Molecular Characterization of Slow-Growing Orange and Key Lime Anthracnose Strains of *Colletotrichum* from Citrus as *C. acutatum*

A. E. Brown, S. Sreenivasaprasad, and L. W. Timmer

First and second authors: research scientists, Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast, BT9 5PX, UK; third author: professor of plant pathology, University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred 33850-2299.

Current address of S. Sreenivasaprasad: Department of Microbial Biotechnology, Horticultural Research International, Wellesbourne, Warwick, CV35 9EF, UK.

Florida Agricultural Experiment Station Journal Series R-04765.

Accepted for publication 5 February 1996.

ABSTRACT

Brown, A. E., Sreenivasaprasad, S., and Timmer, L. W. 1996. Molecular characterization of slow-growing orange and Key lime anthracnose strains of *Colletotrichum* from citrus as *C. acutatum*. Phytopathology 86:523-527.

Three forms of *Colletotrichum* are recognized on citrus: the fast-growing gray (FGG) form, a common saprophyte and postharvest pathogen that is morphologically distinct from the other two; the slow-growing orange (SGO) form, the causal agent of postbloom fruit drop; and the Key lime anthracnose (KLA) form, the cause of lime anthracnose. Pathogenicity tests confirmed that the SGO isolates infected sweet orange flowers only, the KLA isolates infected both sweet orange flowers and Key lime foliage, and the FGG isolates did not infect either of these host tissues. Polymerase chain reaction (PCR) with the *C. acutatum*-specific primer *Ca*Int2 and the internal transcribed spacer (ITS)4 primer (from a conserved sequence of ribosomal DNA [rDNA]) amplified a 490-bp fragment from the SGO and KLA isolates but not from the FGG isolates. PCR with the *C. gloeosporioides*-specific primer *Cg*Int and the ITS4 primer amplified a 450-bp fragment only from the FGG isolates. The nucleotide sequence of the rDNA ITS1 of four SGO and four KLA isolates indicated either complete homology or a 1-base difference from a *C. acutatum* reference isolate but only 80 to 81% homology to a *C. gloeosporioides* reference isolate. The ITS1 sequence of the FGG isolates had 97% homology to the *C. gloeosporioides* reference isolate but only 80% homology to the *C. acutatum* reference isolate. These data confirmed the classification of the SGO and KLA isolates as *C. acutatum* and the FGG isolates as *C. gloeosporioides*. Cluster analysis of rDNA and mitochondrial DNA restriction fragments divided the SGO and KLA isolates into two recognizable groups with genetic similarity of 87%. These two groups were more closely related to each other than to any of the reference isolates used (<80% similarity). It is suggested that the SGO and KLA isolates had a recent common ancestor.

Agostini et al. (1) described the morphology and pathogenicity of the three forms of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. that occur on citrus. The fast-growing gray (FGG) form was typical of *C. gloeosporioides*. The FGG isolates grew rapidly and produced abundant gray mycelium, acervuli with setae, and conidia with both ends rounded. The FGG strain was not pathogenic to sweet orange flowers or to young foliage of Key limes. The slow-growing orange (SGO) and Key lime anthracnose (KLA) strains grew slowly, produced pink-orange colonies, rarely formed setae, and produced conidia that often had one fusiform end. The SGO and KLA isolates differed only slightly in conidial size but had differently shaped appressoria. Both of these strains produced typical postbloom fruit drop (PFD) symptoms on sweet orange flowers, but only KLA isolates caused anthracnose on Key lime foliage.

The FGG (type 1) and SGO (type 2) isolates also could be differentiated on the basis of forms of ribosomal DNA (rDNA) (9). A clone from the nontranscribed spacer region hybridized with type 1 (FGG) but not type 2 (SGO) isolates. Variation in cutinases from the SGO and FGG isolates and genetic markers, such as rDNA restriction fragment length polymorphisms (RFLPs) and chromosomal DNA size, differentiated the two strains (8,9).

Gray and pink isolates of *Colletotrichum* spp. have been described on strawberry (6,13), peach, apple, and pecan (2) and have been identified as *C. gloeosporioides* and *C. acutatum* J.H. Simmonds, respectively. The FGG isolates from citrus share most characteristics with the gray isolates described on the above hosts: rapid growth, gray mycelium, presence of setae, and conidia with rounded ends. The SGO and KLA isolates from citrus are similar to the pink isolates described above: slow growth, pink-orange colonies, and few or no setae. However, most conidia of the SGO and KLA strains have only one end that is fusiform and about one-third have both ends rounded (1). Gray isolates from deciduous tree hosts are highly sensitive to benomyl, whereas pink isolates are relatively insensitive (2). Sonoda and Pelosi (14) and Liyanage et al. (9) found that the FGG strain was sensitive to benomyl but that the SGO strain tolerated much higher levels.

