

Host Range and Genetic Relatedness of *Colletotrichum acutatum* Isolates from Fruit Crops and Leatherleaf Fern in Florida

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ABSTRACT

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Isolates of *Colletotrichum acutatum* were collected from anthracnose-affected strawberry, leatherleaf fern, and Key lime; ripe-rot-affected blueberry; and postbloom fruit drop (PFD)-affected sweet orange in Florida. Additional isolates from ripe-rot-affected blueberry were collected from Georgia and North Carolina and from anthracnose-affected leatherleaf fern in Costa Rica. Pathogenicity tests on blueberry and strawberry fruit; foliage of Key lime, leatherleaf fern, and strawberry; and citrus flowers showed that isolates were highly pathogenic to their host of origin. Isolates were not pathogenic on foliage of heterologous hosts; however, several nonhomologous isolates were mildly or moderately pathogenic to citrus flowers and blueberry isolates were pathogenic to

strawberry fruit. Based on sequence data from the internal transcribed spacer (*ITS*)1-5.8S rRNA-*ITS*2 region of the rDNA repeat, the glutaraldehyde-3-phosphate dehydrogenase intron 2 (*G3PD*), and the glutamine synthase intron 2 (*GS*), isolates from the same host were identical or very similar to each other and distinct from those isolated from other hosts. Isolates from leatherleaf fern in Florida were the only exception. Among these isolates, there were two distinct *G3PD* and *GS* sequences that occurred in three of four possible combinations. Only one of these combinations occurred in Costa Rica. Although maximum parsimony trees constructed from genomic regions individually displayed little or no homoplasy, there was a lack of concordance among genealogies that was consistent with a history of recombination. This lack of concordance was particularly evident within a clade containing PFD, Key lime, and leatherleaf fern isolates. Overall, the data indicated that it is unlikely that a pathogenic strain from one of the hosts examined would move to another of these hosts and produce an epidemic.

Colletotrichum acutatum J. H. Simmonds is a globally distributed species that causes disease on a wide range of plant hosts, including fruit and vegetable crops, pine, and leatherleaf fern (26,33). Simmonds (31) first described the species in a study of *Colletotrichum* spp. that caused fruit rots in Australia, where it was isolated commonly from fruit of papaya, strawberry, and solanaceous crops. *C. acutatum* is distinguished from other *Colletotrichum* spp. by production of ellipsoid conidia, slow growth in culture, and a pink to chromogenic red colony color. An isolate from papaya from northern Australia was established recently as the epitype of this species (40) because Simmonds (31) never properly established a type specimen. *C. acutatum* sensu lato (*s.l.*) includes strains displaying some morphological and genetic variation from the original species description of Simmonds (21). To aid classification of *C. acutatum* (*s.l.*) strains, molecular markers have been used to give strains a genetic group designation (12,17, 21,33). Some of the genetic groups are also differentiable using morphological characters (21). The isolates composing groups are consistent across multiple studies, although the groups have been described using different terminologies. For example, one has been described as group C (17), group A (21), subgroup I (12), or group A3 (33) in different studies. Except as noted, we use the terminology of Guerber et al. (17) to describe groups in this report. For a cross reference of group designations from different studies, refer to Sreenivasaprasad and Talhinhas (33). Four to eight *C. acutatum* groups have been identified using nucleic acid

sequence data (12,17,21,33). Differences in the number of groups identified depended on the number and variability of isolates evaluated and the mutation rate of the loci used for phylogenetic analysis. Some groups probably define a recombining population that is genetically isolated from other groups and, thus, fit the criteria of true biological species. For example, isolates from group C produce the teleomorph of *C. acutatum*, *Glomerella acutata*, when paired with other group C isolates and isolates from only one other genetic group, J4 (16,17). Although they formed fertile perithecia when paired with J4 isolates, the genetic distance between group C and J4 isolates suggests that they do not recombine in nature (17). A teleomorph has also been described from group F isolates (21). Other groups have not been shown to produce a teleomorph, suggesting that strains from these groups reproduce vegetatively and that the genetic groups comprise similar isolates at the termini of strictly clonal lineages. There could still be limited recombination between groups or among strains within genetic groups for which no teleomorph has been described. Analysis of the congruence of gene trees from multiple loci would provide evidence for or against recombination at some point in the history of strains from these groups (15).

Generally, within *C. acutatum*, strains do not appear to be limited to a single host, but there may be exceptions. Groups are typically composed of isolates from multiple hosts (33). Often, a strain can cause disease on a host other than its host of origin, given that genetically indistinguishable or very similar isolates within the same group attack the alternative host. For example, based on rDNA internal transcribed spacer (*ITS*) and β -tubulin 2 nucleotide sequences and arbitrarily primed polymerase chain reaction (ap-PCR) banding patterns, isolates from olive and anemone and a clonal population from strawberry were identical or highly

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similar to one another and isolates from both olive and anemone were pathogenic to strawberry (14,37). Pink-pigmented isolates from apple and peach also were cross pathogenic and displayed identical or similar AT-rich DNA and ap-PCR banding patterns (3,13). Isolates from postbloom fruit drop (PFD)-affected citrus and anthracnose-affected Key lime are very closely related to one another and are included in group J (17). However, PFD isolates did not cause Key lime anthracnose and Key lime anthracnose isolates caused less PFD than isolates from PFD-affected sweet orange (1,25). Thus, it appears that, within this group, strains may have a more limited host range, and consistent differences in DNA markers between isolates responsible for these diseases supports this (25).

Isolates from the same host, such as those from apple, citrus, lupine, olive, pecan, pine, and strawberry, can also be found in more than one group (17,36,37). The occurrence of genetically variable strains on the same host suggests that multiple lineages contribute to epidemics of the same disease. However, in many studies where isolate groups were determined, only the host of origin is reported and not the disease or symptoms caused. It is conceivable that, if pathogenicity tests had been conducted and disease symptoms described, some isolates would not have been classified as pathogens or differences in the symptoms caused by isolates from different groups would be distinguishable. When large samples of isolates that cause PFD on citrus and anthracnose of Key lime, lupine, olive, and anemone at multiple locations were examined, nearly all of the isolates responsible for each disease were genetically uniform (14,25,36,37). There were two distinct populations that were shown to produce anthracnose lesions on strawberry fruit or foliage and one of the populations was genetically uniform throughout the world (6). The occurrence of one or a few broadly distributed genotypes at multiple geographic locations does not support the idea that multiple genetic lineages contribute to disease epidemics caused by *C. acutatum* and indicates that strains of this species are host specific. However, specificity may not always be limited to one host, as demonstrated by the homogenous population that causes disease on strawberry, olive, and anemone (14,37). Because diseases are often associated with a single strain of *C. acutatum* or strains from a limited number of genetic groups, it is important that genetic information such as that provided by group designations be used to describe *C. acutatum* when referring to a disease.

In central Florida, important diseases caused by *C. acutatum* include anthracnose of strawberry (19) and leatherleaf fern (34), PFD of citrus (42), as well as Key lime anthracnose, which is a problem in home garden plantings. Blueberry ripe rot is another important disease caused by *C. acutatum*, but it is not clear if it is caused by *C. acutatum* or by *C. gloeosporioides* in central Florida (32). Crops grown in central Florida display the broad range of symptoms caused by *C. acutatum*. On strawberry, *C. acutatum* causes lesions on fruit and petiole tissues, flower blight, and plants may be stunted or killed if roots are infected (11). On leatherleaf fern, the fungus blights immature fronds and, on Key lime, it blights immature leaf tissue, stems, and flowers (26). PFD symptoms include necrosis of petal tissue, abscission of fruitlets, and persistence of the calyxes after fruit abscission (42). On blueberry, *C. acutatum* typically infects fruit when green and the fruit rots after it matures (26). It may also blight leaves or twigs of this host.

Previous studies indicate that *C. acutatum* strains responsible for PFD and anthracnose of Key lime, leatherleaf fern, and strawberry in Florida belong to at least two previously identified *C. acutatum* groups (17). Isolates from citrus with PFD in the Americas belonged to group J (17) and were all highly similar to one another (25). Groups C and F also contained citrus isolates (17) but the isolates were from New Zealand and no citrus disease caused by *C. acutatum* has been reported from that country. Key lime anthracnose isolates and two isolates from leatherleaf fern

collected in Florida were also in group J (17). Sequences from numerous Key lime isolates from throughout the Americas have been characterized and all of the isolates were very similar, yet distinct from PFD isolates (25). Leatherleaf fern anthracnose was reported first in Costa Rica and it is believed that it was introduced into Florida from that country (24). Molecular analyses using ap-PCR marker and *ITS2* sequence data showed that leatherleaf fern isolates from Costa Rica were distinct from strawberry isolates in that country and that leatherleaf fern isolates were distinct from a citrus isolate based on ap-PCR markers but not on *ITS2* sequence data (30). It is not clear whether the Costa Rican leatherleaf fern isolates are the same as isolates from Florida because sequence data at a common locus is not available for comparison. Strawberry isolates from around the world can be found in groups C, D, F, and J (17). The vast majority of strawberry isolates are from a group referred to as the CA-clonal group by Denoyes-Rothan et al. (6). The CA-clonal group is synonymous with group D (33). Denoyes-Rothan et al. (6) also described a more heterogeneous CA-variable group of isolates. Most of these isolates produced a pink chromogenic color in culture, whereas CA-clonal isolates appeared orange (6). In Florida, strawberry isolates show little genetic variation and colony color is orange, indicating that they belong to the CA-clonal group (43). Characterized *C. acutatum* isolates from blueberry occur in group C (17,33) and a recently described group, A9, from Australia (44). No blueberry isolates from Florida have been characterized. If *C. acutatum* is responsible for blueberry ripe rot in Florida, the number of *C. acutatum* groups most commonly associated with disease on citrus, Key lime, strawberry, leatherleaf fern, and blueberry in this region would likely increase by at least one.

