Two Genetically Distinct Populations of *Colletotrichum gloeosporioides* from Citrus

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ABSTRACT

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Two or more types of *Colletotrichum gloeosporioides* from citrus have been described on the basis of colony morphology and growth in culture. We report here two distinct genetic populations of the fungus from sweet orange (*Citrus sinensis*) and Tahiti lime (*Citrus aurantifolia*); the distinction of the two populations was made on the basis of DNA variation at several genetic loci as well as cultural morphology and growth. Type 1 strains are slow growing, morphologically stable, and contain a single homogeneous form of ribosomal DNA (rDNA) as detected by common *Hin*dIII, *Pst*I, *Sph*I, and *Sst*I fragments hybridizing to cloned *Neurospora crassa* rDNA. Type 2 strains are faster growing, morphologically less stable, and have rDNA repeats distinct from type 1 strains. The rDNA from a type 1 strain was cloned and mapped for 10 restriction enzyme sites and genes coding for large subunit, small subunit, and 5.8S rRNA. A subclone constructed from the nontranscribed spacer region of this rDNA clone hybridizes only to rDNA from type 1 strains. DNA polymorphisms detected by heterologous hybridization with cloned *N. crassa* genes for glutamate dehydrogenase, anthranilate synthetase, histidinol dehydrogenase, and β-tubulin corresponded to type 1 or 2 strains. Chromosome-sized DNAs separated by pulsed-field gel electrophoresis also corresponded to type 1 and type 2 strains defined by restriction fragment length polymorphism. Type 1 strains had five large chromosomal DNAs: 7.6, 7.0. 4.7. 3.7, and 3.3 (or 2.8) million base pairs (Mb) and one or two smaller chromosomes ranging in size from 1.6 to 0.63 Mb. Type 2 strains had three large chromosomal DNAs (7.8, 4.7, and 3.7 Mb) and two to four smaller chromosomal DNAs ranging in size from 0.52 to 0.28 Mb. Type 1 strains all were more tolerant to benomyl than type 2 strains. For every genetic marker examined, polymorphism that corresponded to morphological type was observed.

Citrus diseases caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. have been known to occur in Florida since the fungus was first isolated from citrus plants in 1886 (19,25). Postbloom fruit drop disease affects most commercially grown citrus species and is characterized by necrotic petal lesions, blossom blight, and premature fruit drop, which leave persistent peduncles and calyxes (4,5,7,16,22). By the presence of the persistent flower parts, known as buttons, the disease can be distinguished from physiological thinning of fruits that occurs naturally in citrus after blooming (5). The causal agent of postbloom fruit drop has been identified as *C. gloeosporioides* (5,16,22).

Historically, *C. gloeosporioides* has been described as a variable fungus with many morphological variants (2,27). Burger (3) described morphologically variable mycelial sectors arising within single-spore cultures of *C. gloeosporioides* from citrus. Other workers (1,4,6,22) also have noted strains differing in morphology, growth, and pathogenicity to flowers. Because of the morphological instability of strains and the difficulty with pathogenicity testing on ephemeral tissue in a perennial crop, the exact correspondence between morphology and pathogenicity has yet to be firmly established. However, recent evidence indicates that only certain strains of *C. gloeosporioides* isolated from citrus are the actual causal agents of postbloom fruit drop (1,22).

In this study, we examine the morphological and genetic diversity in *C. gloeosporioides* isolated from sweet orange (*Citrus sinensis*) and Tahiti lime (*Citrus aurantifolia*). We interpreted our results to suggest that two genetically distinct populations of *C. gloeosporioides* are associated with these *Citrus* species.

MATERIALS AND METHODS

Strains of *C. gloeosporioides*. Strain designation, host, geographic location, and year of isolation are listed in Table 1. All strains used in this study were obtained from single-spore cultures. Colony color, growth rate, and sporodochia production were examined in potato-dextrose agar (PDA) or potato-dextrose broth (PDB; Difco Laboratories, Detroit, MI). All cultures were grown in the laboratory at ambient temperature (approximately 21–23 C).

Extraction of fungal DNA. Lyophilized mycelium was ground and mixed with extraction buffer (100 mM Tris, pH 8.0, 50 mM

TABLE 1. Source of strains of *Colletotrichum gloeosporioides* from postbloom fruit drop of citrus

