Postbloom Fruit Drop of Citrus and Key Lime Anthracnose Are Caused by Distinct Phylogenetic Lineages of *Colletotrichum acutatum*

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ABSTRACT

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Colletotrichum acutatum causes two diseases of citrus, postbloom fruit drop (PFD) and Key lime anthracnose (KLA). PFD is a disease restricted to flowers of sweet orange and most other citrus, and symptoms include petal necrosis, abscission of developing fruit, and the formation of persistent calyces. KLA is a disease of foliage, flowers, and fruits of Key lime only, and symptoms include necrotic lesions on leaves, fruits, twigs, flowers, and blight of entire shoots. The internal transcribed spacers 1 and 2 and the gene encoding the 5.8S ribosomal RNA subunit within the nuclear ribosomal cluster (ITS) and intron 2 of the glyceraldehyde-3-phosphate dehydrogenase gene (*G3PD*) were sequenced for isolates from PFD-affected sweet orange and KLA-affected Key limes collected in the United States (Florida), Brazil (São Paulo), Mexico, Belize, Costa Rica, and the Dominican Republic to determine if there are consistent genetic differences between PFD and KLA isolates over the geographic area where these diseases occur. Based on the sequence data, isolates clustered into two well-supported clades with little or no sequence variation among isolates within clades. One clade (PFD clade) contained PFD isolates

from all countries sampled plus a few isolates from flowers of Key lime in Brazil. The other clade (KLA clade) contained KLA isolates from Key lime foliage from all countries sampled and one isolate from flowers of sweet orange in Mexico. In greenhouse inoculations with PFD and KLA isolates from Florida, isolates from both clades produced PFD symptoms on Orlando tangelo flowers, but KLA-clade isolates produced significantly less severe symptoms. PFD-clade isolates were not pathogenic to Key lime foliage, confirming previous studies. The differentiation of PFD and KLA isolates into two well-supported clades and the pathogenicity data indicate that PFD and KLA are caused by distinct phylogenetic lineages of *C. acutatum* that are also biologically distinct. PFD is a recently described disease (first reported in 1979) relative to KLA (first reported in 1912) and it had been proposed that strains causing PFD evolved from strains causing KLA eventually losing pathogenicity to Key lime foliage. We reject the hypothesis that PFD strains have diverged from KLA strains recently based on estimated divergence times of haplotypes and it appears that PFD and KLA strains have been dispersed throughout the Americas independently in association with each host.

Additional keywords: *Gloeosporium limetticolum*, *Colletotrichum gloeosporioides*.

Postbloom fruit drop (PFD) is caused by *Colletotrichum acutatum* J. H. Simmonds (7,31,32). The pathogen infects flower petals causing peach to orange-colored lesions on open flowers and flower buds. Subsequently, the fruitlet abscises leaving the calyx and floral disk attached to the twig. These persistent structures remain attached for the life of the twig, and the leaves around inflorescences are usually twisted and distorted. Fruit abscission and persistent calyces are related to high levels of indoleacetic acid and other hormones in affected flowers (18). PFD affects most citrus species but is most severe on sweet oranges, lemons, and limes, and less severe on grapefruit and tangerines. PFD was described first in Belize in 1979 (8), although there are informal reports of the disease in Belize, Colombia, and Mexico prior to that date (25,41). In the past 25 years, PFD has appeared in all major, humid citrus production areas of the Americas (39) and severe outbreaks have occurred in Florida (United States) and Brazil. The disease is a limiting factor for citrus production in high rainfall areas of Belize, Mexico, Costa Rica, and the Caribbean islands (38).

Key lime anthracnose (KLA) is also caused by *C. acutatum* and affects only Key lime, *Citrus aurantifolia* (Christm.) Swingle

(synonymous with Mexican, West Indian, and Kagzi limes) (11, 38). This pathogen infects flower petals, leaves, twigs, and fruit, producing necrosis varying from small lesions to blight of entire shoots and inflorescences (21,22,38). The disease does not affect other limes such as Tahiti or Persian limes (*C. latifolia* Tan.), but these limes are affected by PFD. KLA was described much earlier than PFD (5). It is a serious disease in the major production area of Key limes in semi-arid western Mexico and causes greatly reduced fruit yields following the rainy season (21,23). Key limes are not grown commercially in humid areas of the Americas due to the devastating effects of this disease (21,23,38).

The causal agent of PFD was described originally as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (8). Subsequently, this pathogen was re-assigned to *C. acutatum* based on sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region of the genome (4). The causal agent of KLA was described originally as *Gloeosporium limetticolum* R. E. Clausen (5). When the genus *Gloeosporium* was revised (2,34), this pathogen was considered to be a form of *C. gloeosporioides*. However, like the PFD pathogen, the KLA pathogen was also reassigned to *C. acutatum* based on sequences from the ITS region (4).