In the present study, *C. acutatum* isolates were obtained from PFD-affected citrus; anthracnose-affected Key lime, strawberry, and leatherleaf fern; and ripe rot-affected blueberry in Florida or neighboring states. The pathogenicity of these isolates was determined on each of the five hosts and the genetic relatedness of isolates was determined from sequence data at three loci. Concordance of gene genealogies was examined to determine if there was a history of recombination among isolates from the different hosts. Isolates from leatherleaf fern in Costa Rica were also compared with those from Florida. The study provides information on the genetic relatedness of *C. acutatum* isolates from cultivated crops in Florida and their potential to attack other hosts.

MATERIALS AND METHODS

Isolates and isolation procedures. Isolates of *C. acutatum* were recovered from strawberry, leatherleaf fern, and Key lime affected by anthracnose; ripe rot-affected blueberry; and sweet orange flowers affected by PFD. Isolates and data collected from each isolate are listed in Table 1. To obtain most of the isolates, tissues were surface sterilized using 0.6% sodium hypochlorite for 1 min, rinsed three times in sterile water, and plated on a general isolation (GI) medium (19.5 g of potato dextrose agar [PDA] 9.5 g of agar, 0.1 g of streptomycin, and 0.25 g of ampicillin per liter of water). To obtain blueberry isolates, fruit with no disease symptoms were collected from two fields in Dover, FL or purchased in the grocery store where the fruit was grown in Florida, Georgia, or North Carolina. The fruit was surface disinfested and placed on a wire mesh in a humid chamber at 22°C. Ripe-rot symptoms developed after 1 week and isolations were made from the affected fruit. Most of the isolates recovered from fruit in Florida were *C. gloeosporioides*, but one isolate of *C. acutatum* was obtained from a field in Dover. *C. acutatum* isolates were recovered commonly from fruit grown in North Carolina and Georgia. All isolates were single spored. Three isolates from each host were selected for pathogenicity experiments and for phylogenetic analysis. Blueberry isolates came from Florida, Georgia, and North Carolina; all other isolates came from central

or south Florida. Gene sequences or restriction fragment length polymorphism (RFLP) banding patterns from amplified sequences were obtained for additional leatherleaf fern isolates from Florida and Costa Rica to compare strains present in these two countries. Due to quarantine restrictions, the leatherleaf fern isolates remained in Costa Rica, where DNA was extracted and shipped to the United States. Seven isolates were deposited in the American Type Culture Collection (ATCC, Manassas, VA) from this study. They are, with accession numbers in parenthesis, PFD isolate ALB-IND-25 (MYA-4396), Key lime isolate Hm-1 (MYA-4397), strawberry isolate 02-163 (MYA-4519), blueberry isolate 05-88 (MYA-4520), and three leatherleaf fern isolates: 05-155 (MYA-4516), 05-161 (MYA-4517), and 05-200 (MYA-4518).

Pathogenicity assays. Conidial suspensions were prepared from 5- to 7-day-old cultures grown at 23°C under constant light on PDA. For inoculations, conidia were washed from dishes, passed through four layers of cheesecloth, and diluted to appropriate concentrations with sterile water. The pathogenicity of isolates on blueberry (*Vaccinium corymbosum*) and strawberry (*Fragaria × ananassa*) was tested using modifications of methods used to screen plants for disease resistance (5,7,27). Blueberry assays were conducted in a greenhouse (10 to 30°C) on cv. Gulf Coast during spring 2006, summer 2006, and spring 2007. Clusters of green fruit approximately 5 mm in diameter were sprayed to runoff with a suspension of 10^6 conidia/ml and covered with a clear plastic bag overnight. After the bags were removed, plants remained in a greenhouse. Fruit were harvested when ripe

and placed in wells of egg cartons, and the egg cartons were placed in humid chambers at 22°C. After incubation for 1 week, fruit were scored for ripe-rot symptoms. On inoculation dates, an equal number of fruit clusters were inoculated with each isolate and all isolates were used for the inoculations.

Cv. Camarosa was used for strawberry pathogenicity assays. For detached fruit assays, white-pink fruit were harvested from the field and surface sterilized for 60 s in 60% ethanol, rinsed once in sterile water, surface sterilized for 6 min in a 0.04% sodium hypochlorite solution, and rinsed twice in sterile water. The fruit were placed in egg carton wells and allowed to dry. A 1.5-μl drop of a suspension of 2×10^5 conidia/ml (300 conidia) was placed on berries when dry and the cartons moved to humid chambers at 22°C. After 8 days, the fruit were evaluated for lesions and the diameter of lesions measured. Detached fruit assays were conducted on three dates using 21 or 22 fruit/isolate. For disease assays on strawberry foliage, emerging succulent leaves were sprayed to runoff with a 10^6 conidia/ml suspension and the inoculated plant placed in a clear plastic bag for 24 h at 26.5°C. Upon removal from the bags, plants were kept at 26.5°C for an additional 3 days and, subsequently, returned to a greenhouse (at 10 to 30°C) and monitored for 4 weeks. At 4 days postinoculation, the number of lesions per plant was counted and lesion sizes estimated and, at 8 days, the number of girdled petioles per plant was counted. At 4 weeks postinoculation, plants were rated on the scale of Denoyes and Baudry (5), where 0 = no lesions, 0.5 = lesion just visible, 1.0 = single developed lesion; 1.5 = two

TABLE 1. Description of *Colletotrichum acutatum* isolates used for pathogenicity and phylogenetic studies

Original host (organ)	Isolate code	Site ^x	Pathogenicity	Sequenced (<i>G3PD</i> , <i>GS</i> , <i>ITS</i>) ^y	RFLP (<i>G3PD</i> , <i>GS</i>)
Blueberry (fruit)	05-88	Dover, FL, USA	*	*, *, *	...
Blueberry (fruit)	05-148	Unknown, NC, USA	*	*, *, *	...
Blueberry (fruit)	05-197	Unknown, GA, USA	*	*, *, *	...
Strawberry (petiole)	02-163	Floral City, FL, USA	*	*, *, *	...
Strawberry (fruit)	02-179	Plant City, FL, USA	*	*, *, *	...
Strawberry (crown)	03-32	Thonotosassa, FL, USA	*	*, *, *	...
Sweet orange (flower)	ALB-IND-25 ^z	Indiantown, FL, USA	*	*, *, *	...
Sweet orange (flower)	OCO-ARC-4 ^z	Arcadia, FL, USA	*	*, *, *	...
Sweet orange (flower)	STF-FTP-10 ^z	Frostproof, FL, USA	*	*, *, *	...
Key lime (leaf)	Ss ^z	Sarasota, FL, USA	*	*, *, *	...
Key lime (leaf)	Hm-1 ^z	Homestead, FL, USA	*	*, *, *	...
Key lime (leaf)	KLA-Anderson ^z	Lake Alfred, FL, USA	*	*, *, *	...
Leatherleaf fern (frond)	05-155	Pierson, FL, USA (FJ)	*	*, *, *	...
Leatherleaf fern (frond)	05-161	Crescent City, FL, USA	*	*, *, *	...
Leatherleaf fern (frond)	05-200	Seville, FL, USA	*	*, *, *	...
Leatherleaf fern (frond)	05-121	Pierson, FL, USA (FJ)	...	*, *	...
Leatherleaf fern (frond)	05-129	Pierson, FL, USA (VP)	...	*, *	...
Leatherleaf fern (frond)	05-130	Pierson, FL, USA (VP)	...	*, *	...
Leatherleaf fern (frond)	05-141	Crescent City, FL, USA	...	*, *	...
Leatherleaf fern (frond)	05-115	Seville, FL, USA	...	*, *	...
Leatherleaf fern (frond)	H3	Cervantes, Cartago, Costa Rica	...	*, *	...
Leatherleaf fern (frond)	H8	Fraijanes, Alajuela, Costa Rica	...	*, *	...
Leatherleaf fern (frond)	H12	Tejar, Cartago, Costa Rica	...	*, *	...
Leatherleaf fern (frond)	H19	Tres Ríos, Cartago, Costa Rica	...	*, *	...
Leatherleaf fern (frond)	H24	San Pablo, Alajuela, Costa Rica	...	*, *	...
Leatherleaf fern (frond)	05-116,05-117,05-119, 05-120,05-201,05-202, 05-203,05-205	Seville, FL, USA	*, *
Leatherleaf fern (frond)	05-123,05-124,05-125	Pierson, FL, USA (FJ)	*, *
Leatherleaf fern (frond)	05-131,05-132,05-133	Pierson, FL, USA (VP)	*, *
Leatherleaf fern (frond)	05-139,05-140,05-142, 05-143, 05-144,05-145, 05-146,05-159,05-160, 05-162,05-163,05-213	Crescent City, FL, USA	*, *
Leatherleaf fern (frond)	H4	Cervantes, Cartago, Costa Rica	*, *
Leatherleaf fern (frond)	H7,H9	Fraijanes, Alajuela, Costa Rica	*, *
Leatherleaf fern (frond)	H13,H15,H16	Tejar, Cartago, Costa Rica	*, *
Leatherleaf fern (frond)	H17,H18,H20,H21	Tres Ríos, Cartago, Costa Rica	*, *
Leatherleaf fern (frond)	H25,H26	San Pablo, Alajuela, Costa Rica	*, *

