

## Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies

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**ABSTRACT** Panama disease of banana, caused by the fungus *Fusarium oxysporum* f. sp. *cubense*, is a serious constraint both to the commercial production of banana and cultivation for subsistence agriculture. Previous work has indicated that *F. oxysporum* f. sp. *cubense* consists of several clonal lineages that may be genetically distant. In this study we tested whether lineages of the Panama disease pathogen have a monophyletic origin by comparing DNA sequences of nuclear and mitochondrial genes. DNA sequences were obtained for translation elongation factor 1 $\alpha$  and the mitochondrial small subunit ribosomal RNA genes for *F. oxysporum* strains from banana, pathogenic strains from other hosts and putatively nonpathogenic isolates of *F. oxysporum*. Cladograms for the two genes were highly concordant and a partition-homogeneity test indicated the two datasets could be combined. The tree inferred from the combined dataset resolved five lineages corresponding to “*F. oxysporum* f. sp. *cubense*” with a large dichotomy between two taxa represented by strains most commonly isolated from bananas with Panama disease. The results also demonstrate that the latter two taxa have significantly different chromosome numbers. *F. oxysporum* isolates collected as nonpathogenic or pathogenic to other hosts that have very similar or identical elongation factor 1 $\alpha$  and mitochondrial small subunit genotypes as banana pathogens were shown to cause little or no disease on banana. Taken together, these results indicate Panama disease of banana is caused by fungi with independent evolutionary origins.

Bananas, interspecific and intraspecific hybrids of *Musa acuminata* and *M. balbisiana* (Zingiberales: Musaceae), are perennial herbs that originated in southeast Asia, but which now have a pantropical distribution (1). Despite their reputation as desert items in the industrialized world, they are of far greater importance as staple foods for subsistence farmers in the tropics. Historically, diseases have been the most important constraint on banana whether for export or subsistence production. Panama disease or Fusarium wilt of banana, incited by the fungus *Fusarium oxysporum* f. sp. *cubense*, was the first serious disease to affect bananas produced for the export trade (2), and it ranks as one of the most destructive plant diseases of all time (3). Panama disease is still a serious threat to subsistence production and significant efforts now are being made to breed resistant hybrids by international agricultural research organizations. Before 1960, the disease had almost completely destroyed the export trade, which was saved only by the introduction of Cavendish cultivars that are generally resistant to this disease. Recent outbreaks of Panama disease

on the Cavendish clones in Southern Asia, however, threaten their continued use by the export trades (4).

*F. oxysporum* is a cosmopolitan soilborne filamentous fungus. A sexual state of the fungus never has been observed (5), and significant gametic disequilibrium reported among isolates of *F. oxysporum* from banana is consistent with the hypothesis that asexual reproduction is the predominant or exclusive reproductive strategy (6). Somatic fusion and heterokaryon formation between individuals can occur independent of sexual reproduction, but usually only among strains with similar genotypes (7). These exclusive networks of strains capable of heterokaryosis have been called vegetative compatibility groups (VCGs) (8).

Many isolates of *F. oxysporum* cause wilt diseases by systemically colonizing and occluding the vascular tissue of their hosts (9). Plants with these diseases typically show symptoms of wilting followed by death, hence the name “Fusarium wilt.” Over 120 host-specific forms of *F. oxysporum*, known as *formae speciales*, have been described (10); each *forma specialis* consists of strains with ability to cause wilt on a unique host or set of plant host species. Because the hosts of a given *forma specialis* usually are closely related, it has been assumed that members of a *forma specialis* are also closely related and may have arisen by descent from a common ancestor (7). However, results from recent work on *F. oxysporum* f. sp. *cubense* have brought these assumptions into question. Koenig *et al.* (6) used anonymous, single-copy restriction fragment length polymorphisms (RFLPs) to define 10 clonal lineages from a worldwide collection of *F. oxysporum* f. sp. *cubense*. These results showed that pathogens of banana could be as closely related to pathogens of other hosts, such as tomato, as they are to one another. Considerable genetic diversity within *F. oxysporum* f. sp. *cubense* also has been inferred from the high level of chromosomal polymorphisms found among strains (11, 12), random amplified polymorphic DNA fingerprints (13), and from the number and geographic distribution of VCGs (4).