The pathogens that cause PFD and KLA are difficult to distinguish from one another based on morphological characters (1). PFD and KLA isolates have been characterized genetically in two previous studies (4,14). These studies suggested that, although PFD and KLA isolates are closely related, they are genetically

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distinct. However, only a few isolates from a limited geographic area were included and the putative center of origin of the pathogens was not sampled (4,14). Also, in one of the studies, there was variation among isolates that appeared to overlap between populations (4). In essence, it has been difficult to draw any firm conclusions based on previous genetic studies regarding whether or not *C. acutatum* strains that cause PFD are genetically distinct from those that cause KLA. However, KLA and PFD isolates can be distinguished from one another based on pathogenicity. Pathogenicity assays conducted by Agostini et al. (1) showed that KLA isolates produced all of the symptoms of PFD when sweet orange flowers were inoculated, whereas PFD isolates were only weakly pathogenic to Key lime foliage (1). When the pathogenicity studies were conducted, it was speculated that PFD isolates may have diverged recently from KLA isolates (1). The proposed mechanism by which this occurred was that a KLA strain became established on the flowers of a non-Key lime citrus host and subsequently lost pathogenicity to Key lime foliage. If this hypothesis is correct, PFD isolates from various geographic regions should have lower genetic diversity relative to KLA isolates and represent a genetic subset of the variation observed among KLA isolates.

In the present study, we sampled isolates of *C. acutatum* causing KLA on Key limes, and PFD on sweet orange and other citrus from Belize, Costa Rica, Mexico, Dominican Republic, Florida (United States), and São Paulo (Brazil). The nuclear ribosomal internal transcribed spacer (ITS) and the intron 2 of the glyceraldehyde-3-phosphate dehydrogenase gene were sequenced for each isolate. The pathogenicity of PFD and KLA isolates from Florida to citrus flowers and Key lime foliage was also determined. The objective of this study was to determine if there were consistent genetic differences between KLA and PFD isolates over broad geographic areas and to determine whether or not there was any evidence that the pathogen that causes PFD may have evolved recently from the KLA population. A preliminary report of this study has been published (26).

MATERIALS AND METHODS

Collection of isolates. All isolates from PFD-affected flowers were collected from sweet orange (*Citrus sinensis* (L.) Osb.) trees in commercial plantings, except for one isolate from Mexico which was collected from Persian lime flowers. Isolates were chosen from different plantings and multiple locations within countries to maximize geographic diversity of the samples. With the exception of Mexico, where Key lime is grown commercially, KLA isolates were collected from lime anthracnose-affected trees from back yard plantings. All collections were made by the authors between 1999 and 2001 except those from Mexico which were provided by Mario Orozco-Santos (INIFAP, Tecoman, Colima, Mexico). Many of the isolates from Florida and Brazil were from collections made for a previous study (27). A chromogenic isolate from blueberry, described originally by Gunnell and Gubler (15), was collected in North Carolina and a second chromogenic blueberry isolate from Michigan was recovered by the authors from ripe rot-affected fruit purchased in a grocery store. Isolate numbers and the information on the sources are presented in Table 1.

For isolations, affected tissues were surface disinfested by placing them in 95% ethanol for 30 s, in 1.0% sodium hypochlorite for 1 min, and rinsing them twice in sterile, distilled water. Small pieces of tissue (approximately 2 mm²) were plated on potato dextrose agar (PDA = 4 g of potato starch, 20 g of dextrose, and 15 g of agar per liter) (Difco Laboratories, Sparks, MD) and incubated at 23 to 28°C for 3 days. Hyphal tips of fungi emerging from tissue pieces were transferred to PDA and grown at 24°C. Cultures were identified initially by cultural characteristics and conidial morphology. All isolates were single-spored

prior to use. Cultures were stored on dry filter papers at -20°C (24).

Pathogenicity tests. Isolates of *C. acutatum* from PFD-affected sweet orange and KLA-affected trees from Florida were grown on PDA for 1 week at 27°C. Only Florida isolates were used for pathogenicity tests due to quarantine restrictions. Conidia were washed from the dishes, passed through four layers of cheesecloth, and diluted in sterile, distilled water to 10⁶ conidia/ml. Tests for pathogenicity to flowers were conducted using potted 5- to 10-year-old grafted Orlando tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.) trees using a method similar to that described by Agostini et al. (1). Flower clusters with buds fully elongated, but not open were chosen for inoculations. Clusters were sprayed to runoff with conidial suspensions, covered with plastic bags for 18 h and held at 15 to 25°C in a greenhouse. Flower clusters were inoculated on seven different dates and on each date all six isolates and a negative water control were used for inoculations. In total, twelve clusters were inoculated per isolate. The number of clusters inoculated on the seven inoculation dates was dependent on availability and ranged from one cluster per isolate to three clusters per isolate. The number of clusters inoculated with each isolate and the water control was the same on each inoculation date to eliminate any systematic bias that the timing of inoculations might introduce. The day after inoculations, the total number of buds for each cluster at any stage of development was counted. The number of open flowers with at least one necrotic lesion and the number of open flowers without any lesions was determined 5 days after inoculation. Thirty days after inoculation, the number of persistent calyxes on each cluster was counted. The number of open flowers with necrosis and the total number of open flowers 5 days postinoculation from the seven inoculation dates were summed and used to calculate the percent of flowers with necrosis. The number of buds counted 1 day post inoculation and the number of persistent calyxes at 30 days postinoculation were also summed over the seven inoculation dates to calculate the percentage of buds that gave rise to persistent calyxes. The percentage of open necrotic flowers relative to the total number of open flowers at day 5 and the percentage of persistent calyxes relative to the total number of buds inoculated for the different isolates or the water control were compared by Fisher's exact test (SAS Institute, Version 9.0, Cary, NC). Differences in the percentage of necrotic flowers or persistent calyxes between groups of isolates categorized as PFD or KLA were assessed using a generalized mixed model. In the model, proportions transformed using the logit link function were described by the fixed effect clade (PFD or KLA) and the random effect isolate nested within the clade. The % GLIMMIX macro for the SAS software program was used for this analysis and variability of disease incidence for individual isolates was assumed to follow a binomial distribution.