^x City, state or province, and country.

^y Sequenced regions: *G3PD* = intron 2 of the glyceraldehyde 3-phosphate dehydrogenase gene, *GS* = intron 2 of the glutamine synthetase gene, and *ITS* = the internal transcribed spacer (*ITS*)1, 5.8S rRNA, and *ITS*2 regions of the rDNA repeat. The *G3PD* and *GS* regions were also analyzed for restriction fragment length polymorphisms (RFLP).

^z Isolate was previously described in Peres et al. (25). Alternative isolate designations are included in that report.

lesions; 2.0 = at least two leaves or petioles with expanded lesions; 2.5 = stunted plant, but not wilted; 3.0 = beginning to wilt; 3.5 = two wilted leaves; 4.0 = most leaves wilted; 4.5 = all leaves wilted, but slightly green; and 5.0 = plant dead. Only the final disease rating was reported for each isolate. Inoculations on strawberry foliage were conducted on four separate dates using one plant per isolate.

For pathogenicity assays on leatherleaf fern (*Rumohra adiantiformis*) and Key lime (*Citrus aurantifolia* (Christm.)), a single fern frond at the fiddlehead stage or two immature shoots on a Key lime cutting were sprayed to runoff with a suspension of 10^6 conidia/ml of each isolate on four different dates. Plants were placed in plastic bags at 22°C following inoculation and they were returned to a greenhouse (10 to 30°C) 24 h postinoculation. At 6 days postinoculation for Key lime and 2 weeks postinoculation for leatherleaf fern, the percentage of inoculated tissue that was necrotic was estimated visually.

To evaluate the ability of isolates to produce PFD symptoms, flower clusters on 5- to 10-year-old grafted Orlando tangelo (*C. reticulata* Blanco \times *C. paradisi* Macf.) trees were sprayed to runoff with a suspension of 10^6 conidia/ml. The flowers were then covered with a plastic bag for 18 h and the plants held in a greenhouse at 15 to 25°C. Flower clusters chosen for inoculation contained a large proportion of fully elongated but unopened buds. Flower clusters were inoculated on seven different dates. In total, 12 flower clusters per isolate were inoculated. All 15 isolates were used for inoculations on each date and an equal number of clusters were inoculated with each isolate. The day after inoculation, all buds at any stage of development were counted on each cluster. Five days after inoculations, the total number of open flowers and the number of open flowers with lesions were counted on each cluster. At 60 days postinoculation, persistent calyxes on each flower cluster were counted. For clusters inoculated with the same isolate, the sum of open flowers with necrosis and the sum of the number of open flowers at 5 days postinoculation were used to calculate the percentage of flowers with necrosis. To calculate the percentage of buds giving rise to persistent calyxes, buds counted the day after inoculation were summed and persistent calyxes at 60 days postinoculation were summed for each isolate. For all assays, flowers were sprayed with sterile water as a control. Results of pathogenicity assays for Key lime and PFD isolates on Key lime foliage and Orlando tangelo flowers were reported previously (25).

Statistical analysis of pathogenicity experiments. Percent necrosis on leatherleaf fern fronds and Key lime foliage and disease ratings for strawberry plants inoculated with each isolate or the control were compared using the nonparametric Wilcoxon rank sum test implemented in SAS (version 9.0; SAS Institute, Cary, NC). All pairwise comparisons were made using this statistic and isolates were grouped if the exact two-sided probability, $P > W$, was >0.05 . Fisher's exact test implemented in SAS was used to compare the percent tangelo flowers with necrosis, the percent flower buds giving rise to persistent calyxes, the percent detached strawberry fruit with anthracnose lesions, and the percent blueberry fruit with ripe rot in assays using flowers or fruit inoculated with each isolate or sprayed with water. Again, all possible pairwise comparisons were made using this test statistic and isolates were grouped if the two-sided table probability was >0.05 . Logistic analysis in SAS was used to determine if inoculation date and inoculation date-isolate effects on flower necrosis, persistent calyx production, and fruit rots might invalidate conclusions drawn from pooled incidence data. Treatments in which $>95\%$ or $<5\%$ of fruit or flowers were diseased on all inoculation dates were excluded from these analyses. Inoculation date and inoculation date-isolate effects were not evident ($P > 0.05$) for any of the dependent variables examined; therefore, their occurrence does not invalidate conclusions from pooled data. PROC MIXED in SAS was used to evaluate lesion diameters on straw-

berry fruit caused by isolates from hosts with the ability to infect this fruit. Diameters were square root transformed prior to analysis. Isolate means were compared using least square means t tests and grouped if $P > t$ exceeded 0.05.

DNA extraction, PCR amplification conditions, and gene sequencing. Mycelia from 2- to 4-day-old cultures grown in 50 ml of Emerson media (4 g of yeast extract, 15 g of soluble starch, 1 g of K_2HPO_4 , and 0.5 g of $MgSO_4$ per liter) was collected by vacuum filtration through Whatman no. 3 filter paper and dried overnight in a centrifugal evaporator. DNA was extracted from 60 mg of the dried mycelia using a previously published cetyltrimethylammonium bromide procedure (46) and quantified by comparing the brightness of sample bands to a known quantity of lambda DNA after electrophoresis through a 1% agarose gel and ethidium bromide staining.

Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify a fragment that includes a 494- to 495-bp region containing *ITS1*, the gene encoding the 5.8S rRNA subunit, and *ITS2* (45). This combined region is described as the *ITS* region. Primers ITS1 and ITS4 anneal to genes encoding the small and large subunit rRNA, respectively, and amplify the region spanning bases 1,729 to 2,310 of a reference nuclear rDNA repeat sequence from *Colletotrichum acutatum* (GenBank accession no. AJ301936) (23). Primers GDF1 (5'-GCCGTCAACGACCCCTTCATTGA-3') and GDR1 (5'-GGGTGGAGTCGTACTTGAGCATGT-3') were used to amplify a region containing the second intron of the glyceraldehyde-3-phosphate dehydrogenase (*G3PD*) gene (17). This intron was 209 to 213 bp long depending on the isolate. The primers annealed to coding regions of the *G3PD* gene and amplified a region spanning bases 1,538 to 1,817 of a reference *C. gloeosporioides* isolate (GenBank accession no. M93427) (38). Primers GSF1 (5'-ATGCGCCGAGTACATCTGG-3') and GSR1 (5'-GAACCGTCGAAGTTCCAC-3') were used to amplify the second intron of the glutamine synthetase (*GS*) gene (17). The length of this region was 894 to 910 bp among *C. acutatum* isolates. Both primers annealed to coding regions of the *GS* and amplified a region spanning bases 326 to 1,340 of a reference *C. gloeosporioides* isolate (GenBank accession no. L78067) (35).

PCR amplifications of the full second intron of the *GS* were inconsistent from template DNA of leatherleaf fern isolates using the GSF1/GSR1 primer set. For this reason, three additional primer pairs were designed to amplify subregions of the second intron of the *GS* from leatherleaf fern isolates for additional sequencing and for RFLP analysis. Primer pairs FernGS1 (5'-GGTACGTTACACAACAACCACC-3')/FernGS3 (5'-CGTGG-AGAACAGTAACGACC-3') and FernGS4 (5'-TGGAGGTCTT-GCGAGTTGCT-3')/FernGS5 (5'-CTCCTTGAGAGTCTATGG-AGGC-3') were used to produce sequencing templates. Primers FernGS1 and FernGS5 overlap the exon/intron and intron/exon junction, respectively. Primers FernGS3 and FernGS4 annealed to regions within the second intron of *C. acutatum* isolates from leatherleaf fern. FernGS1/FernGS3 produced a 702- to 712-bp product and FernGS4/FernGS5 produced a 324- to 332-bp product. A third primer pair, FernGS1 (5'-GGTACGTTAC-ACAACAACCACC-3')/FernGS2 (5'-CGCTATTCCAGCCGG-AAGT-3') was used to amplify 289 to 292 bp of the 5' end of the *GS* second intron from leatherleaf fern isolates for RFLP analysis described below. The primers designed from fern sequence data successfully amplified a product from template DNA of all leatherleaf fern isolates.

