamma shape parameter [G] set at 0.3229); *Lateritum* clade *benA* (HKY model (Hasegawa et al 1985) with transition/transversion ratio (TRatio) set at 2.5363 and G = 0.5481); GFC tef (TrN model with G = 0.3671); GFC *benA* (HKY model with TRatio = 2.4578 and G = 0.4240); and GFC combined (TrN model with proportion of invariable sites [I] set at 0.3221 and G = 0.7292). All models used observed base frequencies. For maximum likelihood analyses, alignment gaps were considered missing sites and heuristic searches were performed using random sequence addition and TBR branch swapping. Bootstrapping was performed using maximum parsimony as the criterion, using random sequence addition, and MAXtrees set at 10 000.

RESULTS

Growth and morphology of isolates.—Eighteen of the 36 isolates analyzed matched the descriptions of *F. xylarioides* by Gerlach and Nirenberg (1982) and Steyaert (1948), and of the "female" strain of *F. xylarioides* described by Booth (1971). Colonies of isolates L-388–397, L-399 and L-400 from Uganda were examined morphologically on PDA, SNA and CLA. Colonies on PDA and SNA were pale to colorless after 3 d and pale orange 4–14 d later depending on the isolate. The orange pigmentation was more intense in the center of the colony and faded to pale orange to white at the growing margin. Colony appearance on the reverse was the same. Orange pionnotes were produced in concentric rings by Day 4. On PDA and SNA, hyaline mycelium was sparse and oppressed. On SNA, 0–3-septate conidia, always curved in the apical cell and often in the basal cell



FIGS. 1–6. Conidia from selected GFC and *Lateritium* clade fusaria. Bar = 10 μm. 1. Sporodochial conidia from *Fusarium xylarioides* isolate L-388. 2. Conidia from the surface of a CLA plate produced by *F. xylarioides* isolate L-388. 3. Sporodochial conidia from *Lateritium* clade isolate L-69. 4. Sporodochial conidia from *Lateritium* clade isolate L-81. 5. Sporodochial conidia from *Lateritium* clade isolate L-110. 6. Sporodochial conidia from *Lateritium* clade isolate L-120.

(FIG. 1), were formed from irregularly branched conidiophores in sporodochia or pionnotes. On CLA, isolates formed abundant protoperithecia and sporodochia on carnation leaf pieces, and abundant, usually 0–1-septate conidia with an exaggerated curve were produced on the agar surface, with the tips of the apical and basal cells sometimes nearly touching each other (FIG. 2). Few aerial mycelia and no chlamydospores were observed after 21 d. Additional isolates from African coffee, L-96, L-102, L-125, L-126 and L-128, were similar morphologically in terms of conidial morphology and protoperithecium production but showed differences in colony color on PDA, ranging from pale, off-white shades to orange.

The remaining 18 isolates from coffee and other hosts varied considerably in their colony and micromorphological characteristics on PDA and CLA, but all produced elongate, often five or more septate sporodochial conidia more reminiscent of *Lateritium* clade fusaria than those described above (FIGS. 3–6).

DNA sequence analysis.—BLAST searches using partial translation elongation factor 1-alpha (tef) sequences showed that 18/36 isolates, comprising the ex-type of *G. xylarioides* CBS 258.52, the 12 isolates from Uganda (L-388–397, L-399–400) and isolates L-96, L-102, L-125, L-126, and L-128, appeared to be part of the *Gibberella fujikuroi* species complex. These isolates have the *F. xylarioides* "female" strain morphology. Two tef alleles were observed among these isolates that differed at 17/690 sites (2.5%). Partial beta-tubulin (*benA*) sequences of these isolates, however, were identical and also showed a clear grouping with the GFC.

Phylogenetics of isolates from coffee associated with the GFC.—tef and *benA* sequences from GFC-associated coffee wilt isolates were added to DNA sequence alignments representing the known phylogenetic breadth of the GFC (O'Donnell et al 1998a, O'Donnell et al 2000) and subjected to phylogenetic analysis using maximum likelihood as the criterion. Heuristic searches identified a single tree (FIG. 7A). The two tef alleles grouped in the African clade of the GFC, in a 98% bootstrap-supported clade with *F. udum, F. phyllophilum* and an undescribed *Fusarium* sp. NRRL 26064 from sorghum seed. However they were not inferred to be one another's closest relatives, given that the tef allele from the ex-type and isolate L-102 were more closely related to an undescribed *Fusarium* sp. NRRL 26064 than to the tef allele from other coffee wilt isolates. As is often the case with the tef locus alone, the African clade *sensu* O'Donnell (1998a) was not inferred to be monophyletic.