The SGO and KLA isolates from citrus have many characteristics of *C. acutatum*, but the conidial shape is atypical for that species. The FGG isolates from citrus clearly fit the description of *C. gloeosporioides*. RFLPs and the nucleotide sequence of the internal transcribed spacer (ITS)1 region of rDNA have been useful in differentiating species of *Colletotrichum* from strawberry and in identifying *C. acutatum* (15,16). In this study, we applied these techniques to determine the identity of isolates of *Colletotrichum* from citrus and the relationship among them.

Corresponding author: L. W. Timmer; E-mail address: lwt@gnv.ifas.ufl.edu

Publication no. P-1996-0226-02R

MATERIALS AND METHODS

Isolates and pathogenicity test. The sources of isolates used in the study are listed in Table 1. For pathogenicity tests, isolates were grown on potato dextrose agar at 27°C for 5 days, and conidia were washed from plates and suspended in sterile distilled water.

To evaluate pathogenicity to sweet orange flowers, three flower clusters on screenhouse-grown, navel orange trees (*Citrus sinensis* (L.) Osbeck) were sprayed with suspensions containing 10^6 conidia per ml for each isolate. Three potted Key lime (*Citrus aurantifolia* (Christm.) Swingle) seedlings with abundant new flushes were similarly inoculated to evaluate pathogenicity to the host. Flower clusters and seedlings were covered with plastic bags for 48 h after inoculation. Flowers were examined for production of orange-brown lesions on petals, which are typical of PFD, or leaf lesions and foliage blight, which are typical of lime anthracnose.

For molecular analysis, fungal mycelium was grown in glucose casamino acid liquid medium, and the DNA was extracted from freeze-dried mycelial powder as described in Sreenivasaprasad et al. (16).

PCR amplification of genomic DNA. Primers *Ca*Int2 (5′GGGGAAGCCTCTCGCGG3′), from the ITS1 region of *C. acutatum* reference isolate 397 (17), *Cg*Int (5′GGCCTCCCGCCTCCGGGCGG3′), from the ITS1 region of *C. gloeosporioides* (10), and ITS4 (5′TCCTCCGCTTATTGATATGC3′), from the conserved region of the 25/28S rDNA gene (23), were synthesized and supplied by Operon Technologies, Alameda, CA. PCR reactions with these primers were as described in Mills et al. (10), except that an annealing temperature of 55°C was used for *Cg*Int/ITS4 as well as *Ca*Int2/ITS4.

Nucleotide sequencing of the ITS1 region. The ITS1 region was amplified with primers ITS1ext (5′GTAACAAGGTTTCCGTAGGTG3′) and ITS2ext (5′ATTTCGCTGCGTTCTTCATCG3′) synthesized on a Millipore oligonucleotide synthesizer (Millipore Corp., Hertfordshire, England). Amplification mixtures contained approximately 100 ng of genomic DNA, *Taq* DNA polymerase buffer, 1.5 μM $MgCl_2$, 200 μM of each dNTP, 1.0 μM of each primer, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). Mixtures were subjected to 30 cycles of 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C on a thermal cycler (Perkin-Elmer Cetus, Warrington, England). PCR products (10 μl) were visualized on 1.4% (wt/vol) agarose gels containing ethidium bromide (0.5 μg ml^{-1}).

To prepare templates for cycle sequencing, PCR-amplified products were purified using the Wizard DNA clean-up system (Promega) according to the manufacturer's instructions. DNA was eluted in 50 μl of sterile distilled water, and the quantity was checked by comparative gel electrophoresis with digested pGEM molecular size markers (Promega). Cycle sequencing reactions were performed in oilfree tubes on a thermal cycler (Perkin-Elmer Cetus) with approximately 500 ng of template DNA and the Prism ready reaction dyedeoxy terminator cycle sequencing kit (Applied Biosystems, Liverpool, England) according to the instructions provided by the manufacturer. The sequencing reaction products were phenol/chloroform extracted and ethanol precipitated. The pellet was dissolved in 3 μl of formamide loading buffer. Denatured (3 min at 95°C) samples were electrophoresed on 6% (wt/vol) acrylamide gel on an automated sequencing apparatus (373 A DNA sequencing system, Applied Biosystems). All reagents, including gel-running buffer, acrylamide solution, and loading buffer, were prepared according to the manufacturer's instructions.

