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Evolutionary Relationships among the *Fusarium oxysporum* f. sp. *cubense* Vegetative Compatibility Groups[▽]

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***Fusarium oxysporum* f. sp. *cubense*, the causal agent of fusarium wilt of banana (*Musa* spp.), is one of the most destructive strains of the vascular wilt fungus *F. oxysporum*. Genetic relatedness among and within vegetative compatibility groups (VCGs) of *F. oxysporum* f. sp. *cubense* was studied by sequencing two nuclear and two mitochondrial DNA regions in a collection of 70 *F. oxysporum* isolates that include representatives of 20 VCGs of *F. oxysporum* f. sp. *cubense*, other formae speciales, and nonpathogens. To determine the ability of *F. oxysporum* f. sp. *cubense* to sexually recombine, crosses were made between isolates of opposite mating types. Phylogenetic analysis separated the *F. oxysporum* isolates into two clades and eight lineages. Phylogenetic relationships between *F. oxysporum* f. sp. *cubense* and other formae speciales of *F. oxysporum* and the relationships among VCGs and races of *F. oxysporum* f. sp. *cubense* clearly showed that *F. oxysporum* f. sp. *cubense*'s ability to cause disease on banana has emerged multiple times, independently, and that the ability to cause disease to a specific banana cultivar is also a polyphyletic trait. These analyses further suggest that both coevolution with the host and horizontal gene transfer may have played important roles in the evolutionary history of the pathogen. All examined isolates harbored one of the two mating-type idiomorphs, but never both, which suggests a heterothallic mating system should sexual reproduction occur. Although, no sexual structures were observed, some lineages of *F. oxysporum* f. sp. *cubense* harbored *MAT-1* and *MAT-2* isolates, suggesting a potential that these lineages have a sexual origin that might be more recent than initially anticipated.**

Fusarium oxysporum Schlechtendahl emend. Snyder and Hansen is a cosmopolitan species (9) comprised of both pathogenic and nonpathogenic isolates (20). The pathogenic isolates of *F. oxysporum* cause fusarium wilt of several agricultural crops, and are accordingly subdivided into formae speciales (3, 26, 55). One of the economically more important and destructive formae speciales is the causal agent of fusarium wilt (Panama disease) of banana (*Musa* spp.), *F. oxysporum* f. sp. *cubense* (E. F. Smith) Snyder et Hansen. This disease has been reported in all banana production regions of the world, except those bordering the Mediterranean, Melanesia, Somalia, and some islands in the South Pacific (66, 77).

A range of approaches are typically employed for the characterization of *F. oxysporum* f. sp. *cubense* isolates. Based on virulence to specific banana cultivars (66, 67), the pathogen may be classified into one of three races (i.e., races 1, 2, and 4), although this designation may be contingent on environmental conditions. For instance, genetically identical isolates of *F. oxysporum* f. sp. *cubense* are classified as race 4 isolates in the subtropics and as race 1 isolates in the tropics because they cause disease to Cavendish bananas under subtropical conditions only (67, 86). Based on vegetative compatibility, *F. oxysporum* f. sp. *cubense* isolates have been separated into 24 so-called vegetative compatibility groups (VCGs) (5, 29, 47, 68). Finally, various DNA-based tools have been used to separate *F. oxysporum* f. sp. *cubense* into a number of clonal lineages that more or less correspond to their grouping based on VCGs (6, 22, 38, 59).

The evolutionary history of *F. oxysporum* f. sp. *cubense* is complex. Based on the results of phylogenetic studies (4–7, 22, 38, 57, 59). *F. oxysporum* f. sp. *cubense* represent multiple unrelated lineages, some of which are more closely related to other *formae speciales* of *F. oxysporum* than to other *F. oxysporum* f. sp. *cubense* lineages (3, 57, 59). This has lead to speculations that new pathogenic forms of *F. oxysporum* may be derived from other pathogenic and nonpathogenic members of this species (21). Factors such as coevolution with the plant host and the spread of virulence determinants via processes such as parasexuality, heterokaryosis, and sexual recombination also have been implicated in the evolution of this pathogen (11, 36, 37, 39, 64, 65, 69). Although parasexuality and heterokaryosis are known to occur in *F. oxysporum* (11, 39), sexual fruiting structures have never been observed in the species and only indirect evidence for sexual recombination has been detected (82). Indeed, the organization of the *F. oxysporum* f. sp. *cubense* mating type locus (*MAT*) is similar to those found in the closely related *Gibberella fujikuroi* (Sawada) Ito in Ito et K. Kimura complex and other heterothallic ascomycetes (2, 90).

Development of appropriate disease management strategies and the selection of *F. oxysporum* f. sp. *cubense*-resistant banana cultivars may benefit from a better understanding of the diversity and evolutionary history of the pathogen. Although most previous DNA-based studies provided knowledge regarding the diversity of *F. oxysporum* f. sp. *cubense*, the genetic relatedness among the lineages identified in these studies re-

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mains uncertain (22). It is also not clear how the different races and VCGs of *F. oxysporum* f. sp. *cubense* are related to one another and to other isolates of *F. oxysporum*. Therefore, the main objective of this study was to resolve the relationships among the *F. oxysporum* f. sp. *cubense* VCGs and determine their relationships with other formae speciales and nonpathogenic members of *F. oxysporum* by using a multigene phylogenetic approach (8, 32, 52, 53, 62, 75, 91). To facilitate the rapid differentiation of the various *F. oxysporum* f. sp. *cubense* lineages, we also aimed to develop a diagnostic PCR-restriction fragment length polymorphism (RFLP) procedure. To evaluate the potential of *F. oxysporum* f. sp. *cubense* to reproduce sexually, sexual crosses among isolates of opposite mating types were attempted after PCR-based detection of the *MAT-1* and *MAT-2* idiomorphs (34).

MATERIALS AND METHODS

Fungal isolates. A global collection of 70 *F. oxysporum* isolates representing 20 of the 24 VCGs, a new *F. oxysporum* f. sp. *cubense* VCG from Vietnam, other formae speciales of *F. oxysporum*, *F. oxysporum* isolates from heliconia (*Heliconia* sp.), and nonpathogenic *F. oxysporum* isolates from the rhizosphere of banana plants in South Africa (48) were included in this study (Table 1). All cultures are maintained in the culture collection (CAV) of the Forestry Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. DNA for cultures that were not available was kindly supplied by Suzy Bentley from the Queensland Department of Primary Industries (QDPI) in Brisbane, Australia.

Pathogenicity tests. To verify that the isolates included in this study are indeed specific pathogens of banana, 27 isolates representing 17 known VCGs of *F. oxysporum* f. sp. *cubense* were selected for pathogenicity tests on banana plants (Table 1). All cultures were grown on 20 g/liter potato dextrose agar (PDA) (Biolab Diagnostics, Wadeville, South Africa) for 10 days. A spore suspension was prepared by washing spores from mycelia with sterile distilled water, followed by filtration through cheese cloth and adjustment of the spore concentration to 1×10^6 spores/ml. Pathogenicity tests were performed with all isolates on Gros Michel tissue culture banana plantlets or Gros Michel and Bluggoe tissue culture banana plantlets for the new VCG from Vietnam and the *F. oxysporum* isolates from heliconia. Five tissue culture banana plantlets were inoculated for each isolate tested. The tests were conducted in a hydroponics system (49), and disease severity was measured after 6 weeks using a disease rating scale developed previously (12).

