Etiology and Population Genetics of *Colletotrichum* spp. Causing Crown and Fruit Rot of Strawberry

A. R. Ureña-Padilla, S. J. MacKenzie, B. W. Bowen, and D. E. Legard

First, second, and fourth authors: University of Florida, Gulf Coast Research and Education Center, Dover 33527; and third author: Department of Fisheries and Aquatic Sciences, University of Florida, Gainesville 32611.
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ABSTRACT

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Isolates of *Colletotrichum* spp. from diseased strawberry fruit and crowns were evaluated to determine their genetic diversity and the etiology of the diseases. Isolates were identified to species using polymerase chain reaction primers for a ribosomal internal transcribed spacer region and their pathogenicity was evaluated in bioassays. Isolates were scored for variation at 40 putative genetic loci with random amplified polymorphic DNA and microsatellite markers. Only *C. acutatum* was recovered from diseased fruit. Nearly all isolates from crowns were *C. gloeosporioides*. In crown bioassays, only isolates of *C. gloeosporioides* from strawberry caused collapse and death of plants. A dendrogram generated from the genetic analysis identified several primary lineages. One lineage included isolates of *C. acutatum* from fruit and was characterized by low diversity. Another lineage included isolates of *C. gloeosporioides* from crowns and was highly polymorphic. The isolates from strawberry formed distinctive clusters separate from citrus isolates. Evaluation of linkage disequilibrium among polymorphic loci in isolates of *C. gloeosporioides* from crowns revealed a low level of disequilibrium as would be expected in sexually recombining populations. These results suggest that epidemics of crown rot are caused by *Glomerella cingulata* (anamorph *C. gloeosporioides*) and that epidemics of fruit rot are caused by *C. acutatum*.

Additional keywords: arbitrarily primed polymerase chain reaction, *Fragaria* × *ananassa*, random amplified polymorphic DNA.

Anthracnose fruit rot and Colletotrichum crown rot are important diseases of strawberry worldwide (9,13,23,24). Symptoms of anthracnose fruit rot include sunken necrotic lesions with abundant conidia in acervuli (4). In contrast, Colletotrichum crown rot is characterized by reddish-brown necrotic areas in the crown, which ultimately cause wilting and death of the plant. In some locations, up to 80% of transplants were infected with *Colletotrichum* spp. before planting (3) or were killed by Colletotrichum crown rot (23). In the United States, losses attributed to fruit rot have been greater than 30% when warm and humid conditions favor serious epidemics (23,42).

Colletotrichum acutatum, *C. fragariae*, and *C. gloeosporioides* (teleomorph *Glomerella cingulata*) are the three main species of *Colletotrichum* that cause strawberry diseases (23). The sexual state of *Colletotrichum* spp. does not have a recognized role in strawberry epidemics and its role in the disease cycle has not been studied (23,25). However, *Glomerella* spp. do play a role in the epidemiology of diseases affecting other annual and perennial crops including other members of the family Rosaceae (37). On apple, where *C. gloeosporioides* and *G. cingulata* produce a disease called bitter rot, ascospores contribute the primary inoculum for epidemics (38). The sexual stage for *C. acutatum* (*G. acutata*) has also been described recently (18).

Species identification for isolates in the genus *Colletotrichum* has traditionally been based on the shape and size of conidia, production of perithecia, morphology of setae (if present), and colony growth characteristics (19,34,37). However, these characteristics can be highly variable in *Colletotrichum* spp. and differentiation based on morphological characteristics may be uncertain (7,37). This situation has lead to numerous cases of incorrect species identification (1,37,39). In California, a group of historical isolates from apple and peach originally identified as *G. cingulata* were later reclassified as *C. acutatum* (19). In Florida, postbloom fruit drop of citrus was originally identified as being caused by slow growing strains of *C. gloeosporioides* before species-specific polymerase chain reaction (PCR) primers revealed that *C. acutatum* was the correct pathogen (1,5). Construction of species-specific primers, especially from the ribosomal DNA internal transcribed spacer (ITS) region, has been proposed as the most efficient and reliable system for detection and differentiation of *Colletotrichum* spp. (15,36). However, strains of *C. fragariae* are still difficult to distinguish from those of *C. gloeosporioides* using morphological characters or ITS primers (6,36). Molecular evidence from mitochondrial DNA, ribosomal DNA, and random amplification of polymorphic DNAs (RAPDs) suggests a relatively close genetic relationship between *C. fragariae* and *C. gloeosporioides* (36,37).

The biology and epidemiology of *Colletotrichum* spp. on strawberry is unclear (23,24). In Florida, clonally produced strawberry plants are grown as an annual winter crop. Green-top transplants from nurseries in the northeastern United States or southeastern Canada are planted in the fall on fumigated plastic-mulched raised beds. Fruit are typically harvested from middle November to early April. Although the sources of primary inoculum for *Colletotrichum* epidemics in Florida are uncertain (23,24), transplants are the likely source (16,23). Transplants infected with *Colletotrichum* spp. have been reported in California (10), Israel (13), and Switzerland (3). In temperate regions, inoculum from plant debris may initiate epidemics each spring (10,42). However, in the southeastern United States, *Colletotrichum* spp. do not survive between strawberry seasons in plant debris (41) or in soil (22).