The nucleotide sequences were read and edited by 373A Gene Scan software and Sequence Navigator (Applied Biosystems), respectively. The sequence data were aligned and analyzed by DNASIS (LKB Products, Bromma, Sweden) and the CLUSTAL V package (7).

DNA digestion, Southern blotting, hybridization, and autoradiography. Genomic DNA (1 to 3 μg) was digested with 25 units of restriction enzyme until completion at 37°C according to the manufacturer's instructions (Promega). Hexameric restriction endonucleases *Bam*HI, *Cla*I, *Eco*RI, *Sac*I, and *Sma*I were used for rDNA RFLP analysis and *Eco*RI, *Hin*cII, *Hin*dIII, *Kpn*I, and *Msp*I were used for mitochondrial DNA (mtDNA) RFLP analysis. Digested DNA was electrophoresed in 0.8% (wt/vol) agarose gels for 13 h at 60 V (electrophoresis buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) with *Hin*dIII-digested λDNA and digested pGEM as molecular size markers (Promega), transferred to a nylon membrane (Hybond N, Amersham Corp., Buckinghamshire, England) by capillary transfer (12), and immobilized by baking at 80°C for 90 min.

For rDNA analysis, Southern blots were probed with the plasmid pMY60 that contained a complete rDNA unit from *Saccharomyces carlsbergensis* (21). For mitochondrial genome probing, mtDNA purified from *C. acutatum* isolate 1073 (15) was used. Probes were labeled with [∝-^{32}P]-deoxyadenosine 5′-triphosphate (Amersham) by the Prime-a-Gene labeling system (Promega) and separated from unincorporated nucleotides on a Sephadex G50

TABLE 1. Sources and pathogenicity of isolates of *Colletotrichum* spp. used in this study

Isolate	Species or strain[v]	Location	Host plant	Tissue	Pathogenicity	
					Sweet orange flowers	Key lime foliage
LA-5	FGG	Lake Alfred, FL	Sweet orange	Senescent flowers	–	–
Ar-FGG	FGG	Arcadia, FL	Sweet orange	Senescent flowers	–	–
Im	SGO	Immokalee, FL	Sweet orange	PFD-affected flowers[w]	+	–
In-1	SGO	Indiantown, FL	Sweet orange	PFD-affected flowers	+	–
VB	SGO	Vero Beach, FL	Sweet orange	PFD-affected flowers	+	–
FtP (cs)	SGO	Ft. Pierce, FL	Sweet orange	PFD-affected flowers	+	–
Ss	KLA	Sarasota, FL	Key lime	Leaf-anthracnose	+	+
ftP	KLA	Ft. Pierce, FL	Key lime	Leaf-anthracnose	+	+
Hm-1	KLA	Homestead, FL	Key lime	Leaf-anthracnose	+	+
Hm-3	KLA	Homestead, FL	Key lime	Leaf-anthracnose	+	+
CA 397	*C. acutatum*	Florida	Strawberry	ND[x]	ND	ND
PD 85/694[y]	*C. acutatum*	Netherlands	Chrysanthemum	ND	ND	ND
NI 90	*C. acutatum*	Northern Ireland	Strawberry	ND	ND	ND
302a[z]	*C. acutatum*	United Kingdom	Nandina	ND	ND	ND
394	*C. acutatum*	Florida	Strawberry	ND	ND	ND
CG231[z]	*C. gloeosporioides*	Florida	Strawberry	ND	ND	ND

[v] FGG = fast-growing gray; SGO = slow-growing orange; and KLA = Key lime anthracnose strains.
[w] PFD = postbloom fruit drop.
[x] ND = not assessed for pathogenicity on citrus.
[y] Culture collection: Plantenziektenkundige Dienst, Wageningen, Netherlands.
[z] Culture collection: MAFF Plant Pathology Laboratory, Harpenden, England.

column. Hybridization was performed in a hybridization oven (Techne, Cambridge, England) at 65°C following the procedures of Sambrook et al. (12). Hybridization conditions were as described in Sreenivasaprasad et al. (15).

Data analysis. Each fragment generated from the five rDNA and five mtDNA RFLP analyses was treated as a separate character. DNA fragments of the same size were assumed to represent the same genetic locus and were scored as either present or absent. The cluster analysis of the data was done based on a similarity matrix derived from the formula: number of shared characters/total number of characters. The dendrogram was generated by the unweighted pair group method with arithmetic mean (UPGMA), using pairwise Jaccard's similarity coefficients on the program GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station, England).