The objectives of this study were to determine the phylogenetic relationships among strains of *F. oxysporum* that cause Fusarium wilt of banana, and to determine whether the collection of strains with specificity to banana have descended from an exclusive common ancestor. To address these questions a subset of strains from different lineages, as assigned by RFLP data (6), were chosen for direct DNA sequence analysis together with isolates of *F. oxysporum* that were either pathogenic on other hosts or putatively nonpathogenic. Results of

Abbreviations: EF, elongation factor; mtSSU, mitochondrial small subunit; VCG, vegetative compatibility group; RFLP, restriction fragment length polymorphism; MPT, most parsimonious tree.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF008446–AF008515, U34509, U34519, and U61608).

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these studies indicate that strains of *F. oxysporum* that cause disease in banana are not monophyletic and appear to have multiple evolutionary origins. The implications of these results and their bearing on the *forma specialis* concept are discussed.

## MATERIALS AND METHODS

**Biological Materials.** Fungal strains listed in Table 1 were derived from single microconidia and are stored cryogenically at  $-175^{\circ}\text{C}$  in the Agricultural Research Service (NRR) Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL). Genomic DNA, was prepared by using a hexadecyltrimethylammonium bromide protocol as described previously (14). Pathogenicity of isolates to banana was determined by the method of Ploetz and Shepard (15) with minor modifications using tissue-culture-derived plantlets of the banana cultivars Gros Michel, Bluggoe, and Pisang awak. Experiments were terminated 6 weeks after planting, at which time plants were dissected to determine the extent of internal symptom development.

Table 1. Strains used in this study

Taxon <sup>a</sup> [C# = clonal lineage] <sup>b</sup>	NRR strain number <sup>c</sup>	Host or substrate <sup>d</sup>
<i>F. inflexum</i>	20433	<i>Vicia faba</i>
<i>F. oxysporum</i>	25356	Soil
<i>F. oxysporum</i>	25357	Soil
<i>F. oxysporum</i>	25369	<i>Terminalia ivorensis</i>
<i>F. oxysporum</i>	26374	<i>Homo sapiens</i>
<i>F. oxysporum</i> f. sp. <i>batatas</i>	26409	<i>Ipomoea batatas</i>
<i>F. oxysporum</i> f. sp. <i>canariensis</i>	26035	<i>Phoenix canariensis</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C5]	25367	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C2]	25603	<i>Musa acuminata</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C2]	25605	<i>Musa acuminata</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C1]	25607	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C5]	25609	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C3]	26022	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C4]	26024	<i>M. acuminata</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C4]	26029	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>cubense</i> [C2]	26038	<i>Musa acu.</i> × <i>M. bal.</i>
<i>F. oxysporum</i> f. sp. <i>erythroxyli</i>	26574	<i>Erythroxylum coca</i>
<i>F. oxysporum</i> f. sp. <i>glycines</i>	25598	<i>Glycine</i> sp.
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26034	<i>Solanum esculentum</i>
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26037	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26200	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26202	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26203	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	26383	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>melonis</i>	26178	<i>Cucumis melo</i>
<i>F. oxysporum</i> f. sp. <i>melonis</i>	26406	<i>C. melo</i>
<i>F. oxysporum</i> f. sp. <i>passiflorae</i>	22549	<i>Passiflora edulis</i>
<i>F. oxysporum</i> f. sp. <i>perniciosum</i>	22550	<i>Albizia julibrissin</i>
<i>F. oxysporum</i> f. sp. <i>radicislycopersici</i>	26033	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>radicislycopersici</i>	26379	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>radicislycopersici</i>	26380	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>radicislycopersici</i>	26381	<i>S. esculentum</i>
<i>F. oxysporum</i> f. sp. <i>tuberosi</i>	22555	<i>S. tuberosum</i>
<i>F. oxysporum</i> f. sp. <i>vasinfectum</i>	25420	<i>Gossypium hirsutum</i>
<i>Fusarium</i> sp.	22903	<i>Pseudotsuga mensiesii</i>
<i>Fusarium</i> sp.	25184	<i>Vitis vinifera</i>

<sup>a</sup>EF-1 $\alpha$  and mtSSU rDNA sequences deposited in GenBank (<http://www.ncbi.nlm.nih.gov>) under accession numbers AF008446–AF008515, U34509, U34519, and U61608.