Evaluation of the genetic structure and relationships among lineages of *Colletotrichum* spp. infecting strawberry should pro-

Corresponding author: D. E. Legard; E-mail address: legard@ufl.edu

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vide insights into their biology and the etiology of the diseases. For asexually reproducing fungi such as *C. acutatum* and *C. gloeosporioides*, detection of subdivisions among populations within a particular area can indicate the source or sources of initial inoculum, reveal host or tissue specificity, or test assumptions about the role of the teleomorphic state in the epidemiology of the diseases (32,35). The purpose of this study was to characterize intra- and interspecies isolates responsible for fruit rot and crown rot epidemics in Florida, to evaluate the genetic relationships among isolates of these pathogens, and to provide insights into the biology of the pathogens and etiology of Colletotrichum crown rot and anthracnose fruit rot. A preliminary report of this research has been made (40).

MATERIALS AND METHODS

Isolates. Monoconidial isolates of *Colletotrichum* spp. causing anthracnose fruit rot (44 isolates) and Colletotrichum crown rot (132 isolates) of strawberry were evaluated (Table 1). The isolates were collected from different locations and different seasons and were representative samples from epidemics of fruit rot and crown rot in the Plant City-Dover strawberry production region in west central Florida. Isolates from a majority of commercial cultivars grown in the region during the late 1990s are represented in this collection. One field was sampled for crown rot three times in the same season (Table 1; samples Crown-9b, Crown-9c, and Crown-9d) to obtain a large collection of isolates from one farm for population genetic analysis. A large sample of anthracnose fruit rot isolates from a single farm was also collected for genetic comparisons (Fruit-5a). Six isolates of *C. fragariae* (Table 1) from strawberry crown rot lesions previously isolated and characterized were included to confirm differences between this species and *C. gloeosporioides* in the genetic analysis. Ten isolates of *C. gloeosporioides* and three isolates of *C. acutatum* from nonstrawberry hosts (Table 2) were included in the study as putative nonpathogenic controls in the bioassays. The five isolates from citrus (four *C. gloeosporioides* and one *C. acutatum*) were also included in the genetic analysis to provide out-group information within those species. Cultures were stored in 20% glycerol in a freezer at –85°C.

TABLE 1. Isolates of *Colletotrichum* spp. from strawberry used for genetic analysis

Tissue[a]	Source location[b]	Year collected	Number of isolates	Species[c]
Crown	Crown-1	1999	1	*C. gloeosporioides*
	Crown-2	1997	1	*C. gloeosporioides*
	Crown-3	1996	1	*C. gloeosporioides*
	Crown-4	1995	1	*C. gloeosporioides*
	Crown-5b	1997	1	*C. gloeosporioides*
	Crown-6a	1988	3	*C. fragariae*
	Crown-6b	1996	3	*C. gloeosporioides*
	Crown-6c	1997	1	*C. gloeosporioides*
	Crown-6d	1999	2	*C. gloeosporioides*
	Crown-7	1995	1	*C. gloeosporioides*
	Crown-8	1996	1	*C. gloeosporioides*
	Crown-9b	1998	25	*C. gloeosporioides*
	Crown-9c	1998	23	*C. gloeosporioides*
	Crown-9d	1999	11	*C. gloeosporioides*
	Crown-10	1999	1	*C. gloeosporioides*
	Crown-11a	1995	10	*C. gloeosporioides*
	Crown-11b	1997	3	*C. gloeosporioides*
	Crown-11c	1999	2	*C. gloeosporioides*
	Crown-12	1999	1	*C. gloeosporioides*
	Crown-13	1997	1	*C. gloeosporioides*
	Crown-14	1997	1	*C. acutatum*
	Crown-15	1996	2	*C. gloeosporioides*
	Crown-16a	1996	1	*C. gloeosporioides*
	Crown-16b	1997	1	*C. gloeosporioides*
	Crown-17	1996	1	*C. gloeosporioides*
	Crown-18	1999	1	*C. gloeosporioides*
	Crown-19	1995	3	*C. gloeosporioides*
	Crown-20	1999	1	*C. gloeosporioides*
	Crown-21	1997	15	*C. gloeosporioides* and *C. acutatum*[d]
	Crown-22	1998	15	*C. gloeosporioides*
	Crown-23	1996	1	*C. gloeosporioides*
	Crown-24	?	3	*C. fragariae*
Fruit	Fruit-5a	1996	25	*C. acutatum*
	Fruit-6b	1996	4	*C. acutatum*
	Fruit-9a	1995	7	*C. acutatum*
	Fruit-24	?	2	*C. acutatum*
	Fruit-25	1995	1	*C. acutatum*
	Fruit-26	?	1	*C. acutatum*
	Fruit-27	1996	4	*C. acutatum*

[a] Isolates were collected from crowns with Colletotrichum crown rot or fruit with anthracnose fruit rot.
[b] Locations with the same number and different letters were collected from the same farm but on different dates.
[c] All isolates were identified to species using internal transcribed spacer primers, except for C. *fragariae* isolates, which were previously identified historical isolates.
[d] Fourteen isolates were identified as *C. gloeosporioides* and one as *C. acutatum.*