Strain	Host	Location
H-1	Tahiti lime	Immokalee, Florida, 1989
H-3	Tahiti lime	Immokalee, 1989
H-9	Tahiti lime	Homestead, Florida, 1988
H-25B	Tahiti lime	Homestead, 1989
H-35[a]	Sweet orange	Ft. Pierce, Florida, 1989
IMB-3[b]	Tahiti lime	Immokalee, 1990
LP-1[b]	Sweet orange	Lake Placid, Florida, 1990
Maran[b]	Sweet orange	Indiantown, Florida, 1990
OCO[b]	Sweet orange	Arcadia, Florida, 1990
H-4	Tahiti lime	Immokalee, 1989
H-11	Tahiti lime	Homestead, 1989
H-12	Tahiti lime	Homestead, 1988
H-46[a]	Sweet orange	Vera Cruz, Mexico, 1989
H-47	Sweet orange	Vera Cruz, 1989
H-48[a]	Sweet orange	Vera Cruz, 1989
189269[c]	Sweet orange	Belize, Mexico
226802[c]	Sweet orange	Belize

[a]Strains were provided by R. Sonoda, Agricultural Research and Education Center, Fort Pierce, Florida.
[b]Strains provided by L. W. Timmer, Citrus Research and Education Center, Lake Alfred, Florida. All the other strains were obtained from the culture collection at Tropical Research and Education Center, Homestead, Florida.
[c]Strains obtained from Commonwealth Institute of Mycology, Kew, London, U.K., provided by R. Sonoda.

EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], in H_2O) to make a slurry and incubated at 65 C for 30 min. One-half volume of 5 M potassium acetate (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of H_2O) was added to samples and incubated on ice for 30 min. The supernatant was collected by centrifugation at 12,000 *g* for 15 min and was treated with 30–50 μg/ml of DNase-free RNase (30 min at 37 C). After RNase (Sigma Chemical Co., St. Louis, MO) treatment 200–250 μg/ml of Proteinase K (Sigma Chemical Co.) was added, and the supernatant was incubated for an additional 20 min. Samples were purified by phenol/isoamyl alcohol/chloroform (25:1:24 by volume) extraction, and DNA was precipitated by addition of a twofold volume of absolute ethanol. DNA pellets were recovered by centrifugation at 12,000 *g* for 15 min and dissolved in 100 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). The DNA was further purified by precipitation with 0.7 vol of PEG-NaCl (20% PEG 8000 in 2.5 M NaCl; Sigma Chemical Co.) for 20–30 min on ice. Precipitated DNAs were collected at 12,000 *g* for 15 min, resuspended in TE, and stored at −20 C.

Molecular markers and Southern hybridization. Genes for the major forms of ribosomal RNA were detected by heterologous hybridization to plasmid pMF2 containing the cloned rDNA unit repeat from *Neurospora crassa* (9). Plasmids containing *N. crassa* genes for anthranilate synthetase (pNC2; 21), glutamate dehydrogenase (pJR2; 11), histidinol dehydrogenase (pNH60; 13), and β-tubulin (pSV50; 26) were used in Southern hybridizations to detect DNA polymorphisms. Clones of *N. crassa* genes were obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). Southern hybridization of ^{32}P-labeled probes were carried out according to methods described by Sambrook et al (20). Hybridization and washing of blots were carried out at 68 C. First and second washes were with 2× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), 0.1% SDS, and 0.1× SSC, 0.1% SDS, respectively. Restriction enzymes used in this study were purchased from either New England Bio Labs (Beverly, MA) or Bethesda Research Laboratories (BRL, Gaithersburg, MD). Restriction digests were conducted according to manufacturers' recommendations at 37 C for 6–12 h.

Cloning and mapping of rDNA from *C. gloeosporioides*. The rDNA of *C. gloeosporioides* was identified by heterologous hybridization with pMF2 (9). Total DNA from *C. gloeosporioides* strain H-25B was digested with restriction enzyme *Pst*I and fractionated on a 0.7% agarose (FMC BioProducts, Rockland, ME) gel in 0.5× TBE (Tris-borate EDTA). The piece of the gel containing the 7- to 10-kb DNA range was cut out, and the DNA was eluted by the freeze-squeeze method (24). The DNA was ligated to *Pst*I-cut pUC119 (30) and transformed into *Escherichia coli* strain ER1647 (*E. coli* K-12 *mcrB*$^-$; 29). Ligation, preparation of competent cells, and transformation were carried out according to Sambrook et al (20). Clones hybridizing to pMF2 were identified and restriction-mapped. Single and double restriction enzyme digestion of one presumptive rDNA clone, called pCGR1, and size fractionation in 1% agarose gels in 0.5× TBE were carried out to construct a restriction map of the clone. Regions of pCGR1 coding for large subunit and small subunit rRNA were mapped by Southern hybridization to heterologous probes constructed from *N. crassa* (14). A probe to detect the 5.8S rRNA gene was prepared by polymerase chain reaction with primers flanking the gene. The primers 5′-TCCGTAGGTGAACCTGCGC-3′ and 5′-GCTGCGTTCTTCATCGATGC-3′ amplify a 290-bp fragment that includes the transcribed spacer of the 3′ end of the small subunit rRNA gene and the entire 5.8S gene (28).