Morphological characterization. To confirm that the isolates included in this study represent *F. oxysporum*, their cultural and morphological characteristics were studied using the procedures described by Nelson et al. (50) and Leslie and Summerell (41). All *F. oxysporum* f. sp. *cubense* isolates were cultured on PDA (40 g/liter) and carnation leaf agar (41) and incubated at 25°C under white and near-UV fluorescent light for 12 days. Morphological features such as the presence and abundance of micro- and macroconidia, chlamydospores, and the size and shape of the macroconidia produced on carnation leaf agar were examined using light microscopy. Colony color and colony diameter were recorded after 3, 7, and 10 days of growth on PDA, and the presence of sclerotia and sporodochia was documented after 12 days.

DNA isolation, PCR amplification, and sequencing. Isolates of *F. oxysporum* f. sp. *cubense* and *F. oxysporum* were grown on 20 g/liter PDA medium for 7 days. DNA was isolated from the isolates as described previously (22). For phylogenetic analyses, we targeted the translation elongation factor-1α (TEF) and the mitochondrial small subunit (MtSSU) rRNA genes, as well as the rRNA intergenic spacer (IGS) region and a repeat region encoded in the mitochondrial genome (MtR) (T. Gordon, unpublished data). For this purpose, we used the primer sets EF1 and EF2 (59), MS1 and MS2 (88), and PNFo and PnF22 (17) to amplify regions of TEF, MtSSU, and IGS, respectively. To target the MtR region, primers R117 (5′-GTCAACCAGGAGCAGACTG-3′) and U9 (5′-GTA ACCTCTGACTCACCG-3′) were used. Each amplification reaction mixture contained ~5 ng/μl DNA, 0.3 μM of each primer, 250 μM deoxynucleoside triphosphates (dNTPs; Fermantas, Nunningen, Switzerland), 0.04 U/μl *Taq* DNA polymerase (Roche Molecular Biochemicals, Manheim, Germany), and PCR buffer with $MgCl_2$ (Roche). Cycling conditions consisted of 35 cycles at 94°C for 45 s, 60°C (TEF), 53°C (MtSSU), 50°C (IGS), or 59°C (MtR) for 45 s and 72°C for 90 s. Each PCR was preceded by an initial denaturation step at 94°C for 2 min and concluded with a final extension step at 72°C for 5 min. PCR products were purified using the High Pure PCR product purification kit (Roche Applied Biochemicals) and sequenced using the Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 377 automated sequencer (Applied Biosystems). In the case of IGS, we also designed and used an internal reverse primer, IGS2 (5′-GCCGGATTTGCTCCCTTCT-3′), for sequencing of the entire 1,500-bp fragment.

Phylogenetic analysis. Multiple sequence alignments were constructed using MAFFT, version 5.85 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/), with the L-INS-i option effective (30, 33). This option utilizes an iterative refinement method with various algorithms for optimization of local and pairwise alignments (30). Four data sets were constructed for the sequenced gene regions, where three of these comprised the MtR, IGS, and combined TEF-plus-MtSSU sequences of *F. oxysporum* f. sp. *cubense* generated in this study. The fourth data set represented an extended TEF-MtSSU data set that included *F. oxysporum* f. sp. *cubense* sequences, as well as sequences for other formae speciales of *F. oxysporum*, nonpathogenic *F. oxysporum* from South Africa, *F. oxysporum* isolates from heliconia, and sequences for *F. oxysporum* isolates that were obtained from GenBank. All ambiguously aligned sites, including a 148- or 156-bp insertion/deletion (indel) within MtR, were excluded from these analyses.

To test for the combinability of data sets, the partition homogeneity test (18) implemented in PAUP*, version 10b (79), was used on parsimony informative sites only (14, 16, 40). These tests were based on 1,000 repartitions and heuristic searches using 100 rounds of random sequence additions with tree bisection reconnection branch swapping. Phylogenies based on maximum parsimony (MP), Bayesian inference (BI), and maximum likelihood (ML) methods were inferred for the different data sets using PAUP*, version 10b, MrBayes, version 3.b.4 (27), and PhyML, version 2.4.3 (23), respectively. For these analyses, the best-fit models of evolution, as indicated by MrModeltest 2.2 (54) and Modeltest 3.7 (71), were used. For the *F. oxysporum* f. sp. *cubense* TEF-MtSSU data set, the BI analysis used separate parameters for each gene (27), where the Hasegawa, Kishino, and Yano (HKY) model (25) plus proportion invariable sites (I) were used for TEF and Felsenstein's model (19) for MtSSU. For ML analysis of the *F. oxysporum* f. sp. *cubense* TEF-MtSSU data set, the transitional model with equal base frequencies (TIMef) (81) was used. The IGS data set used the general time reversible (GTR) model (81) plus I and gamma correction for among-site variation (G) for BI and Kimura's (35) three-parameter model for the ML analysis. For the MtR data set, the BI analysis used Felsenstein's model (19), while ML used Tumara and Nei's (TN) (84) model with equal base frequencies (TNef). BI of the extended TEF-MtSSU data set utilized HKY plus I for TEF and HKY plus I plus G for MtSSU, while ML analysis of this data set utilized TN plus G. BI trees were constructed using the Metropolis-coupled Monte Carlo Markov chain with 2,000,000 generations, after which Bayesian posterior probabilities were calculated. MP and ML bootstrap confidence values were based on 1,000 replications and the same parameters described above.

DNA-based diagnosis of the *F. oxysporum* f. sp. *cubense* lineages. All sequences were screened for VCG- or lineage-specific polymorphisms in BioEdit, version 6.0.7 (24). As the IGS region contained polymorphisms for the different *F. oxysporum* f. sp. *cubense* lineages, five restriction enzymes were used for diagnostic PCR-RFLP purposes. These enzymes included AvaI (New England BioLabs, Hitchin, England), BbvI (New England BioLabs), BceAI (New England BioLabs), BsrDI (New England BioLabs), and Csp6I (Fermentas). All enzymes were used separately in PCR-RFLP digestion reactions and consisted of 5 μl IGS PCR product, 2 U of the restriction enzyme, and 2 μl of the supplied restriction buffer. After incubation at 37°C for 3 h, the restricted fragments were separated by agarose (3% [wt/vol]) gel electrophoresis (72).

Mating type diagnoses and mating studies. Mating types of the various *F. oxysporum* f. sp. *cubense* isolates were determined by PCR using the primer set Falpha 1 and Falpha 2 for *MAT-1* (2), and the primers GFmat2c (76) and FF1 (87) for *MAT-2*. PCR conditions were similar to those described above, apart from the use of annealing temperatures of 55°C for *MAT-1* and 54°C for *MAT-2*. Selected *MAT-1* and *MAT-2* products were also sequenced in both directions with the original PCR primers, as described above and compared to those in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN. Once the mating type of the isolates was known, two *F. oxysporum* f. sp. *cubense* isolates in each VCG were crossed in all possible combinations with isolates of the opposite mating type in other VCGs by procedures described by Leslie and Summerell (41). For the *F. oxysporum* f. sp. *cubense* lineages containing both mating types, all isolates of opposite mating type were crossed with each other. For all of the crosses, isolates were treated as males and females. *F. oxysporum* f. sp. *cubense* isolates were also crossed with the mating type tester of *Fusarium circinatum* (MRC 6213 or MRC 7488) (10) for control purposes, and crosses between the *F. circinatum* tester isolates were included as positive controls.