benA sequences were identical in all coffee wilt isolates from the GFC, and these sequences also

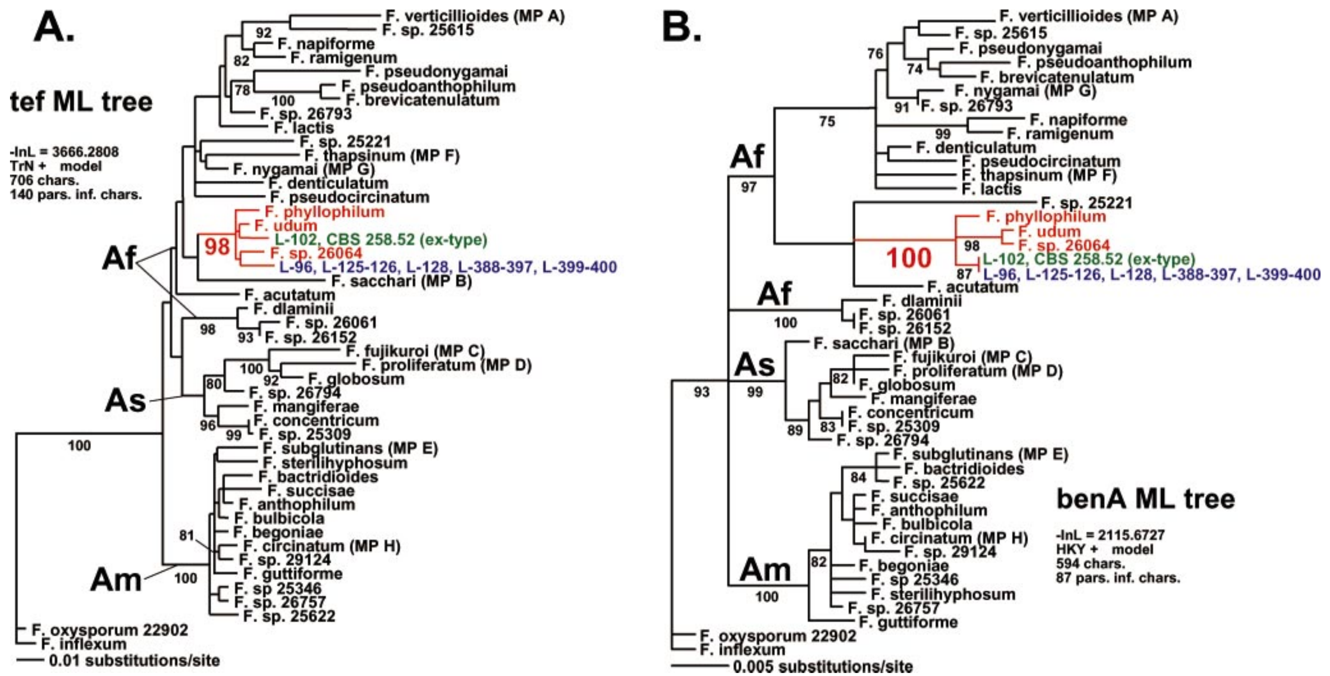


FIG. 7. Maximum likelihood phylograms of the *Gibberella fujikuroi* species complex including isolates from coffee, based on the A. tef and B. *benA* gene regions. The clade including *F. udum* and its relatives is highlighted in red, with the two coffee-associated tef alleles highlighted in green and blue. Bootstrap values (>70% shown) based on maximum parsimony analysis are given below the branches. Clades corresponding to African (Af), Asian (As) and American (Am) *sensu* O'Donnell (1998a, 2000) are labeled. MP = mating population or biological species.

grouped in the same part of the African clade of the GFC in a maximum likelihood analysis, which yielded a single tree (FIG. 7B). This tree also indicated a close phylogenetic relationship among these coffee wilt isolates and *F. udum*, *F. phyllophilum* and *Fusarium* sp. NRRL 26064, with 100% bootstrap support.