Pathogenicity to Key lime was assessed using 6-month to 1-year-old rooted cuttings, as described by Agostini et al. (1). Two newly emerged shoots on each plant were inoculated with a 10⁶ conidia/ml spore suspensions of each isolate or sprayed with sterile water. After inoculation, plants were covered with a plastic bag for 18 h at 24°C. A visual estimate of the percentage of tissue on the two shoots that was necrotic was made 6 days after inoculation. Inoculations were conducted on four different dates and on each of these dates one plant was inoculated with each isolate or sprayed with a water control. Percentages of leaf necrosis on Key lime caused by the different isolates or the water control were compared using the nonparametric Wilcoxon rank sum test (Statistix, Version 8.1, Analytical Software, Tallahassee, FL).

DNA extraction and sequencing. For production of mycelium for DNA extractions, isolates were grown for 2 days on PDA dishes covered with cellophane at 24°C. Mycelium was collected from the surface of the cellophane, freeze dried, and ground in liquid nitrogen. Genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA) according to the instructions of the

manufacturer. DNA concentrations were estimated visually in 0.7% ethidium bromide-stained agarose gels by comparing band intensity with known quantities of lambda DNA/*Hind*III markers (Promega, Madison, WI).

The nuclear ribosomal DNA repeat including ITS1, 5.8S rRNA, ITS2, and portions of the genes encoding both small and large subunit rRNAs was amplified using forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-

TABLE 1. Codes and origins of isolates of *Colletotricum acutatum* used in pathogenicity and sequencing studies

Code number	Original name (reference)	Country of origin	Host variety (tissue)
BZ-PFD-1	DLR-A1	Belize	Sweet orange (flowers)
BZ-PFD-2	MTR-SWO-A1	Belize	Sweet orange (flowers)
BZ-PFD-4	MDX-SWO-1	Belize	Sweet orange (flowers)
BZ-PFD-7	BTC-B1	Belize	Sweet orange (flowers)
BZ-PFD-8	SRV-1	Belize	Sweet orange (flowers)
BZ-PFD-10	DLR-B1	Belize	Sweet orange (flowers)
BR-PFD-1	CIT-CA-ITP-20 (27)	São Paulo, Brazil	Sweet orange (flowers)
BR-PFD-4	CIT-CA-ITP-1 (27)	São Paulo, Brazil	Sweet orange (flowers)
BR-PFD-7	SLG-MGG-2 (27)	São Paulo, Brazil	Sweet orange (flowers)
BR-PFD-10	FZR-PRD-2 (27)	São Paulo, Brazil	Sweet orange (flowers)
BR-PFD-11	FSH-CLB-2 (27)	São Paulo, Brazil	Sweet orange (flowers)
CR-PFD-1	Cartago-1	Costa Rica	Sweet orange (flowers)
CR-PFD-4	Pital-1	Costa Rica	Sweet orange (flowers)
CR-PFD-6	Los Chiles	Costa Rica	Sweet orange (flowers)
CR-PFD-7	Cachi-1	Costa Rica	Sweet orange (flowers)
CR-PFD-10	Pocora-4	Costa Rica	Sweet orange (flowers)
DR-PFD-1	AD F1 C3-1	Dominican Republic	Sweet orange (flowers)
DR-PFD-3	JJB-1	Dominican Republic	Sweet orange (flowers)
DR-PFD-6	AD F3 C51-2	Dominican Republic	Sweet orange (flowers)
DR-PFD-7	AD F4 C70-1	Dominican Republic	Sweet orange (flowers)
DR-PFD-9	AD F1 C3-3	Dominican Republic	Sweet orange (flowers)
DR-PFD-10	AD F3 C51-3	Dominican Republic	Sweet orange (flowers)
FL-PFD-1	RCO-IMK-1 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-3	OCO-ARC-1 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-5	VTP-IND-1 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-7	SGR-FTP-2 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-8	CLR-IMK-2 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-9	RCO-IMK-2 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-14	OCO-ARC-4 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-15	ALB-IND-25 (27)	Florida, USA	Sweet orange (flowers)
FL-PFD-16	STF-FTP-10 (27)	Florida, USA	Sweet orange (flowers)
MX-PFD-1	A39NVTab (23)	Tabasco, Mexico	Sweet orange (flowers)
MX-PFD-2	A47NVTam (23)	Tamaulipas, Mexico	Sweet orange (flowers)
MX-PFD-7	A125NVVer (23)	Veracruz, Mexico	Sweet orange (flowers)
MX-PFD-8	A115LPVer (23)	Veracruz, Mexico	Persian lime (flowers)
MX-PFD-9	A40NVTab (23)	Tabasco, Mexico	Sweet orange (flowers)
BZ-KLA-1	PBP-KLA	Belize	Key lime (tissue?)
BZ-KLA-2	MTR-KLA-A1	Belize	Key lime (tissue?)
BZ-KLA-3	MTR-KLA-B1	Belize	Key lime (tissue?)
BZ-KLA-5	GVL-1	Belize	Key lime (leaves)
BZ-KLA-6	ADS	Belize	Key lime (tissue?)
BZ-KLA-8	CREI	Belize	Key lime (flowers)
BR-KLA-1	KLA-MGG-1	São Paulo, Brazil	Key lime (flowers)
BR-KLA-2	KLA-BTU	São Paulo, Brazil	Key lime (leaves)
BR-KLA-4	KLA-CRD-CV-1	São Paulo, Brazil	Key lime (leaves)
BR-KLA-6	KLA-MGG-5	São Paulo, Brazil	Key lime (flowers)
BR-KLA-7	KLA-MGG-7	São Paulo, Brazil	Key lime (flowers)
CR-KLA-1	Ciudad Colon	Costa Rica	Key lime (leaves)
CR-KLA-2	Rio Frio	Costa Rica	Key lime (leaves)
CR-KLA-3	Aguas Zarca	Costa Rica	Key lime (leaves)
CR-KLA-4	S.F. Palmera	Costa Rica	Key lime (tissue?)
CR-KLA-5	Horquetas	Costa Rica	Key lime (leaves)
CR-KLA-6	Orosi	Costa Rica	Key lime (tissue?)
DR-KLA-1	SBN-1	Dominican Republic	Key lime (leaves)
DR-KLA-2	SBN-2	Dominican Republic	Key lime (leaves)
DR-KLA-4	MPL-KLA-2	Dominican Republic	Key lime (leaves)
DR-KLA-5	RICA	Dominican Republic	Key lime (leaves)
DR-KLA-6	JJB	Dominican Republic	Key lime (tissue?)
FL-KLA-1	GL-PIKE	Florida, USA	Key lime (tissue?)
FL-KLA-5	KLA-Anderson	Florida, USA	Key lime (leaves)
FL-KLA-6	KLA-Jonathan, Hm-1 (4)	Florida, USA	Key lime (tissue?)
FL-KLA-7	Lowes	Florida, USA	Key lime (leaves)
FL-KLA-8	KLA-DPI, Ss (4)	Florida, USA	Key lime (leaves)
MX-KLA-1	A 54 LM Tam (23)	Tamaulipas, Mexico	Key lime (leaves)
MX-KLA-3	A 04 LM Oax (23)	Oaxaca, Mexico	Key lime (leaves)
MX-KLA-4	A 15 LM Col (23)	Colima, Mexico	Key lime (leaves)
MX-KLA-5	A 19 LM Col (23)	Colima, Mexico	Key lime (leaves)
MX-KLA-8	A 50 LM Tam (23)	Tamaulipas, Mexico	Key lime (leaves)
MX-KLA-10	A 06 LM Gro (23)	Guerrero, Mexico	Key lime (leaves)
MI-BLU-1	Mi-BB-2, 04-52	Michigan, USA	Blueberry (ripe fruit)
NC-BLU-1	Gc-13-1, 96-58 (15)	North Carolina, USA	Blueberry (ripe fruit)