<sup>b</sup>The number in parenthesis following the nine strains identified as *F. oxysporum* f. sp. *cubense* indicates the clonal lineage.

<sup>c</sup>Only four strains included in this study (25356, 25357, 25369, and 26374) are not known to be plant pathogens.

<sup>d</sup>*Musa acu.* × *M. bal.* = *Musa acuminata* × *M. balbisiana*.

**DNA Amplification and Sequencing.** PCR amplification and sequencing of the mitochondrial small subunit (mtSSU) rDNA was performed by using reagents and primers described in White *et al.* (16) and O'Donnell and Cigelnik (17). The 5' portions of translation elongation factor (EF) 1 $\alpha$  coding region and introns were amplified with primers EF-1 and EF-2, which prime within conserved exons (Fig. 1). In addition to the amplification primers, one forward (EF-11) and two reverse internal sequencing primers (EF-21 and EF-22) were used with the fluorescent-labeled DyeDeoxy protocol (Perkin-Elmer) on an Applied Biosystems model 373 automated sequencer.

**Phylogenetic Analysis.** Phylogenetic analyses were performed by using PAUP 4.0d59 (18) on DNA sequences of the mtSSU rDNA and EF-1 $\alpha$  gene, both as individual and as a combined dataset for the 36 taxon matrix. Indels were coded as single events. Unweighted parsimony analyses were performed on the individual datasets, excluding uninformative characters, using the heuristic search option with 1,000 random addition sequences with MULPARS on and tree bisection-reconnection branch swapping. Maximum parsimony analysis of the combined dataset was by the branch-and-bound option in PAUP (18) for exact solutions. The two outgroup species selected for rooting the gene trees represent a putative sister group to the *F. oxysporum* complex (17, 19). Clade stability was assessed by 1,000 parsimony bootstrap replications (20) and decay indices calculated with TREEROT (21). Neighbor-joining gene trees also were inferred with uncorrected ("P") and maximum-likelihood distance methods (18). Concordance of the mtSSU rDNA and EF-1 $\alpha$  gene datasets was evaluated with the partition-homogeneity test implemented with PAUP (18), using 1,000 random repartitions, with MAXTREES set to 5,000. The Kishino-Hasegawa likelihood test implemented in PAUP (18) was used to compare various constrained and unconstrained topologies (Table 2). Alternative topologies were rejected with 95% confidence if they were >1.96 SD less likely than the most parsimonious tree (MPT). Distribution of substitutions within the EF-1 $\alpha$  gene (Fig. 1) was traced with MACCLADE (22).

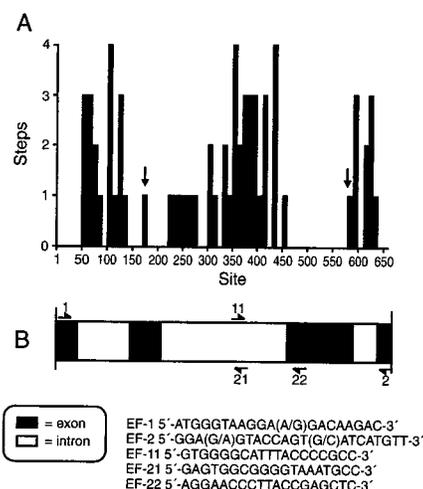


FIG. 1. (A) Distribution of phylogenetically informative nucleotide positions across the EF-1 $\alpha$  gene exons and introns. Each bar represents the number of steps (= base substitutions) per 10-bp interval traced by MACCLADE (22) from the MPT (Fig. 2A) for the 36 taxa studied. The two arrows identify the only informative substitutions within exons. (B) Map of the region of the EF-1 $\alpha$  gene sequenced showing exons and introns. Numbered half-arrows indicate the position of PCR and sequencing primers. Degenerate oligonucleotide primers used for PCR were based on conserved nucleotide sequences in the EF-1 $\alpha$  gene shared by the filamentous fungi *Trichoderma reesei* and *Histoplasma capsulatum* (GenBank accession nos. Z23012 and U14100).