TABLE 1. Origin, pathogenicity, VCG, and mating type of isolates of *Fusarium oxysporum* f. sp. *cubense*, *F. oxysporum*, and *F. circinatum* selected for a multigene phylogenetic comparison

Isolate no.[a]	Other identification no.[a]	Species and forma specialis	VCG	Host or cultivar	Origin	Collector(s)	Pathogenicity[b]	Mating type[c]
CAV 009		*F. oxysporum* f. sp. *cubense*	0120	Cavendish	South Africa	A. Viljoen	Not tested	*MAT-2*
CAV 045		*F. oxysporum* f. sp. *cubense*	0120	Cavendish	South Africa	A. Viljoen	Not tested	*MAT-2*
CAV 105		*F. oxysporum* f. sp. *cubense*	0120	Cavendish	South Africa	A. Viljoen	Not tested	*MAT-2*
CAV 293	IC-1	*F. oxysporum* f. sp. *cubense*	0120	Dwarf Cavendish	Canary Islands	J. Hernandez	Pathogenic	*MAT-2*
CAV 294	34661	*F. oxysporum* f. sp. *cubense*	0120	Highgate	Honduras	R. Ploetz, American Type Culture Collection	Pathogenic	*MAT-2*
CAV 296	STH1	*F. oxysporum* f. sp. *cubense*	0120	Highgate	Honduras	R. Ploetz, R. H. Stover	Not tested	*MAT-2*
CAV 298	BR18	*F. oxysporum* f. sp. *cubense*	0120/15	Banana	Brazil	Unknown	Not tested	*MAT-2*
CAV 299	PD14-1	*F. oxysporum* f. sp. *cubense*	0120/15	Gros Michel	Nigeria	C. Pausberg-Gauhl	Not tested	*MAT-2*
CAV 612	RPCR1-1	*F. oxysporum* f. sp. *cubense*	01215	Gros Michel	Costa Rica	Unknown	Pathogenic	*MAT-2*
CAV 607	RP13	*F. oxysporum* f. sp. *cubense*	0122	Cavendish	Philippines	R. Ploetz	Pathogenic	*MAT-1*
CAV 605	RP14	*F. oxysporum* f. sp. *cubense*	0122	Cavendish	Philippines	R. Ploetz	Pathogenic	*MAT-1*
CAV 613	Phil 7	*F. oxysporum* f. sp. *cubense*	0126	Latundan	Philippines	Unknown	Pathogenic	*MAT-2*
CAV 793	Indo 33	*F. oxysporum* f. sp. *cubense*	0126	Pisang Rubus	Indonesia	R. Shivas	Pathogenic	*MAT-2*
CAV 794	Indo 38	*F. oxysporum* f. sp. *cubense*	0126	Pisang Rubus	Indonesia	Unknown	Not tested	*MAT-2*
CAV 1051	RP52	*F. oxysporum* f. sp. *cubense*	01210	Apple	United States	R. Ploetz	Pathogenic	*MAT-1*
CAV 632	RP26	*F. oxysporum* f. sp. *cubense*	01210	Highgate	Honduras	R. Ploetz	Pathogenic	*MAT-1*
CAV 847	Indo 35	*F. oxysporum* f. sp. *cubense*	01219	Pisang Raja Sereh	Indonesia	H. Stover	Pathogenic	*MAT-1*
CAV 195	Indo 25	*F. oxysporum* f. sp. *cubense*	01219	Pisang Ambon	Indonesia	N. Moore	Not tested	*MAT-1*
	RP7	*F. oxysporum* f. sp. *cubense*	0121	Cavendish	Taiwan	R. Ploetz	Pathogenic	*MAT-2*
	RP8	*F. oxysporum* f. sp. *cubense*	0121	Cavendish	Taiwan	R. Ploetz	Not tested	*MAT-2*
	RP9	*F. oxysporum* f. sp. *cubense*	0121	Cavendish	Taiwan	R. Ploetz	Not tested	*MAT-2*
CAV 810	Indo 34	*F. oxysporum* f. sp. *cubense*	01213	Pisang Berangan	Indonesia	I. Buddenhagen, J. C. Barlett	Pathogenic	*MAT-1*
CAV 811	Indo 30	*F. oxysporum* f. sp. *cubense*	01213	Pisang Susu	Indonesia	H. Stover	Pathogenic	*MAT-1*
CAV 300	CV-1	*F. oxysporum* f. sp. *cubense*	01213	Valery	Indonesia	Jepara Banana Plantation	Not tested	*MAT-1*
CAV 312	RPML 25	*F. oxysporum* f. sp. *cubense*	01213/16	Pisang udang	Malaysia	R. Ploetz	Pathogenic	*MAT-1*
CAV 313	RPML 47	*F. oxysporum* f. sp. *cubense*	01213/16	Pisang awak legor	Malaysia	R. Ploetz	Not tested	*MAT-1*
CAV 814	Indo 47	*F. oxysporum* f. sp. *cubense*	01216	Cavendish	Indonesia	I. Buddenhagen, R. Shivas	Pathogenic	*MAT-1*
CAV 815	Indo 56	*F. oxysporum* f. sp. *cubense*	01216	Cavendish	Indonesia	I. Buddenhagen, G. P. Salingay	Pathogenic	*MAT-1*
CAV 604	Indo 50	*F. oxysporum* f. sp. *cubense*	01216	Cavendish	Indonesia	Unknown	Not tested	*MAT-1*
CAV 602	23534	*F. oxysporum* f. sp. *cubense*	0124	Lady finger	Australia	Unknown	Pathogenic	*MAT-2*
CAV 609	23538	*F. oxysporum* f. sp. *cubense*	0124	Lady finger	Australia	Unknown	Pathogenic	*MAT-2*
CAV 786	23734	*F. oxysporum* f. sp. *cubense*	0124	Lady finger	Australia	K. Pegg	Not tested	*MAT-2*
	8611	*F. oxysporum* f. sp. *cubense*	0125	Lady finger	Australia	Unknown	Pathogenic	*MAT-2*
	23480	*F. oxysporum* f. sp. *cubense*	0125	Lady finger	Australia	Unknown	Not tested	*MAT-2*
	23487	*F. oxysporum* f. sp. *cubense*	0125	Lady finger	Australia	Unknown	Not tested	*MAT-2*
CAV 1097	22993	*F. oxysporum* f. sp. *cubense*	0128	Blue Java	Australia	Unknown	Pathogenic	*MAT-2*
CAV 1096	22994	*F. oxysporum* f. sp. *cubense*	0128	Bluggoe	Australia	Unknown	Pathogenic	*MAT-2*
	24211	*F. oxysporum* f. sp. *cubense*	01220	Cavendish	Australia	Unknown	Not tested	*MAT-2*
	24219	*F. oxysporum* f. sp. *cubense*	01220	Cavendish	Australia	Unknown	Not tested	*MAT-2*
CAV 957	Thai 37	*F. oxysporum* f. sp. *cubense*	0123	Kluai Namwa	Thailand	S. Kooariyakul	Not tested	*MAT-2*
CAV 929	PHIL 13	*F. oxysporum* f. sp. *cubense*	0123	Latundan	Philippines	L. Magnaye	Pathogenic	*MAT-2*
CAV 933	Thai 2-1	*F. oxysporum* f. sp. *cubense*	0123	Kluai Namwa	Thailand	N. Singburaudom	Pathogenic	*MAT-2*
	23510	*F. oxysporum* f. sp. *cubense*	0129	Lady finger	Australia	Unknown	Not tested	*MAT-2*
CAV 1100	23518	*F. oxysporum* f. sp. *cubense*	0129	Lady finger	Australia	K. Pegg	Pathogenic	*MAT-2*
	23631	*F. oxysporum* f. sp. *cubense*	01211	SH3142	Australia	Unknown	Not tested	*MAT-2*
	RP58	*F. oxysporum* f. sp. *cubense*	01212	Ney poovan	Tanzania	Unknown	Not tested	*MAT-2*
CAV 189	RPMW 40	*F. oxysporum* f. sp. *cubense*	01214	Harare	Malawi	R. Ploetz	Pathogenic	*MAT-1*
CAV 871	MAL 7	*F. oxysporum* f. sp. *cubense*	01217	Pisang Rastali	Malaysia	Unknown	Pathogenic	*MAT-2*
CAV 791	Indo 5	*F. oxysporum* f. sp. *cubense*	01218	Pisang Siem	Indonesia	N. Moore	Pathogenic	*MAT-1*
CAV 1107	Viet 6	*F. oxysporum* f. sp. *cubense*	0129/11	Chuoi xiem	Vietnam	I. Buddenhagen, N. Moore, S. Bentley	Not tested	*MAT-2*
CAV 1020	Viet 19	*F. oxysporum* f. sp. *cubense*	Unknown	Chuoi xiem	Vietnam	I. Buddenhagen, N. Moore, S. Bentley	Pathogenic	
CAV 1788		*F. oxysporum*		*Heliconia* sp.	South Africa	S. Tween	Nonpathogenic	
CAV 1787		*F. oxysporum* *F. circinatum*		*Heliconia* sp.	South Africa	S. Tween	Nonpathogenic	
CAV 211		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 273		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 202		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 261		*F. oxysporum* nonpathogenic		soil	South Africa	B. Nel		
CAV 246		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 275		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 231		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 274		*F. oxysporum* nonpathogenic		Soil	South Africa	B. Nel		
CAV 330	CBS 413.90	*F. oxysporum* f. sp. *lycopersici*		Tomato	Israel	R. Cohn		
CAV 337	CBS 411.90	*F. oxysporum* f. sp. *vasinfectum*		Cotton	Israel	J. Katan		
CAV 342	CBS 101.97	*F. oxysporum* f. sp. *lupine*		Lupinus	The Netherlands	M. Guranowska		
CAV 343	CBS 424.90	*F. oxysporum* f. sp. *melonis*		Melon	Israel	J. Katan		

