Genetic and Pathogenic Analyses of *Colletotrichum gloeosporioides* Isolates from Strawberry and Noncultivated Hosts

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Accepted for publication 20 January 2004.

ABSTRACT

Xiao, C. L., Mackenzie, S. J., and Legard, D. E. 2004. Genetic and pathogenic analyses of *Colletotrichum gloeosporioides* from strawberry and noncultivated hosts. Phytopathology 94:446-453.

Colletotrichum crown rot of strawberry in Florida is caused primarily by *Colletotrichum gloeosporioides*. To determine potential inoculum sources, isolates of *Colletotrichum* spp. from strawberry and various noncultivated plants growing in the areas adjacent to strawberry fields were collected from different sites. Species-specific internal transcribed spacer primers for *C. gloeosporioides* and *C. acutatum* were used to identify isolates to species. Random amplified polymorphic DNA (RAPD) markers were used to determine genetic relationships among isolates recovered from noncultivated hosts and diseased strawberry plants. Selected isolates also were tested for pathogenicity on strawberry plants in the greenhouse. In all, 39 *C. gloeosporioides* and 3 *C. acutatum* isolates were recovered from diseased strawberry crowns, and 52 *C. gloeosporioides* and 1 *C. acutatum* isolate were recovered from noncultivated hosts. In crown inoculation tests, 18 of the 52 *C. gloeosporioides* isolates recovered from noncultivated hosts were pathogenic to strawberry. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from noncultivated hosts into two separate clusters. One cluster contained 50 of the 52 isolates and a second cluster contained 2 isolates that were homothallic in culture. Isolates from strawberry were interspersed within the cluster containing the 50 isolates that were recovered from noncultivated hosts. The results are not inconsistent with the hypothesis that *C. gloeosporioides* isolates obtained from strawberry and noncultivated hosts adjacent to strawberry fields are from the same population and that noncultivated hosts can serve as potential inoculum sources for Colletotrichum crown rot of strawberry.

Additional keyword: anthracnose.

Anthracnose of strawberry collectively refers to diseases of strawberry caused by *Colletotrichum* spp. (23). Three major species of *Colletotrichum*, *C. acutatum* (29), *C. fragariae* (5), and *C. gloeosporioides* (18), are associated with Colletotrichum diseases on strawberry. Symptoms of *Colletotrichum* infection include crown rot (15), fruit rot (16,19), root necrosis (10), irregular or black leaf spot (18), and spotting of petioles and runners (5,7). Necrosis of the crown tissue by *Colletotrichum* spp., which we will refer to as Colletotrichum crown rot (15,23), typically results in the wilting and death of plants. Although all three *Colletotrichum* spp. pathogenic to strawberry have been isolated from crowns of collapsed strawberry plants, a recent study has indicated that *C. gloeosporioides* is the primary cause of Colletotrichum crown rot in Florida (36).

C. gloeosporioides isolated from strawberry displays considerable diversity in random amplified polymorphic DNA (RAPD) banding patterns (11,36) and there is little evidence for linkage disequilibrium among loci (36), suggesting that *C. gloeosporioides* from strawberry may derive from a relatively large recombining population. Under west-central Florida climatic conditions, *C. gloeosporioides* does not appear to have the capacity to survive on strawberry plant debris in soil during the hot summer (20,37). In Florida, strawberry plants are grown as an annual crop in a hill plasticulture system. Soil is fumigated with a mixture of methyl bromide and chloropicrin before bare-root transplants are set in the fall (3). Thus, the carry-over of inoculum between strawberry production seasons is unlikely. Inoculum for the initiation of Colletotrichum crown rot epidemics in Florida must come from sources outside of production fields. Infected transplants are one potential source and there are some apparent correlations between crown rot epidemics in Florida production fields with the nursery providing transplants (22). Another potential source of inoculum is alternate host species growing in the vicinity of strawberry fields. In Florida, strawberry fields generally are surrounded by noncultivated trees, shrubs, and herbaceous plants. Our observations indicated that, in some fields, strawberry plants with Colletotrichum crown rot symptoms were aggregated near the edge of the field adjacent to noncultivated plants. Therefore, we hypothesized that noncultivated plants can provide sources of inoculum for strawberry crown rot.

C. gloeosporioides has been found on a broad range of hosts (24). The cross-infection potential for strains of *C. gloeosporioides* isolated from different cultivated plants has been reported (1,12). *C. gloeosporioides* from noncultivated host species also has been shown to be pathogenic on black locust (40). *C. fragariae*, although infrequently isolated from collapsed crowns in Florida, has been both reported on a noncultivated host (17) and shown to produce disease symptoms on an alternate host (38). Based upon ribosomal DNA sequence analysis, *C. gloeosporioides* isolates cluster into two distinct groups (21,28). However, analysis of more rapidly evolving regions of the genome provided by RAPD, restriction fragment length polymorphism, and isozyme analysis have identified genetically distinct subgroups within groups identified using ribosomal DNA sequence information (14,32). Isolates of *C. gloeosporioides* recovered from plant material of seven tropical fruit species tend to be more pathogenic on leaves of the plant species from which they are

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Publication no. P-2004-0317-01R

isolated. Molecular data, however, did not indicate that isolates from different hosts are derived from genetically distinct populations (1).