TCCTCCGCTTATTGATATGC-3') (44). The region spans bases 1729 to 2310 of a reference nuclear rDNA repeat sequence from *C. acutatum* (GenBank accession no. AJ301936) (20). The ITS region 1, the 5.8S ribosomal RNA gene, and ITS region 2 were annotated from sequences reported for the small subunit (GenBank accession no. X04971) (33) and 5.8S subunit rRNA (GenBank accession no. M10692) (30) of *Neurospora crassa* and the large subunit rRNA of *Saccharomyces cerevisiae* (GenBank accession J01355) (12). Regions flanking the ITS regions of *C. acutatum* and these other species were highly conserved. The complete ITS1, 5.8S rRNA, and ITS2 region was either 494 or 495 bp long for *C. acutatum* isolates in the present study. Intron 2 of the glyceraldehyde 3-phosphate dehydrogenase (*G3PD*) gene was amplified with the forward primer GDF (5'-GCCGTCAA-CGACCCCTTCATTGA-3') and reverse primer GDR (5'-GGG-TGGAGTCGTA CTTGAGCATGT-3') (14). These primers anneal within coding regions of the *G3PD* gene from nucleotide 1538 to 1817 relative to the *G3PD* gene of *C. gloeosporioides* (GenBank accession no. M93427) (36). Intron 2 includes the 216-bp region spanning bases 1574 to 1789 of the reported sequence. For *C. acutatum* isolates in the present study, this region was either 209 or 213 bp long. Amplifications for sequencing used 5 ng of template DNA and were carried out in a 120- μ l volume containing 1 $\times$ reaction buffer (10 mM Tris [pH 9], 50 mM KCl, and 2 mM MgCl₂), 200 μ M dNTP, 5 units of *Taq* polymerase, and 60 pmol of each primer/reaction. Cycling parameters consisted of a 4-min denaturing step at 94°C followed by 34 cycles at 94°C for 45 s, 52°C (rDNA primers) or 60°C (*G3PD* primers) for 45 s, and 72°C for 1 min. Prior to sequencing, DNA was concentrated to 20 ng/ μ l, using a Microcon YM-30 centrifugal filter (Millipore, Billerica, MA) and 15- μ l samples were submitted to the University of Florida Interdisciplinary Center for Biotechnology Research in Gainesville. Sequence data were generated from fluorescent cycle sequencing reactions using an automated sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA). Sequence data were generated in forward and reverse directions and alignments were done using CLUSTAL W (37).