PCR reactions to prepare template for sequencing were carried out in a 120- μ l volume with 20 ng of genomic DNA template and, to prepare product for RFLP analysis, reactions were carried out in a 25- μ l volume with 4 ng of genomic DNA template. For the ITS1/ITS4 and GDF1/GDR1 primer sets, reactions contained 1 \times reaction buffer (10 mM Tris [pH 9], 50 mM KCl, and 2 mM $MgCl_2$) with 200 μ M dNTP, 500 nM concentration of both

primers, and *Taq* DNA polymerase at 0.04 U/μl. Cycling parameters consisted of a 4-min denaturing step at 94°C; followed by 34 cycles at 94°C for 45 s, 52°C (ITS1/ITS4) or 60°C (GDF1/GDR1) for 45 s, and 72°C for 1 min; and a single step at 72°C for 5 min. For the GSF1/GSR1 primer set, the reactions contained 1× reaction buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin) with 200 μM dNTP, 400 nM concentration of both primers, and *Taq* DNA polymerase at 0.04 U/μl added during the denaturing step. The cycling parameters for GSF1/GSR1 consisted of a 4-min denaturing step at 94°C; followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and a single step at 72°C for 5 min. For the primer pairs FernGS1/FernGS2, FernGS1/FernGS3, and FernGS4/FernGS5, reactions contained 1× reaction buffer (10 mM Tris[pH 9], 50 mM KCl, and 2 mM MgCl₂) with 200 μM dNTP, 400 nM concentration of both primers, and *Taq* DNA polymerase at 0.04 U/μl. Cycling parameters consisted of a 4-min denaturing step at 94°C; followed by 34 cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min; and a single step at 72°C for 5 min. PCR product used for sequencing was concentrated to 20 ng/μl with a Microcon YM-30 centrifugal filter (Millipore, Billerica, MA) and 15-μl samples were submitted to the University of Florida Interdisciplinary Center for Biotechnology Research in Gainesville for sequencing. Sequence data were generated in forward and reverse directions from fluorescent cycle sequencing reactions using an automated sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA). All of the reported PCR primers were also used as sequencing primers, with the exception of primers FernGS2, FernGS3, and FernGS4. Two additional sequencing primers, SeqFernGS1 (5'-AGCAACTCGCAAGACCTCCA-3') and SeqFernGS2 (5'-GGCGCGTCTTCGGTGAA-3'), were used to obtain sequence data from FernGS1/FernGS3 and FernGS4/FernGS5 PCR products, respectively. These additional primers were used to alleviate sequence ambiguities caused by the occurrence of a cytosine repeat within the *GS* of leatherleaf fern isolates. Accession numbers of sequences reported to GenBank (National Center for Biotechnological Information, Bethesda, MD) are presented in Table 2.

Gene trees. Only sequence data from the *C. acutatum* isolates evaluated for pathogenicity were used for construction of gene trees. Sequence alignments were done using CLUSTAL W (41). *C. gloeosporioides* is a closely related species to *C. acutatum* and *ITS*, *G3PD*, and *GS* sequences were available from GenBank for *C. gloeosporioides* isolate NC329 (*G3PD* accession no.

DQ792849, *GS* accession no. DQ792872, and *ITS* accession no. DQ003087) (9,22). In a preliminary analysis, the potential of using this isolate as an outgroup to root trees constructed with *C. acutatum* isolates was evaluated. Inclusion of NC329 substantially elevated homoplasy and retention index (RI) values and may have interfered with proper alignment of *G3PD* and *GS* sequences from *C. acutatum* isolates. For these reasons, the isolate was excluded from final trees and the trees were midpoint rooted. However, a comparison of the position of the midpoint root to the outgroup root was reported. A heuristic search implemented in PAUP 4.0 (Sinauer Associates, Inc., Sunderland, MA) was used to find maximum parsimony (MP) trees. The search employed 1,000 random stepwise addition replicates and the tree-bisection-reconstruction branch-swapping algorithm. Gaps were not used for tree construction, and transitions and transversions were weighted equally. Support for nodes of parsimony trees were obtained from 1,000 bootstrapped data sets. Maximum likelihood (ML) trees for *ITS*, *G3PD*, and *GS* sequence data were determined from a heuristic search employing the tree bisection-reconnection algorithm in PAUP 4.0. Bayesian posterior probabilities of ML tree nodes were determined with MrBayes 3.1.2 (29). Four chains were run for each analysis using MrBayes, one cold and three heated. The temperature parameter was set to 0.2. In all, 600,000 generations were run and trees sampled every 400 generations. The burn-in period consisted of the first 200,000 generations. Sequence evolution models were determined using FINDMODEL (Los Alamos National Laboratory, Los Alamos, NM), a web implementation of MODELTEST (28). For *ITS* sequences, the F81 model (10) was used; for *G3PD* sequences, the K80 model (20); and for *GS* sequences, an HKY + gamma model (18). ML trees had the same topology as MP trees and are not reported. Bayesian posterior probability estimates are reported at nodes on MP trees. A partitioned matrix that includes aligned *ITS*, *G3PD*, and *GS* sequences for isolates used to construct trees and the actual trees depicted in Figure 1 were deposited in TreeBASE (matrix accession no. M4118 and study accession no. S2171).

Group designation. Isolates were assigned to *C. acutatum* groups by comparing generated sequences with sequences reported in public data bases for isolates already assigned a group designation. Primarily *ITS* sequences were available for comparison. Guerber et al. (17) included isolates from all five hosts in their study that identified genetic groups within *C. acutatum* from *GS* and *G3PD* sequence data, but these sequences were not deposited in a database. *ITS* sequences from some isolates in that study were available and used for comparisons. The *G3PD* and *ITS* sequences of Key lime and PFD isolates were previously compared with isolates included in the Guerber et al. (25) study. Colony color and conidia shape was also used to assign isolates to genetic groups.

Test for recombination among isolates. The partition homogeneity test (PHT), used before to detect recombination in *Aspergillus flavus* (15), was applied to test the null hypothesis that there was no evidence for recombination among *C. acutatum* isolates used to construct gene trees. The PHT creates artificial data sets in which polymorphic nucleotides are swapped randomly between loci of individuals. Subsequently, MP trees are made from the artificial data sets and the tree lengths at each locus summed. In the absence of any recombination, gene trees from different loci should be perfectly concordant and swapping nucleotides should not increase the sum of the tree lengths. Recombination events among loci would tend to introduce homoplasy into the data set. The increase in homoplasy is then detected by an increase in the sum of tree lengths in the artificial data set when compared with the original. The PHT was conducted in PAUP 4.0. The probability of obtaining the summed branch length observed for original trees in swapped data sets was based on 1,000 repetitions. Parsimony trees were determined using the same search algorithm as described earlier for *ITS*, *G3PD*, and *GS*

TABLE 2. Accession numbers for sequences from *Colletotrichum acutatum* isolates deposited into GenBank^a

Isolate code	Locus ^z		
	<i>ITS</i>	<i>G3PD</i>	<i>GS</i>
05-88	EU647299	EU647312	EU647325
05-148	EU647300	EU647313	EU647326
05-197	EU647301	EU647314	EU647327
02-163	EU647302	EU647315	EU647328
02-179	EU647303	EU647316	EU647329
03-32	EU647304	EU647317	EU647330
ALB-IND-25	EU168901	EU168905	EU647331
OCO-ARC-4	EU647305	EU647318	EU647332
STF-FTP-10	EU647306	EU647319	EU647333
Ss	EU647307	EU647320	EU647334
Hm-1	EU168903	EU168907	EU647335
KLA-Anderson	EU647308	EU647321	EU647336
05-155	EU647309	EU647322	EU647337
05-161	EU647310	EU647323	EU647338
05-200	EU647311	EU647324	EU647339

^a Sequences for an isolate were deposited into GenBank if all three loci were sequenced and the pathogenicity profile of the isolate was determined.