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TABLE 1—*Continued*

Isolate no.[a]	Other identification no.[a]	Species and forma specialis	VCG	Host or cultivar	Origin	Collector(s)	Pathogenicity[b]	Mating type[c]
CAV 336	CBS 488.76	*F. oxysporum* f. sp. *raphanai*		Radish	Germany	W. Gerlach		
CAV 341	CBS 794.70	*F. oxysporum* f. sp. *perniciosum*		Silktree	Iran	W. Gerlach		
CAV 329	CBS 259.51	*F. oxysporum* f. sp. *lini*		Flax	Canada	J.W. Groves		
CAV 328	CBS 137.97	*F. oxysporum* f. sp. *gladioli*		Freesia	The Netherlands	E. J. A. Roebroeck, L. B. O. Lisse		
CAV 335	CBS 101587	*F. oxysporum* f. sp. *radicis-lycopersici*		Tomato		G. V. Bloemberg		

[a] CAV, Culture Collection at FABI, University of Pretoria, South Africa; numerals only, Culture Collection of the Queensland Department of Primary Industries, Brisbane, Australia; RP, Culture Collection of Randy Ploetz at the University of Florida, Homestead. DNA was supplied by Suzy Bentley from the Queensland Department of Primary Industries, Brisbane, Australia.

[b] The pathogenicity test was conducted in a hydroponics system (49), and disease severity was measured with a previously developed rating scale (12).

[c] Mating types were determined by PCR using the primer set Falpha 1 and Falpha 2 for *MAT-1* (2) and the primers GFmat2c (76) and FF1 (87) for *MAT-2*.

Nucleotide sequence accession numbers. Sequences determined in this study have been submitted to Genbank under accession no. FJ664901 to FJ66531.

RESULTS

Pathogenicity tests. All isolates designated *F. oxysporum* f. sp. *cubense* caused disease symptoms typical of fusarium wilt on Gros Michel and/or Bluggoe plantlets. After symptom development, the inoculated pathogens were reisolated from randomly selected plants to confirm Koch's postulates. The two *F. oxysporum* isolates obtained from heliconia, which were previously designated as *F. oxysporum* f. sp. *cubense* race 3 (68, 78), did not cause any symptoms on the respective banana hosts.

Morphological characterization. Isolates of *F. oxysporum* f. sp. *cubense* developed cultural and morphological traits typical of those described for *F. oxysporum* (41, 50). No significant differences were found in growth rate between isolates representing different VCGs, lineages, or clades of *F. oxysporum* f. sp. *cubense*. No sclerotium-like structures or sporodochia were produced by any of the isolates after 12 days. Microconidia were produced in false heads on short monophialides and were mostly single celled and kidney shaped. All isolates produced microconidia in abundance, with the exception of isolates associated with *F. oxysporum* f. sp. *cubense* VCGs 0126, 01210, and 01219, which produced few microconidia. Thin, sickle-shaped macroconidia were sparse or absent in most isolates, except for one isolate representing each of *F. oxysporum* f. sp. *cubense* VCGs 01210, 0126, and 0123. Chlamydospores were produced by *F. oxysporum* isolates from heliconia after 12 days, and for the *F. oxysporum* f. sp. *cubense* isolates only after 4 weeks, and in some cases after 6 weeks. Chlamydospores were formed singly and sometimes in pairs with a coarse protective wall.

Sequence and phylogenetic analysis. In this study, we sequenced ~650 bp, 700 bp, and 1,500 bp of the regions encoding TEF, MtSSU, and IGS, respectively. The length of our *F. oxysporum* f. sp. *cubense* MtR sequences ranged between 1,100 and 1,250 bp. Isolates associated with the same VCG had identical sequences for all of these regions. The partition homogeneity test supported the combination of the TEF and MtSSU datasets ($P = 0.9$) but rejected all of the possible combinations of the other regions ($P \leq 0.01$). Phylogenetic analyses of the combined *F. oxysporum* f. sp. *cubense* TEF-MtSSU data set separated the 48 isolates into two distinct clades (A and B) and eight lineages (I to VIII) (Fig. 1). Clade A included lineage I (VCG 01219), lineage II (VCGs 0126 and 01210), lineage III (VCGs 0129, 0129/11, and 01211), lineage IV (VCGs 0120, 01215, 0120/15, and 0122), and lineage V (VCGs 0121, 01216, 01213, and 01213/16). Clade B included lineage VI (VCGs 0123, 01217, and 01218), lineage VII (VCGs 0124, 0125, 0128, 01220, and 01212), and lineage VIII (VCG 01214).

The IGS and MtR (Fig. 2) data did not support the two main clades revealed by the TEF-MtSSU data. The MtR data did, however, cluster the 48 *F. oxysporum* f. sp. *cubense* isolates into groups that match the TEF-MtSSU-based lineages (Fig. 1). The IGS sequences also allowed separation of the isolates into groups that broadly match those based on the TEF-MtSSU and MtR data sets. The only exceptions were the divergent placement of *F. oxysporum* f. sp. *cubense* VCGs 0121, 0122, 01210, and 01214 in the IGS phylogeny. *F. oxysporum* f. sp. *cubense* VCG 0121 formed part of lineage V, based on the TEF-MtSSU data set, whereas it is associated with an exceptionally long branch in the IGS tree and was not related to other lineage V members. In the TEF-MtSSU tree, *F. oxysporum* f. sp. *cubense* VCGs 0122 and 01210 formed part of lineages IV and II, respectively, but grouped together in the IGS tree separate from isolates representing TEF-MtSSU-based lineages IV and II. In the IGS and MtR trees, the lineage VIII taxon, *F. oxysporum* f. sp. *cubense* VCG 01214, was nested in a clade of lineage VI isolates but displayed a sister group relationship with this lineage based on the TEF-MtSSU data.