The goal of this study was to determine whether noncultivated hosts adjacent to strawberry fields serve as potential sources of *C. gloeosporioides* inoculum for infection of strawberry crown. The specific objectives were to (i) collect and identify isolates of *Colletotrichum* spp. from noncultivated plant species adjacent to strawberry fields; (ii) evaluate *C. gloeosporioides* isolates from noncultivated hosts for their pathogenicity on strawberry; and (iii) determine the genetic relatedness of *C. gloeosporioides* isolates from noncultivated plant species to isolates recovered from diseased strawberry plants.

MATERIALS AND METHODS

Fungal isolate collections. From 1995 to 1998, isolates of *Colletotrichum* spp. with morphology consistent with descriptions for *C. gloeosporioides* (13,30) were collected from various noncultivated hosts growing adjacent to strawberry production fields and from diseased strawberry plants in west-central Florida (Table 1). Isolations were made from diseased tissues, such as foliar and fruit lesions of noncultivated host plants and diseased crowns of strawberry plants. Diseased plant tissues were surface disinfested for 5 min with 0.525% sodium hypochlorite and plated onto either potato dextrose agar (PDA) amended with neomycin sulfate (20 mg/liter), chloramphenicol (6.5 mg/liter), tetracycline hy-

TABLE 1. Collection sites and host species from which *Colletotrichum* spp. were recovered

Species, collection site[a]	Host (species code)[b]	Common name	Number of isolates
Colletotrichum gloeosporioides			
NC1/96	*Quercus* spp. (sp12)	Oak	2
	Smilax rotundifolia (sp14)	Smilax	1
	S. rotundifolia, fruit (sp14B)	Smilax berry	3
	Vitis rotundifolia (sp16)	Wild grape	8
NC1/97	*Callicarpa americana*, fruit (sp3)	Beauty berry	1
	Dioscorea bulbifera (sp4)	Air potato	4
	Ipomoea spp. (sp5)	Morning glory	1
	Liquidambar styraciflua (sp6)	Sweet gum	2
	Myrica cerifera L. (sp10)	Wax myrtle	2
	Parthenocissus quinquefolia (sp11)	Virginia creeper	3
	Quercus spp. (sp12)	Oak	1
	S. rotundifolia, fruit (sp14B)	Smilax berry	1
	Urena lobata (sp15)	Caesar weed	1
NC2	*Momordica charantia* L. (sp9)	Balsamapple	2
	Richardia brasiliensi (sp13)	Brazilian pusley	1
NC3	*Magnolia virginiana* L. (sp7)	Magnolia, sweet bay	2
	Myrica cerifera L. (sp10)	Wax myrtle	1
	Quercus spp. (sp12)	Oak	1
	S. rotundifolia (sp14)	Smilax	2
	S. rotundifolia, fruit (sp14B)	Smilax berry	1
	V. rotundifolia (sp16)	Wild grape	1
	Unknown species (sp17)	…	2
NC4	*V. rotundifolia* (sp16)	Wild grape	1
NC5	*Asclepias* spp. (sp1)	Milkweed	2
	Bidens bipinnata (sp2)	Bidens	1
	C. Americana, fruit (sp3)	Beauty berry	1
	Melia acustralis Sweet (sp8)	China berry	2
	S. rotundifolia (sp14)	Smilax	2
S1	*Fragariae* × *ananassa*	Strawberry crown	1
S2/95	*Fragariae* × *ananassa*	Strawberry crown	11
S2/97	*Fragariae* × *ananassa*	Strawberry crown	9
S3	*Fragariae* × *ananassa*	Strawberry crown	1
S4	*Fragariae* × *ananassa*	Strawberry crown	1
S5	*Fragariae* × *ananassa*	Strawberry crown	1
S6	*Fragariae* × *ananassa*	Strawberry crown	1
S7	*Fragariae* × *ananassa*	Strawberry crown	1
S8	*Fragariae* × *ananassa*	Strawberry crown	1
S9	*Fragariae* × *ananassa*	Strawberry crown	1
S10	*Fragariae* × *ananassa*	Strawberry crown	2
S11	*Fragariae* × *ananassa*	Strawberry crown	2
S12	*Fragariae* × *ananassa*	Strawberry crown	1
S13	*Fragariae* × *ananassa*	Strawberry crown	1
S14	*Fragariae* × *ananassa*	Strawberry crown	3
S15	*Fragariae* × *ananassa*	Strawberry crown	2
Lake Alfred, FL	*Citrus* spp.	Citrus	4
Homestead, FL	*Mangifera indica*	Mango	3
Colletotrichum acutatum			
NC5	*Callicarpa americana*, fruit (sp3)	Beauty berry	1
S9	*Fragariae* × *ananassa*	Strawberry crown	1
S16	*Fragariae* × *ananassa*	Strawberry crown	1
S17	*Fragariae* × *ananassa*	Strawberry crown	1
Dover, FL	*Fragariae* × *ananassa*	Strawberry Fruit	1
Lake Alfred, FL	*Citrus* spp.	Citrus	1

[a] Sites beginning with NC are areas with noncultivated plants in close proximity to strawberry fields, whereas sites beginning with S are strawberry fields. Numbers following / represent year sample was collected for sites collected more than once. All sites except those in which mango and citrus isolates were obtained are in west-central Florida.