Phylogenetic analysis. Sequences from two isolates of *C. acutatum* from blueberry were included in the analysis to identify a node where KLA and PFD clades diverged. The blueberry isolates were chromogenic and belonged to a clade previously shown to form an outgroup from *C. acutatum* isolates from citrus and Key lime based on sequence data from intron regions of the *G3PD* gene and glutamine synthase gene (14). However, the blueberry isolates were not assumed to be an outgroup in our analysis and we report midpoint-rooted trees. An obvious outgroup species would have been *C. gloeosporioides*, but *G3PD* sequences from KLA and PFD isolates did not align well with sequences from *C. gloeosporioides*, and therefore sequence data from this species were not included. Maximum parsimony trees and a consensus tree were obtained using PAUP 4.0 (Sinauer Associates, Inc., Sunderland, MA). For the determination of branch lengths, transitions and transversions were weighted equally and gaps treated as a fifth character. An heuristic search employing the tree-bisection-reconnection branch swapping algorithm was used to obtain maximum parsimony trees. Starting trees for branch swapping were constructed by random stepwise-addition. One hundred random addition replicates were performed for each search. Support for nodes of parsimony trees were obtained from 1,000 bootstrapped data sets. Isolates were labeled according to host and site in reported trees. The consensus tree represented the strict consensus of trees constructed from *G3PD* intron 2 and rDNA sequence data. Bayesian posterior probabilities of sampling nodes were determined with MrBayes 3.1.2 (29). The posterior probabilities are based on maximum likelihood phylogenies assuming the HKY model of sequence evolution for *G3PD* intron 2 and the F81 model for the rDNA sequence data. Models were selected using the online program FINDMODEL (Los Alamos

National Laboratory, Los Alamos, NM), a web implementation of the program MODELTEST (28). For the tree constructed using combined data, the data were partitioned and both models used to estimate posterior probabilities. Four chains were run for each analysis, one cold and three heated. The temperature parameter was set to 0.2. Five hundred thousand generations were run and trees sampled every 100 generations. The burn-in period consisted of the first 100,000 generations. A maximum likelihood tree was also constructed in PAUP for each sequence using the same models of sequence evolution described above and both sequences combined assuming the HKY model of evolution. These trees are not reported, as the overall topology and the branch placement of the midpoint root was the same in these trees and reported maximum parsimony trees.

The depth of the node where KLA and PFD isolates diverged in trees constructed using ITS and *G3PD* sequence data was estimated using the mean path length method (3). Gaps were excluded from this analysis because only substitution rate estimates were available for these regions. A 95% confidence interval for each mean path length was estimated from 1,000 bootstrapped maximum parsimony trees in PAUP. To test the hypothesis that the PFD population diverged from the KLA population very recently (decades), upper and lower estimates for the mean path length were converted to dates using substitution rates for Eurotiomycete fungi published by Kasuga et al. (17) and the equation for divergence time was: divergence time = mean path length/length of gene/substitution rate. Reported substitution rates vary depending on the algorithm used to determine them and the estimate of the Eurotiomycetes/Sordariomycetes divergence used as a calibration point. The most recent estimate for the KLA/PFD divergence from ITS sequence data was calculated using the lower mean path length estimate and the fastest substitution rate estimated for the combined ITS1, ITS2, and 5.8S rDNA region (9.76×10^{-9} substitutions/site/year). The early divergence estimate was calculated using the high mean path length estimate and the slowest substitution rate (3.3×10^{-10} substitutions/site/year). Recent and early boundaries were calculated for the *G3PD* using the same methodology, only the fastest (92.09×10^{-9} substitutions/site/year) and slowest (0.51×10^{-9} substitutions/site/year) reported substitution rates for both introns and synonymous sites were used.

Isolate and sequence deposits. From each of three resolved clades a single isolate originating from the United States was deposited in the American Type Culture Collection (Manassas, VA). Original strain designations of deposited isolates with accession numbers in parenthesis are ALB-IND-25 (MYA-4396), Hm-1 (MYA-4397), and Gc-13-1 (MYA-4398). Based on sequence data from intron 2 of the *G3PD* gene and the ITS1, 5.8S subunit, and ITS2 of the ribosomal DNA repeat combined, there were four distinct haplotypes. Sequence data from one isolate with each haplotype was deposited into GenBank (National Center for Biotechnology Information, Bethesda, MD). Original strain designations with *G3PD* accession numbers in parentheses are ALB-IND-25 (EU168905), MTR-KLA-A1 (EU168906), Hm-1 (EU168907), and Gc-13-1 (EU168908). Original strain designations with rDNA accession numbers in parentheses are ALB-IND-25 (EU168901), MTR-KLA-A1 (EU168902), Hm-1 (EU168903), and Gc-13-1 (EU168904).