Table 2. Likelihood analysis of constrained and unconstrained trees from combined EF-1 $\alpha$  gene and mtSSU rDNA dataset

Tree <sup>a</sup>	Tree length (steps) <sup>b</sup>	No. of trees <sup>c</sup>	Log likelihood (L) test results <sup>d</sup>			
			ln L	Difference <sup>e</sup>	SD <sup>f</sup>	P <sup>g</sup>
MPT (Fig. 3)	85	1	-333.63405	(Best)		
f. sp. <i>lycopersici</i> monophyletic	97 (+12)	10	-369.85437	-36.22032	9.3271	0.0003
f. sp. <i>melonis</i> monophyletic	102 (+17)	4	-376.70075	-43.06671	8.1074	<0.0001
f. sp. <i>radicis-lycopersici</i> monophyletic	92 (+7)	10	-357.69116	-24.05711	8.3201	0.0057
f. sp. <i>cubense</i> monophyletic	112 (+27)	120	-409.36083	-75.72679	11.1467	<0.0001

<sup>a</sup>Monophyly constraints enforced with PAUP (18).

<sup>b</sup>Under indel coding excluding uninformative characters. Numbers in parentheses indicate difference in length between the MPT and constrained trees.

<sup>c</sup>Only the best tree from each constraint was included in this test.

<sup>d</sup>Transition/transversion ratio estimated via maximum likelihood for Kishino-Hasegawa test.

<sup>e</sup>Difference in ln likelihood (L) between best tree and suboptimal tree.

<sup>f</sup>SD of log likelihood.

<sup>g</sup>Probability of obtaining a more extreme T-value, using the two-tailed test, with the null hypothesis being that there is no difference between the two trees. All values are significant at  $P = <0.05$ .

**Electrophoretic Karyotypes.** Chromosome-sized DNAs from strains of *F. oxysporum* f. sp. *cubense* were analyzed by pulsed field electrophoresis as described previously (12). Chromosome number values for strains were determined as described previously (11), and distributions for groups of *F. oxysporum* f. sp. *cubense* were compared by a Kolmogorov-Smirnov test (NPARIWAY) using SAS for Windows release 6.12 (SAS Institute, Cary, NC).

## RESULTS

Initially, a total of 23 strains of *F. oxysporum* f. sp. *cubense* were chosen for study. Each had been tested for pathogenicity to banana and represented a unique genotype reflecting the range of genotypic diversity inferred from single copy RFLP data of a larger ( $n = 165$ ) worldwide collection (6). Also included were putatively nonpathogenic isolates of *F. oxysporum* as well as pathogenic strains from other hosts including *Cucumis melo* (cantaloupe, f. sp. *melonis*), *Solanum* (synonym = *Lycopersicon*; ref. 23) *esculentum* (tomato, f. spp. *lycopersici* and *radicis-lycopersici*), and a range of other plant species. Two independent loci were examined representing, respectively, the nuclear (EF-1 $\alpha$  gene) and mitochondrial (mtSSU rDNA) genomes. Although 23 strains of *F. oxysporum* f. sp. *cubense* were sequenced, the entire range of genotypes for the mtSSU rDNA and EF-1 $\alpha$  gene is represented by the nine strains listed in Table 1. Thus only these nine strains are shown in the phylogenetic analyses.

The EF-1 $\alpha$  gene dataset consisted of 656 nucleotide characters, 39 of which were cladistically informative. All of the informative sites were within EF-1 $\alpha$  introns (Fig. 1) except for two synonymous third-position C-T transitions. In contrast to exons, which lacked insertions and deletions, EF-1 $\alpha$  introns possessed nine separate 1- to 3-bp indels. Sequences comprising the 694-bp mtSSU rDNA dataset contained 26 synapomorphies. GC base composition for the EF-1 $\alpha$  gene and mtSSU rDNA was 51.6% and 39.5%, respectively. Results of the partition-homogeneity test ( $P = 0.108$ ) indicated that the EF-1 $\alpha$  and mtSSU rDNA gene trees reflect the same underlying phylogeny. Therefore these datasets were combined and analyzed by using several tree-building programs.