[b] Species codes given in parentheses are combined with site numbers to identify the sample location and host for isolates displayed in Figure 1.

drochloride (25 mg/liter), and erthromycin (7.5 mg/liter) or a semiselective Colletotrichum medium (16 g of Difco potato dextrose broth, 14 g of Difco agar, 250 mg of ampicillin, 150 mg of streptomycin sulfate, 5 mg of iprodione, 100 µl of Tergitol, and 1,000 ml of deionized water). Isolation plates were incubated under continuous fluorescent light at room temperature (≈22°C) or in an incubator at 24°C for 3 to 7 days. Cultures of *Colletotrichum* spp. were single spored and stored in 20% glycerol at –85°C.

In all, 53 *Colletotrichum* spp. isolates were obtained between 1996 and 1997 from noncultivated plants growing on land adjacent to strawberry fields at five locations in west-central Florida (Table 1). Among these sites there were 16 known and two unknown noncultivated hosts from which *Colletotrichum* spp. were isolated. One host species, *Smilax rotundifoli*, had *Colletotrichum* spp. growing on both foliar tissue and fruit. Isolates coming from strawberry or noncultivated hosts were identified by the letter "S" for strawberry or "NC" for noncultivated at the beginning of the isolate code (Table 1). Approximately half of the isolates included in the noncultivated population were collected from site NC1. This site was sampled once in 1996 and again in 1997. The population of *Colletotrichum* spp. obtained from infected strawberry crowns consisted of 42 isolates collected from 17 sites between 1995 and 1998 (Table 1). Site S2 was sampled in both 1995 and 1997. Approximately half of the strawberry crown rot isolates evaluated in the study were from this site. If a site was collected more than once, the site number is followed by a slash and the year of collection. For isolates from noncultivated hosts, the host species are indicated by the letters "sp" followed by a number referring to the particular host species (Table 1 lists species corresponding to the code number). Three *C. gloeosporioides* isolates from mango, four *C. gloeosporioides* isolates from citrus, and one *C. acutatum* isolate from citrus were used as representative outgroup populations for genetic comparisons. These isolates are believed to be from or have previously been shown to be distinct from populations on strawberry (14,37). One *C. acutatum* isolate from strawberry fruit also was included as an outgroup in the genetic analysis and used to confirm the identity of any *C. acutatum* isolates that may have been isolated from crown tissue.

Extraction of fungal DNA. Total fungal DNA was extracted from mycelia obtained from cultures grown in 100 ml of Emerson media (yeast extract at 4 g/liter, soluble starch at 15 g/liter, K_2HPO_4 at 1 g/liter, and $MgSO_4$ at 0.5 g/liter) for 2 to 4 days at room temperature (≈22°C). Mycelium was harvested from the liquid cultures by vacuum filtration through Whatman no. 3 filter paper and transferred into a 15-ml tube. Mycelia then were dried overnight in a centrifugal evaporator and subsequently ground into a fine powder using a sterile glass rod. Dried powder of each isolate (60 mg) was suspended in 750 µl of DNA extraction buffer consisting of 700 mM NaCl, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 1% cetyltrimethylammonium bromide, and 1% β-mercaptoethanol for 2 h with periodic shaking at 65°C. Particulate material was pelleted by centrifugation at 10,000 rpm for 10 min, and the supernatant removed and extracted once with chloroform/isoamyl alcohol (24:1). Two volumes of 100% ethanol were added to the aqueous extract and the mixture was incubated at room temperature for 10 min. Nucleic acids were pelleted from the ethanol solution by centrifugation at 10,000 rpm for 10 min. The pellet was washed with 100% ethanol and suspended in 400 µl of 1× Tris-EDTA (TE) buffer containing 10 RNase at µg/ml for 1 h at 37°C. Ribonuclease was removed from the nucleic acid solution by extraction with 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1). To the aqueous extract, 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added to precipitate the DNA. This solution was incubated at –20°C for 1 h and the DNA was pelleted at 10,000 rpm for 10 min. The DNA pellet was washed once with 1 ml of 80% ethanol, dried, suspended in 50 to 500 1× TE buffer and stored at –20°C.

Species identification. A species-specific internal transcribed spacer region 1 (ITS1) primer and the conserved universal primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) encoded in the 28S ribosomal subunit were used in pairs to identify isolates to species (39). The ITS1 primers used were either the *C. gloeosporioides*-specific ITS primer 5′-GACCCTCCCGGCCTCCCGCC-3′ or the *C. acutatum*-specific ITS primer 5′-GGGGAAGCCTCTCGCGG-3′ (31,33). The *C. acutatum*-specific primer was included as a negative control for *C. gloeosporioides* identifications and to differentiate any *C. acutatum* isolates that may have been included in the sample. Isolates where assigned to the species group for which a positive amplification with a specific ITS1 primer was obtained. Amplifications were carried out under mineral oil in a 20-µl volume containing 1× reaction buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin), 200 µM dNTP, 1 unit of *Taq* polymerase, and 10 ρmol of each primer/reaction. The reaction buffer for the *C. acutatum*-specific primer also contained 5% glycerol. Temperature cycling parameters for the *C. gloeosporioides*-specific/ITS4 pair consisted of a denaturing step for 5 min at 94°C, followed by 26 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. Temperature cycling parameters for the *C. acutatum*-specific/ITS4 pair consisted of a denaturing step for 5 min at 94°C followed by 32 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. The amplified products were separated by electrophoresis through a 2% agarose gel containing 1× Tris-acetate-EDTA (TAE) buffer. Gels were photographed on a UV transilluminator after ethidium bromide staining.