Mycelium production and DNA extraction. Isolates were grown on potato dextrose agar (PDA) at 24°C under continuous fluorescent light for 5 to 7 days. Conidial suspensions were prepared from the cultures in sterile distilled water and 5 ml of the suspension, which was transferred with sterile pipettes to 250-ml flasks containing 100 ml of Emerson Media (4 g of yeast extract, 15 g of soluble starch, 1 g of potassium phosphate, 0.5 g of magnesium sulfate, and distilled water up to 1 liter). Cultures were incubated at room temperature under continuous shaking (150 rpm). After 3 days, mycelium was harvested, rinsed with sterile distilled water, filtered through cheesecloth, and freeze-dried with a centrifugal evaporator (Jouan, Inc., Winchester, VA). The dried mycelium was ground into a powder with sterile glass rods in 15-ml Falcon tubes and suspended in 5 ml of extraction buffer (700 mM NaCl; 50 mM Tris-HCl, pH 8.0; 1% β-mercaptoethanol; and 1% hexadecyltrimethyl-ammonium bromide). Suspensions were incubated at 65°C for 60 min, 8 ml of chloroform/isoamyl alcohol (24:1, vol/vol) was added, and the mixture was emulsified by gently shaking the tubes by hand. After centrifugation at 4°C for 25 min, the supernatant was re-emulsified by hand with 8 ml of chloroform/isoamyl alcohol and centrifuged at 4°C for 25 min. The resulting supernatant was mixed with 0.8 volume of isopropanol and centrifuged for 6 min. The DNA precipitate was air dried and resuspended in 2 ml of Tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 7.4). The DNA was reprecipitated with 0.5 volume of 7.5 M ammonium acetate (NH_4OAc) and 2 volumes of ethanol. After centrifuging (6 min), the pellet was resuspended in 400 µl of TE buffer with RNase (10 µg of RNase A per ml). After 30 min, DNA was precipitated with 0.5 volume of 7.5 M NH_4OAc and 2 volumes of ethanol and centrifuged at 14,000 rpm for 10 min. The DNA pellets were resuspended in 50 to 500 µl of TE buffer and stored at –20°C.

ITS primers. All species determinations were made by PCR amplification using species-specific primers from the ribosomal DNA ITS region (35,36). PCR amplifications were carried out in mixtures containing 2 µl of DNA in TE buffer, 2 µl of 10× reaction buffer (No. 1779; Idaho Technology, Salt Lake City), 1.2 µl of 1.5 mM $MgCl_2$, 2 µl of dNTP (2.0 mM; Pharmacia PL Biochemicals, Milwaukee, WI), 0.2 µl of *Taq* polymerase (5 units/µl), and 10.6 µl of sterile distilled water. Ten micromoles (1 µl) each of the conserved 28S ribosomal subunit primer (5′-TCCTCCGCTTATTGATATGC-3′) and the species-specific primer CaInt2 (5′-GGGGAAGCCTCTCGCGG-3′) for *C. acutatum* or the specific primer Cg/f Int1 (5′-GACCCTCCCGGCCTCCCGCC-3′) for *C. gloeosporioides* was used for identification (36,37). DNA was amplified using an initial step of 4 min at 94°C followed by 34 cycles at 94°C for 1 min, 59°C for 2 min, and 72°C for 2 min.

DNA was separated by electrophoresis in a 0.7% (wt/vol) LE agarose (Promega, Madison, WI) gel in 0.5× Tris-borate-EDTA (TBE) buffer (0.045 M Tris-borate and 0.001 M EDTA, pH 8.0), stained with ethidium bromide, and photographed.

Pathogenicity tests. The pathogenicity of representative strawberry isolates of *Colletotrichum* from crown rot (62 isolates) and fruit rot (20 isolates), isolates of *C. acutatum* and *C. gloeosporioides* from nonstrawberry hosts (13 isolates), and four isolates of *C. fragariae* from strawberry were determined in laboratory and greenhouse bioassays. Isolates for the assays included representatives from all major lineages within the dendrogram constructed from RAPD and microsatellite markers for all isolates. Sterile distilled water was used for a control treatment. Each bioassay was conducted with a conidial suspension produced from 7-day-old colonies grown on PDA at 24°C under continuous fluorescent light and adjusted to 1×10^6 conidia per ml in sterile deionized water.

The ability of each isolate to produce symptoms characteristic of Colletotrichum crown rot was evaluated by inoculating three greenhouse-grown strawberry plants. Because field and greenhouse bioassay experiments with four commercial cultivars and six isolates of *C. gloeosporioides* found no evidence of cultivar–isolate interactions (S. J. MacKenzie, *unpublished data*), a single cultivar was used for the greenhouse bioassays. Highly susceptible cv. Camarosa was selected because symptoms developed faster on it than other cultivars.

To inoculate plants, a syringe needle was used to wound and inject 0.1 ml of freshly made conidial suspension into the lower leaf axil of each crown. After inoculation, the plants were placed inside a moisture chamber at 24 to 30°C for 48 h and maintained in a greenhouse at the same temperature for 4 weeks. Plants were evaluated weekly for symptoms characteristic of Colletotrichum crown rot (i.e., wilting and collapse of plant). An isolate was considered pathogenic if at least two of three plants wilted and collapsed within 28 days of inoculation. Crown sections from diseased plants were surface sterilized, sectioned, and plated onto a Colletotrichum semiselective medium (16 g of Difco potato dextrose broth, 14 g of Difco agar (Becton Dickinson, Franklin Lakes, NJ), 250 mg of ampicillin, 150 mg of streptomycin sulfate, 5 mg of iprodione, 100 µl of Tergitol, and deionized water to 1 liter) for re-isolation and confirmation of the pathogen. All isolates were evaluated in at least two separate inoculation experiments.

To evaluate fruit pathogenicity, laboratory assays were conducted using detached, fully ripe fruit of cv. Camarosa. For each isolate, 10 detached fruit were inoculated by pipetting 10 µl of a conidial suspension (10^6 conidia per ml) onto the surface of the fruit. Fruit were placed inside plastic containers (32 × 26 × 10 cm) on wire racks suspended above 100 ml of water. Each container was wrapped with plastic film (Reynolds Film 910) to maintain high humidity conditions. Fruit were examined for lesion development after 2, 3, and 4 days of incubation at 25°C.