Pulsed-field gel electrophoresis. The strains of *C. gloeosporioides* were grown for 7 days in 20% (w/v) V8 juice (Campbell Co., Camden, NJ) at 250 rpm on an orbital shaker (Lab-Line Instruments Inc., Melrose Park, IL), and conidia were collected by centrifugation at 7,000 *g* for 5 min. Approximately 10^9 spores per milliliter were resuspended in 50 ml of PDB and incubated at room temperature (23–25 C) for 16–24 h at 200 rpm. The germlings were collected by centrifuging at 7,000 *g* for 5 min. Protoplasts were made by adding germlings to a 10-ml solution containing NovoZym 234 (Novo Industries, Bagsvaerd, Denmark), a complex mixture of wall-degrading enzymes. The NovoZym solution was prepared by mixing 1.5 ml of 1 M sorbitol and 50 mM sodium citrate containing 0.2 g of NovoZym 234 with 8.5 ml of 1.4 M $MgSO_4$ and 50 mM sodium citrate, pH 5.8. Germlings were incubated in this solution with gentle rocking on a Bellco rocker (Bellco Biotechnology, Vineland, NJ) at 4 rpm for 3–6 h at ambient temperature until most cells were protoplasts. The protoplasts were filtered through four layers of cheesecloth for removal of cell debris and undigested germlings. The filtrate was centrifuged at 3,000 *g* for 25 min at room temperature. Protoplasts were removed from the top and washed three times with 1 M sorbitol and 50 mM EDTA, pH 8.0. Protoplast inserts for pulsed-field gel electrophoresis were made as described by method 1 of Orbach et al (17). We employed a commercially available apparatus (BioRad CHEF DRII, Richmond, CA) with different pulse time combinations to separate chromosome-sized DNAs. Most electrophoresis was done with 0.6% FastLane agarose (FMC BioProducts) gels in 0.25× TBE buffer (20) at 4 C with rapid circulation of the buffer. The gels were run at 40 V for 6–10 days. Pulse times were "ramped" for various times ranging from 10 to 180 min. For the separation of smaller chromosome-sized DNA, 1% SeaKem agarose (FMC BioProducts) with 0.5× TBE buffer was used. These gels were run at 200 V for 24 h with pulse times of 30–60 s or 50–90 s.

Benomyl tolerance. The growth of *C. gloeosporioides* strains was measured in PDA medium containing 0, 2, and 10 μg/ml of benomyl. Growth was measured as the length of the mycelial colony radius over a 10-day period. Growth rates in millimeters per hour were estimated by the slopes obtained by linear regression analysis of the growth curve. We made a comparison of slopes by using analysis of variance. Each treatment was repeated once and replicated two to five times.

Pathogenicity. All the strains were tested for their ability to infect flowers of Tahiti lime under natural conditions in the field as well as in the laboratory. Strains were grown in 20% V8 juice for 7 days at 250 rpm on an orbital shaker, and suspensions containing 10^7 spores per milliliter of water were prepared. Tahiti lime flowers were sprayed to wetness with a spore suspension or water, and symptom development was observed at 12- to 16-h intervals for 3 days.

RESULTS

Morphology and growth. The *C. gloeosporioides* strains examined can be grouped into two major categories on the basis of morphology and growth characteristics. Type 1 strains (H-1, H-3, H-9, H-25B, H-36, IMB-3, LP-1, Maran, and OCO) produce morphologically stable and relatively slow-growing mycelial colonies in PDB. The colonies are orange and have appressed mycelia with abundant sporodochia. Type 2 strains (H-4, H-12, H-46, H-48, 189269, and 226802) grow faster and produce mostly gray, fluffy mycelial colonies. The type 1 strains grow at a rate of 0.008–0.10 mm/h, which is significantly slower than type 2 strains, which grow at 0.12–0.15 mm/h as calculated by the slopes of linear regression data. The strain types also differ in culture stability as determined by their ability to produce sectors of different color, morphology, and growth habit. To quantitate these levels of instability, we isolated 100 conidia from each of three type 1 strains and two type 2 strains and tested for morphological stability. All 100 single-spore cultures from strains H-1, H-3, and H-25B (type 1) grown in PDB produced identical colonies. All 100 single-spore cultures from strain H-12 and H-48 produced sectoring colonies. These colonies varied in colony color from dark gray, gray, white, or orange with different growth rates. Sporodochia production was scattered or inhibited but could be stimulated by mechanical injury. One-hundred induced spores from the H-48 gray mycelial sector produced 50 sectoring colonies, 24 dark gray colonies with no sporodochia, and 26 orange colonies with scattered sporodochia production.