The presence of a 148- or 156-bp indel at nucleotide position 901 relative to the R117 primer position in the IGS sequences separated the *F. oxysporum* f. sp. *cubense* isolates into three groups. One group of isolates representing *F. oxysporum* f. sp. *cubense* VCGs 0120, 0120/15, 01215, 0126, 01210, 01219, 0121, 01213, 01213/16, 01216, and 01218 lacked an insertion at this position and mostly corresponded to those included in clade A (Fig. 1). Isolates representing *F. oxysporum* f. sp. *cubense* VCGs 0124, 0125, 0128, 01220, 01212, 0123, and 01214 harbored a 148-bp insertion at this position, while the third group of isolates harbored a 156-bp insertion and included isolates representing VCGs 01211, 0122, 0129, and 0129/11 and corresponded to those in clade B. The only exceptions are the clade B taxon, VCG 01218, which lacked an insertion, and the clade

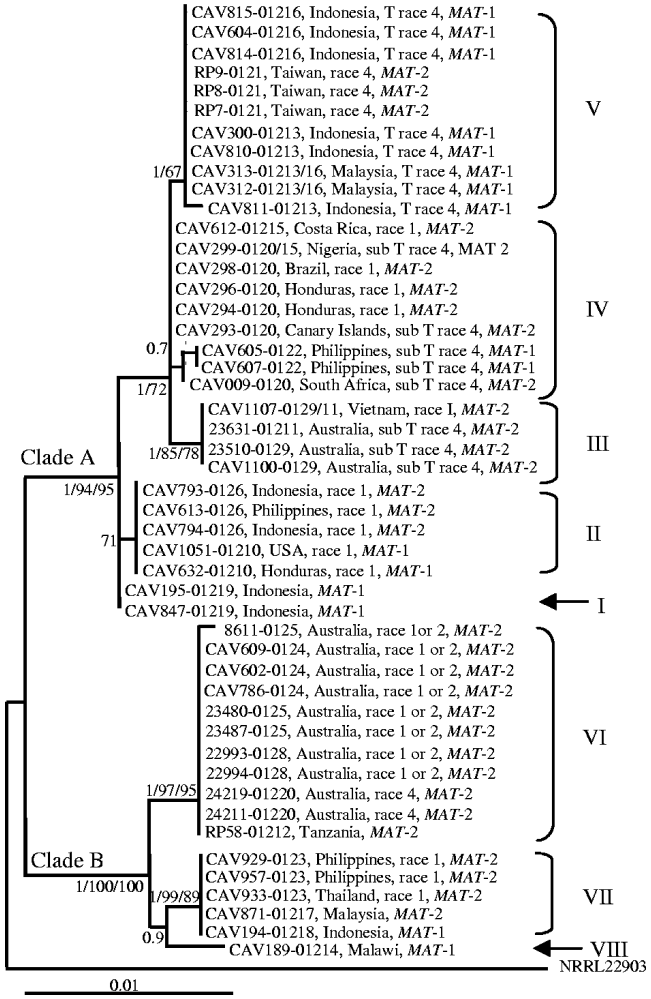

FIG. 1. ML phylogenetic tree of *F. oxysporum* f. sp. *cubense* inferred from combined TEF and MtSSU rRNA sequence data. A tree with a similar topology was generated using BI and MP (tree scores, confidence interval = 0.882 and retention index = 0.988). The two major clades are indicated at their respective branches with A and B, while the various *F. oxysporum* f. sp. *cubense* lineages (I to VIII) are indicated to the right of the tree. For each taxon, VCG and race designation, geographic origin, and mating type are indicated. Bayesian posterior probabilities (>0.7) and bootstrap values (>70%) for the ML analyses and MP are indicated in that order at the internodes. The tree is rooted with *Fusarium* sp. strain NRRL 22903.

A VCGs 0122, 01211, 0129/11, and 0129, which contained the 156-bp insert.

The extended TEF-MtSSU data set that included the *F. oxysporum* f. sp. *cubense* sequences, as well as those for other *F. oxysporum* isolates determined in this study and those obtained from GenBank, separated the isolates into four well-supported clades (Fig. 3). Two of these clades (A and B) correspond with those identified using the smaller TEF-MtSSU data set (Fig. 1) and included a representative set of all of the *F. oxysporum* f. sp. *cubense* isolates examined here. We also included isolates from the phylogenetic analysis by O'Donnell et al. (59), which allowed for a direct comparison between the two studies. Lineages C1, C2, C3, C4, and C5 identified in the previous study (59) correspond to our lineages VII, I, VI, II, and VIII, respectively. However, our results show that *F. oxysporum* f. sp. *cubense* is even more diverse than initially thought as it includes at least three additional lineages (lineages I, III, and V). Clade C included human pathogens, as well as formae speciales of *F. oxysporum* other than *F. oxysporum* f. sp. *cubense*. Clade D included the *F. oxysporum* isolates from heliconia and a single *F. oxysporum* isolate from human tissue. Although clade A consisted predominantly of *F. oxysporum* f. sp. *cubense* isolates, it also included isolates of *F. oxysporum* f. sp. *canariensis* and *F. oxysporum* f. sp. *perniciosum*. Clade B included a number of nonpathogenic *F. oxysporum* isolates, two *F. oxysporum* isolates from human tissue, *Fusarium inflexum*, and several formae speciales of *F. oxysporum.* Within clade B, an *F. oxysporum* f. sp. *cubense* isolate typed as VCG 01214 appeared to be more closely related to nonpathogenic *F. oxysporum* isolates; other formae speciales of *F. oxysporum* such as *raphanai*, *vasinfectum*, *melonis*, and *dianthi*; and *F. inflexum* than to *F. oxysporum* f. sp. *cubense*. Isolate CAV 1020, representing a novel *F. oxysporum* f. sp. *cubense* VCG from Vietnam, was included in clade B but was also more closely related to nonpathogenic isolates than to known *F. oxysporum* f. sp. *cubense* VCGs.

IGS PCR-RFLP. Of the four regions sequenced, IGS was most useful for identifying the different TEF-MtSSU-based lineages of *F. oxysporum* f. sp. *cubense*, and we selected five restriction enzymes to apply for diagnostic purposes (Fig. 4). Enzyme AvaI allowed separation of clades A and B (Fig. 4A). Within clade B, BbvI separates lineage VII from lineages VI and VIII (Fig. 4B). Among the clade A lineages, BceAI separates lineage V from lineages I, II, III, and IV (Fig. 4C), while Csp6I separates lineages I and II and lineages III and IV (Fig. 4D), and BsrDI separates lineages III and IV (Fig. 4E). No restriction enzyme was able to separate isolates of lineages VI and VIII from one another as well as from lineages I and II. However, isolates from lineage VIII (VCG 01214) harbor a 94-bp deletion within the MtR gene region at position 747 with respect to the forward primer and can therefore be separated from lineage VI by means of conventional agarose gel electrophoresis.

Mating type diagnoses and mating studies. The mating types of the *F. oxysporum* f. sp. *cubense* isolates were identified as *MAT-1* and *MAT-2* based on the presence of 370- and 700-bp fragments, respectively. Only one *MAT* amplicon was present per *F. oxysporum* f. sp. *cubense* isolate. *MAT-1* was present in *F. oxysporum* f. sp. *cubense* VCGs 0122, 01210, 01219, 01213, 01213/16, 01216, 01214, and 01218, and *MAT-2* was present in *F. oxysporum* f. sp. *cubense* VCGs 0120, 01215, 0120/15, 0126, 0121, 0124, 0124/5, 0125, 0128, 01220, 0123, 0129, 01211, 01212, 01217, and 0129/11 (Table 1 and Fig. 1 to 3). Both *MAT* amplicons were present within clades A and B, as well as within lineages II, IV, V, and VII.