Pathogenicity tests. Pathogenicity of the 53 *Colletotrichum* isolates recovered from noncultivated plants was evaluated on the susceptible strawberry cv. Camarosa in the greenhouse. Conidial suspensions were made from 7- to 10-day-old PDA cultures grown at 24°C under continuous fluorescent light and adjusted to 1×10^6 conidia/ml in sterile deionized water. Runners of cv. Camarosa were rooted in pots filled with a potting mix and maintained in the greenhouse. Plants at four to six leaves were used for inoculation tests. To inoculate plants, a 25G1 syringe needle was used to wound and inject approximately 0.1 ml of conidial suspensions at the leaf axils in the crown area. For controls, plants were wounded and treated with sterile water. Inoculated plants (six plants [one in each pot] for each isolate) were placed in a moisture chamber at 24 to 30°C for 48 h, and then maintained in a greenhouse at 24 to 30°C for 4 weeks. Strawberry plants were evaluated weekly for the development of Colletotrichum crown rot symptoms (i.e., wilting and collapse of plant). An isolate was considered to be pathogenic to strawberry if at least four of six inoculated plants collapsed within the 4 weeks after inoculation. Each isolate was tested at least twice in separate inoculation experiments. In addition, 10, 3, and 4 isolates of *C. gloeosporioides* recovered from diseased strawberry plants, mango, and citrus, respectively, also were tested for pathogenicity on strawberry plants in the same manner as described previously.

RAPD markers. Initially, 34 primers were evaluated for their ability to generate RAPD. Five primers, including two tetra-nucleotide repeat primers, $(ACTG)_4$ and $(GACA)_4$, the trinucleotide repeat primer $(TCC)_5$, and two short oligonucleotides, 5′-GTGAGGCGTC-3′ (OPC-2) and 5′-GATGACCGCC-3′ (OPC-5) (Operon Technologies, Alameda, CA), were selected for the population studies based on their ability to consistently amplify bands that demonstrated a high level of fluorescence under UV light. DNA amplifications were carried out under mineral oil in a 20-µl volume containing 1× reaction buffer (50 mM Tris [pH 8.3], bovine serum albumin at 0.25 mg/ml, 2 mM $MgCl_2$, 0.5% Ficoll, and 1 mM Tartrazine), 200 µM dNTP, 1 unit of *Taq* polymerase, and 20 ρmol of primer/reaction (primers $(ACTG)_4$, $(GACA)_4$, and $(TCC)_5$) or 8 ρmol of primer/reaction (primers OPC-2 and

OPC-5). Cycling parameters for the polymerase chain reaction (PCR) consisted of a 5-min denaturing step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 48°C, and 2 min at 72°C for primers $(ACTG)_4$ and $(GACA)_4$; 34 cycles of 1 min at 95°C, 1 min at 46°C, and 1.5 min at 72°C for primer $(TCC)_5$, or 38 cycles of 1 min at 95°C, 1 min at 35°C, and 2 min at 72°C for primers OPC-2 and OPC-5. The amplified products were separated by electrophoresis through a 1.5% high resolution blend agarose (3:1) gel containing 1× TAE buffer. Gels were photographed on a UV transilluminator after ethidium bromide staining.

Statistical analyses. The SPSS 8.0 statistics package (SPSS Inc., Chicago) was used to perform Fisher's exact tests to test for the association between the pathogenicity of the isolates and the site or noncultivated host species from which the isolates were recovered. The genetic relationship of isolates to one another was summarized in a dendrogram constructed from dice similarity coefficients using the unweighted pair group method with arith-