RESULTS

Pathogenicity tests. PFD isolates from Florida were highly pathogenic to flowers of Orlando tangelo and affected nearly all open flowers 5 days after inoculation (Table 2). KLA isolates were also pathogenic to flowers of Orlando tangelo but caused significantly less disease than PFD isolates ($P < 0.001$). Persistent calyxes typical of PFD were formed following inoculation with both PFD and KLA isolates. Although there were differences in the percent of calyxes produced between isolates, the clade from

which the isolate was derived (KLA versus PFD) did not account for these differences ($P = 0.89$). PFD isolates produced virtually no necrosis on Key lime leaves, whereas KLA isolates caused necrosis of about 30 to 60% of the leaf area inoculated (Table 2).

Phylogenetic analysis. The phylogeny estimated using sequence data from the ITS1-5.8S rDNA-ITS2 region was based on eight parsimony informative sites and the phylogeny estimated from *G3PD* sequence data was based on 24 parsimony informative sites. No homoplasy was observed in either phylogeny. In both phylogenies, citrus isolates formed two clades distinct from that of the blueberry isolates (Fig. 1A and B). A strict consensus of the ITS and *G3PD* phylogenies separated citrus isolates into two clades, indicating that clades identified from either rDNA or *G3PD* sequence data were the same (Fig. 1C). Bootstrap support for both clades was high based on either rDNA or *G3PD* sequence data alone. However, when sequence data from both regions of the genome was combined, support for clades was much stronger (Fig. 1D). The phylogram constructed using combined sequence data was based on 32 parsimony informative sites and no homoplasy was detected. One clade contained all the PFD isolates except one of five PFD isolates from Mexico (MX-PFD-2). This clade also contained three KLA isolates from Brazil: BR-KLA-1, BR-KLA-6, and BR-KLA-7. There was no haplotype variation among isolates of this clade for either sequence. The second clade contained all of the KLA isolates from Belize, Costa Rica, Dominican Republic, United States, and Mexico, and two of the five KLA isolates from Brazil. The *G3PD* intron 2 sequence was identical for all isolates within this group, but there was a single nucleotide substitution within the gene encoding the 5.8S rRNA subunit in four of the six isolates from Belize that differed from the others. Between the two clades, there were four nucleotide differences in the 2nd intron of the *G3PD* gene and four to five nucleotide or insertion/deletion differences in the rDNA repeat. Blueberry sequences for both genes were identical and distinct from sequences for the PFD and KLA isolates. Within the *G3PD* 2nd intron, there were 21 and 23 nucleotide or insertion/deletion differences between blueberry isolates and isolates from the PFD and KLA clusters. There were four to six nucleotide or insertion/deletion differences between blueberry and citrus isolates within the rDNA repeat (Table 3).

The 95% confidence interval for the mean path length from the node where the two identified clades diverged was 0.061 to 3.652 substitutions/locus for the rDNA repeat and 0.530 to 3.757 substitutions/locus for the *G3PD* intron 2 locus. Based on these estimates and published substitution rates for the combined ITS1, ITS2, and 5.8S rDNA region, synonymous sites and introns, the 95% confidence interval for the divergence of the rDNA haplotype groups is 12,569 to 22,402,159 years before present and for the divergence of the *G3PD* haplotype groups 27,552 to 44,628,952 years before the present.

DISCUSSION

PFD and KLA isolates sampled throughout the Americas were differentiated into two well-supported phylogenetic lineages. One clade (PFD) contained almost exclusively PFD isolates and the second clade (KLA) almost exclusively KLA isolates. PFD appears to be of recent origin having been first described in Belize in 1979 (8). Agostini et al. (1) suggested that movement of a *C. acutatum* strain from Key lime might have been responsible for the sudden emergence of the disease. It is obvious from the current study that PFD did not evolve on this timescale. Neither the ITS haplotype nor the *G3PD* haplotype of PFD-type isolates was observed among isolates from Key lime foliage, which would be expected if the pathogen recently migrated from this host. The confidence intervals for the estimated time that haplotypes diverged were wide, reflecting the uncertainty of estimates. Mean path lengths were based on only a few nucleotide changes and as a result the 95% confidence interval for their estimations were wide. In the case of the rDNA region, the upper boundary was 60-fold greater than the lower boundary. There were also large differences in the estimated substitution rates over the two regions. For example, the fastest estimated rate for introns and synonymous sites was 180-fold greater than the slowest rate. However, even with the large error in divergence time estimates, the probability that the PFD and KLA clades diverged within the last several thousand years is extremely low. Preliminary results from a study examining a larger number of hosts suggest that hybridization events may occur among related *C. acutatum* isolates (S. J. MacKenzie and N. A. Peres, unpublished data). We also sequenced a third region of the genome, 903 bp of the 2nd intron of the glutamine synthase gene used by Guerber et al. (14), from the Florida isolates employed in pathogenicity tests. There were 27 polymorphic nucleotide sites between the PFD and KLA clades for this gene (S. J. MacKenzie and N. A. Peres, unpublished data). This provides additional support that the PFD population is not derived from a recent hybridization event involving the KLA population, although significant sequence differentiation at a greater number of rapidly evolving loci would be required to conclusively rule out that a recent hybridization event did not occur.