No sexual fruiting structures were produced in any of the crosses between *F. oxysporum* f. sp. *cubense* isolates 8 weeks after incubation. Protoperithecium-like structures were, however, formed in some crossing combinations. These protoperithecial structures were dark purple to black and superficially resembled the perithecia (9) that were produced by crosses between the *F. circinatum* tester strains. Structures that were too small to be protoperithecia were also observed. Protoperithecia were abundantly produced when individuals from the

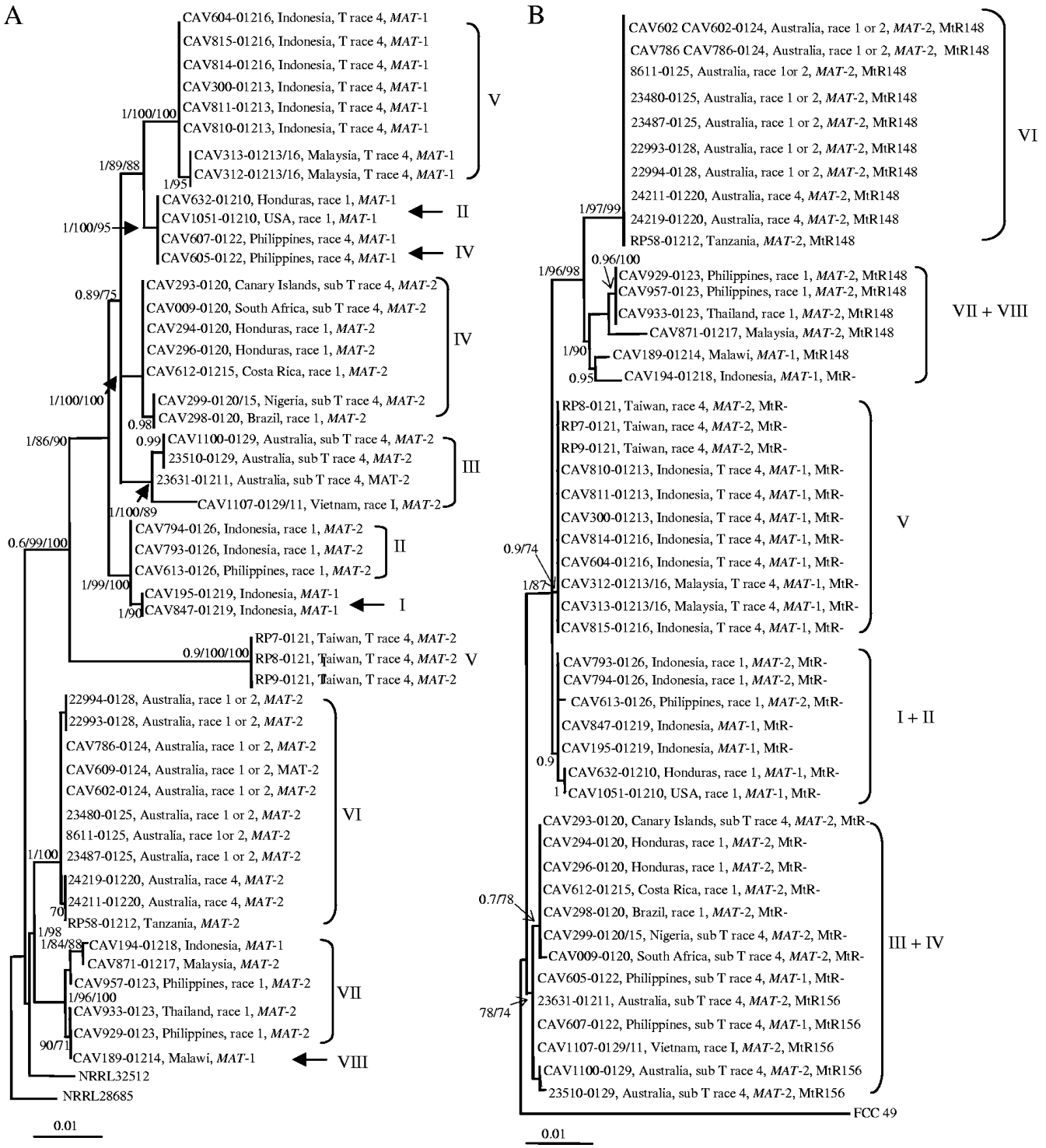

FIG. 2. ML phylogenetic tree of *F. oxysporum* f. sp. *cubense* inferred from sequences for the IGS region of the rRNA operon data (A) and the MtR (B). A tree with a similar topology was generated using BI and MP (tree scores, confidence interval [CI] = 0.779 and retention index [RI] = 0.956 for IGS and CI = 0.85 and RI = 0.98 for MtR). The TEF-MtSSU-based *F. oxysporum* f. sp. *cubense* lineages (I to VIII) identified in Fig. 1 are indicated to the right of the tree. For each taxon, VCG and race designation, geographic origin, and mating type are indicated. Isolates haboring the 156-bp and 148-bp MtR insertions are indicated by "MtR156" and "MtR148," respectively, and those lacking an insertion are indicated by "MtR−". Bayesian posterior probabilities (>0.7) and bootstrap values (>70%) for the ML analyses and MP are indicated in that order at the internodes. The tree is rooted with *Fusarium* sp. strain NRRL 28687 for IGS and *F. circinatum* for MtR.

FIG. 3. ML phylogenetic tree of *F. oxysporum* f. sp. *cubense* (*Foc*) and other isolates in the *F. oxysporum* complex inferred from combined TEF and MtSSU rRNA sequence data. A tree with a similar topology was generated using BI and MP (tree scores, confidence interval = 0.73 and retention index = 0.95). The three main clades are indicated at their respective branches with A, B, C, and D to the right of the tree. For each *F. oxysporum* f. sp. *cubense* taxon, VCG and race designation, geographic origin, and mating type are indicated. Taxa representing other *F. oxysporum* isolates are indicated as a human pathogen or nonpathogenic or with the specific forma specialis. Bayesian posterior probabilities (>0.7) and bootstrap values (>70%) for the ML analyses and MP are indicated in that order at the internodes. The tree is rooted with *Fusarium* sp. strains NRRL 22903 and NRRL 25184.