^z *ITS* = the *ITS1*, 5.8S rRNA, and *ITS2* regions of the rDNA repeat; *G3PD* = intron 2 of the glyceraldehyde 3-phosphate dehydrogenase gene; and *GS* = intron 2 of the glutamine synthetase gene.

sequences. The PHT test, under certain conditions, has a higher than expected type I error (2,8). These conditions include excess homoplasy in at least one or more data sets, unequal branch lengths, and rate variance among data sets (2). At noise levels observed within trees used for this test (RI > 0.98), the type I error rate should not have exceeded expected values using all of the sequence data from Florida isolates (2). However, analyses were also conducted without the blueberry isolates and *ITS* sequence data. The blueberry isolates were excluded to reduce branch-length variation and to determine the effect of excluding group C isolates, which produce a teleomorph, on the results of the PHT. The *ITS* region was excluded because the mutation rate of this sequence was lower than that of the others.

Comparison of leatherleaf fern isolates from the United States and Costa Rica. Among leatherleaf fern isolates from the United States used in pathogenicity assays, there were two distinct *G3PD* and *GS* sequences. To further evaluate the genetic diversity at these two loci within the leatherleaf fern population, the *G3PD* and *GS* region was sequenced from an additional isolate obtained at each of the three sites in the United States where the isolates used for pathogenicity assays were obtained, two isolates from a second fernery in Pierson, FL, and five isolates from different ferneries in two provinces of Costa Rica. MP and ML trees were then constructed using sequence from the *G3PD* and *GS* regions of these isolates and isolates previously evaluated. The tree construction methodology was the same as that reported above. One to twelve additional leatherleaf fern isolates from each of the nine sites in the United States and Costa Rica were evaluated by RFLP analysis. Restriction digestion of the *GDF1/GDR1* PCR product with the enzyme *RcaI* yielded

either a 273-bp uncut product or bands 208 and 65 bp in length. Restriction digestion of the *FernGS1/FernGS2* PCR product with *BstUI* yielded either a 292-bp uncut product or bands 149 and 140 bp in length. Restriction digests were done according to the manufacturer's instructions and bands separated through a 2% agarose gel.

RESULTS

Cross-pathogenicity of isolates. The incidence of anthracnose lesions on strawberry fruit was highest for fruit inoculated with strawberry isolates (Table 3). Fruit inoculated with all three blueberry isolates had a higher incidence of anthracnose than control fruit, although only one of the three blueberry isolates (05-148) had an incidence similar to any strawberry isolate. In addition to the higher incidence, lesions on fruit infected with strawberry isolates were larger than those on fruit infected with blueberry isolates (Table 3). The low level of disease in the control (1.6%) resulted from natural infections in the field where strawberry fruit were harvested. Strawberry fruit inoculated with only one isolate, PFD isolate ALB-IND-25, from a host other than blueberry or strawberry had an anthracnose incidence higher than the control. Given that none of the other PFD isolates had more disease than the control, the relatively low disease incidence for fruit inoculated with ALB-IND-25, and the numerous pairwise comparisons with the control that were conducted, the statistically higher disease incidence for this isolate may have resulted from chance. Blueberry ripe-rot incidence was higher than the control only for fruit inoculated with isolates from blueberry (Table 3). For inoculations on strawberry, leatherleaf fern, and Key lime foliage,

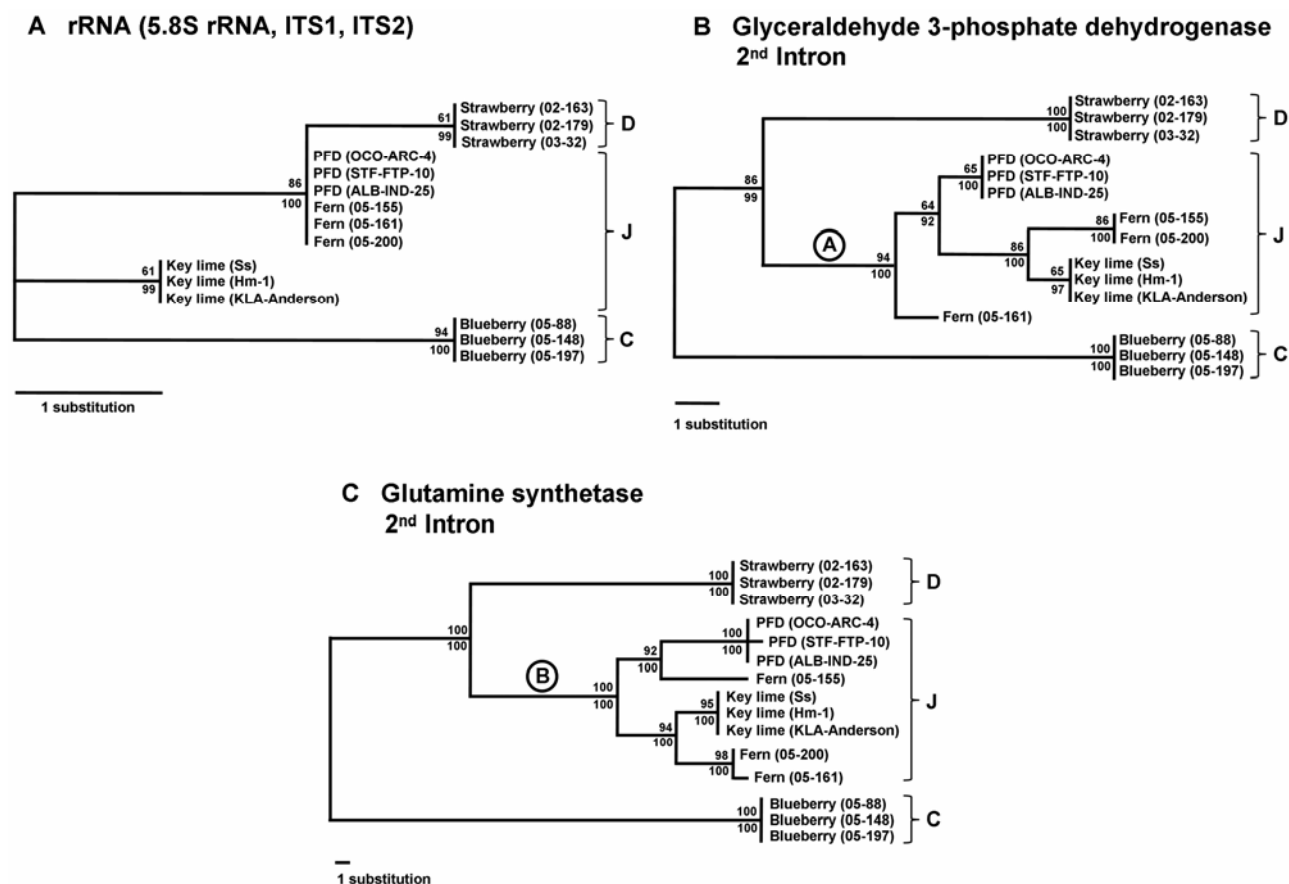


Fig. 1. Maximum parsimony (MP) trees based on sequence from the **A**, internal transcribed spacer (*ITS*), 5.8S, and *ITS2* regions of the ribosomal DNA; **B**, the second intron of the glyceraldehyde-3-phosphate dehydrogenase gene (*G3PD*); and **C**, the second intron of the glutamine synthetase (*GS*) gene. The numbers above nodes are the bootstrap values determined for MP trees (shown) and numbers below nodes are Bayesian posterior probabilities for maximum likelihood trees that had the same topologies as MP trees (not shown). Letters D, J, and C to the right of trees show group designations for isolates to the left. The circled letters A and B identify branches leading into clades depicted in Figure 2.

only plants inoculated with isolates from the homologous host had disease ratings or percentages of necrotic tissue higher than levels observed for the controls (Table 4). The percentage of open tangelo flowers inoculated with PFD, Key lime, blueberry, and strawberry isolates that were necrotic at 5 days postinoculation was higher than for control flowers (Table 5). The percentage of necrotic flowers in clusters inoculated with leatherleaf fern isolates was no greater than in control clusters. Among the isolates that produced necrosis on flowers, PFD isolates had the highest percentage of flowers necrotic (90.2 to 100%). Among isolates from blueberry, strawberry, and Key lime, there was no trend suggesting that isolates from any particular host were more or less pathogenic than isolates from the others. Based on percentages of buds that gave rise to persistent calyces, isolates were not easily separated (Table 5). Persistent calyx formation tended to be greatest for buds inoculated with PFD isolates but the highest percentage of flower buds that gave rise to persistent calyces was for a Key lime isolate. Isolates from the other hosts that caused symptoms on flower petals (Key lime, blueberry, and strawberry) also produced persistent calyces at percentages comparable with PFD isolates.