Because no strongly supported discordances were observed between the two gene trees, the data were combined and subjected to a phylogenetic analysis. This analysis yielded a single tree (FIG. 8), grouping all GFC coffee wilt isolates with *F. udum, F. phyllophilum* and the undescribed *Fusarium* sp. NRRL 26064 with 100% bootstrap support. Monophyly of the GFC coffee wilt isolates was inferred based on the combined data, albeit with weak (72%) bootstrap support. The beta-tubulin and combined trees both showed the three major biogeographic clades proposed by O'Donnell (1998a) to be monophyletic, including the African clade (FIGS. 7, 8).

Phylogenetics of isolates from coffee associated with the *Lateritium* clade.—BLAST searches of tef sequences from the remaining 18 isolates from the FRC collection listed as *F. lateritium, F. stilboides* or *F. xylarioides* from coffee showed highest similarity to that of *F. lateritium* isolate L-55 (= NRRL 13622 from elm, Louisiana, USA). A phylogenetic analysis of tef sequences from 56 diverse *Fusarium* isolates with *Gibberella* teleomorphs confirmed a close relationship between these isolates and *F. lateritium* isolate L-55 (results not shown).

Maximum likelihood analysis of the tef and *benA* datasets for the 18 *Lateritium* clade-associated coffee isolates, with isolates of *F. oxysporum* and *F. inflexum* used as outgroup sequences, yielded trees with nearly identical topologies and four strongly supported groups (FIG. 9). These groups did not correlate well with the previous species identifications. The first group (I) consisted of coffee isolates from around the world, including western, southern and eastern Africa, Brazil, New Guinea and New Caledonia. These isolates had been identified previously either as *F. lateritium, F. stilboides* or *F. xylarioides*. The next two groups (IIA and IIB) were closely related and consisted of citrus isolates from New Zealand, Philippines and New Caledonia, and Philippines soil and coffee, previously identified either as *F. lateritium* or '*F. lateritium/stilboides*'. The fourth distinct group (III) consisted of two isolates originally identified as *F. lateritium* that had nearly identical sequences from coffee in Papua, New Guinea. The tef allele shared by these two isolates was more similar to that *F. lateritium* reference isolate L-55 than those of other coffee isolates, but they differed from L-55 at ~2.3% of the tef sites.

FIG. 8. Maximum likelihood phylogram of the *Gibberella fujikuroi* species complex including isolates from coffee, based on a combined analysis of the tef and *benA* gene regions. The clade including *F. udum* and its relatives is highlighted in red, with the two coffee-associated tef alleles highlighted in green and blue. Bootstrap values (>70% shown) based on maximum parsimony analysis are given below the branches. Clades corresponding to African (Af), Asian (As) and American (Am) clades *sensu* O'Donnell (1998a, 2000) are labeled. MP = mating population or biological species.

Isolates in Group I produced a red pigment on PDA, with circadian rings of white aerial mycelium. Sprododochial conidia in this group were long, only slightly curved, with beaked apical cells and a nearly blunt basal cell. Abundant spindle-shaped 1–2-septate aerial conidia also were produced, and some isolates produced polyphialides. At this time a molecular or morphological connection has not been made between this group and a described *Fusarium* species beyond its placement within the *Lateritium* clade.

Isolates in Groups IIA and IIB also produced a red pigment on PDA, with pinkish aerial mycelium. Few aerial conidia were produced. Included in Group IIA was isolate L-405 (= NRRL 25485 = BBA 63887) from citrus in New Zealand. This isolate was described as a member of *F. stilboides* by Gerlach and Nirenberg (1982), and our observations of Groups IIA and IIB were consistent with their description of this species.

Isolates in Group III produced an orange pigment on PDA and some with aerial mycelium. Zero to two-septate aerial conidia were produced abundantly from a mixture of mono- and polyphialides, in addition to long, thin sporodochial conidia typical of *F. lateritium*.

DISCUSSION

Fusarium xylarioides *is a member of the GFC.*—Eighteen of the 36 isolates analyzed were members of the African clade of the GFC as defined by O'Donnell et al (1998a) with strong support in both genes, individually and in combination. Both genes also indicated *F. udum, F. phyllophilum* and an undescribed *Fusarium* sp. NRRL 26064 were their closest relatives, with consistently high bootstrap support. Included in this group was the ex-type of *G. xylarioides* (CBS 258.52 = NRRL 25486), the assumed sexual stage of this fungus. Morphologically these isolates matched the original description of *F. xylarioides* (Steyaert 1948) as well as that of the so-called "female" strains of this species described by Booth (1971), producing abundant 0–3-septate, highly curved conidia. On CLA, these isolates produced abundant protoperithecia, suggesting the possibility of an active sexual stage, consistent with *F. xylarioides* whose *Gibberella* sexual state is often observed in the field. We conclude that *F. xylarioides* Steyaert is a member of the GFC. This is consistent with the placement of *F. xylarioides* in section *Liseola* (Heim 1950).