RAPD and microsatellite markers. Thirty-four primers were evaluated for their ability to generate RAPD or microsatellite markers. Four primers were selected for use in this study: $(GACA)_4$; $(ACTG)_4$; $(TCC)_5$; and OPC-5 (GATGACCGCC) (Operon Technologies, Alameda, CA). PCR amplifications were done in mixtures containing 13.2 µl of sterile distilled water, 2 µl of 10× reaction buffer (500 mM Tris at pH 8.3, 2.5 mg of bovine serum albumin per ml, 20 mM $MgCl_2$, 5% Ficoll 400, 10 mM Tartrazine; Idaho Technology), 0.4 µl of dNTP (2 mM; Pharmacia Biochemicals), 2 µl of DNA, 2 µl (20 µM) of primer, and 0.2 µl of *Taq* polymerase (5 units/µl).

Samples were overlaid with 50 µl of mineral oil, and DNA was amplified in a programmable thermocycler (M.J. Research, Watertown, MA) with an initial step of 5 min at 95°C followed by 34 cycles of 1 min at 94°C, 1 min at 40°C for primer $(GACA)_4$, 46°C for primers $(ACTG)_4$ and $(TCC)_5$ or 2 min at 37°C for primer OPC-5, and 1.5 min [primers $(GACA)_4$, $(ACTG)_4$, and $(TCC)_5$] or 2.0 min (primer OPC-5) at 72°C. PCR products were separated by electrophoresis as described previously using 2% (wt/vol) agarose-1000 (Invitrogen Corporation, Carlsbad, CA) gels in 0.5× TBE buffer. All RAPD and microsatellite primers were tested at least three times for each isolate. Forty DNA fragments that were consistently amplified between replicates were scored as present or absent and used for genetic analyses.

Data analysis. A dendrogram displaying genetic distances among populations classified according to host, isolate species identification, and host tissue were generated from RAPD allele frequencies using the TFPGA program (M. P. Miller, Northern Arizona University). Genetic distances between populations were estimated using Nei's D, and clustering was performed by the unweighted pair-group method with arithmetic average (UPGMA) algorithm (29). Each population was treated as an operational taxonomic unit. Statistical support for nodes was obtained from 1,000 bootstrapped samples.

At the species level, dendrograms showing the relationship between individual isolates were constructed using the NTSYS program. The dendrograms show relationships of isolates based

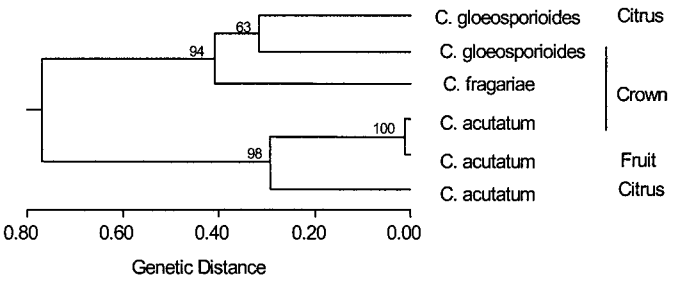

Fig. 1. Dendrogram showing genetic distances between *Colletotrichum* spp. isolated from strawberry and citrus. Isolates from strawberry are labeled by the tissue they were isolated from (i.e., crown or fruit). Citrus isolates were included for comparison. Numbers at branch points indicate the percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped dendrograms. Only branches occurring in at least 50% or more of bootstrapped dendrograms are labeled.

TABLE 2. Isolates of *Colletotrichum* spp. from nonstrawberry hosts used for bioassay and genetic comparisons with isolates of *Colletotrichum* spp. from strawberry crown and fruit

Location	Host	Symptom	Isolates collected	Species
Arkansas	Weeds[a]	Unknown	2	*C. gloeosporioides*
Dover, Florida	Sweet gum[b]	Unknown	1	*C. gloeosporioides*
Homestead, Florida	Mango	Unknown	3	*C. gloeosporioides*
Lake Alfred, Florida	*Citrus* spp.	Unknown	1	*C. acutatum*
Lake Alfred, Florida	*Citrus* spp.	Unknown	4	*C. gloeosporioides*
South Carolina	Peach	Fruit rot	1	*C. acutatum*
South Carolina	Grape	Fruit rot	1	*C. acutatum*