Analysis of gene trees. There was one MP tree constructed from the *ITS*, *G3PD*, and *GS* regions of the isolates used for pathogenicity studies (Fig. 1). Within the *ITS* region, there were seven parsimony informative sites and no uninformative sites. The MP tree constructed from sequences in this region required seven nucleotide transitions, the homoplasy index (HI) was 0.0, and the RI was 1.0. There were 27 parsimony informative sites and one uninformative site within the *G3PD* intron. The MP tree for this region required 30 transitions, the HI = 0.033, and the RI = 0.99. Within the *GS* intron, there were 85 parsimony informative and four uninformative sites. The MP tree for this region required 95 transitions, the HI = 0.042, and the RI = 0.98. ML trees constructed for each region had the same topology as MP trees (not shown). Overall statistical support for the occurrence of clades observed using both tree construction methods was strong, as demonstrated by high bootstrap values and Bayesian posterior probabilities. However, support for clades was reduced in the tree constructed from *ITS* sequence data and in the portion of the tree constructed from *G3PD* sequence data containing PFD, leather-

leaf fern, and Key lime isolates. This was likely due to the lower substitution rate of the *ITS* region and the relatively close relationship between the PFD, leatherleaf fern, and Key lime isolates. In the trees constructed from *ITS*, *G3PD*, and *GS* sequences, isolates from strawberry, Key lime, and blueberry grouped into three different clades, with isolates from the same host composing each clade (Fig. 1A to C). There was no sequence variation within these clades. Based on *ITS* sequence data, PFD and leatherleaf fern isolates grouped together (Fig. 1A), although there was an insertion or deletion that was not used for phylogenetic analysis that distinguished the isolates. PFD isolates formed a distinct clade in trees constructed from the *G3PD* and *GS* intron regions (Fig. 1B and C). There was a single nucleotide difference in the *GS* region between isolates from PFD-affected citrus (Fig. 1C). Leatherleaf fern isolates did not occur in the same clade in trees constructed from the *G3PD* and *GS* sequences (Fig. 1B and C). Based on *G3PD* sequence data, isolates 05-155 and 05-200 grouped together and, based on the *GS* sequence data, isolates 05-161 and 05-200 grouped together. Six substitutions in the *G3PD* differentiated isolate 05-161 from isolates 05-155 and 05-200, and 13 to 14 substitutions and four insertion or deletion events in the *GS* region differentiated isolate 05-155 from isolates 05-161 and 05-200.

Inclusion of *C. gloeosporioides* isolate NC329 as an outgroup rooted *G3PD* and *GS* trees on the branch separating blueberry isolates from all other isolates, producing *C. acutatum* isolate clades identical to those of the reported midpoint-rooted trees for these genes (Fig. 1B and C). For the *ITS* tree, inclusion of *C. gloeosporioides* isolate NC329 rooted the tree on the branch separating Key lime and blueberry isolates from PFD, leatherleaf fern, and strawberry isolates. This differed from the reported *ITS* tree which did not group Key lime and blueberry isolates into a clade (Fig. 1A).

Group designations. The blueberry isolates were all group C isolates; those from strawberry were group D isolates; and the PFD, Key lime, and Leatherleaf fern isolates were group J isolates (Fig. 1A to C). The *ITS* region from blueberry isolates was identical to the *ITS* region of isolates from multiple hosts reported in GenBank. Among the isolates were two well-characterized isolates, PCN5 (GenBank accession no. AF272786) from pecan and

TABLE 3. Anthracnose incidence and mean lesion diameter on detached strawberry fruit 8 days after inoculation and ripe rot incidence of blueberry fruit 7 days after harvest^w

Host or disease (code) ^x	Strawberry			Blueberry	
	Fruit inoculated (n)	Decayed fruit (%) ^y	Lesion diameter (mm) ^z	Fruit inoculated (no.)	Decayed fruit (%) ^y
Strawberry (02-163)	64	64.1 a	12.1 a	62	3.2 c
Strawberry (03-32)	63	50.8 a	11.6 a	57	8.8 c
Strawberry (02-179)	65	49.2 ab	10.6 ab	67	7.4 c
Blueberry (05-148)	59	32.2 bc	6.3 c	69	39.1 b
Blueberry (05-197)	62	25.8 cd	6.9 c	75	42.7 b
Blueberry (05-88)	62	19.4 cde	7.9 bc	64	62.5 a
PFD (ALB-IND-25)	61	14.8 def	...	62	3.2 c
Key lime (Ss)	63	11.1 efg	...	80	1.3 c
Key lime (KLA-Anderson)	61	6.6 efg	...	59	3.4 c
PFD (STF-FTP-10)	63	6.4 fg	...	55	1.8 c
Fern (05-200)	64	6.3 fg	...	70	2.9 c
PFD (OCO-ARC-4)	63	4.8 fg	...	51	2.0 c
Fern (05-155)	62	3.2 g	...	57	7.0 c
Fern (05-161)	62	3.2 g	...	73	1.4 c
Control	61	1.6 g	...	102	2.0 c
Key lime (Hm-1)	63	1.6 g	...	52	1.9 c

^w Blueberry fruit were inoculated on the plant when green. *Colletotrichum acutatum* isolates from different hosts were used for inoculations or fruit were sprayed with water as a control.

^x Original host or disease and isolate code. Isolates from postbloom fruit drop-infected citrus are identified as PFD isolates. All other isolates are identified by the common name of their host.

^y *P* value obtained from Fisher's exact test comparing means followed by the same letter exceeded 0.05.

^z *P* value obtained from least squares means *t* tests comparing lesion diameters followed by the same letter exceeded 0.05. Lesion diameters of isolates from Key lime, fern, and PFD-affected citrus were not compared due to the low incidence of fruit with lesions. Reported means for lesion diameter were calculated by backtransforming means for the square root of lesion diameter.

isolate ATCC MYA-662 (GenBank accession no. DQ286121) from apple. The pink-pigmented isolate PCN5 was described as a subgroup I isolate by Freeman et al. (12), a group that corresponds to group C of Guerber et al. (17,33). Isolate ATCC MYA-662 was also characterized as a group C isolate (17). Group C is composed primarily of fruit-rot isolates from hosts such as apple, peach, blueberry, almond, pecan, and strawberry (12,17). Isolates from this group are typically pink in culture and have fusiform conidia (3,12). The blueberry isolates obtained in the present study were chromogenic pink in culture and had fusiform conidia, similar to blueberry isolates from other regions of the United States (6,32). There was no evidence that the blueberry isolates belonged to a new group, designated group A9, which is composed of isolates from blueberry as well as a number of other hosts in Australia (44). The *ITS* region of strawberry isolates also matched that of isolates from multiple hosts in GenBank. Among the isolates was the strawberry isolate TUT-5954 (GenBank accession no. AF207794), described by Denoyes-Rothan et al. (6) as a CA-clonal group isolate and strawberry isolate S2 (39) (GenBank accession no. DQ454018) that groups with the recently described epitype for *C. acutatum* (40). Isolate TUT-5954 possessed an arbitrarily primed PCR DNA banding pattern identical to a strawberry isolate, TUT-7E, described as a group D isolate (11,17). The Florida strawberry isolates produced fusiform conidia and were orange in culture. Isolates from the globally distributed CA-clonal group have these same morphological traits (6). Sequences of the *ITS* and *G3PD* regions for the three PFD and three Key lime isolates were previously compared with a population of PFD and Key lime isolates that included isolates from Guerber et al. (17) and were found to be group J isolates (25). The *ITS* region of Florida leatherleaf fern isolates was the same as that of leatherleaf fern isolates from Costa Rica (30). Sequences for fern isolates from the United States were not present in any public database; however, leatherleaf fern isolates were included in published trees constructed from *GS* and *G3PD* sequences (17). In trees constructed from both regions, leatherleaf fern isolates fell into the group J clade along with PFD and Key lime isolates, and the distance between Key lime isolates and leatherleaf fern isolates was less than that between Key lime and PFD isolates. Most of the leatherleaf fern isolates occupied this same position (Fig. 1B and C). Also, isolates from group J generally do not produce

TABLE 4. Strawberry anthracnose disease ratings and the percentage of immature leatherleaf fern and Key lime foliage that was necrotic after inoculation with *Colletotrichum acutatum* isolates from different hosts or sprayed with water as a control^y

Host or disease (code) ^z	Disease rating (0–5)		Necrosis (%)	
	Strawberry	Fern	Key lime	
Strawberry (02-179)	3.8 a	0.0 c	0.0 b	
Strawberry (03-32)	3.5 a	0.0 c	0.0 b	
Strawberry (02-163)	2.9 a	0.5 c	0.1 b	
Key lime (Hm-1)	0.4 b	0.0 c	60.6 a	
Fern (05-161)	0.1 b	78.8 a	0.0 b	
Fern (05-155)	0.1 b	17.5 b	0.0 b	
Key lime (Ss)	0.1 b	0.0 c	31.8 a	
Fern (05-200)	0.0 b	61.3 ab	0.0 b	
Blueberry (05-197)	0.0 b	0.3 c	0.0 b	
Key lime (KLA-Anderson)	0.0 b	0.0 c	29.9 a	
PFD (STF-FTP-10)	0.0 b	0.0 c	0.1 b	
PFD (ALB-IND-25)	0.0 b	0.0 c	0.0 b	
Blueberry (05-88)	0.0 b	0.0 c	0.0 b	
Blueberry (05-148)	0.0 b	0.0 c	0.0 b	
PFD (OCO-ARC-4)	0.0 b	0.0 c	0.0 b	
Control	0.0 b	0.0 c	0.0 b	

^y *P* value obtained from the Wilcoxon rank sum test comparing disease ratings among isolates with means followed by the same letter exceeded 0.05.