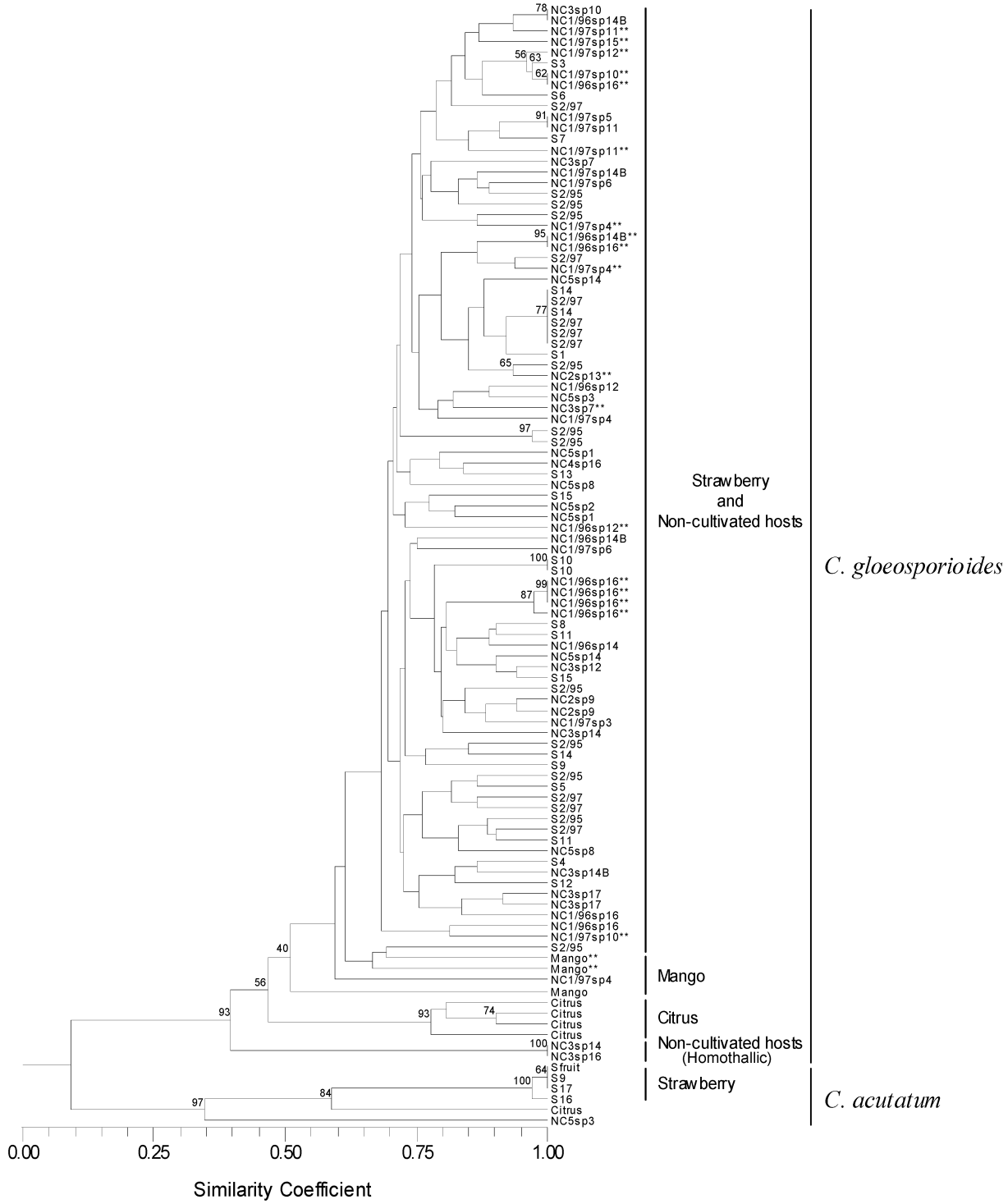

Fig. 1. Unweighted pair group method with arithmetic averages dendrogram showing similarity (Dice) between *Colletotrichum gloeosporioides* and *C. acutatum* isolates from noncultivated plants, strawberry crowns, citrus, and mango. Numbers at nodes are the percentage of occurrence of the cluster to the right of the branch in 1,000 bootstrapped samples. All bootstrapped values are reported for clusters that are less than 0.50 similar to other isolates or clusters. For all other clusters, only bootstrapped values greater than 50 are reported. Nonstrawberry *C. gloeosporioides* isolates with asterisks (**) indicate they were pathogenic on strawberry in greenhouse inoculation tests. Isolates are identified in Table 1.

metic averages clustering algorithm (NTSYS, PC version 2.0; Exeter Software, Setauket, NY). Statistical support for branches was based on 1,000 bootstrapped samples using Winboot (9,26). The probability of obtaining identical genotypes among strains in the sample population, assuming a random distribution of alleles, was determined using a custom-written QBASIC program which shuffles alleles among strains at each loci to mimic recombination and subsequently determines the frequency of the most common genotype observed in the shuffled data set (34,35). Probabilities of obtaining clone frequencies were based on analysis of 10,000 randomized data sets. Population differentiation was examined using an exact test for population differentiation at each locus and Fisher's combined probability test to obtain a probability estimate over all loci (27). Similarity between the strawberry population and noncultivated host populations was quantified using Nei's genetic identity (25). Tests for population differentiation and calculations of Nei's genetic identity were performed using the software program TFPGA (Utah State University, Logan).

RESULTS

Identification of isolates. Of the 53 isolates recovered from noncultivated hosts, 52 produced a characteristic PCR product when the *C. gloeosporioides*-specific primer was used; therefore, they were identified as *C. gloeosporioides*. One isolate produced an amplification product when the *C. acutatum* species-specific primer was used (Table 1). The *C. acutatum* isolate obtained from the noncultivated host came from the fruit of *Callicarpa americana.* It was genetically distinct from any *Colletotrichum acutatum* isolates from strawberry (Fig. 1) and did not produce either crown rot (Table 2) or fruit lesions (S. J. MacKenzie, *unpublished data*) when inoculated on strawberry. Of the 42 isolates recovered from diseased strawberry crowns, 39 were identified as *C. gloeosporioides* and three as *C. acutatum*. The species identities of *Colletotrichum* isolates from mango and citrus also were confirmed with *C. gloeosporioides* or *C. acutatum* species-specific primers (Table 1).