[a] Unidentified weed hosts.
[b] Sweet gum: *Liquidambar styraciflua.*

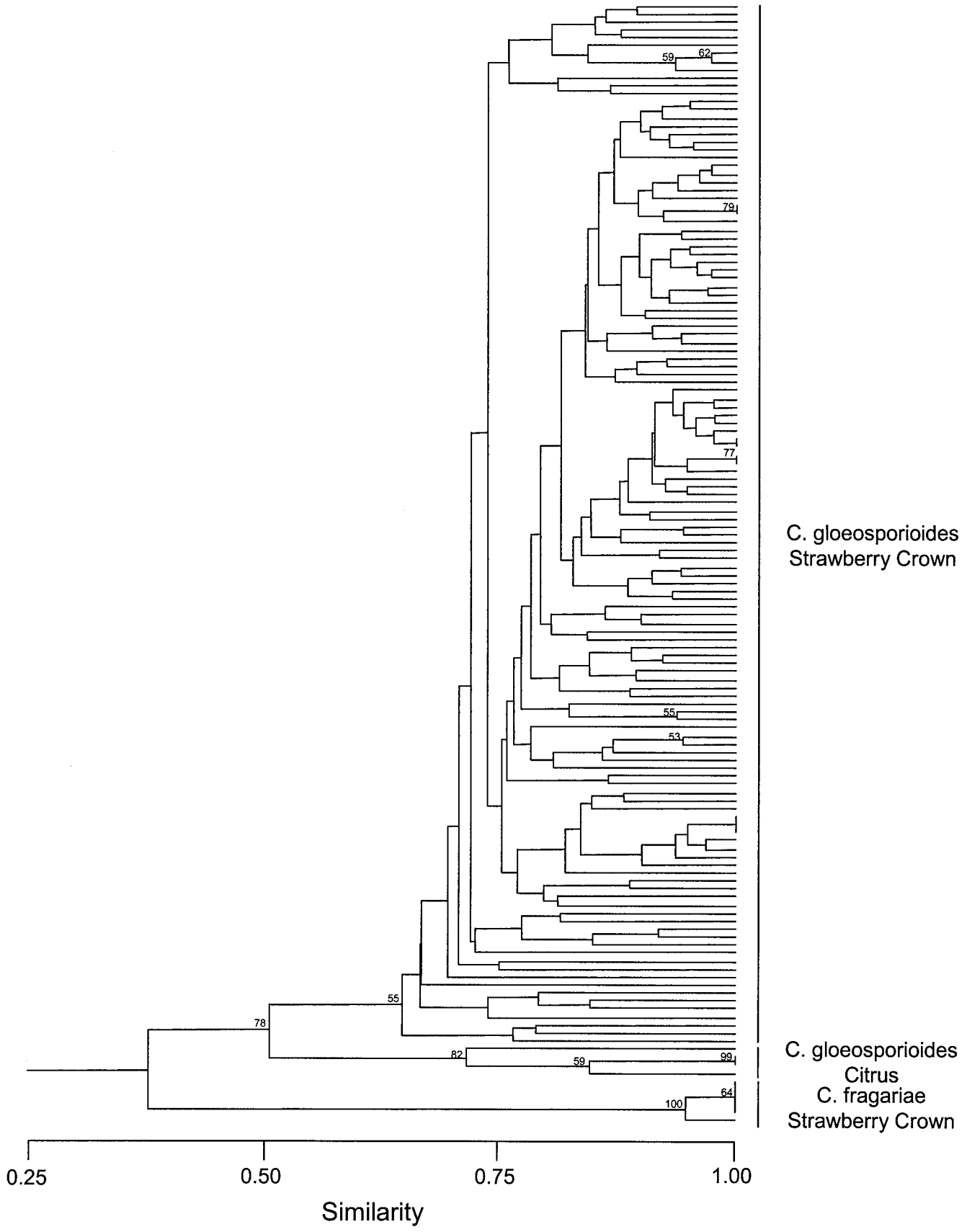

Fig. 2. Dendrogram showing similarity (Dice) between *Colletotrichum gloeosporioides* from strawberry crowns, *C. gloeosporioides* from citrus, and *C. fragariae* from strawberry crowns. Numbers at branch points indicate the percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped dendrograms. Only branches occurring in at least 50% or more of bootstrapped dendrograms are labeled.

on Dice similarity coefficients calculated from RAPD bands using the UPGMA algorithm. Statistical support for branches was based on 1,000 bootstrapped samples using Winboot (12,31). Genetic diversity measurements (H_t) were calculated for each species isolated from strawberry (30). Only loci with at least one positive amplification product from isolates within the species were included in the analysis.

Further examination of genetic diversity in *C. gloeosporioides* crown rot and *C. acutatum* fruit rot populations was conducted on four subpopulations (Crown-9b, Crown-9c, Crown-9d, and Fruit-5a) with more than 10 isolates per subpopulation. Subpopulations labeled Crown-9b-d consisted of crown isolates taken from the same farm on three separate dates, whereas the subpopulation Fruit-5a consisted of fruit rot isolates collected on a single date. Allele frequencies were estimated within each of these four subpopulations (mean weighted by population sample size) and the population substructure was evaluated by analysis of molecular variance (AMOVA; 11). For AMOVA, the total genetic variance was partitioned into between population and between individuals within population components. Linkage disequilibrium between pairs of loci was evaluated to test the hypothesis that sexual recombination may be occurring in the sampled populations. Linkage disequilibrium was evaluated using a likelihood ratio test (ARLEQUIN version 2.000; Genetics and Biometry Laboratory, University of Geneva, Switzerland). This test compares the likelihood that pairs of polymorphic loci are in linkage equilibrium with the likelihood that they are in linkage disequilibrium. To increase the definition power of the test, parameters in the program were set so that only polymorphic loci with at least three copies of each possible allele were evaluated. All AMOVA and linkage disequilibrium analyses were performed using the ARLEQUIN program.

RESULTS

Species-specific primers. All 176 isolates from strawberry were identified as either *C. gloeosporioides* or *C. acutatum* by amplification of a 450- or a 490-bp DNA fragment with one of the species-specific primers (Cg/f Int1 or CaInt2). All but 2 of 132 isolates from strawberry crowns produced a characteristic amplification product when the *C. gloeosporioides*-specific primer set (Cg/f Int1/ITS4) was used. The remaining two isolates and all 44 fruit rot isolates produced an amplification product when the *C. acutatum* primer set (CaInt2/ITS4) was used. The six *C. fragariae* isolates also produced an amplification product with the Cg/f Int1/ITS4 primer set. Species identities of isolates from nonstrawberry hosts used in pathogenicity tests and molecular analysis were also confirmed by the ITS primers (Table 2).