Benomyl tolerance. All type 2 strains were completely inhibited by 2 or 10 μg/ml of benomyl in PDA, whereas type 1 strains were more tolerant (Table 2). Average growth rates for individual type 1 and type 2 strains are listed in Table 2. Analysis of variance showed that benomyl concentration had a significant ($P < 0.01$) effect on type 1 strains. The benomyl concentration effect was strain-dependent as indicated by a significant interaction between strains and concentration ($P < 0.01$).

Ribosomal DNA polymorphism. Polymorphisms in DNA corresponding to the genes for ribosomal RNAs also coincided with morphological types. Southern hybridizations of *Pst*I-digested DNA from *C. gloeosporioides* strains detected polymorphic fragments hybridizing to ^{32}P-labeled pMF2 (9), which contained the genes for *N. crassa* rRNA. Type 1 strains had only a single 8.4-kb *Pst*I fragment hybridizing to pMF2, whereas type 2 strains either had 8.4- and 6.8-kb *Pst*I fragments (strain H-48) or a single 6.8-kb *Pst*I fragment that hybridized (Fig. 1, left).

TABLE 2. Effect of benomyl concentration on radial growth rate (mm/h) of *Colletotrichum gloeosporioides* type 1 and type 2 strains

Strain	Benomyl concentration (μg/ml)		
	0	2	10
Type 1			
H-1	0.10	0.033	0.029
H-3	0.10	0.033	0.033
H-9	0.10	0.045	0.041
H-25B	0.041	0.041	0.041
H-36	0.095	0.037	0.033
IMB-3	0.008	0.008	0.004
LP-1	0.10	0.050	0.041
Maran	0.041	0.029	0.033
OCO	0.095	0.037	0.033
Type 2			
H-4	0.141	0.00	0.00
H-11	0.125	0.00	0.00
H-12	0.121	0.00	0.00
H-46	0.133	0.00	0.00
H-47	0.133	0.00	0.00
H-48	0.145	0.00	0.00
189269	0.133	0.00	0.00
226802	0.150	0.00	0.00

The 8.4-kb *Pst*I fragment was cloned from the type 1 strain, H-25B, to create pCGR1. The plasmid was mapped for 10 restriction enzymes (Fig. 2) and compared with the genomic restriction fragments of the strain H-25B hybridizing to pMF2 for seven restriction enzymes. The maps of the clone and the genomic fragments were identical. The map of pCGR1 was compared to restriction fragments that hybridized to pMF2 from the DNA of other type 1 strains. DNAs from all strains were digested either with *Hin*dIII, *Sph*I, or *Sst*I. The hybridization of the fragments to pMF2 agreed with the map of pCGR1 for the three enzymes for all type 1 strains.

Ribosomal DNA among type 2 strains was polymorphic for *Pst*I (Fig. 1, left), *Sph*I, and *Sst*I (not shown) restriction enzyme sites. However, *Hin*dIII digests show a similar pattern for all type 2 strains. A few subcloned fragments from the nontranscribed spacer (NTS) region of pCGR1 were tested for specific hybridization to DNA from type 1 strains. A 0.4-kb *Kpn*I-*Pst*I subclone (pCGRN1) from the NTS region hybridized specifically to DNA from type 1 strains (Fig. 1, right). The 8.4-kb *Pst*I band from type 2 strain H-48 did not hybridize to pCGRN1.

Other DNA polymorphisms. We used four clones of *N. crassa* genes as heterologous probes to identify additional genetic loci in *Hin*dIII-digested DNA from *C. gloeosporioides* strains. The probe pSV50 (26), containing the gene for β-tubulin, hybridized to a 3.2-kb fragment in type 1 strains but a 5.0-kb fragment in type 2 strains (Fig. 3). The probe pJR2 (11), containing the gene for glutamate dehydrogenase, hybridized to a 3.2-kb fragment only in type 2 strains, whereas only diffuse hybridization was observed in type 1 strains. The probe pNH60 (13), containing the gene for histidinol dehydrogenase, hybridized to 3.8- and 4.3-kb fragments in type 1 strains but hybridized to 3.3- and 4.8-kb fragments in type 2 strains. The probe pNC2 (21), containing a gene for anthranilate synthetase, hybridized to a 3.4-kb band in type 2 strains. Type 1 strains showed differences with this probe. Maran, H-36, and OCO hybridized to a 3.2-kb fragment, but all the other type 1 strains hybridized to a 2.7-kb fragment.