There were a few isolates from PFD-affected flowers and Key limes that did not fall into expected clades including a single PFD isolate from Mexico in the KLA clade and three KLA isolates from Brazil in the PFD clade. Based on the pathogenicity data, it would not be surprising to obtain isolates with the ability to cause KLA from necrotic flowers of citrus species other than Key lime. Also, *C. acutatum* strains that cause PFD have the ability to infect Key lime flowers (S. J. MacKenzie and N. A. Peres, unpublished data) and as a result it would not be surprising to find PFD-type isolates from Key lime flowers. However, one would not expect to

TABLE 2. Pathogenicity of *Colletotrichum acutatum* isolates from Florida causing postbloom fruit drop (PFD) and Key lime anthracnose (KLA) to flowers of Orlando tangelo and to foliage of Key lime

Isolates	Orlando tangelo			Key lime leaves
	Open flowers, total buds	Necrosis (%)	Persistent calyxes (%)	Necrosis (%)
PFD				
FL-PFD-15	80,155 ^x	100.0 a ^y	6.5 bc ^y	0.0 b ^z
FL-PFD-14	93,158	97.8 a	13.3 ab	0.0 b
FL-PFD-16	92,199	90.2 b	11.6 ab	0.1 b
KLA				
FL- KLA-6	86,187	46.5 c	17.6 a	60.6 a
FL- KLA-8	76,157	34.2 cd	2.5 c	31.8 a
FL- KLA-5	92,189	30.4 d	5.3 c	29.9 a
Control	99,214	3.0 e	0.0 d	0.0 b

^x Total number of open flowers evaluated to determine the percentage of necrotic flowers after inoculation and the total number of buds inoculated used to determine the percentage of buds forming persistent calyxes.

^y Treatments followed by the same letter had $P > 0.05$ in pairwise comparisons using Fisher's exact test.

^z Treatments followed by the same letter had $P > 0.05$ in pairwise comparisons using the Wilcoxon rank sum test.

Fig. 1. Maximum parsimony phylogenetic relationships among isolates of *Colletotrichum acutatum* causing postbloom fruit drop (PFD) and Key lime anthracnose (KLA) from Belize (BZ), São Paulo, Brazil (BR), Costa Rica (CR), Dominican Republic (DR), Florida, USA (FL), and Mexico (MX) based on sequences of **A**, the ITS1, 5.8S, and ITS2 regions of ribosomal DNA and **B**, the 2nd intron of the glyceraldehyde-3-phosphate dehydrogenase gene (*G3PD*). **C**, Strict consensus phylogeny based on ribosomal DNA sequences and the 2nd intron of *G3PD* and **D**, phylogeny based on the combined sequence of both the ribosomal DNA and the *G3PD* 2nd intron. Numbers above nodes are bootstrap values determined for the maximum parsimony phylogenies (shown) and numbers beneath nodes are Bayesian posterior probabilities for maximum likelihood phylogenies (identical topologies to parsimony trees, not shown).

find PFD-type isolates on Key lime foliage because they fail to produce symptoms on this tissue. The Brazilian KLA isolates that grouped with PFD isolates were recovered from symptomatic flowers (Table 1). Flowers were sampled at the site where these isolates were found because no symptoms were present on Key lime foliage. The Key lime trees at this site were also situated close to a sweet orange grove where PFD was endemic. Based on this history, there is a reasonable probability that these isolates were disseminated from another citrus host. The PFD isolate from Mexico, MX-PFD-2, in the KLA clade, was collected from sweet orange flowers on an experiment station in Ciudad Victoria, Tamaulipas, Mexico, where numerous Key limes with anthracnose were also present. We presume that the MX-PFD-2 isolate represents a KLA isolate from a chance infection of sweet orange flowers. The isolate also grouped with KLA isolates based on random amplified polymorphism DNA (RAPD) analysis, was pathogenic to Key lime foliage, and had appressoria typical of KLA isolates (22). No PFD-type isolates were recovered from Key lime foliage as would be expected based on pathogenicity results. Given that only one KLA-type isolate was obtained from a non-Key lime flower and the site where the isolate was found was in close proximity to a sweet orange grove, it also appears that

incursions of KLA-type isolates to sweet orange flowers are not sustainable. PFD isolates can survive between blooms as appressoria on leaf surfaces (39,40,45). Possibly, KLA isolates lack this ability and that may be the reason that they are not isolated more frequently from non-Key lime flowers.

Although it is clear that PFD and KLA isolates are phylogenetically distinct, it is not clear how the pathogens became established and evolved on their citrus hosts or if these lineages can infect hosts other than citrus. PFD and KLA are important diseases of citrus only in the Americas (39). To our knowledge, lime anthracnose has been reported outside of the Americas only in Zanzibar (42,43). Citrus originated in Asia and it is possible that both diseases occur there and simply have not been reported. A survey of citrus species within Asia for PFD and KLA would help ascertain whether PFD and KLA strains were introduced into the Americas on host material or if citrus plants became infected through a host shift from some unknown host or hosts in the New World. *C. acutatum* is often described as a taxonomic species (or group species) comprised of multiple, genetically isolated populations (6,10,14,19,25) and at least some of these populations can be distinguished from one another based on morphological characteristics that correlate with host range (9,16,19,31,32).