Pathogenicity tests. Sixty-four crown isolates (60 of *C. gloeosporioides* and four of *C. fragariae*), 22 *C. acutatum* isolates (two from strawberry crowns and 20 from strawberry fruit), and 13 isolates from nonstrawberry hosts were evaluated for their ability to cause disease in greenhouse bioassays. When inoculated into crowns, all 60 isolates of *C. gloeosporioides* and the four isolates of *C. fragariae* obtained from crowns, produced symptoms characteristic of Colletotrichum crown rot (i.e., rapid wilt and plant death). The two isolates of *C. acutatum* isolated from diseased crowns did not produce typical crown rot symptoms. These isolates, and two additional isolates of *C. acutatum* obtained from strawberry fruit, caused a slow decline in plant vigor, wilting and dying of older leaves, and stunting. Eighteen other isolates of *C. acutatum* obtained from strawberry fruit, and 12 of 13 isolates of *Colletotrichum* spp. obtained from nonstrawberry hosts did not produce symptoms in crowns. Although some plants were severely affected, all of the plants inoculated with *C. acutatum* isolates in the greenhouse bioassays were still alive after 4 weeks. No symptoms developed in the controls inoculated with sterile water. *C. gloeosporioides* and *C. fragariae* were re-isolated and re-characterized from symptomatic crowns.

All isolates of both *C. gloeosporioides* and *C. acutatum* produced typical symptoms of anthracnose rot on inoculated detached ripe fruit, i.e., limited sunken necrotic lesions and orange-colored conidial masses, within 4 days of inoculation. No symptoms developed in the water-inoculated controls. *C. gloeosporioides* and *C. acutatum* were re-isolated and characterized from infected fruit.

RAPD and microsatellite markers. Forty RAPD or microsatellite markers were identified for population genetic comparisons of the isolates. DNA markers ranged in size from ≈200 to 1,500 bp. Primers OPC-5, $(TCC)_5$, $(ACTG)_4$, and $(GACA)_4$ produced 12, 11, 10, and 7 scorable alleles, respectively.

A dendrogram was constructed using Nei's genetic distance examining subgroups based on species identification and the host tissue the pathogen was isolated from (Fig. 1). Bootstrap analysis supported groupings of isolates based on ITS1 primer amplifications and morphological criteria. There is also support for *C. acutatum* isolates from strawberry forming a distinct cluster from the citrus isolates.

Separate dendrograms of each species were constructed to facilitate the presentation of genetic relationships among isolates (Figs. 2 and 3). Bootstrap analysis values are presented in these figures. The first dendrogram includes all isolates of *C. gloeosporioides* (as identified by the Cg/f Int1 and the ITS4 primers) collected from strawberry crown rot tissue, four isolates from citrus and six isolates of *C. fragariae* from strawberry (Fig. 2). The overall diversity (H_T) among *C. gloeosporioides* strawberry crown rot isolates was 0.224, and a majority of isolates had unique genotypes, revealing the relatively high level of diversity within *C. gloeosporioides* from strawberry. The four citrus isolates of *C. gloeosporioides* formed a distinct subgroup, with 0.50 similar to the isolates from strawberry. The *C. fragariae*

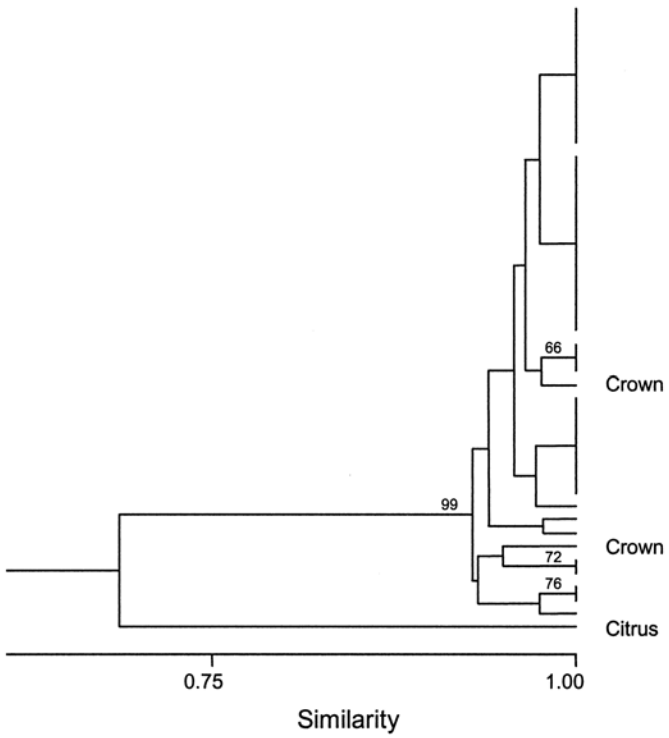

Fig. 3. Dendrogram showing similarity (Dice) between *Colletotrichum acutatum* isolates from strawberry fruit, strawberry crown, and citrus. Fruit isolates are unlabeled. Numbers at branch points indicate the percent occurrence of the cluster to the right of the branch in 1,000 bootstrapped dendrograms. Only branches occurring in at least 50% or more of bootstrapped dendrograms are labeled.

isolates were 38% similar to the *C. gloeosporioides* group. This third lineage consisted of a mostly clonal population of historical isolates of *C. fragariae*. This species does not appear to play an important role in recent *Colletotrichum* epidemics on strawberry, because no isolates of *C. fragariae* were collected during this study.