One of the closest relatives of *F. xylarioides, F. udum,* is also an unusual member of the GFC. It too produces abundant 0–3-septate conidia that often are curved, although it also produces larger, 4–5-septate conidia. It also produces densely and irregularly branched conidiophores that are reminiscent of *F. xylarioides* (Gerlach and Nirenberg 1982). This species was assigned to GFC based on molecular phylogenetics (O'Donnell et al 1998a). In previous treatments, *F. udum* was placed in sections *Eupionnotes* (Wollenweber and Reinking 1935), *Lateritium* (Booth 1971) and *Elegans* (Gerlach and Nirenberg 1982). Like *F. xylarioides, F.udum* also causes a vascular wilt of a woody host, *Cajanus cajan* (pigeon pea or red gram), and has been observed to produce its sexual stage in the field (Rai and Upadhyay 1982). Although GFC comprises a diverse assemblage of plant pathogens, these two species are the only members of this complex associated with true vascular wilt diseases. Fusarial vascular wilt diseases are most commonly associated with the *F. oxysporum* complex, which approximates *Fusarium* section *Elegans*. Traditional sections *Elegans* and *Liseola* were distinguished by morphological characters that included

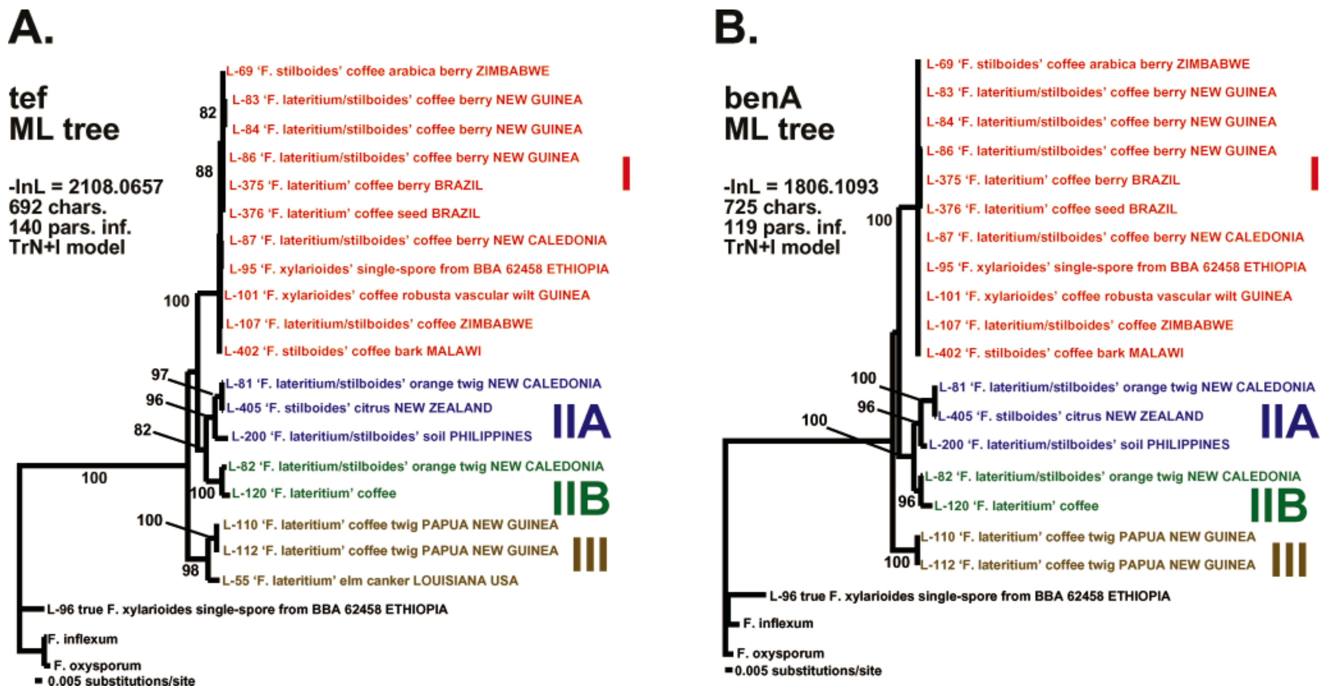


FIG. 9. Maximum likelihood phylograms of *Lateritum* clade fusaria primarily from coffee, based on the A. tef and B. *benA* gene regions. Clade I is highlighted in red, Clade IIA in blue, Clade IIB in green and Clade III in brown. Bootstrap values (>70% shown) based on maximum parsimony analysis are given below the branches. Taxon labels represent the species identifications listed in the FRC culture collection. Many names obviously are incorrect.