^z Original host or disease and isolate code. Isolates from postbloom fruit drop-infected citrus are identified as PFD isolates. All other isolates are identified by the common name of their host.

fusiform conidia (4,23) and this was true for the PFD, Key lime, and leatherleaf fern isolates.

Evidence for recombination. The topology of trees in the area that included the strawberry, PFD, leatherleaf fern, and Key lime isolates was not consistent. For example, strawberry isolates occupied a different position relative to PFD, leatherleaf fern, and Key lime in the tree constructed from *ITS* data and the position of leatherleaf fern isolates 05-161 and 05-200 was not consistent in the trees constructed from *G3PD* and *GS* introns. These discrepancies in tree topologies suggested a history of recombination among the sampled isolates. Assuming a complete absence of any historical recombination, an MP tree constructed from sequences of each region combined should have the same number of steps as the sum of steps for trees constructed from regions individually because recombination would not introduce additional homoplasy. The number of steps required to construct a tree when all sequences were combined was nine steps greater than the sum of individual tree lengths for the three loci (Table 6). When *ITS*

TABLE 5. Postbloom fruit drop symptoms on Orlando tangelo flower clusters inoculated with *Colletotrichum acutatum* isolates from different hosts or sprayed with water

Host or disease (code) ^x	Open flowers, total buds ^y	Necrotic flowers (%) ^z	Persistent calyxes (%) ^z
PFD (ALB-IND-25)	80, 155	100.0 a	6.5 bcde
PFD (OCO-ARC-4)	93, 158	97.9 a	13.3 ab
PFD (STF-FTP-10)	92, 199	90.2 b	11.6 ab
Key lime (Hm-1)	86, 187	46.5 c	17.6 a
Blueberry (05-197)	73, 177	45.2 cd	6.8 bcd
Blueberry (05-88)	67, 178	41.8 cde	12.9 ab
Blueberry (05-148)	73, 159	38.4 cde	7.6 bc
Strawberry (03-32)	59, 124	37.3 cde	4.8 cdef
Key lime (Ss)	76, 157	34.2 cde	2.5 cdef
Strawberry (02-163)	90, 210	33.3 cde	1.0 fg
Key lime (KLA-Anderson)	92, 189	30.4 de	5.3 cde
Strawberry (02-179)	71, 172	26.8 e	2.9 cdef
Fern (05-155)	55, 147	10.9 f	2.0 defg
Fern (05-200)	86, 234	4.7 f	2.6 def
Fern (05-161)	70, 129	4.3 f	1.6 efg
Water control	99, 214	3.0 f	0.0 g

^x Original host or disease and isolate code. Isolates from postbloom fruit drop-infected citrus are identified as PFD isolates. All other isolates are identified by the common name of their host.

^y Total number of open flowers evaluated to determine the percentage of flowers with necrosis and the total number of buds inoculated used to determine the percentage of buds that became persistent calyxes.

^z *P* value obtained from Fisher's exact test comparing means followed by the same letter exceeded 0.05.

TABLE 6. Actual and minimum possible maximum parsimony tree lengths for trees constructed from sequences at three loci separately and combined

Excluded isolates, locus ^y	Tree length	Minimum tree length	Excess steps
None			
<i>ITS</i>	7	7	0
<i>G3PD</i>	30	29	1
<i>GS</i>	95	91	4
<i>ITS</i> , <i>G3PD</i> , <i>GS</i> combined	141	132 ^z	9
<i>G3PD</i> , <i>GS</i> combined	132	125 ^z	7
Blueberry			
<i>ITS</i>	4	4	0
<i>G3PD</i>	18	18	0
<i>GS</i>	56	53	3
<i>ITS</i> , <i>G3PD</i> , <i>GS</i> combined	85	78 ^z	7
<i>G3PD</i> , <i>GS</i> combined	81	74 ^z	7

^y Host of the isolates shown in Figure 1. *ITS* = the *ITS1*, 5.8S rRNA, and *ITS2* regions of the rDNA repeat; *G3PD* = intron 2 of the glyceraldehyde 3-phosphate dehydrogenase gene; and *GS* = intron 2 of the glutamine synthetase gene.

^z The minimum possible length for the tree constructed from loci combined was the sum of the actual tree lengths for trees constructed using sequence from each locus individually.

sequences or the blueberry isolates were excluded from the analysis, the number of steps required to construct a tree when all sequences were combined was seven greater than the sum of individual tree lengths. These results suggest that combining sequences introduced additional homoplasy and that most of the observed incongruence was between *GS* and *G3PD* trees in the clade that included isolates from strawberry, citrus, and leatherleaf fern. The PHT was used to evaluate the null hypothesis that there is no history of recombination among lineages statistically. After nucleotide swapping between loci, the probability of obtaining a sum of tree lengths equal to or less than that observed was 0.001 when all loci and isolates were included in the analysis. When the *ITS* locus or sequence from blueberry isolates was excluded from the analysis, this probability did not change. The *P* values for the PHT test indicate that the null hypothesis (i.e., there is no history of recombination among lineages) should be rejected.

Comparison of leatherleaf fern isolates from the United States and Costa Rica. The occurrence of dissimilar genotypes within the *G3PD* and *GS* regions of the three isolates initially characterized from Florida and the presence of multiple combinations of the *G3PD* and *GS* genotypes suggested that this population might be highly variable and evidence for recombination easily detected. The different *G3PD* genotypes could be distinguished from one another with the restriction enzyme *RcaI* and the *GS* genotypes by the restriction enzyme *BstUI*. The three observed genotype combinations are referred to as *GD-RcaI*(-)/*GS-BstUI*(+), *GD-RcaI*(-)/*GS-BstUI*(-), and *GD-RcaI*(+)/*GS-BstUI*(-). The *G3PD* and *GS* regions from five additional isolates from Florida were sequenced along with five isolates from different sites in Costa Rica. Costa Rican leatherleaf fern isolates

were identical to one another, with the exception of a single-nucleotide polymorphism in the *G3PD* region, and only differed from isolate 05-161 from Florida by one nucleotide in the *GS* region and either zero or one nucleotide in the *G3PD* region (Fig. 2). These isolates were all *GD-RcaI*(+)/*GS-BstUI*(-). No new genotypes were observed among the five additional Florida isolates from which sequence data were obtained. Restriction enzyme analysis of 26 additional isolates from Florida and 12 from Costa Rica did not reveal the presence of the fourth genotype, *GD-RcaI*(+)/*GS-BstUI*(+), that could occur by recombination of *G3PD* and *GS* regions, even though isolates with both *GD-RcaI* and *GS-BstUI* alleles were present at sampling sites in Florida (Table 7). Also, analysis of the additional 12 isolates from Costa Rica did not reveal the presence of the two other genotypes observed in Florida (Table 7).

DISCUSSION

Pathogenicity assays indicated that there are clear pathological differences between the genetically differentiable strains that explain the lack of migration of isolates between hosts to cause disease. Only isolates from group C and the recently described group A9 (44) have been isolated from blueberry. The present study indicates that isolates from Key lime, leatherleaf fern, PFD-affected citrus, and strawberry, from groups J and D, lack the ability to infect fruit of this host. Only isolates from blueberry and strawberry infected strawberry fruit. The blueberry isolates in the present study were group C isolates that are commonly isolated from fruit of hosts such as peach, apple, pecan, and strawberry in addition to blueberry (3,17,33). In fruit-rot assays, group C isolates from apple and peach produced lesions on a wide variety