Maximum parsimony searches, excluding uninformative characters, yielded a single MPT for the EF-1 $\alpha$  gene (Fig. 2A), mtSSU rDNA (Fig. 2B), and the combined dataset (Fig. 3). Neighbor-joining gene trees topologically concordant with the MPT from the combined dataset were found using the uncorrected ("P") and maximum-likelihood distance options in PAUP (18). Parsimony phylogenies inferred from EF-1 $\alpha$  gene introns/exons and mtSSU rDNA were highly concordant but differ in the degree to which they resolved some clades. Clade stability, as assessed by 1,000 parsimony bootstrap replications,

identified more strongly supported clades in the EF-1 $\alpha$  gene tree (17 nodes >50%, Fig. 2A) than in the mtSSU rDNA phylogeny (eight nodes >50%, Fig. 2B). Monophyly of the ingroup in both datasets was strongly supported by bootstrapping (100%, Fig. 2A and B) as were the same two major subclades (EF-1 $\alpha$  gene, 89 and 95%, Fig. 2A; mtSSU rDNA, 94 and 85%, Fig. 2B). Multiple independent groups of *F. oxysporum* f. sp. *cubense* strains were resolved within the EF-1 $\alpha$  and mtSSU rDNA gene trees.

Overall there was an increase in bootstrap support when the combined dataset was used (21 nodes >50% with 12 of these  $\geq 84%$ ; Fig. 3). Strains from banana (f. sp. *cubense*), cantaloupe (f. sp. *melonis*), and tomato (f. spp. *lycopersici* and *radicis-lycopersici*) did not form exclusive groups within the molecular phylogeny. Instead, strains from banana and cantaloupe appeared to have independent evolutionary origins within clades 1 and 2, and clades 2 and 3, respectively. At least five independent lineages of *cubense* were embedded in a deeper phylogeny in which many of the *cubense* strains were more closely related to strains from other hosts, or from soil, than to each other. A similar paraphyletic set of relationships is exhibited by the tomato pathogens, *lycopersici* and *radicis-lycopersici*. Topological constraints forcing the monophyly of each *forma specialis* were significantly worse than the uncon-

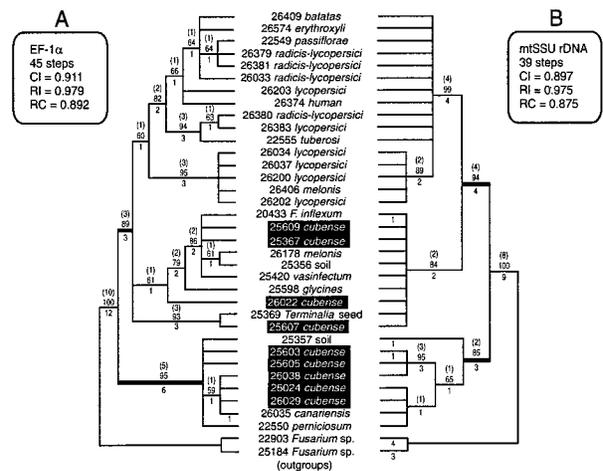


FIG. 2. Concordance of single most-parsimonious cladograms inferred from individual (A) EF-1 $\alpha$  gene and (B) mtSSU rDNA datasets for the *F. oxysporum* complex rooted by the outgroup method. Bootstrap replication frequencies >50% and decay indices (in parentheses) are indicated above nodes. Edge length is indicated below nodes and branches. The node subtending the two major subclades resolved by each dataset is indicated with bold lines.