Electrophoretic separation of chromosomes. Chromosome-sized DNAs (henceforth called chromosomes) from type 1 and type 2 strains were separated by pulsed-field gel electrophoresis along with *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosome size standards (BioRad Laboratories,

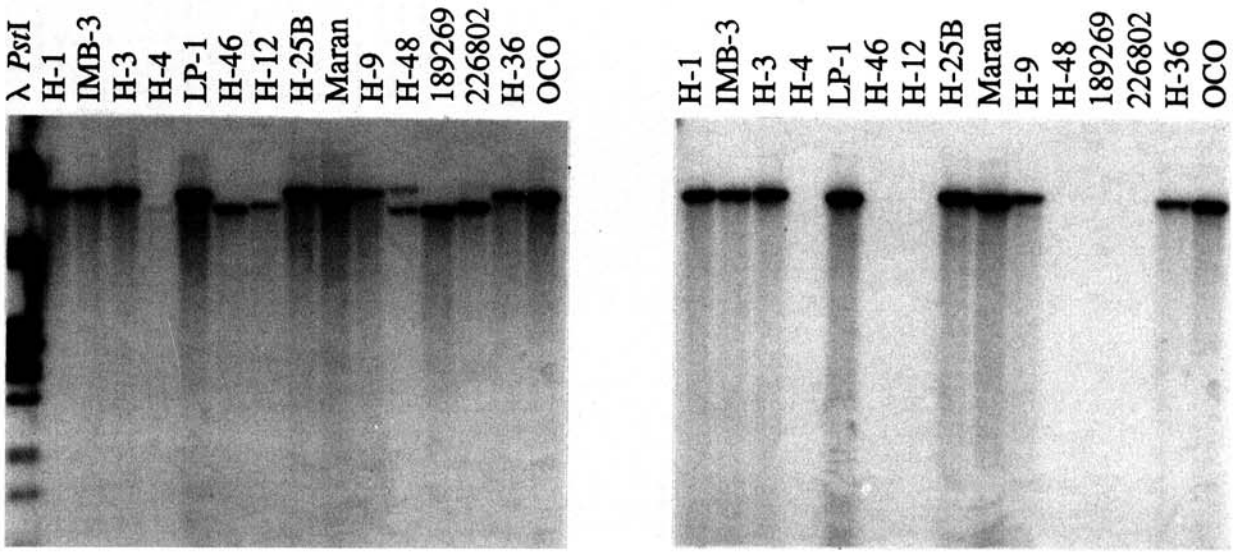

Fig. 1. Ribosomal DNA in *Colletotrichum gloeosporioides* strains. Left, genomic DNA of the indicated strains was digested with *Pst*I and hybridized to pMF2, the cloned rDNA unit repeat from *Neurospora crassa*. The probe also contained a small amount of bacteriophage λ DNA. The lane on the far left contains λ DNA digested with *Pst*I used as a size marker. (The two bands visible for H-48 persist even after prolonged incubation with *Pst*I. However, digestion of H-48 DNA with *Hin*dIII, *Sph*I, or *Sst*I indicated that only a single-sized rDNA repeat was present. The two *Pst*I bands may represent sequence heterogeneity within spacer regions that do not hybridize to the heterologous probe.) Right, a 0.4-kb *Pst*I-*Kpn*I fragment from the nontranscribed spacer region of cloned rDNA unit repeat from *C. gloeosporioides* strain H-25B detected only the 8.4-kb rDNA form in type 1 strains.

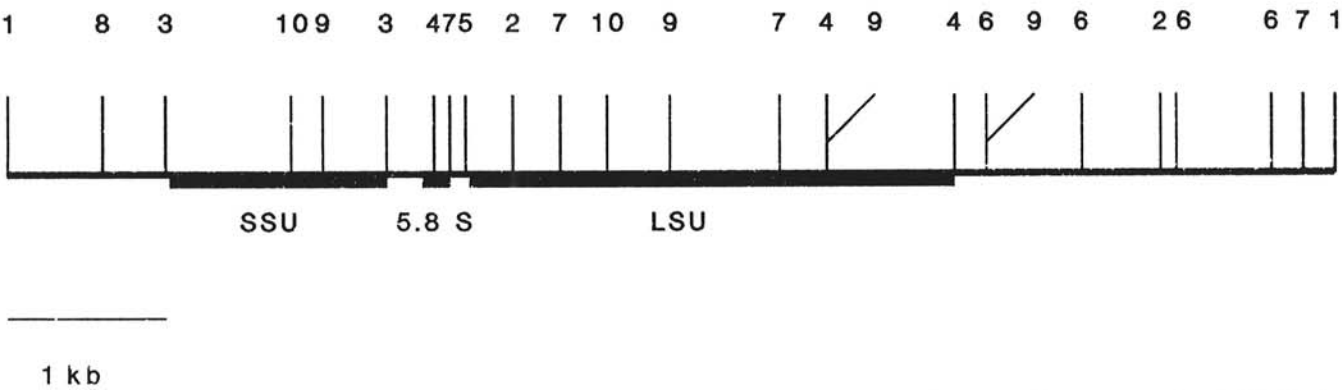

Fig. 2. Restriction enzyme map for the cloned rDNA unit repeat (pCGR1) from *Colletotrichum gloeosporioides* type 1 strain, H-25B. Regions hybridizing to large subunit rRNA (LSU), small subunit rRNA (SSU) from *Neurospora crassa*, and the polymerase chain reaction amplified 5.8S rRNA gene from *C. gloeosporioides* are indicated by solid boxes. Restriction enzyme sites are *Pst*I (1), *Hin*dIII (2), *Sph*I (3), *Eco*RI (4), *Bam*HI (5), *Kpn*I (6), *Hin*cII (7), *Xba*I (8), *Sma*I (9), and *Sst*I (10).