RESULTS

Pathogenicity of *Colletotrichum* isolates from citrus. Pathogenicity tests confirmed that the SGO isolates infected sweet orange flowers but did not cause anthracnose symptoms on Key lime leaves. The KLA isolates infected both of these hosts (Table 1). The two FGG isolates did not infect either sweet orange flowers or Key lime foliage.

PCR amplification of genomic DNA. The *C. acutatum*-specific primer *Ca*Int2, in conjunction with the ITS4 primer, amplified a 490-bp fragment from genomic DNA of the SGO and KLA isolates and the reference *C. acutatum* isolate (397) but not from the two FGG isolates (Fig. 1A). In contrast, a 450-bp fragment was amplified from genomic DNA of the FGG isolates and the reference *C. gloeosporioides* isolate (231) with the *C. gloeosporioides*-specific primer *Cg*Int and the ITS4 primer. *Cg*Int did not amplify a product from the DNA of the SGO and KLA isolates (Fig. 1B).

Sequencing of the ITS1 region. Nucleotide sequences of the ITS1 region of the SGO, KLA, and FGG isolates were compared with those of the *C. acutatum* reference isolate 397 and the *C. gloeosporioides* reference isolate 231 (16) (Fig. 2). The ITS1 sequence of the SGO isolates Im, In-1 (cs), and VB and the KLA isolate Hm-1 was identical to that of the *C. acutatum* isolate 397, whereas that of the SGO isolate FtP (cs) and the KLA isolates Ss, FtP, and Hm-3 had a 1-base difference. The SGO and KLA isolates showed only 80 to 81% homology to *C. gloeosporioides* isolate 231. The ITS1 sequence of the FGG isolates LA-5 and ArFGG had 97% homology to this *C. gloeosporioides* isolate and only 80% identity to *C. acutatum* isolate 397.

Restriction fragment patterns in rDNA and mtDNA. Genomic DNA from the SGO and KLA isolates digested with *Sac*I and hybridized with the rDNA probe pMY60 revealed similar restriction fragment banding patterns (Fig. 3). These isolates all yielded a band of 2.2 kb, and all but KLA isolate FtP and SGO isolate Im yielded a second band at 6.5 kb. Isolates FtP and Im yielded a fractionally larger band of 6.7 kb. The banding patterns of these isolates were different from those of the four *C. acutatum* reference isolates, although all had the 2.2-kb band.

Minor variations among the SGO and KLA isolates were observed after digestion of the genomic DNA with *Bam*HI, *Cla*I, *Eco*RI, and *Sma*I and hybridization with pMY60 (data not shown). Again, no banding patterns identical to those of the reference isolates were observed, although common bands were present, e.g., a 2.0-kb *Cla*I fragment was common to all isolates, except reference isolate PD85/694 and a 2.45-kb *Eco*RI fragment.

The two FGG isolates had an identical banding pattern with *Sac*I that was distinct from those of the SGO and KLA isolates (Fig. 3). Except for the 2.0-kb band with the *Cla*I fragment (data not shown), the FGG isolates did not share any common bands with the *C. acutatum* isolates with the five restriction enzymes used.

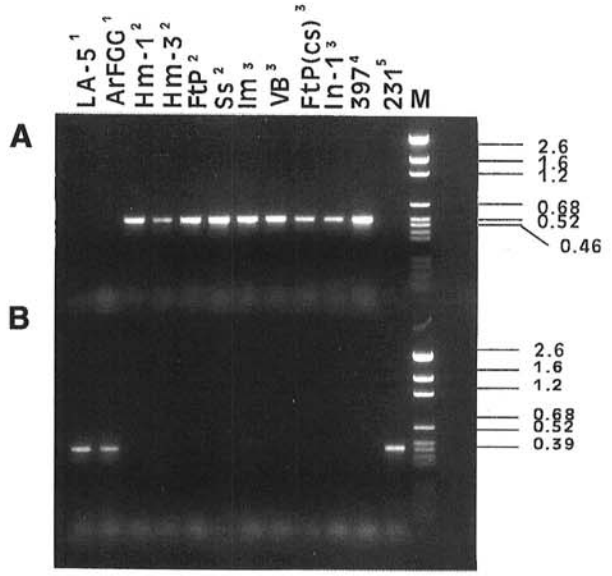

Fig. 1. Amplification of specific fragments from fungal DNA with target primers **A,** *Ca*Int2 and internal transcribed spacer (ITS)4 and **B,** *Cg*Int and ITS4 (superscripts: 1 indicates fast-growing gray isolates; 2 indicates Key lime anthracnose isolates; 3 indicates slow-growing orange isolates; 4 indicates *Colletotrichum acutatum* reference isolate 397; and 5 indicates *C. gloeosporioides* reference isolate 231). Lane M, digested pGEM was used as the molecular size marker.