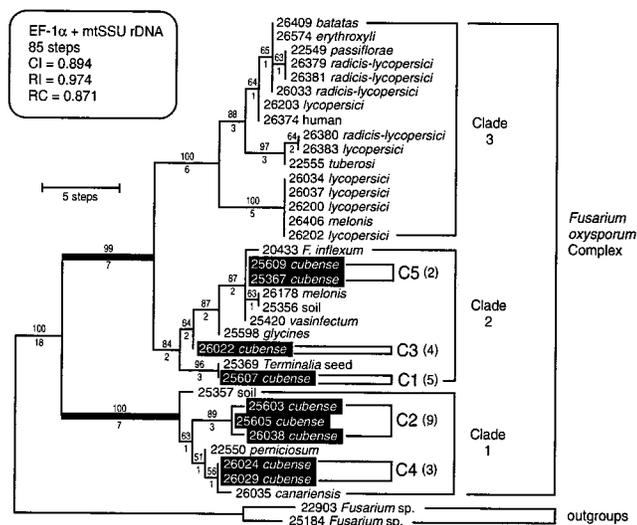


FIG. 3. Single most-parsimonious phylogram by parsimony branch-and-bound search implemented in PAUP (18) based on the combined EF-1 $\alpha$  gene and mtSSU rDNA dataset for the *F. oxysporum* complex rooted with sequences from *Fusarium* sp. NRRL 22903 and 25184. Numbering of the five clonal lineages (C1–C5) of “*F. oxysporum* f. sp. *cubense*” is according to the frequency with which each has been recovered from diseased bananas (6). The number of strains sequenced from each of the five clonal lineages is indicated in parentheses. Bootstrap values (1,000 replications) are indicated above nodes. Decay indices calculated with TREEROT (21) are indicated below nodes.

strained MPT when subjected to the Kishino–Hasegawa likelihood test ( $P < 0.05$ ) (Table 2).

Several isolates from other hosts or from soil also were found to have EF-1 $\alpha$  and mtSSU rDNA genotypes very similar or identical to strains obtained from banana. To determine whether these isolates could cause disease on banana, pathogenicity tests were performed with six strains not originally isolated from banana (NRRL 20433, NRRL 25356, NRRL 25598, NRRL 25369, NRRL 25357, and NRRL 22550). Each of these strains caused significantly less or no disease on banana cultivars Bluggoe and Gros Michel than did the four authentic strains of *F. oxysporum* f. sp. *cubense* tested ( $P < 0.05$ ) (NRRL 25609, NRRL 26022, NRRL 25607, and NRRL 26024). However, one strain from soil (NRRL 25357) and one from soybean (NRRL 25598) caused slight, but significant, vascular discoloration on the Pisang awak cultivar. Overall, the six strains from other sources were judged to be nonpathogenic on banana because of the lack of or very slight symptoms that they caused during these trials.

Because the strains of *F. oxysporum* f. sp. *cubense* examined also were used in a previous RFLP study (6), results of the two studies can be compared directly. The five clonal lineages of banana pathogens defined by EF-1 $\alpha$  and mtSSU rDNA gene sequences corresponded almost completely with lineages defined by RFLP analysis (6). In general, RFLP-based methods for designating clonal lineages resolved more taxa than parsimony analysis of the DNA sequence data. VCGs also corresponded both with sequence and RFLP-based taxa. The 23 strains of *F. oxysporum* f. sp. *cubense* analyzed here represented 12 different VCGs. Strains in the same VCG usually had identical EF-1 $\alpha$  and mtSSU rDNA genotypes and always were in the same clonal lineage.

Variation in chromosome number has been widely reported for filamentous fungi (24), including *F. oxysporum* f. sp. *cubense*, where chromosome number as determined from electrophoretic karyotypes ranged from 9 to 14 (11). In addition, chromosome number distribution in *F. oxysporum* f. sp. *cubense* is strongly associated with VCG (11). Because

clonal lineages defined by analysis of the EF-1 $\alpha$  and mtSSU rDNA gene sequences also correspond with VCG, they, too, may be expected to differ in chromosome number distribution. To test this, chromosome number estimates were pooled for 44 strains belonging to the three VCGs found in clonal lineage C1, and 47 strains belonging to the five VCGs found in clonal lineage C2. The chromosome number of strains in clonal lineage C1 ranged from 9 to 12 whereas the chromosome number for strains in clonal lineage C2 ranged from 10 to 14 (Fig. 4). The null hypothesis that chromosome number distributions were equal for the two lineages was evaluated by using a Kolmogorov–Smirnov test. Despite having an overlapping range of chromosome values, chromosome number distribution for the two clonal lineages was not equal ( $P < 0.0001$ ).