the presence of chlamydospores (never observed in section *Liseola*) and the production of polyphialides (never observed in section *Elegans*). Molecular phylogenetic analyses have demonstrated that both of these characters are likely plesiomorphic in the *F. oxysporum* and *G. fujikuroi* species complexes (Geiser et al 2001, O'Donnell et al 1998a), as now appears to be the case with the ability to cause true vascular wilt diseases.

Isolates identified as *F. xylarioides* all had identical *benA* sequences, but two distinct tef alleles were observed. The two tef alleles were 2.5% divergent and were not inferred as monophyletic (FIG. 7A). This finding leaves open the distinct possibility that *F. xylarioides* as discussed here might comprise multiple related cryptic species that the *ben*A locus failed to resolve. The two tef alleles observed were associated with geography, with L-102 and the *G. xylarioides* ex-type isolate CBS 258.52 possessing one allele and both coming from western Africa (Guinea and Ivory Coast respectively) and the remaining isolates with the alternate allele coming from countries in eastern Africa (Ethiopia, Uganda). Additional studies are under way to explore species boundaries in the group, using morphology, mating, and multilocus DNA sequence data.

O'Donnell et al (1998a) defined the "African" clade of the GFC based on the large number of species in the clade with African hosts, positing an African origin for this diverse group. There are exceptions to this trend, particularly *F. verticillioides,* which is known mostly in association with maize (American in origin) and is cosmopolitan in its distribution. All isolates of authentic *F. xylarioides* analyzed in this study were African in origin, and robusta and arabica coffee varieties are native to Africa (Simpson and Ogorzaly 1995), overall suggesting a common biogeographic origin for *F. xylarioides* and its only known host.

Coffee-associated fusaria related to Fusarium lateritium.—The remaining 18 isolates studied appeared to be diverse, but all showed a phylogenetic connection to the *Lateritium* clade. The majority (12/18) of isolates fell into one major clade, Clade I (FIG. 9A, B). These isolates did not exhibit morphological and molecular phylogenetic characteristics that allow a strong connection to a described species beyond *F. lateritium* broadly defined, and might represent a previously unnamed taxon. However all these geographically diverse isolates came from coffee, either from berries, bark or vascular tissue, and had been identified previously as *F. lateritium, F. stilboides* or *F. xylarioides.*

Isolate L-96 (= MRC 1853), an authentic member of *F. xylarioides* as delimited here, was derived from

a single macroconidium from a culture of BBA 62458, an isolate considered in the description of *F. xylarioides* contained in Gerlach and Nirenberg (1982). Derived from a single spore from the same culture at the same time was isolate L-95 (= MRC 1845). Isolates L-95 and L-96 are distinct morphologically, with the latter showing characteristics of the "female" morphology described by Booth (1971). They also are distinct phylogenetically, with L-95 being a member of Clade I of the *Lateritium* clade and L-96 being a member of the GFC.

Clades IIA and IIB in the *Lateritium* clade include isolates from citrus and coffee in New Caledonia, New Zealand and the Philippines. Isolate L-405 (= BBA 63887, NRRL 25485, CBS 746.79) in Clade IIA was described by Gerlach and Nirenberg (1982) as a member of *F. stilboides* var. *stilboides,* which is known to cause bark and fruit rot diseases of citrus and coffee in the tropics and subtropics (Booth 1971, Gerlach and Nirenberg 1982). These isolates were variable morphologically but generally fit the description of *F. stilboides* (Gerlach and Nirenberg 1982). Relationships of the five isolates comprising Clades IIA and IIB were identical within the tef and *benA* gene trees. However, because these clades differed at up to 4.7% of their nucleotide sites over both genes, we suspect that they comprise *F. stilboides* and one or more cryptic species.