Isolates	Nucleotide Sequences		
397	CTGAGTTACC	GCTCTATAAC	CCTTTGTGAA
3, 4, 5, 8	----------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	-------TA-	----------	----------
1, 2	-------TA-	-------C--	----------
397	CATACCTA**	ACCGTTGCTT	CGGCGGGCAG
3, 4, 5, 8	-G--------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	-------TA	--T-------	-------T--
1, 2	-------TA	--T--- ---	-------T--
397	GGGAAGCCTC	TCGCGGGCCT	CCCCTCCCGG
3, 4, 5, 8	----------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	--****T---	*-----A****	*---------
1, 2	--****T---	*-----A****	*---------
397	CGCCGGCCCC	CACCACGGGG	ACGGGGCGCC
3, 4, 5, 8	----------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	--CT--**--G-	--T--G*---C-	GGTC------
1, 2	--CT--**--G-	--T--G*---C-	AGTC------
397	CGCCGGAGGA	*AACCAAACT	CTATTTACAC
3, 4, 5, 8	----------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	----------	T---------	--GA--TA--
1, 2	----------	T---------	--GA--TA--
397	GACGTCTCTT	CTGAGTGGCA	CAAGCAAATA
3, 4, 5, 8	----------	----------	----------
6, 7, 9, 10	----------	----------	----------
231	-----T----	---------T-	----------
1, 2	-----T----	---------T-	----------
397	ATTA		
3, 4, 5, 8	----		
6, 7, 9, 10	----		
231	----		
1, 2	--C-		

Fig. 2. Nucleotide sequences of the ribosomal DNA internal transcribed spacer (ITS)1 of 10 isolates of *Colletotrichum* from citrus aligned with previously characterized isolates of *C. acutatum* (isolate 397) and *C. gloeosporioides* (isolate 231). 1 and 2 = fast-growing gray isolates LA-5 and Ar-Fgg, respectively; 3 through 6 = slow-growing orange isolates Im, In-1, VB, and FtP(cs), respectively; and 7 through 10 = Key lime anthracnose isolates Ss, Hm-1, FtP, and Hm-3, respectively. ITS1 nucleotide sequences of *C. acutatum* isolate 397 and *C. gloeosporioides* isolate 231 are reproduced from Sreenivasaprasad et al. (15). Dash (-) indicates identity with the sequence of isolate 397; asterisk (*) indicates alignment gaps and base deletions.

Based on mtDNA restriction fragment banding patterns produced with *Eco*RI (Fig. 4A), *Kpn*I, *Hinc*II, and *Msp*I (data not shown), using the *C. acutatum* mtDNA probe, the SGO and KLA isolates formed two distinct groups with only slight variation within each group. Only with *Hin*dIII were there differences in the banding pattern of KLA isolate Ss (largest band at <9 kb) from those of the other three KLA isolates (largest band at >12 kb). There also was a slight variation in the size of bands between 6.0 and 6.2 kb among the SGO and KLA isolates (Fig. 4B). Both the FGG isolates had identical banding patterns with *Eco*RI and *Hin*dIII that were distinctly different from the SGO and KLA isolates (Fig. 4A and B).

A dendrogram (Fig. 5), produced from rDNA and mtDNA RFLP data for the SGO and KLA isolates and the four reference *C. acutatum* isolates, was used to assess relatedness among isolates. Three of the four SGO isolates, VB, FtP (cs), and In-1, were virtually identical, with a genetic similarity level of 97%; the fourth, isolate Im, showed some variation, with 92% similarity to the other three. Two of the KLA isolates, Hm-3 and Hm-1, also were virtually identical (96% similarity), with the isolates FtP and Ss showing some variation (91% similarity). The SGO and KLA isolates were much more similar to one another (87% similarity) than they were to any of the reference isolates (<80% similarity). Analysis of mtDNA bands alone also revealed that SGO and KLA were two distinct groups, whereas separation was less apparent with the rDNA data alone.