The second dendrogram included all the *C. acutatum* isolates (identified with the CaInt2 and the ITS4 primers) from strawberry and the one isolate from citrus (Fig. 3). The overall minimum similarity among *C. acutatum* from strawberry was 0.92 and H_T was equal to 0.0730. In marked contrast to isolates identified as *C. gloeosporioides*, 72% of the *C. acutatum* isolates belonged to just three clonal groups (similarity coefficient of 1.0). The two isolates of *C. acutatum* obtained from crown tissue and identified as *C. acutatum* with ITS primers grouped with the strawberry fruit rot isolates. The isolate from citrus was only 0.78 similar to the isolates from strawberry.

AMOVA of allele frequencies for three *C. gloeosporioides* crown rot populations (Crown-9b, Crown-9c, and Crown-9d) revealed significant differentiation among these subpopulations of *C. gloeosporioides* ($F_{ST} = 0.15$; $P \leq 0.05$). Because of the significant differentiation among subpopulations, pairwise linkage disequilibrium was calculated individually for each subpopulation of *C. gloeosporioides*. Twenty-three polymorphic loci (those that had at least three copies of each allele at loci within a subpopulation) were used in linkage disequilibrium tests and percentages of loci in disequilibrium with each individual locus ($P \leq 0.05$) are presented in Table 3. Of the 23 putative loci evaluated among crown rot isolates, 17 in subpopulation Crown-9b, 15 in Crown-9c, and 6 in Crown-9d were tested. For crown rot isolates, the percentage of loci in disequilibrium with individual loci ranged from 0% (unlinked locus) to 28.6% (Table 3). Only 3 loci out of 23 could be analyzed for fruit rot isolates from Fruit-5a and their level of disequilibrium ranged between 0 and 50%.

TABLE 3. Percentage of linked loci for 23 putative molecular loci generated with random amplified polymorphic DNA (RAPD) and microsatellite markers of three subpopulations of *Colletotrichum gloeosporioides* (Crown-9b, Crown-9c, and Crown-9d) from diseased strawberry crowns and one population of *C. acutatum* (Fruit-5a) from diseased fruit

RAPD or microsatellite primer and locus size (kbp)	Population or subpopulation[a]			
	Crown-9b (n = 25)	Crown-9c (n = 23)	Crown-9d (n = 11)	Fruit-5a (n = 24)
$(GACA)_4$				
1.2	–	7.1	–	–
1.0	–	7.1	–	–
0.7	6.2	14.3	20.0	–
$(ACTG)_4$				
2.1	0	0	–	–
1.2	0	14.3	–	–
1.0	6.2	0	–	–
0.8	12.5	–	–	–
0.6	–	0	20.0	–
0.5	12.5	21.4	–	0
0.4	0	7.1	–	–
0.3	0	–	–	–
OPC-5				
2.8	18.8	–	–	–
2.1	25.0	–	–	–
1.7	25.0	–	20.0	–
1.5	–	–	20.0	–
1.2	6.2	7.1	–	–
0.8	6.2	14.3	0	50.0
0.65	–	–	–	50.0
0.6	–	21.4	–	–
$(TCC)_5$				
1.8	12.5	7.1	0	–
1.05	6.2	7.1	–	–
0.4	18.8	28.6	–	–
0.3	6.2	–	–	–
No. of polymorphic loci	17	15	6	3

[a] n indicates sample size for each population or subpopulation; – indicates not determined.

DISCUSSION

The results of this study support the hypothesis that epidemics of anthracnose fruit rot are caused by *C. acutatum* and epidemics of Colletotrichum crown rot are caused by *G. cingulata*, the teleomorph of *C. gloeosporioides*. The high diversity and low linkage disequilibrium of populations of *C. gloeosporioides* on strawberry suggest that they undergo genetic recombination.

The ITS primers could not differentiate between *C. gloeosporioides* and *C. fragariae* isolates, due to the uniform size of the amplification fragments of genomic DNA (490 bp). The difficulty in identifying isolates of *C. gloeosporioides* and *C. fragariae* with ITS primers was expected due to the lack of significant differences between the two species in the region of the ribosomal DNA that was amplified (35,36). Our identification of the crown rot isolates from strawberry as *C. gloeosporioides* is supported by the separate grouping of the historical isolates of *C. fragariae* shown in Figures 1 and 2. The population of *C. fragariae* in this study also had a clonal structure expected for a species with no known teleomorph (25).

All isolates of *C. gloeosporioides* from strawberry but none of the isolates of *C. acutatum* produced typical crown rot symptoms in greenhouse bioassays. However, two isolates of *C. acutatum* caused a slow decline and a crown necrosis in the bioassays. The slower decline differentiated these symptoms from the rapid plant decline caused by *C. gloeosporioides.* In addition to causing fruit rot, *C. acutatum* can also cause petiole lesions, irregular leaf spot, root rot (13,25), and a slow decline and death of strawberry plants (D. E. Legard, *unpublished data*). However, the slow decline disease is easily distinguished in the field from the rapid plant wilt and death caused by *C. gloeosporioides* and *C. fragariae* (22,24). Only petiole, crown, and stolon infections were reported in the original descriptions of strawberry diseases caused by *C. fragariae* and *C. gloeosporioides* (4,8). Serious epidemics of fruit rot developed in the United States after the apparent introduction of *C. acutatum* in the 1980s (23,33). Most early descriptions of anthracnose fruit rot identify *C. acutatum* as the responsible pathogen (9,10,23). Reports of epidemics of fruit rot caused by *C. gloeosporioides* or *C. fragariae* may be attributed to persistent problems in identifying species of *Colletotrichum* based on morphological characteristics (15,36).