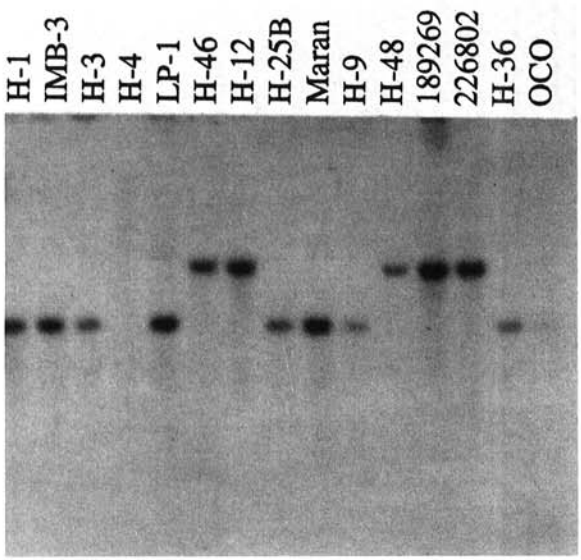

Fig. 3. DNA polymorphisms in type 1 and type 2 strains detected by Southern hybridization with a ^{32}P-labeled clone (pSV50) containing the *Neurospora crassa* β-tubulin gene as a probe. Type 1 strains hybridize to a 3.2-kb *Hin*dIII fragment, whereas type 2 strains hybridize to a 5.0-kb fragment. DNA from strain H-4 was degraded.

Richmond, CA). Type 1 strains had chromosomes distinguishable from type 2 strains (Table 3). The chromosomes of *C. gloeosporioides* isolated from *Stylosanthes* have been classified by Manners and co-workers (15) into larger, similar-sized chromosomes, and smaller (<1.3 Mb) variable-sized elements called "minichromosomes". A similar arrangement is noted for strains isolated from sweet orange and Tahiti lime. Type 1 strains possessed five chromosomes and an additional one or two minichromosomes (Fig. 4; Table 3). Type 2 strains possessed three chromosomes in addition to two to four minichromosomes, depending on the strain. Within each type, strains showed variations in chromosome number and size. However, type 2 strains showed more total variation in chromosome and minichromosome sizes (Fig. 4; Table 3). A Southern blot, separating larger chromosome-sized DNAs, was hybridized with a ^{32}P-labeled ribosomal DNA probe. The rDNA was associated with a 4.7-Mb chromosome in type 1 strains and with a 7.8-Mb chromosome in type 2 strains (Fig. 5).

Pathogenicity to Tahiti lime flowers. Brown lesions developed in flowers inoculated individually with all strains of the pathogen 24 h after inoculation. The petals were blighted completely at 36 h and had dropped at 48 h. Flowers sprayed with water alone were not blighted after 72 h. The fungal strains reisolated from infected tissues were found to be morphologically like the original strains. The relative virulence of strains was not measured in this study.

TABLE 3. Estimated sizes for chromosomal DNAs from *Colletotrichum gloeosporioides* type 1 and type 2 strains

Strain	Estimated size (Mb)[a]						
	I	II	III	IV	V	VI	VII
Type 1							
H-1	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-3	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-9	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-25B	7.6	7.0	4.7	3.7	3.3	1.1	0.63
H-36	7.6	7.0	4.7	3.7	3.3	0.77	0.63
IMB-3	7.6	7.0	4.7	3.7	3.3	1.1	0.63
LP-1	7.6	7.0	4.7	3.7	3.3	1.6	0.61
Maran	7.6	7.0	4.7	3.7	2.8	...[b]	0.63
OCO	7.6	7.0	4.7	3.7	2.8	...	0.65
Type 2							
H-4	7.8	4.7	3.7	0.42	0.38	...	...
H-12	7.8	4.7	3.7	0.46	0.38	...	...
H-46	7.8	4.7	3.7	0.52	0.47	0.42	0.27
H-48	7.8	4.7	3.7	0.46	0.43	0.40	...
189269	7.8	4.7	3.7	0.43	0.41	0.39	...
226802	7.8	4.7	3.7	0.44	0.42	0.39	0.37

[a] *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* size standards were used for the calculation of sizes (megabase pairs). Sizes greater than 5.6 Mb were obtained by extending the calibration curve beyond the largest size standard and, therefore, may be subject to greater error. The number of chromosomes should be considered a minimum estimate because two or more comigrating chromosome-sized bands may have been considered a single chromosome. Because bands differed so greatly in size, not all could be resolved in a single gel. Therefore, size estimates were calculated from 16 gel runs with different pulse times. Values for individual bands are the mean determined from two or more gels.