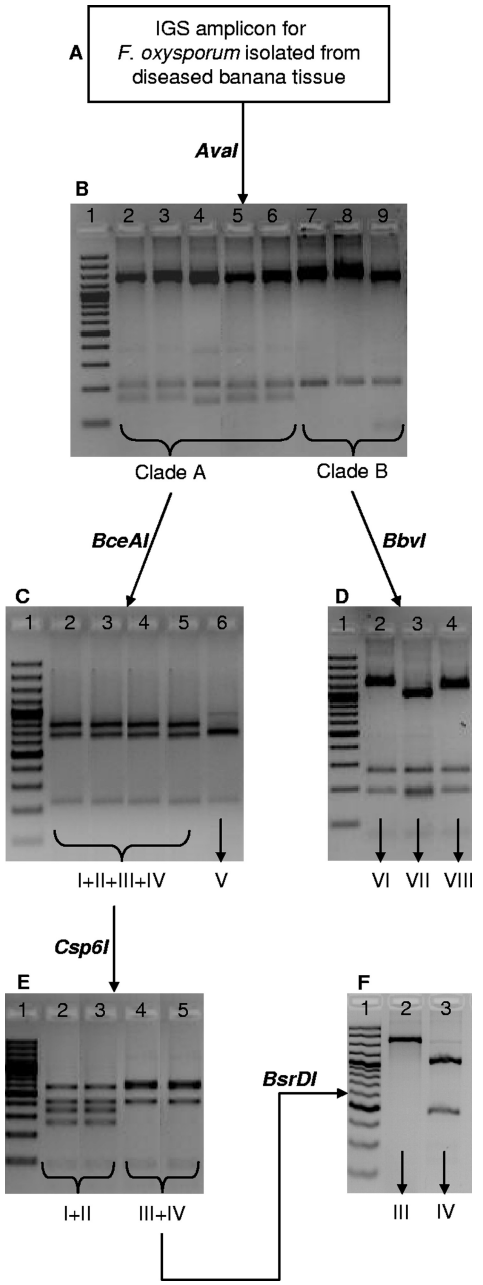

FIG. 4. PCR-RFLP analysis of the rRNA IGS region for differentiating the lineages of *F. oxysporum* f. sp. *cubense*. For this procedure, DNA obtained from a putative *F. oxysporum* f. sp. *cubense* isolate is used as a template for amplication of the IGS region (A), after which the amplicon is sequentially subjected to digestion with restriction enzymes AvaI (B), BceAI (C), BbvI (D), Csp6I (E), and BsrDI. (F) (B) Lane 1, 100-bp marker; lane 2, CAV 847 (lineage I); lane 3, CAV 613 (lineage II); lane 4, CAV 1100 (lineage III); lane 5, CAV 009 (lineage IV); lane 6, CAV 810 (lineage V); lane 7, CAV 957 (lineage VI); lane 8, CAV 608 (lineage VII); lane 8, CAV 189 (lineage VIII). (C) Lane 1, 100-bp marker; lane 2, CAV 794 (lineage I); lane 3, CAV 815 (lineage II); lane 4, CAV 294 (lineage III); lane 5, CAV 933 (lineage IV); lane 6, CAV 1098 (lineage V); lane 7, CAV 189 (lineage VI); lane 8, CAV (lineage VII); lane 8, CAV (lineage VIII). (D) Lane 1, 100-bp marker; lane 2, CAV 957 (lineage VI); lane 3, CAV 608 (lineage VII); lane 4, CAV 189 (lineage VIII). (E) Lane 1, 100-bp marker; lane 2, CAV 847 (lineage I); lane 3, CAV 613 (lineage II); lane 4, CAV 1100 (lineage III); lane 5, CAV 009 (lineage IV); lane 6, CAV 810 (lineage V). (F) Lane 1, 100-bp marker; lane 2, CAV 794 (lineage I); lane 3, CAV 815 (lineage II); lane 4, CAV 294 (lineage III); lane 5, CAV 933 (lineage IV).

opposite mating type within the same lineage were crossed with one another. No protoperithecium-like structures were produced when individuals from clade A were mated with those in clade B and vice versa. One or two protoperithecium-like structures were observed when *F. oxysporum* f. sp. *cubense* isolates were crossed with the two *F. circinatum* tester strains. The tester strains, when crossed with each other, produced abundant perithecia with fertile ascospores.

DISCUSSION

This study considered the evolution of the causal agent of fusarium wilt of banana, *F. oxysporum* f. sp. *cubense*, and its various VCGs and races. Based on the DNA sequence information of two nuclear (TEF and IGS) and two mitochondrial (MtSSU and MtR) regions, we demonstrate that *F. oxysporum* f. sp. *cubense*'s ability to cause disease on banana has emerged multiple times, independently in the *F. oxysporum* complex. Within the *F. oxysporum* phylogenetic framework, relationships between the VCGs and races of *F. oxysporum* f. sp. *cubense* are complex, which is consistent with pathogenicity to a specific banana cultivar being a polyphyletic trait. Also, as described more fully below, our data suggest that factors such as coevolution with the banana host, horizontal gene transfer events, and sexual reproduction may have played important roles in shaping the evolutionary history of the causal agent of fusarium wilt of banana.

Species concepts applicable to filamentous fungi (74) may be roughly divided into two broad categories: tree based, such as the phylogenetic species concept, and non-tree based, such as the biological and morphological species concepts (74). Among the taxa under study, the morphological species concept has no utility because the *F. oxysporum* f. sp. *cubense* isolates examined could not be differentiated based on morphological characters. Likewise, the biological species concept is not applicable because no fertility was observed in any crosses between isolates of opposite mating type, and a teleomorph stage for *F. oxysporum* has never been reported (41). On the other hand, a multigene phylogeny separated *F. oxysporum* f. sp. *cubense* isolates into eight distinct and mostly unrelated lineages (Fig. 1 and 3), which is consistent with results from previous studies (8, 37, 55, 59–61). These findings suggest that various *F. oxysporum* f. sp. *cubense* and other *F. oxysporum* lineages probably constitute distinct species for which discriminatory morphological properties may never be identified. The taxonomy of this group therefore requires extensive reevaluation using DNA-based measures of relationships.

The separation of *F. oxysporum* f. sp. *cubense* VCGs into distinct phylogenetic lineages consisting of clusters of related VCGs (Fig. 1, 2, and 3) correlates well with earlier studies using DNA fingerprinting techniques such as RFLPs (38), randomly amplified polymorphic DNAs and DNA amplification fingerprints (4–6), and amplified fragment length polymorphisms (22). This is also true for previous DNA-based phylogenetic studies (59). For all three of the *F. oxysporum* f. sp. *cubense* data sets (TEF-MtSSU, IGS, and MtR) used in the current study, isolates associated with the same VCG had identical sequences and clustered together irrespective of their geographic origin. All three of these data sets also consistently

clustered the same sets of VCGs into each of the *F. oxysporum* f. sp. *cubense* lineages, with the notable exceptions of *F. oxysporum* f. sp. *cubense* VCGs 0121, 0122, and 01210 in the IGS tree (Fig. 2). These discrepancies may potentially be associated with the specific nature of the IGS region as a relatively quickly evolving region with the potential for more than one sequence to reside within a single genome (1). This could preclude the inference of the true phylogenetic history from relationships based solely on IGS. A similar topological conflict between TEF and IGS was reported in a recent study of *F. oxysporum* isolates (58). In some *Fusarium* species, the inference of the true phylogenetic relationships using nucleus-encoded rRNA regions may also be complicated by the presence of multiple nonorthologous copies (56). Nevertheless, despite these potential limitations, the IGS region has proven to be an excellent marker for diagnoses of *Fusarium* spp. (31, 32, 73), as was apparent from this study in which lineage-specific PCR-RFLP fingerprints could be developed for *F. oxysporum* f. sp. *cubense* (Fig. 4).

The IGS PCR-RFLP fingerprinting method developed in this study presents a quick, easy, and accurate method to identify the lineages of *F. oxysporum* f. sp. *cubense*. These fingerprints also allow separation of *F. oxysporum* f. sp. *cubense* from nonpathogenic isolates of *F. oxysporum* (47). They could, therefore, be used for the early detection and characterization of *F. oxysporum* f. sp. *cubense* in infected planting material, whether symptomatic or not, in water, and in the soil. In laboratories without sequencing facilities and where VCG testers of *F. oxysporum* f. sp. *cubense* cannot be used because of national quarantine regulations, these fingerprints could be of great value in the characterization of the fusarium wilt pathogen of banana. It is, specifically, its ability to rapidly and accurately detect *F. oxysporum* f. sp. *cubense* "tropical" race 4 VCG 01213, 01216, and 01213/16 isolates from lineage V in new regions where this pathogen is introduced that could be invaluable in the isolation and management of this most devastating form of *F. oxysporum* f. sp. *cubense*.