Pathogenicity tests. Of the 52 *C. gloeosporioides* isolates from noncultivated hosts tested, 18 produced typical symptoms of Colletotrichum crown rot (i.e., wilting and collapse of plants) on inoculated plants. Therefore, these isolates were considered to be pathogenic to strawberry crowns. The 18 pathogenic isolates were recovered from nine different noncultivated host species from three separate sites (Tables 2 and 3). There was a significant association between the pathogenicity of the isolates and the site from which the isolates were collected (Table 2, Fisher's exact test $P = 0.02$). Of the 18 pathogenic isolates, 16 came from a single site (NC1). Half of the pathogenic isolates from this site were collected in 1996 and the other half in 1997. There was no significant association between the pathogenicity of isolates and the noncultivated host species from which the isolates were recovered (Table 3; $P = 0.23$).

Of 10 isolates that were recovered from diseased strawberry plants, 9 produced typical crown rot symptoms on inoculated strawberry plants. Two of the three isolates from mango and none of the four citrus isolates were pathogenic to strawberry.

RAPD analyses. RAPD amplifications using five primers yielded 60 scorable bands from all isolates examined. Forty-one scorable bands were amplified from *C. gloeosporioides* isolates recovered from strawberry and noncultivated hosts. When isolates from citrus and mango were included, 44 scorable bands were obtained. Thirty scorable bands were amplified from *C. acutatum* isolates. Isolates identified as *C. gloeosporioides* grouped into three clusters with between-cluster similarities less than 0.50 (Fig. 1). All of the *C. gloeosporioides* isolates from citrus had a level of similarity greater than 0.75 and formed a cluster in 93% of bootstrapped dendrograms. A second cluster consisted of two clonal isolates from different noncultivated host species at site NC3 and occurred in 100% of bootstrapped trees. The two isolates in this cluster were homothallic (S. J. MacKenzie, *unpublished data*). Self-fertility was not observed for any other isolates, although it is possible that low numbers of fertile perithecia may have gone unobserved.

A third cluster contained *C. gloeosporioides* from noncultivated hosts, strawberry, and mango. This cluster occurred in only 41% of bootstrapped trees, giving weak support for a monophyletic origin of this cluster. A large amount of the genetic variation within this cluster can be attributed to the mango isolates. The three mango isolates were distinct from most strawberry and noncultivated host isolates based on Dice similarity coefficients, but isolates from this host did not compromise a group of closely related organisms. When mango isolates are excluded from the analysis, the cluster containing strawberry and nonhomothallic, noncultivated host isolates occurred in 59% of bootstrapped samples.

TABLE 2. Isolates of *Colletotrichum* spp. recovered from noncultivated hosts summarized according to sampling site and pathogenicity on strawberry plants

Species, collection site[a]	Number of isolates		
	Pathogenic	Nonpathogenic	Total
Colletotrichum gloeosporioides			
NC1/96	8	6	14
NC1/97	8	8	16
NC2	1	2	3
NC3	1	9	10
NC4	0	1	1
NC5	0	8	8
All sites combined	18	34	52
C. acutatum			
NC5	0	1	1

[a] Sites beginning with NC are areas with noncultivated plants in close proximity to strawberry fields in west-central Florida. There was a significant association ($P = 0.02$) between the pathogenicity of the *C. gloeosporioides* isolates and the site from which the isolates were collected, based on a Fisher's exact test ($P = 0.05$).

TABLE 3. Isolates of *Colletotrichum* spp. recovered from noncultivated hosts summarized according to host species and pathogenicity on strawberry plants

Fungal species, host species[b]	Number of isolates[a]		
	Path	Nonpath	Total
Colletotrichum gloeosporioides			
Asclepias spp. (sp1)	0	2	2
Bidens bipinnata (sp2)	0	1	1
Callicarpa americana, fruit (sp3)	0	2	2
Dioscorea bulbifera (sp4)	2	2	4
Ipomoea spp. (sp5)	0	1	1
Liquidambar styraciflua (sp6)	0	2	2
Magnolia virginiana L. (sp7)	1	1	2
Melia acustralis Sweet (sp8)	0	2	2
Momordica charantia L. (sp9)	0	2	2
Myrica cerifera L. (sp10)	2	1	3
Parthenocissus quinquefolia (sp11)	2	1	3
Quercus spp. (sp12)	2	2	4
Richardia brasiliensi (sp13)	1	0	1
Smilax rotundifolia (sp14)	0	5	5
S. rotundifolia, fruit (sp14B)	1	4	5
Urena lobata (sp15)	1	0	1
Vitis rotundifolia (sp16)	6	4	10
Unknown species (sp17)	0	2	2
All host species combined	18	34	52
Colletotrichum acutatum			
Callicarpa americana, fruit (sp3)	0	1	1

[a] Path = pathogenic, Nonpath = nonpathogenic.

[b] There was no significant association ($P = 0.23$) between the pathogenicity of the *C. gloeosporioides* isolates and the host species from which the isolates were collected, based on a Fisher's exact test ($P = 0.05$). Species codes given in parentheses are combined with site numbers to identify the sample location and host for isolates displayed in Figure 1.

Bootstrapping did not provide support for strawberry isolates forming an evolutionary lineage distinct from noncultivated isolates, because strawberry isolates were interspersed among these isolates in the dendrogram. However, clustering did occur among subsets of strawberry and noncultivated host isolates. Several of these clusters were supported by relatively high bootstrap values (Fig. 1). Seven clusters of nonhomothallic isolates contained two or more clonal individuals based on RAPD profiles. Of these seven clonal lineages, two consisted of isolates from strawberry and five contained isolates from noncultivated hosts. Two of the clonal groups contained three or more isolates. One clonal genotype on strawberry was found at two sites (four isolates at site S2/97; two isolates at S14). Of the five clonal genotypes found on noncultivated hosts, four contained only isolates from the same field and in only one of these fields were all of the isolates from the same host species. Pathogenicity phenotypes were the same among all individuals possessing identical genotypes. Based on probabilities obtained by repeated shuffling of the data set, two or more individuals having the same genotype occurred in 4.39% of the randomized data sets and three or more individuals with the same genotype did not occur in any of the 10,000 randomized data sets examined.