[b] Not detected in any of the gels.

DISCUSSION

Grouping of *C. gloeosporioides* strains on the basis of morphological and physiological observations was first attempted by Burger (3) in 1921. However, morphologically based groups of *C. gloeosporioides* strains were inconsistently described in that study and subsequent studies (2,3,23). Fagan (6), Denham and Waller (4), and Sonoda and Pelosi (22) reported morphological variations associated with this fungus isolated from citrus. The type 1 strains in our study show similarities in morphology, growth, and sporodochia production to strains designated *cgp* by Fagan's description (6) and correspond to the slow-growing orange (SGO) colonies described by Agostini et al (1) and Sonoda and Pelosi (22). The more diverse type 2 strains show similarities to the *cgm* and *cgc* strains of Fagan and correspond to fast-

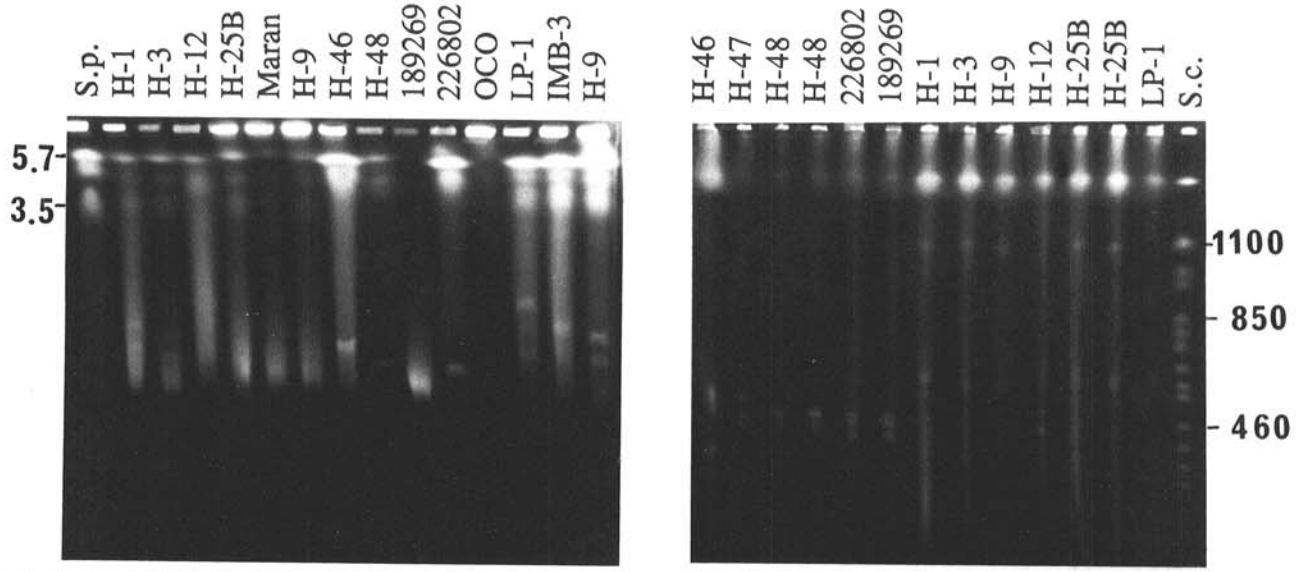

Fig. 4. Separation of chromosome-sized DNA by CHEF-gel electrophoresis (BioRad CHEF DRII, Richmond, CA). Left, a gel showing separation of DNAs smaller than 5.7 Mb. The gel was run for 168 h at 40 V with a ramped 50–100 min pulse time; 0.25× Tris-borate-EDTA buffer and 0.6% SeaKem FastLane agarose (FMC BioProducts, Rockland, ME) were used. Numbers to the left indicate the size of *Schizosaccharomyces pombe* (S.p.) chromosomes in megabase pairs. Right, a gel showing separation of minichromosomes. The gel was run for 24 h at 200 V with a ramped 60–90 s pulse time; 0.5× Tris-borate-EDTA buffer and 1% SeaKem LE agarose were used. Numbers to the right indicate the size of selected *Saccharomyces cerevisiae* (S.c.) chromosomes in kilobases.