Our results show that isolates representing *F. oxysporum* f. sp. *cubense* races 1 and 2 are scattered among the lineages in clades A and B, while isolates representing *F. oxysporum* f. sp. *cubense* race 4 are restricted to clade A (lineages IV and V), with the exception of VCG 01220 (clade B, lineage IV) from Australia, which caused disease in stressed Cavendish bananas (Fig. 1 and 3) (63). Our phylogeny therefore does not reflect the current race designation within *F. oxysporum* f. sp. *cubense.* Race designations in *F. oxysporum* f. sp. *cubense* and other *F. oxysporum* formae speciales are based on field evaluation and are generally known not to produce stable classifications (13, 15, 46). The classification of *F. oxysporum* f. sp. *cubense* into races in the greenhouse is even more difficult, as virulence is influenced by variables such as temperature, host age, and method of inoculation (13), and different pathogenicity tests used in different laboratories around the world could easily generate discordant results (15). Once universally acceptable greenhouse inoculation techniques have been developed and new potentially differential banana cultivars have been selected for race designation in *F. oxysporum* f. sp. *cubense*, the lineages in this study could serve as candidates for developing a new race structure.

In general, the formae speciales of *F. oxysporum* are not monophyletic (3, 22, 38, 59, 61) (Fig. 3). This is evident from phylogenetic trees where isolates representing different formae speciales grouped together, rather than with representatives from the same forma specialis. In our study, for example, *F. oxysporum* f. sp. *cubense* lineages I and II form part of clade A, where they are more closely related to *F. oxysporum* f. sp. *canariense* and *F. oxysporum* f. sp. *perniciosum* than to other isolates of *F. oxysporum* f. sp. *cubense* (Fig. 3). Also, within clade B, lineage VIII forms part of a group containing formae speciales such as *lini*, *dianthi*, *melonis*, *vasinfectum*, and *rhaphania*. In contrast, isolates of formae speciales such as *F. oxysporum* f. sp. *albedinis* (80), *F. oxysporum* f. sp. *ciceris* (28), or *F. oxyposrum* f. sp. *canariensis* (70) always represent each others' closest relatives, thus representing some of the few known instances of monophyletic formae speciales. However, from a genetic point of view, the polyphyletic nature of formae speciales is not surprising. This informal taxonomic rank is based on pathogenicity toward a specific plant host (36) and is largely dependent on the products of the avirulence genes harbored by the fungus (51). Recent studies have shown that these genes are generally subject to strong selection and horizontal gene transfer (83, 85, 89). As a result, the grouping of isolates based solely on host pathogenicity would commonly hide genetic diversity and biological differences (36) and may also artificially cluster unrelated isolates together, as has been demonstrated here and elsewhere (3, 22, 38, 59, 61). This highlights the importance of knowledge regarding pathogen diversity for development of reliable/durable plant resistance.

The occurrence of both mating types in *F. oxysporum* f. sp. *cubense* is reported for the first time in this study. Our results therefore confirm that *F. oxysporum* f. sp. *cubense* would be heterothallic should sexual reproduction take place, as either *MAT-1* or *MAT-2* sequences (never both) were detected in each of the isolates examined. The fact that in some cases, both *MAT-1* and *MAT-2* individuals were detected in a single closely related group of isolates implies that different lineages of the fusarium wilt pathogen have sexual origins that could be more recent than initially anticipated. These results, therefore, support the hypothesis that all fungi were originally sexual (42, 43, 82) and that sexual recombination may be followed by phases of clonal propagation of opportunistic varieties (44).

The results of all previous phylogenetic studies (22, 38, 59) demonstrate multiple origins for the evolution of *F. oxysporum* f. sp. *cubense* as a pathogen of bananas. However, the results presented here suggest that coevolution with the plant host in its center of origin in Wallace's Indo-Malayan region in Southeast Asia (69) has played an important role during this process. For example, the majority of *F. oxysporum* f. sp. *cubense* isolates in clade B originate from banana cultivars that represent *Musa balbisiana* × *Musa acuminata* hybrids with at least one chromosome derived from *M. balbisiana* (e.g., Lady finger and Bluggoe), while those in clade A mostly originate from banana cultivars with pure "A" genomes (i.e., all chromosomes derived from *M. acuminata*; e.g., Cavendish and Gros Michel) (7). It is therefore possible that *F. oxysporum* f. sp. *cubense* lineages I to V derived their ability to cause disease on banana, specifically on *M. acuminata*, from the ancestor of clade A. The ancestor of clade B, on the other hand, appears to have potentially

imparted to its descendants the ability to cause disease to banana cultivars of pure and hybrid background, as well as to plants in the related genus *Ensete*. This is because one member of clade B lineage VIII (VCG 01214) has the capacity to cause disease not only to enset, but also banana cultivars with pure A and mixed A-B genomes (Fig. 1).

In addition to coevolution with the banana host in its center of origin, the evolution of *F. oxysporum* f. sp. *cubense* might also have been influenced by other factors. Although *F. oxysporum* is considered to be strictly mitotic (20, 82), previous research has suggested that genetic exchange among and within individual lineages might occur more frequently than originally thought (82). This possibility is further emphasized by the results of the current study showing that one of the mitochondrial and nuclear regions examined (MtR and IGS, respectively) supported phylogenies that were highly incongruent with the *F. oxysporum* f. sp. *cubense* TEF-MtSSU tree. Therefore, the fact that some *F. oxysporum* f. sp. *cubense* VCGs cluster together in the IGS tree and separate in the TEF-MtSSU tree potentially reflects ancient recombination or genetic exchange beween *F. oxysporum* f. sp. *cubense* lineages. Such genetic exchange or recombination could be due to parasexuality, a nonsexual mode of genetic exchange, or heterokaryosis, a process that is initiated by fusion of vegetative hyphae (anastomosis) between individuals with very similar genomes and that has been shown to occur in *F. oxysporum* (11, 39). Taylor et al. (82) also demonstrated the possibility of recombination within some of the *F. oxysporum* f. sp. *cubense* clonal lineages. In their study, they reanalyzed previous RFLP data (38) and showed that recombination within some of the clonal lineages may exist. They also concluded that the lack of association between DNA amplification fragment-based DNA fingerprint groups (6) and VCGs is further evidence for recombination. The findings presented in the current study also support this notion as both mating types were detected in some *F. oxysporum* f. sp. *cubense* lineages, and crosses between many pairs of isolates of the opposite mating type resulted in the production of structures resembling immature perithecia.

Inclusion of isolates representing formae speciales other than *F. oxysporum* f. sp. *cubense*, nonpathogenic *F. oxysporum* isolates, and *F. oxysporum* isolates from human tissue in our phylogenetic analyses illustrates the great diversity that exists within the *F. oxysporum* complex. In this study, a single isolate from Vietnam (CAV 1020) that was confirmed as pathogenic to banana and shown to be associated with a novel VCG of *F. oxysporum* f. sp. *cubense* (Fig. 4) (6) grouped separately from all other *F. oxysporum* f. sp. *cubense* isolates (Fig. 3.). This result suggests that many distinct lineages of *F. oxysporum* f. sp. *cubense* may remain to be discovered and that additional surveys and research are needed for the full appreciation of the evolution of this pathogen. It also demonstrates that focusing on a single agricultural crop may lead to an overestimation of the clonality (45). Therefore, in order to pinpoint potential species boundaries within *F. oxysporum* and to elucidate the true relationships among the VCGs and lineages of *F. oxysporum* f. sp. *cubense*, the diversity of the *F. oxysporum* complex needs to be fully characterized.

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