Allele frequencies were significantly ($P < 0.05$) different between strawberry and nonhomothallic, noncultivated host isolates at only 4 of 36 (11%) polymorphic loci based on exact tests with α, the level of type I error, equal to 0.05 (Table 4). A test for population differentiation combining all polymorphic loci was not significant ($P = 0.29$). The 1.6-kb band amplified with primer OPC-5, one of four loci displaying allele frequency differences between these isolates, also displayed frequency differences between pathogenic and nonpathogenic isolates when only isolates from noncultivated hosts were compared (frequency = 0.50 among pathogenic isolates and 0.06 among nonpathogenic isolates). There were no significant differences in allele frequencies between pathogenic and nonpathogenic isolates from noncultivated hosts at the three other loci. The value of Nei's genetic identity comparing the strawberry population with the nonhomothallic, noncultivated host population was 0.98, indicating that they were highly similar. The values of Nei's genetic identity comparing the homothallic population with the strawberry population and the nonhomothallic, noncultivated host population were 0.68 and 0.65, respectively.

DISCUSSION

In this study, we found that approximately one-third of the *C. gloeosporioides* isolates recovered from noncultivated hosts grown in the areas adjacent to strawberry fields were pathogenic to strawberry in greenhouse tests. Phylogenetic analysis of RAPD data and tests for genetic differentiation between *C. gloeosporioides* from noncultivated hosts and those from diseased strawberry crowns suggest that they were from a single population. These results indicate that noncultivated hosts growing adjacent to strawberry fields may serve as a source of inoculum for epidemics of strawberry crown rot caused by *C. gloeosporioides.*

Isolates of *C. gloeosporioides* from a wide range of temperate, subtropical, and tropical fruits have shown cross-infection potential (1,12). However, pathogenicity tests in artificial inoculation experiments are not conclusive evidence to support that cross-infection occurs under natural conditions. For this reason, in the present study, in addition to pathogenicity tests, the genetic relationship between isolates from strawberry and different noncultivated hosts was determined. Based upon bootstrap analysis of a dendrogram constructed using RAPD markers, isolates of *C. gloeosporioides* from noncultivated hosts fell into two genetically distinct populations. The largest population consisted of 50 isolates and the smaller population consisted of two genotypically identical isolates. The populations from noncultivated hosts also could be distinguished from one another based upon the presence of homothallism in isolates from the smaller population. Homothallic isolates of *C. gloeosporioides* from apple also have been shown to be genetically divergent from interbreeding heterothallic isolates (6).

Isolates of *C. gloeosporioides* from diseased strawberry crowns had a high level of diversity and were not genetically distinct

TABLE 4. Frequencies of random amplified polymorphic DNA bands for *Colletotrichum gloeosporioides* isolates from strawberry and noncultivated hosts

	Host		
		Noncultivated isolates	
Primer, length (kb)	Strawberry ($n = 39$)[a]	Nonhomothallic ($n = 50$)	Homothallic ($n = 2$)[b]
$(ACTG)_4$			
2.145	0.49	0.52	0
1.9	0.97	1	0
1.55	0	0	1
1.5	1	1	0
1.12	0.85	0.84	0
0.6	0.92	0.84	1
0.4	0.33	0.38	1
$(GACA)_4$			
1.5	0.05	0.02	0
1.35	0.95	0.96	0
1.3	0	0	1
1.2	0.97	0.96	0
1.15	0.03	0.04	0
0.95	0.69	0.74	0
0.9	0.46	0.38	1
0.8	0.13	0.3	0
0.75	0	0.02	0
0.5	1	0.96	0
$(TCC)_5$			
2	0.03	0.1	0
1.9	0.62	0.62	1
1.55	0.23	0.28	0
1.15	1	0.98	1
0.9	0.03	0.02	0
0.75	0.13	0.22	0
OPC-2			
2.2	0.08	0.06	0
1.9	0.62	0.64	0
1.7	0.03	0	0
1.2	0.08	0.02	0
1.1	1	1	1
0.5	0.03	0	0
OPC-5			
2.7	0.18	0.34	0
2.6	0	0	1
2.5	0.21	0.44[c]	0
2.15	0.59	0.82[c]	1
2	0.21	0.22	0
1.8	0.18	0.16	1
1.75	0.08	0.08	0
1.65	0.1	0.30[c]	0
1.6	0.74	0.22[cd]	1
1.55	0.33	0.32	0
1.4	0.03	0.06	0
1.2	0.38	0.22	0

[a] Exact test for population differentiation between strawberry and noncultivated host isolates (nonhomothallic) over all loci was not significant ($P = 0.29$). The value of Nei's genetic identity comparing the strawberry population with the noncultivated host (nonhomothallic) population was 0.98, indicating that they were highly similar.