Clade III was represented by two nearly identical isolates, L-110 and L-112, both isolated from perithecia on coffee twigs in Papua New Guinea. These isolates produced long, often six or more septate sporodochial conidia reminiscent of *F. lateritium.* They showed a strongly supported connection to *F. lateritium* isolate L-55 in their tef alleles (98% bootstrap support). However these two isolates differed from L-55 by 2.6%, suggesting these two groups are possibly not conspecific.

Unlike the well characterized GFC (O'Donnell et al 1998a, O'Donnell et al 2000), no comprehensive multilocus molecular phylogenetic studies have been performed on the *Lateritium* clade. The results here suggest that there might be a number of cryptic species within this group, warranting additional scrutiny of isolates from hosts other than coffee and additional substrates.

Sexual stages and associated dimorphism.—Previous reports of sexual dimorphism in *F. xylarioides* appear to be a matter of mistaken identity. So-called "female" isolates clearly correspond to *F. xylarioides,* while "male" isolates correspond to members of the *Lateritium* clade. Booth's (1971) description of the so-called "male" isolate of *F. xylarioides* included long, thin, 5–7 or more septate sporodochial conidia with a beaked basal cell, a characteristic we observed in all of the *Lateritium* clade studied from coffee. Gerlach (1978) stated that the "male" strain corresponded to a mutant or variety of *F. stilboides.* Different degrees of male and female sexual tendency frequently are observed in isolates of *Fusarium* species (Leslie and Klein 1996), but this is not known to correspond to dimorphism in morphological characteristics. Confusion about sexual dimorphism may have stemmed from the study of mixed cultures, as appears to have occurred where cultures of L-95 (GFC) and L-96 (*Lateritium* clade) apparently were derived from a single isolate. This might be the result of frequent co-occurrence of these very different fusaria on coffee plants, which in turn might lead to frequent co-isolation.

Are G. xylarioides *and* F. xylarioides *the same species?*—In addition to reported sexual dimorphism, a frequently observed sexual stage, *Gibberella xylarioides,* has been assigned to *F. xylarioides.* Previous evidence suggested that *G. xylarioides* indeed is the teleomorph of *F. xylarioides.* Gerlach and Nirenberg (1982) noted that isolates derived from single ascospores corresponded consistently with their concept of *F. xylarioides* and the so-called female morphology. In this study, the ex-type culture of *G. xylarioides* was identical in sequence to L-102 (= BBA 62721 = CBS 749.79), an isolate with clear *F. xylarioides* morphology, further suggesting a connection. However we have not gained access to type material associated with *F. xylarioides* and can only note similarity between the observed morphology of *F. xylarioides* as delimited here and the illustrations in its original description (Steyaert 1948). The discovery of two divergent tef alleles that might track with Western versus Eastern African origins, suggesting a possible cryptic species boundary, leaves open the possibility that the two names refer to two different species. The type of *F. xylarioides* was taken from a diseased coffee plant sent from Bangui, Central African Republic, and Steyaert inferred a connection between this collection and the fungus causing coffee wilt disease, in what was then French Equatorial Africa as well as the border region between Congo and Sudan (Steyaert 1948). Because no connection can be made between the type of *F. xylarioides* and these two potential cryptic species, we prefer to maintain the anamorph-teleomorph connection between *G. xylarioides* and *F. xylarioides* based on previous and current morphological observations.

Fusaria from coffee and coffee wilt.—The question is left open as to the differential roles of *F. xylarioides* and true *Lateritium* clade fusaria in coffee wilt. It appears that both groups can be isolated from coffee

wilt-inflicted plants. Isolates of the true *F. xylarioides* from Uganda cause coffee wilt when inoculated onto coffee plants, and the same fungus was cultured successfully from the diseased plants (Lewis Ivey et al 2003). Future studies should focus on the differential roles of *Lateritium* clade and GFC fusaria in this disease, both alone and in co-infection. *Lateritium* clade fusaria are known widely as wound pathogens of woody hosts, causing a wide variety of diseases (Gerlach and Nirenberg 1982). Members of this clade might be secondary invaders or opportunists on dead or dying plant parts that have been stricken with coffee wilt by infection with *F. xylarioides*. In addition the recognition of *F. xylarioides* as a member of the GFC might aid research on this pathogen, opening up the potential for knowledge transfer from its genetically well studied relatives such as *F. verticillioides* (Kroken et al 2003, Schoch et al 2003).

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