## DISCUSSION

This study presents a direct test of opposing hypotheses that advocate either a monophyletic (2) or polyphyletic (3) evolutionary origin of the Panama disease pathogen of banana, *F. oxysporum* f. sp. *cubense*. Hypotheses of the monophyly of *F. oxysporum* f. sp. *cubense* and three other *formae speciales* (i.e., *lycopersici*, *radicis-lycopersici*, and *melonis*) were strongly rejected by the Kishino–Hasegawa test as implemented in PAUP (18), which indicates that significant differences exist between each of the monophyly constraint trees and the unconstrained MPT. In agreement with data analysis of random amplified polymorphic DNAs (13), production of volatiles (25), anonymous single-copy RFLPs (6), mtDNA haplotypes (6), electrophoretic karyotypes (11), and putative geographic origin based on host genotype (26), cladistic analyses of EF-1 $\alpha$  and mtSSU rDNA gene sequences revealed two major lineages that included strains identified as *F. oxysporum* f. sp. *cubense*. Results of the molecular phylogeny suggest that *F. oxysporum* f. sp. *cubense* may be composed of up to five independent clonal lineages (C1–C5, Fig. 3): C2 and C4 within clade 1, and C1, C3 and C5 within clade 2. The excellent correspondence between trees inferred from DNA sequence data and VCG suggests that the clonal lineages may be long-lived. This idea is bolstered by the fact that the lineages also have significant differences in chromosome number distribution (Fig. 4).

Surprisingly, a direct test of *forma specialis* monophyly has never been conducted. Previous studies have focused on a single *forma specialis* rather than relationships among strains in different *formae speciales*. Even so, *F. oxysporum* f. spp. *cubense* and *melonis* were suspected of being polyphyletic because of their high genetic diversity (6, 27) and because RFLP analysis revealed that members of two of the *cubense* *forma specialis* lineages were as genetically similar to *F. oxysporum* f. sp. *lycopersici* as they were to each other (6).

Our study used nucleotide sequences from EF-1 $\alpha$  gene introns for molecular phylogenetics. Previously, EF-1 $\alpha$  protein and exon nucleotide sequences were used to investigate, respectively, deep divergences within eukaryotic and prokary-

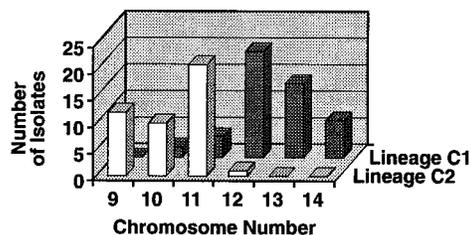


FIG. 4. Frequency distribution histogram for chromosome number from strains assigned to clonal lineage C1 or C2 of *F. oxysporum* f. sp. *cubense*. Chromosome number estimates were made as previously described (11). Strains were assigned to lineages on the basis of corresponding VCGs as described in the text.

otic domains (28, 29) and relationships within the Insecta (30, 31). Results from the present study demonstrate that the EF-1 $\alpha$  gene, with 95% of the signal derived from intron sequences, possesses 50% more phylogenetic information than the mtSSU rDNA. In sharp contrast to the exons, which lack indels and possess only two phylogenetically informative sites within the 36 taxon matrix, EF-1 $\alpha$  introns appear to be under relaxed evolutionary constraints as inferred from the substitutional pattern (Fig. 1A) that includes indels. However, the low level of homoplasy observed, coupled with the concordance of EF-1 $\alpha$  and mtSSU rDNA gene trees, indicate that informative sites within the EF-1 $\alpha$  gene are not saturated.