[b] Values of Nei's genetic identity comparing the noncultivated host, homothallic population with the strawberry population and the noncultivated host, nonhomothallic population were 0.68 and 0.65, respectively.

[c] Exact test for population differentiation between strawberry and noncultivated host isolates (nonhomothallic) was significant at specified loci ($P < 0.05$).

[d] Exact test for population differentiation between pathogenic and nonpathogenic, noncultivated host isolates (nonhomothallic) was significant at specified loci ($P < 0.05$).

from noncultivated host isolates that were not homothallic in culture when all polymorphic loci were included in the analysis. Tests for differences in allele frequencies at a single locus did reveal a 1.6-kb OPC-5 amplification product that occurred at a higher frequency in the strawberry population relative to the noncultivated host population and also occurred at a higher frequency among pathogenic isolates from the noncultivated host population relative to nonpathogenic isolates. Allele frequencies from this locus did not provide strong evidence for population subdivision, given that the frequency of the vast majority of allelic markers, assumed to be neutral, were not different between populations. The positive correlation between the 1.6-kb OPC-5 product and pathogenicity on strawberry does, however, suggest that it may be linked to a genetic factor conferring pathogenicity on strawberry. In this study, we also observed that some isolates from noncultivated hosts were not pathogenic to strawberry, though strawberry isolates were interspersed among these isolates in the dendrogram (Fig. 1). It is very possible for there to be populations that are highly similar for neutral markers, but very different for pathogenicity.

Although isolates with the same genotype occurred on either strawberry or noncultivated hosts, no identical genotypes were found on both strawberry and noncultivated hosts. Assuming a randomly mating population, the occurrence of even two isolates with identical genotypes would be a relatively rare event given the sample size and polymorphic loci examined in this study. In total, there were seven genotypes detected more than once in isolates from the nonhomothallic strawberry and noncultivated host population. This overrepresentation of specific genotypes is consistent with the important role of clonal reproduction in this species (8). Genetic bottlenecks created by recent colonizing events in spatially subdivided populations also can result in overrepresentation of genotypes at specific sites. However, this is unlikely because the sites from which clonal genotypes were collected contained a substantial amount of genetic diversity. Genetically isolated pathogen subpopulations also can arise relatively rapidly from interbreeding fungal populations due to asexual reproduction and may serve as a mechanism to preserve particularly virulent gene combinations on specific hosts (4). The data provided from this study does not support the hypothesis that this has occurred with *C. gloeosporioides* from either strawberry or noncultivated hosts because clonal isolates tended to occur at specific sites and not on specific hosts. There was, however, one genotype that was isolated from six different strawberry crowns at two separate sites (S2/97 and S14). This was the most common genotype observed. This suggests that selection may be preserving some combinations of genes for pathogenicity on strawberry. Alternatively, the occurrence of this genotype may result from selection for a clone at a nursery supplying transplants to farms in the area investigated.

Although a defined group of species harboring pathogenic isolates could not be identified among the nonpathogenic hosts sampled, there was a strong correlation between sampling site and pathogenicity on strawberry (Table 2). Because not all of the host species were present at each sample site, there may be some bias in the tests for association. This bias might make it difficult to discern whether or not isolates from particular hosts or particular sites differed in pathogenicity. However, 11 of 19 isolates from site NC1 that had hosts identical to those found at sites NC2, NC3, NC4, and NC5 were pathogenic to strawberry, whereas only 2 of the 22 isolates from these four other sites were pathogenic on strawberry. This result indicates that the analyses were correct, in that pathogenicity correlated primarily with the site from which isolates were recovered and not the host species from which they were isolated. Variation in levels of pathogenicity among noncultivated host sampling sites may result from different levels of migration from strawberry fields, where selection for virulence on strawberry would likely occur.

Three *C. gloeosporioides* isolates from mango and four from citrus were included in the dendrogram (Fig. 1). The mango isolates examined in this study did not appear to form a subgroup genetically distinct from *C. gloeosporioides* isolated from strawberry or noncultivated hosts. These findings were not expected because a study of *C. gloeosporioides* isolates from mangos collected from around the world found the isolates to be relatively homogeneous and genetically distinct from those recovered from other fruit species (14). In the present study, however, only one mango isolate from Florida was included. The Florida isolate had a slightly smaller rDNA size compared with isolates from other sites. Repeated sampling of *C. gloeosporioides* from mango in Sri Lanka also revealed a greater amount of diversity in rDNA and mtDNA restriction fragment length polymorphisms than was previously thought to exist in this population (2). Also of interest is that the two mango isolates pathogenic on strawberry in greenhouse inoculation tests were more closely related to strawberry isolates than they were to the third mango isolate that was not pathogenic on strawberry. The citrus isolates used in this study were demonstrated previously to be genetically distinct from strawberry isolates (36). Whether or not this genetic divergence is due to geographic isolation or sexual incompatibility has not been examined, but test crosses of citrus isolates to apple reference strains have been unsuccessful (6). A *C. acutatum* isolate also was obtained from a noncultivated host in this study. This isolate was not pathogenic on strawberry crowns and was genetically distinct from strawberry crown isolates, indicating that this strain of *C. acutatum* is not responsible for Colletotrichum crown rot of strawberry.

ACKNOWLEDGMENTS

We thank J. Sumler for assistance in identifying noncultivated plant species.

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