DISCUSSION

The causal agent of PFD of citrus, now known as the SGO strain (1,14), originally was described by Fagan (5) as a strain of *C. gloeosporioides*. The causal agent of Key lime anthracnose, now known as the KLA strain (1), originally was described as *Gloeosporuim limetticolum* (4) and subsequently was transferred to *C. gloeosporioides* (18,22). Agostini et al. (1) found that the SGO and KLA strains differed considerably from the FGG strain, a saprophyte and postharvest pathogen of citrus that has characteristics typical of *C. gloeosporioides*. Most of the characteristics of the SGO and KLA strains were typical of *C. acutatum*, except that conidia were rounded on at least one end, whereas *C. acutatum* is described as having conidia that are fusiform at both ends (19). In the present study, a fragment was amplified by PCR from the SGO and KLA isolates with a primer specific for *C. acutatum* but not with the *C. gloeosporioides*-specific primer. The nucleotide sequences of the ITS1 region of the two strains were identical to or had only a 1-base difference from that of the reference isolate of *C. acutatum*. The sequences differed considerably (19 to 20%) from that of the *C. gloeosporioides* reference isolate. These data clearly demonstrate that the SGO and KLA isolates are *C. acutatum* and not *C. gloeosporioides* as is suggested by the morphology of their conidia. The teleomorphs of these isolates have been observed in only one instance (1), and we have been unable to produce that stage subsequently. Thus, it has not been possible to determine the taxonomic relationships on the basis of the teleomorphic stage. This is another example of the use of molecular analyses to distinguish 'atypical' strains of *C. acutatum* from *C. gloeosporioides* (16) and confirms that, as with strawberry (13), stone and pome fruits (2), and rubber (3), citrus is affected by more than one species of *Colletotrichum*.

Analysis of rDNA and mtDNA restriction fragment banding patterns revealed a similarity of 87 to 97% among the citrus *C. acu-*

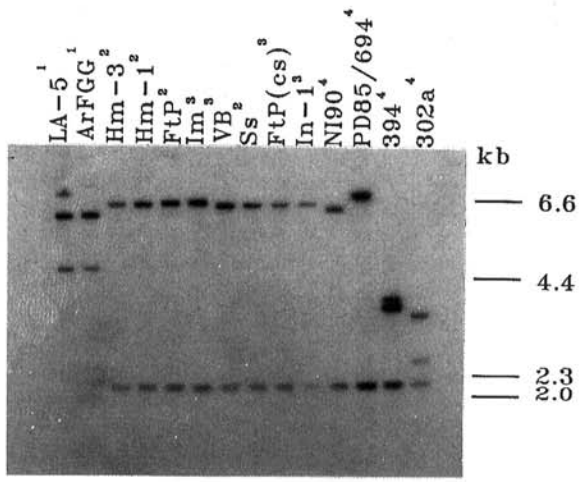

Fig. 3. Restriction fragment patterns of ribosomal DNA (rDNA) from citrus isolates of *Colletotrichum gloeosporioides* and *C. acutatum* and reference isolates of *C. acutatum* digested with *Sac*I and hybridized with the yeast rDNA probe pMY60 (superscripts: 1 indicates fast-growing gray isolates; 2 indicates Key lime anthracnose isolates; 3 indicates slow-growing orange isolates; and 4 indicates reference isolates of *C. acutatum*). *Hin*dIII-digested λDNA was used for molecular size markers.