Although visual inspection of the MPT inferred from the individual datasets showed striking similarities, we have taken a conditional combination approach (32) by implementing the partition homogeneity test in PAUP 4.0d59 (18) to determine whether the partitions are homogeneous. Results of this statistical test strongly indicate the two datasets represent an arbitrary subdivision of one large dataset. The single MPT topology inferred from the combined dataset (Fig. 3) is presented here as the strongest current hypothesis of phylogenetic relationships with the *F. oxysporum* complex for the following reasons: (i) concordance among the datasets provides strong evidence that the hierarchical pattern observed accurately reflects the underlying phylogeny; (ii) the datasets are informative at different hierarchical levels, which is reflected by more nodes with higher measures of clade support in the combined dataset; and (iii) the phylogenetic results are concordant with results from several other molecular studies that identified the same two major lineages within this complex (6, 13, 33). The combined EF-1 $\alpha$  gene and mtSSU rDNA dataset provided much better resolution of relationships among and within lineages than the other loci we tested, which provided little ( $\beta$ -tubulin and calmodulin genes, not shown) or no resolution (internal transcribed spacer region of rDNA and 5' most portion of the nuclear 28S rDNA, not shown). Published parsimony analyses of 28S rDNA sequences provided only 37% bootstrap support for the monophyly of this lineage (34) whereas all of the molecular data reported here and by O'Donnell and Cigelnik (17) demonstrate monophyly of the *F. oxysporum* complex.

The basal split between clade 1 and the remaining taxa suggests that this lineage may be descended from one of the earliest divergences within the *F. oxysporum* complex. The basal most divergence includes Asian host genera (i.e., *Musa*, *Albizia*, and *Phoenix*), which suggests that this may be the ancestral area of the *F. oxysporum* complex and *F. oxysporum* f. sp. *cubense*. Phylogeny reconstructions from the remaining taxa suggest an early divergence between clades 2 and 3, which are strongly supported as monophyletic sisters. Hypotheses of independent evolutionary origins of *F. oxysporum* f. sp. *cubense*, such as clade 1 in Southeast Asia (26) and clonal lineage C5 (VCG 01214) in East Africa (6), are concordant with the molecular phylogeny and available biogeographical data. Because sampling within the *F. oxysporum* complex has just begun, explicit biogeographical interpretation of the three major clades of the *F. oxysporum* complex must await future research.

## CONCLUSIONS

EF-1 $\alpha$  and mtSSU rDNA gene sequences have proven to be excellent phylogenetic markers for resolving relationships within the *F. oxysporum* complex. The utility of EF-1 $\alpha$  gene sequences for species-level systematics also has been demonstrated by its ability to recover well-corroborated phylogenetic relationships within the *Gibberella fujikuroi* complex of *Fusarium* (ref. 19; K.O. and E.C., unpublished work). A more general conclusion is that molecular phylogenetics of discrete nucleotide data will provide essential diagnostic tools needed

to investigate species boundaries and to correlate the host range, biogeography, and mycotoxin potential of these agronomically and medically important fungi because morphological apomorphies currently are not available.

Results of the present study highlight an apparent paradox concerning the population structure of the *F. oxysporum* complex. The available data indicate that this complex is composed of mitotic clonal lineages that are predicted to be short-lived because of the accumulation of deleterious mutations by Müller's Ratchet and the Mutation-Load-Reduction theory (35, 36). However, the current panglobal distribution of the *F. oxysporum* complex, and its genetic diversity as reflected by over 120 host-specific forms, indicate that it is one of the most successful groups within *Fusarium*. We suspect that when phylogenetic analyses are applied to address the population biology of the *F. oxysporum* complex they may reveal, as they have for fungi pathogenic to humans (37–39), that some populations are both clonal and recombining.

Finally, several important findings emerge from this study regarding the phylogeny and classification of the *F. oxysporum* complex. This complex is strongly supported as monophyletic; however, many *formae speciales* are polyphyletic (K.O. and E.C., unpublished work), suggesting that host pathogenicity has evolved convergently. If this is the case for *F. oxysporum* f. sp. *cubense*, then each clonal lineage might be expected to have unique pathogenic properties. Perhaps reflecting this, isolates of different clonal lineages have been recovered in vastly different frequencies from different genotypes of banana, even when those banana cultivars are planted in the same field (40). Consequently, our results challenge the *forma specialis* naming system because, as exemplified by the polyphyly of *F. oxysporum* f. spp. *cubense* and *melonis*, it potentially obscures the communication of critical information concerning the genetic diversity of pathogens urgently needed for effective breeding programs and disease control efforts.

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