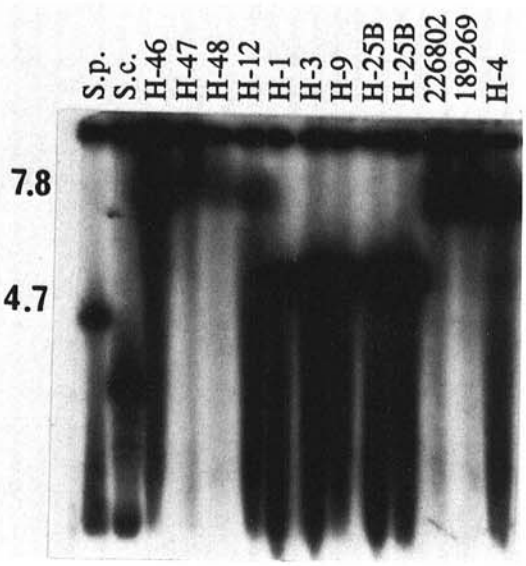

Fig. 5. Southern hybridization of labeled pMF2, a clone of the ribosomal DNA from *Neurospora crassa*, to a blot containing chromosome-sized DNAs from *Colletotrichum gloeosporioides* strains. The gel was run for 264 h at 40 V with a ramped pulse time of 10–120 min; 0.6% SeaKem LE agarose in 0.5× Tris-borate-EDTA buffer was used. Numbers to the left indicate the size of bands in megabases.

growing gray (FGG) colonies described by Agostini et al (1) and Sonoda and Pelosi (22). We recommend for consistancy that our type 1 and type 2 strains in the future be referred to as SGO and FGG strains, respectively.

Type 1 and type 2 strains were isolated from sweet orange as well as Tahiti lime. Both types were pathogenic to Tahiti lime flowers by inoculation tests, confirming previous results (22). No variation was found in the ability of type 1 and type 2 strains to cause disease on Tahiti lime flowers. However, only a single high inoculum concentration was used for pathogenicity studies. Pathogenicity tests with a lower inoculum density found that only the SGO strains could cause flower symptoms and button formation on intact plants (1). Others have also concluded (1,22) that SGO strains were the actual causal agents of postbloom fruit drop, because only these strains could be consistently isolated from diseased petals in the field, whereas the FGG strains were isolated primarily from stems and fruit.

Despite confusion associated with morphological definitions of SGO and FGG strains because of the dramatic changes brought about by sectoring, genetically there are distinct differences between these two types. The rDNA repeat unit appeared to be the same for all type 1 strains. Additionally, pCGRN1, a clone containing a 0.4-kb *Pst*I-*Kpn*I fragment from the NTS region of the ribosomal unit repeat of strain H-25B, hybridized only to type 1 strains. Therefore, type 1 strains, additionally, may be defined as those having a rDNA map similar to pCGR1 and hybridizing to pCGRN1. Morphologically variable type 2 strains contained a degree of diversity at the rDNA level. Ribosomal DNA polymorphisms were seen among type 2 strains when DNA was digested with either *Pst*I, *Sph*I, or *Sst*I. Attempts to find a region of the NTS of the rDNA specific for type 2 strains were unsuccessful. However, in a separate experiment, it was found that a cutinase gene from *C. gloeosporioides* hybridized only to the DNA of type 2 strains (H. D. Liyanage et al, *unpublished*). DNA polymorphisms detected by hybridization to three of four "housekeeping" genes from *N. crassa* also correspond directly to type 1 and type 2 strains. Only type 1 strains showed an additional level of polymorphism within the type as indicated by hybridization to pNC2.

Pulsed-field gel electrophoresis has allowed detection of chromosomal variation within species of phytopathogenic fungi (10,12,15). Similarly, variation was observed in chromosome-sized DNA in *C. gloeosporioides* from sweet orange and Tahiti lime. Two distinct electrophoretic patterns for types 1 and 2 can be described. These patterns show similarities to those described by Masel et al (15) for type A and type B strains of *C. gloeosporioides* from *Stylosanthus*. Similar to type A, type 1 strains described here have five large chromosomes, whereas type B was similar to type 2 strains in that it has three large chromosomes. However, minichromosomes vary in number and size within each type. Type 2 strains had a greater diversity in size and number of minichromosomes. The differences in the size of the larger chromosomes among types are best illustrated by the different sizes of molecules hybridizing to pMF2.

Another difference found in this study was tolerance to the

fungicide benomyl between type 1 and type 2 strains. Type 2 strains were completely inhibited by the levels of benomyl tested, whereas type 1 strains were tolerant although their growth rate was significantly reduced. The benomyl tolerance of type 1 strains may have practical consequences for the control of this disease. Current control measures include spraying benomyl to control postbloom fruit drop (8). If type 1 strains are confirmed as the primary causal disease agent, spraying in the field may only partially inhibit the virulent pathogen but completely eliminate the less virulent form. While slowing the epidemic in the short run, this practice may have the long-term effect of selecting for the most virulent form of the fungus. In response to this potential problem, further research should be carried out on the relative virulence and survival of type 1 and type 2 strains in the field. This study provides the tools to unambiguously identify the two types in the future.

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