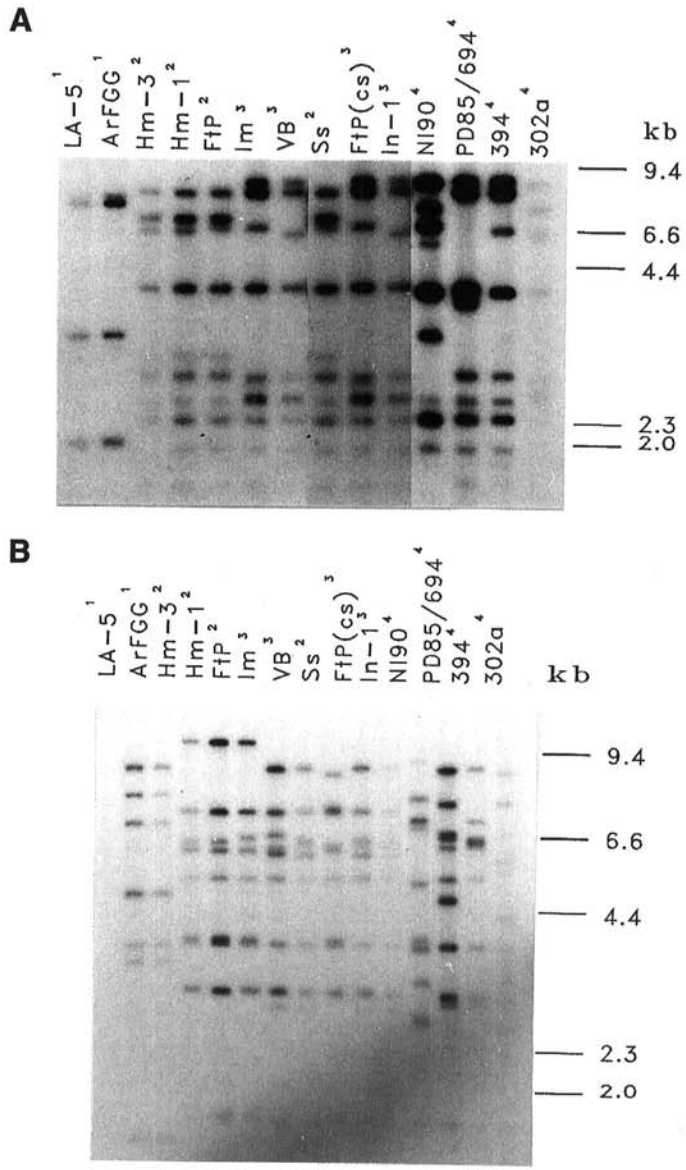

Fig. 4. Mitochondrial DNA (mtDNA) restriction fragment length polymorphism patterns of citrus isolates of *Colletotrichum gloeosporioides* and *C. acutatum* and reference isolates of *C. acutatum* digested with **A,** *Eco*RI and **B,** *Hin*dIII and probed with mtDNA from *C. acutatum* (superscripts: 1 indicates fast-growing gray isolates; 2 indicates Key lime anthracnose isolates; 3 indicates slow-growing orange isolates; and 4 indicates reference isolates of *C. acutatum*). Molecular size markers were *Hin*dIII-digested λDNA and digested pGEM.

tatum isolates and indicated some divergence of these isolates from the reference *C. acutatum* isolates (<80% similarity). The reference isolates used in this study represented four distinct RFLP groups of *C. acutatum* (15). In light of the high degree of intraspecific variation among *C. acutatum* isolates reported by Sreenivasaprasad et al. (15), the relatively high level of similarity among the citrus isolates might suggest a close relationship and a possible common origin. The mtDNA restriction fragment banding patterns, despite having most bands in common, also had polymorphic bands that split the SGO and KLA isolates into two recognizable subgroups, reflecting their variation in pathogenicity.

PFD was first described in Belize in 1979, although it was probably present many years earlier (5). During the 1980s it was reported from several locations in Central America, Mexico, the Caribbean, and Florida and as far away as Argentina and Brazil (20). Some of these may represent new reports of a disease that had been present for some time. However, considering the severity of the PFD problem, it is unlikely that the disease went unnoticed for long periods in commercial citrus areas. Rapid spread of a pathogen that lacks airborne spores throughout the hemisphere seems improbable. After we determined that the Key lime anthracnose pathogen was capable of causing PFD, we speculated that the lime anthracnose pathogen had infected sweet oranges and other citrus, causing PFD (1). Considering the similarity of RFLP patterns and nucleotide sequences of the KLA and SGO strains, this remains a viable hypothesis.

Key lime anthracnose has been recognized in the humid areas of tropical America since the early 1900s and is a limiting factor in lime production (11). As a consequence, the crop in these areas is restricted to backyards and small, commercial plantings where it grows alongside other fruit crops. Under these circumstances, the lime anthracnose pathogen may have spread to flowers of other citrus species, causing PFD, and eventually lost the ability to infect Key lime foliage, producing the SGO strain. Some of the spread of the pathogen throughout the Americas may have occurred by transport of citrus materials from one area to another; however, PFD may have arisen independently in more than one location. Molecular analysis of SGO isolates from many areas may help to resolve this question. In conclusion, the SGO and KLA strains from citrus should now be considered pathotypes of *C. acutatum*, whereas the FGG strain should remain as *C. gloeosporioides*.