Although the results of the crown bioassays clearly distinguished isolates of *C. gloeosporioides* from isolates of *C. acutatum* from strawberry, all the isolates caused lesions in bioassays on fruit. It is likely that ripe, detached strawberry fruit are not reliable for determining pathogenicity of *Colletotrichum* spp. Other researchers (9,14,34) have had similar difficulties in distinguishing pathogens from nonpathogens when using detached fruit for bioassays. Maas and Howard (26) found that isolates of *C. coccodes* caused lesions on fruit even though this species is not a pathogen of strawberry. Therefore, we conclude that our results from the fruit bioassay are not indicative of the pathogenic capability of *C. gloeosporioides* under field conditions. Furthermore, because no isolates of *C. gloeosporioides* were recovered from anthracnose fruit lesions during this study, and that only *C. acutatum* was recovered from samples of anthracnose fruit rot processed by the strawberry diagnostic clinic at Gulf Coast Research and Education Center (GCREC, Dover, FL; D. E. Legard, *unpublished data*), we conclude that *C. gloeosporioides* does not cause epidemics of anthracnose fruit rot of strawberry under field conditions.

The analyses of DNA markers strongly support our conclusion that *C. gloeosporioides* is the causal agent for epidemics of Colletotrichum crown rot and *C. acutatum* is the causal agent for epidemics of anthracnose fruit rot. With the exception of two

isolates of *C. acutatum* (which did not produce typical crown rot symptoms), primary lineages in the dendrogram associate fruit rot with *C. acutatum* and crown rot with *C. gloeosporioides*. The historical *C. fragariae* isolates formed a distinctive lineage in the dendrogram but because few samples were available and no new isolates were obtained from recent samples, the only conclusions available from the genetic analysis are the observation of low genetic diversity.

Genetic diversity among isolates of *C. acutatum* causing fruit rot was notably lower than that observed among isolates of *C. gloeosporioides* from crown rot. The low diversity in RAPD and microsatellite markers may be attributed to the highly clonal nature of *C. acutatum*. Because of the lack of diversity among fruit rot isolates (*C. acutatum*), the analysis of linkage disequilibrium was limited to 3 out of a putative 40 loci. The lower diversity among strawberry isolates of *C. acutatum* in comparison with *C. gloeosporioides* isolates has been reported in the past using different approaches such as isozyme analysis, arbitrarily primed PCR, A+T-rich DNA, and morphological and cultural characteristics (2, 9,17). In contrast, the high level of diversity within the *C. gloeosporioides* crown rot population coupled with a lack of linkage among loci is indicative of a genetically recombining population. Recently, the sexual stage of *C. gloeosporioides* (*Cingulata glomerella*) has been observed on strawberry petioles collected from the field (S. J. MacKenzie, *unpublished data*; 27). High levels of variation among *C. gloeosporioides* isolates from strawberry and other hosts have been reported previously (6,7,14,21). Considerable diversity among isolates of *C. gloeosporioides* in restriction banding patterns for rDNA and mtDNA have been described for isolates infecting avocado and mango (21). Isolates of *C. gloeosporioides* pathogenic on *Stylosanthes* spp. were also highly variable when analyzed with RAPD markers (7).

Sexual recombination is not the only possible explanation for high diversity in *C. gloeosporioides*. Population genetic diversity is also a function of effective population size (N_e): under neutrality theory, large populations maintain higher levels of allelic variation (20). Diversity is also a function of molecular evolutionary rate; higher mutation rates can produce higher levels of genetic diversity. The age of populations can also influence genetic diversity, because a recently colonized habitat will harbor less diversity than an older population, if the young population was founded by a few colonizers (bottleneck effect). The genetic diversity reported in our study could also be explained by long periods of independent evolution from a single strain that gained the ability to infect a particular host, or by assuming that pathogenicity on a certain host may have been acquired by a large number of genetically distinct strains (7,21). None of these explanations can be excluded with finality. However, asexual reproduction by conidia results in clonal population structures that have distinctive features, such as widespread occurrence of identical genotypes and linkage between independent sets of genetic markers (28).

The relatively high values for the coefficients of similarity for *C. acutatum* and *C. fragariae* support the suggestion that their primary means of reproduction is asexual. The relatively low values for the coefficient of similarity and the relatively large number of loci in equilibrium in the linkage disequilibrium tests of *C. gloeosporioides* isolates from strawberry crowns suggest that some of the molecular markers have been under a state of random gametic association or sexual recombination (linkage equilibrium) (20). However, the sexual stage (*Colletotrichum* spp.) has been rarely been observed on strawberry (S. J. MacKenzie, *unpublished data*) (6,9,10,25). Perithecial production in *G. cingulata* is very sensitive to environmental conditions (37), and suitable conditions may not commonly occur in fruit production fields but may be occurring in nurseries.

The realization that epidemics of anthracnose fruit rot and Colletotrichum crown rot are caused by different species, *C. acutatum* and *G. cingulata* (anamorph *C. gloeosporioides*), respectively, and the large differences in genetic diversity found within the different species has implications on quarantine and disease management decisions. Resistant cultivars are one of the most effective means of controlling disease (9,25). The expression of disease resistance to *C. acutatum* may be dependent on the strain of the pathogen (1,7). When screening breeding lines for resistance to anthracnose fruit rot, it is probably advisable that several strains of *C. acutatum* should be used. For *C. gloeosporioides*, a broad selection of isolates should be used to effectively screen germ plasm for resistance. Some cultivars of strawberry have shown immunity to anthracnose fruit rot caused by *C. acutatum*, but no completely resistant cultivars are known for Colletotrichum crown rot (8,9,16, 23,25).

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