TABLE 3. Polymorphic sites in intron 2 of the glyceraldehyde-3-phosphatedehydrogenase gene and in the internal transcribed spacer 1 (ITS1), the 5.8S subunit, and the ITS2 of the ribosomal DNA repeat for all isolates of *Colletotrichum acutatum* used in the study

Isolate code no.	Glyceraldehyde-3-phosphate dehydrogenase 2nd intron																							
Consensus site no.	15	31	48	51	66	73	80	92	93	98	109	111	113	115	120	136	137	138	150	173	181	183	187	190
BR-KLA-1,6,7	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
BZ-PFD-1,2,4,7,8,10	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
BR-PFD-1,4,7,10,11	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
CR-PFD-1,4,6,7,10	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
DR-PFD-1,3,6,7,9,10	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
FL-PFD-1,3,5,7,8,9,14,15,16	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
MX-PFD-1,7,8,9	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
BZ-KLA-1,5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
BZ-KLA-2,3,6,8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
BR-KLA-2,4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
CR-KLA-1,2,3,4,5,6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
DR-KLA-1,2,4,5,6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
FL-KLA-1,5,6,7,8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
MX-KLA-1,3,4,5,8,10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
MX-PFD-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.
MI-BLU-1	C	A	T	A	A	A	G	A	G	T	C	.	G	A	.	C	G	T	T	.	C	A	.	T
NC-BLU-1	C	A	T	A	A	A	G	A	G	T	C	.	G	A	.	C	G	T	T	.	C	A	.	T
Consensus	–	C	C	G	G	C	C	G	A	C	T	T	T	C	A	–	–	–	C	A	G	G	A	C

Isolate code no.	ITS1			5.8S		ITS2			
Consensus site no.	32	99	8	39	40	135	144	149	
BR-KLA-1,6,7	A	–	.	.	.	.	.	T	
BZ-PFD-1,2,4,7,8,10	A	–	.	.	.	.	.	T	
BR-PFD-1,4,7,10,11	A	–	.	.	.	.	.	T	
CR-PFD-1,4,6,7,10	A	–	.	.	.	.	.	T	
DR-PFD-1,3,6,7,9,10	A	–	.	.	.	.	.	T	
FL-PFD-1,3,5,7,8,9,14,15,16	A	–	.	.	.	.	.	T	
MX-PFD-1,7,8,9	A	–	.	.	.	.	.	T	
BZ-KLA-1,5	.	.	.	.	.	.	C	.	
BZ-KLA-2,3,6,8	.	.	C	.	.	.	C	.	
BR-KLA-2,4	.	.	.	.	.	.	C	.	
CR-KLA-1,2,3,4,5,6	.	.	.	.	.	.	C	.	
DR-KLA-1,2,4,5,6	.	.	.	.	.	.	C	.	
FL-KLA-1,5,6,7,8	.	.	.	.	.	.	C	.	
MX-KLA-1,3,4,5,8,10	.	.	.	.	.	.	C	.	
MX-PFD-2	.	.	.	.	.	.	C	.	
MI-BLU-1	.	.	.	C	G	T	.	.	
NC-BLU-1	.	.	.	C	G	T	.	.	
Consensus	G	C	T	A	C	A	A	C	

Freeman et al. (10) divided *C. acutatum* into four subgroups based on arbitrarily primed PCR, A+T-rich DNA and ITS sequence analysis. Each subgroup contained isolates from diverse hosts such as apple, peach, and pecan, suggesting that genetically similar isolates can have wide host range. No citrus isolates were included in the study. Guerber et al. (14) identified distinct isolate subgroups based on sequence data from two gene introns, mtDNA restriction fragment length polymorphisms (RFLP), intron RFLPs, and mating compatibility. Citrus isolates from New Zealand fell into mt-DNA haplotype F8 and those from sweet orange and Persian lime from Florida were in haplotype J2, whereas those from Key lime were in haplotype J3. Isolates with J2 and J3 haplotypes were very closely related based on intronic regions of the glutamine synthetase and *G3PD* genes, but were also distinct from one another based on sequences from these genes. Based on this information, the authors recognized that specialization may occur on some hosts such as Key lime. They also concluded that many populations, such as those in their clades C and J4, infect a wide range of hosts. Using mitochondrial RFLP analysis, Brown et al. (4) found that citrus isolates could be clearly distinguished from isolates from other hosts such as strawberry, nandina, and chrysanthemum. They also found that four KLA and four PFD isolates from Florida clustered separately from one another based on mitochondrial RFLP patterns. Overall, these studies indicate that there is host specificity within phylogenetic lineages of *C. acutatum*. However, it is not clear if the host specificity is

limited to a single host species or multiple host species. It is very likely that the host specificity of phylogenetic lineages lie on a continuum from very broad host specificity to single-host specificity, but due to the large number of potential hosts and limited time to conduct pathogenicity assays, it is difficult to state where on this continuum a given pathogen occurs.

Taylor et al. (35) proposed that a clonal species should include all individuals that evolved subsequent to the last common ancestor between the clonal population and a recombining population. Apparently, isolates from both clades reproduce only vegetatively because the teleomorph of *C. acutatum*, *Glomerella acutata*, has never been observed on citrus, and Guerber et al. (13,14) did not report successful crosses involving J2 and J3 mitochondrial haplotype isolates. Given that isolates from the KLA and PFD clades are more closely related to one another than they are to a known recombining population (14), at the present time, classifying isolates from the KLA and PFD clades as distinct species does not appear to be warranted based on the above definition of a clonal species.

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