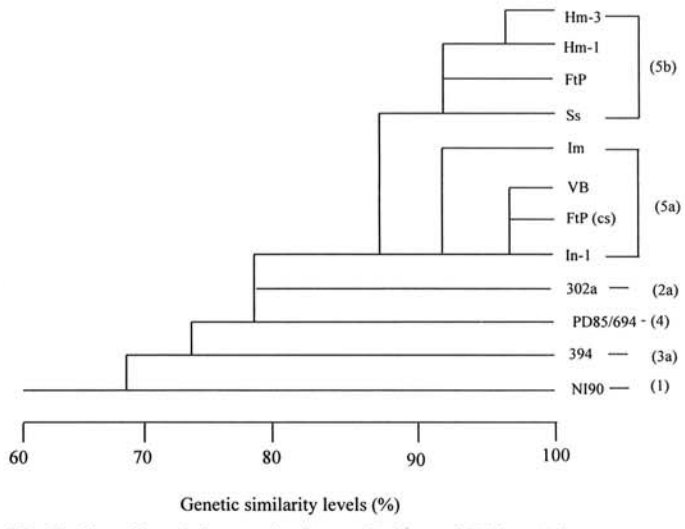

Fig. 5. Clustering of citrus and reference isolates of *Colletotrichum acutatum* from pairwise comparison of ribosomal and mitochondrial DNA restriction fragment banding patterns by the 'group average' method. Group numbers are in parenthesis; 1, 2a, 3a, and 4 refer to designated groups in Sreenivasaprasad et al. (15); 5a refers to slow-growing isolates; and 5b refers to Key lime anthracnose isolates.

LITERATURE CITED

1. Agostini, J. P., Timmer, L. W., and Mitchell, D. J. 1992. Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. Phytopathology 82:1377-1382.
2. Berstein, B., Zehr, E. I., Dean, R. A., and Shabi, E. 1995. Characteristics of *Colletotrichum* from peach, apple, pecan, and other hosts. Plant Dis. 79:478-482.
3. Brown, A. E., and Soepena, H. 1994. Pathogenicity of *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* on leaves of *Hevea* spp. Mycol. Res. 98:264-266.
4. Clausen, R. E. 1912. A new fungus concerned in wither tip of varieties of *Citrus medica*. Phytopathology 2:217-236.
5. Fagan, H. J. 1979. Postbloom fruit drop, a new disease of citrus associated with a form of *Colletotrichum gloeosporioides*. Ann. Appl. Biol. 91:13-20.
6. Gunnell, P. S., and Gubler, W. D. 1992. Taxonomy and morphology of *Colletotrichum* species pathogenic to strawberry. Mycologia 84:157-165.
7. Higgins, D. G., Bleasby, A. J., and Fuchs, R. 1992. CLUSTAL V: Improved software for multiple sequence alignment. CABIOS 8:189-191.
8. Liyanage, H. D., Köller, W., McMillan, R. T., Jr., and Kistler, H. C. 1993. Variation in cutinase from two populations of *Colletotrichum gloeosporioides* from citrus. Phytopathology 83:113-116.
9. Liyanage, H. D., McMillan, R. T., Jr., and Kistler, H. C. 1992. Two genetically distinct populations of *Colletotrichum gloeosporioides* from citrus. Phytopathology 82:1371-1376.
10. Mills, P. R., Sreenivasaprasad, S., and Brown, A. E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiol. Lett. 98:137-144.
11. Nolla, J. A. B. 1926. The anthracnoses of citrus fruits, mango and avocado. J. Dep. Agric., Puerto Rico 10:25-63.
12. Sambrook, J., Fritch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Smith, B. J., and Black, L. L. 1990. Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. Plant Dis. 74:69-76.
14. Sonoda, R. M., and Pelosi, R. R. 1988. Characteristics of *Colletotrichum gloeosporioides* from lesions on citrus blooms in the Indian River area of Florida. Proc. Fla. State Hortic. Soc. 101:36-38.
15. Sreenivasaprasad, S., Brown, A. E., and Mills, P. R. 1992. DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. Physiol. Mol. Plant. Pathol. 41:265-281.
16. Sreenivasaprasad, S., Mills, P. R., and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. Mycol. Res. 98:186-188.
17. Sreenivasaprasad, S., Sharada, K., Brown, A. E., and Mills, P. R. PCR-based detection of *Colletotrichum acutatum* on strawberry. Plant Pathol. In press.
18. Sutton, B. C. 1980. The Coelomycetes. Commonwealth Mycological Institute, Kew, England.
19. Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 1-26 in: *Colletotrichum*—Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, England.
20. Timmer, L. W., Agostini, J. P., Zitko, S. E., and Zulfiqar, M. 1994. Postbloom fruit drop, an increasingly prevalent disease of citrus in the America's. Plant Dis. 78:329-334.
21. Verbeet, M. P., Klootwijk, J., Heerikhuizen, H., Fontijn, R., Vrengdenhil, E., and Planta, R. J. 1983. Molecular cloning of the rDNA of *Saccharomyces rosei* and comparison of its transcription initiation region with that of *Saccharomyces carlsbergensis*. Gene 23:53-63.
22. von Arx, J. A. 1970. A revision of the fungi classified as *Gloeosporium*. Bibl. Mycol. 24:1-203.
23. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols. A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J Sninsky, and T. J. White. Academic Press, San Diego, CA.