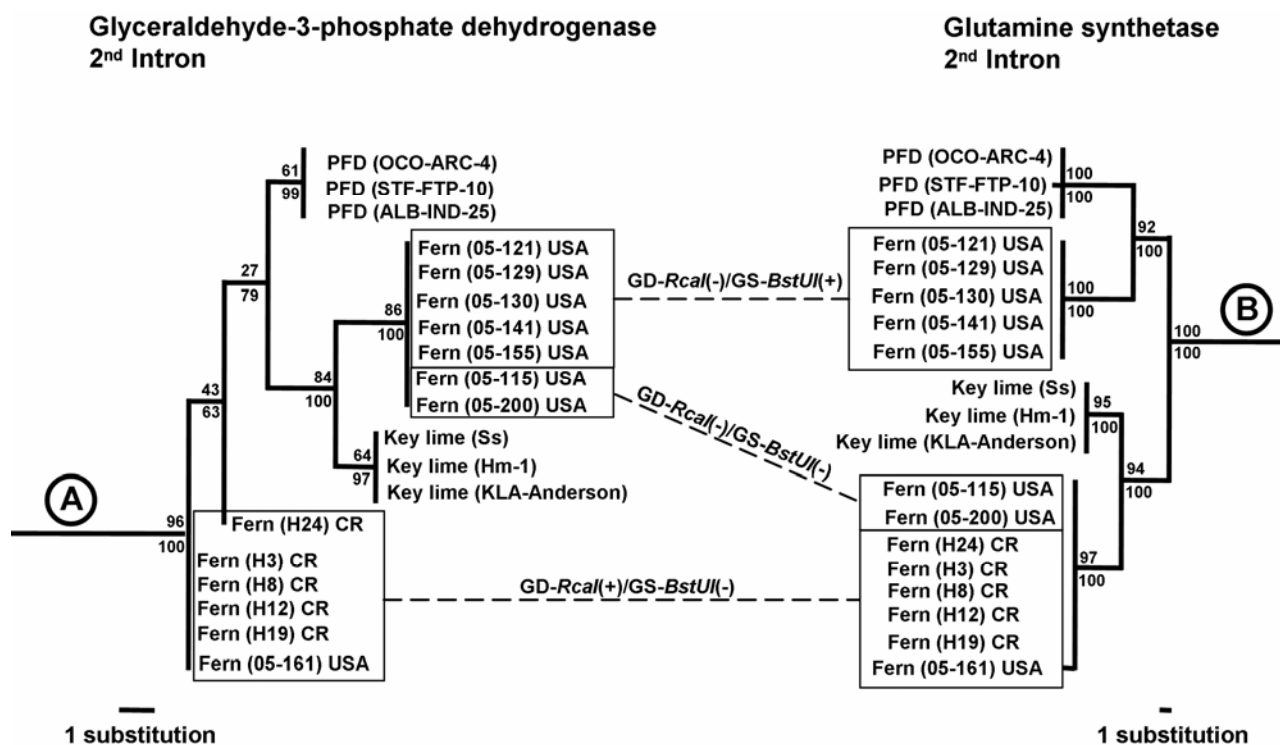


Fig. 2. Clade containing postbloom fruit drop (PFD), Key lime, and leatherleaf fern isolates in maximum parsimony (MP) trees constructed from the either the second intron of the glyceraldehyde-3-phosphate dehydrogenase gene (*G3PD*) or the second intron of the glutamine synthetase gene (*GS*). Branches labeled with circled letters A and B correspond to branches with the same label in Figure 1 with five leatherleaf fern isolates from the United States and five leatherleaf fern isolates from Costa Rica added. The clade identified by the letter A was observed in four MP trees. The position of isolate Fern (H19) CR was different in these four trees. The depicted MP tree had the same topology as a maximum likelihood (ML) tree constructed with the same data. The clade identified by the letter B occurred in the single MP tree which had the same topology as an ML tree constructed from the same data. The numbers above nodes are the bootstrap values determined for MP trees (shown) and numbers below nodes are Bayesian posterior probabilities for ML trees (not shown). Boxes connected by dashed lines contain leatherleaf fern isolates with one of three restriction fragment length polymorphism genotypes: *GD-RcaI*(+)/*GS-BstUI*(+), *GD-RcaI*(-)/*GS-BstUI*(+), or *GD-RcaI*(-)/*GS-BstUI*(-).

of fruit (13), suggesting that isolates from this group have the ability to cross-infect fruit from multiple hosts. Group C contains at least some members of the CA-variable group on strawberry (6,12,17,33), providing additional evidence that the blueberry isolates could cross-infect strawberry in Florida. However, isolates from the CA-variable group have not been found on strawberry in Florida (43). The incidence of strawberry fruit infected by blueberry isolates was lower and lesion size was smaller; therefore, the progression of an epidemic caused by a blueberry isolate would be slower than an epidemic caused by a CA-clonal isolate. Blueberry isolates also failed to cause disease on strawberry foliage and, therefore, epidemics might not be sustainable in the absence of fruit. Another possible explanation for the absence of the blueberry strain on strawberry in Florida is that the climate is unfavorable. *C. acutatum* does not appear to survive very well on blueberry fruit in Florida, where most of the ripe rot appears to be caused by *C. gloeosporioides*, but this is not the case in temperate regions further north (32).

On foliage of strawberry, Key lime, and leatherleaf fern, only homologous host isolates caused disease. These results suggest that the range of isolates capable of causing foliar diseases on these hosts is limited and they are consistent with previous studies examining the genetic relatedness of isolates from these hosts and cross-pathogenicity studies using some of these hosts. In one study, there was evidence for host differentiation among group J isolates obtained from PFD-affected citrus flowers, Key lime, and leatherleaf fern (17). PFD isolates have also been shown to either fail to produce disease or produce substantially less disease on Key lime (1,25). A single genotype responsible for anthracnose on strawberry appears to have been distributed throughout most of the world, which suggests that strains able to reproduce on this host are few (6).

On Orlando tangelo flowers, isolates from four of five hosts caused PFD symptoms. However, the isolates from PFD-affected citrus were more aggressive. Consistent with the pathogenicity data, Key lime anthracnose isolates have been recovered from PFD-affected orange flowers in areas where this host is grown close to Key lime (25), and the ability of Key lime anthracnose isolates to infect citrus flowers and cause persistent calyx formation has been demonstrated (1). In addition to Key lime isolates, isolates from blueberry and strawberry produced flower lesions and increased the incidence of persistent calyxes relative to the control. These isolates, based on gene sequences, tended to be less similar to the PFD isolates than leatherleaf fern isolates which did not produce lesions on flowers. Even though isolates from other hosts cause PFD symptoms, isolates from PFD-affected flowers are exclusively PFD-type isolates, with the exception of KLA-type isolates near Key lime plantings (25). It may be that differences in aggressiveness between strains explain this phenomenon, although the ability of strains to survive between seasons on nonreproductive tissue might also be a contributing factor.

A teleomorph has been associated with only group C, F, and J4 isolates (17,21), which includes only the blueberry isolates from the present study. It is presumed that the mode of reproduction for other groups is vegetative. The results of the PHT indicated that, although these other groups may reproduce vegetatively, there is evidence for recombination. More tangible evidence for recombination came from the genotypes observed for leatherleaf fern isolates. The *G3PD* and *GS* sequences occurred in three as opposed to two combinations. Neither of the two divergent *G3PD* and *GS* sequences within the leatherleaf fern population grouped together into a clade that did not include an isolate from at least one other host, which excludes the possibility that different rates of evolution within clonal lineages accounted for the different genotypes. The absence of a fourth potential combination suggests that recombination is not a frequent event, which is consistent with the absence of any descriptions of a teleomorph produced from group J isolates. Both *G3PD* and *GS* sequences co-occurred at two sites; thus, geographic separation of the different strains cannot account for the absence of this fourth genotype. The lack of homoplasy in trees constructed from individual loci also suggests that recombination is not frequent. The different position occupied by the strawberry isolates relative to the group J isolates in the tree constructed from the *ITS* region also indicates that recombination might occur between more distantly related strains than the group J isolates.

The β -tubulin-2 gene, the *ITS* region, the *G3PD* second intron, and the *GS* second intron have all been used by others to identify groups within *C. acutatum* (17,33,44). Generally, isolate groups determined from gene trees for each genomic region contain the same set of isolates; however, the relationship between some isolate groups differs. Trees reported in the literature from all four regions were in good agreement with the trees generated from the Florida sample in that group D and J isolates consistently form a clade with group C isolates as an outgroup. Conflicts in the reported trees occur with respect to the position of J4 and F isolates, two groups that were not found in the Florida sample. Recombination among groups might account for the differences.

It has been suggested that the *C. acutatum* strains responsible for anthracnose of leatherleaf fern in Florida were imported into the state from Costa Rica, where the disease first appeared (24). However, this hypothesis was not supported by the observation that only one of three leatherleaf fern genotypes present in Florida was present among isolates from Costa Rica. In a previous study, based on universally primed (UP)-PCR fingerprints, there were 10 different *C. acutatum* genotypes on leatherleaf fern in Costa Rica within a sample population of 40 isolates at four sites. This indicates that there is genetic variation within the Costa Rican population and that this variation might be reflected in sequences from the *G3PD* and *GS* regions (30). Possibly, the Costa Rica sample that was compared with the Florida sample was not large enough to detect all three genotypes observed in Florida. However, the sample contained 17 isolates from five sites and should

TABLE 7. Combinations of glyceraldehyde 3-phosphate dehydrogenase intron 2 restriction site polymorphism, GD-*RcaI* (+ or –), and glutamine synthetase intron 2 restriction site polymorphism, GS-*BstUI* (+ or –), at four sites in Florida and five sites in Costa Rica

Site	No. of isolates	GD- <i>RcaI</i> (+) GS- <i>BstUI</i> (+)	GD- <i>RcaI</i> (+) GS- <i>BstUI</i> (–)	GD- <i>RcaI</i> (–) GS- <i>BstUI</i> (+)	GD- <i>RcaI</i> (–) GS- <i>BstUI</i> (–)
Crescent City, FL, USA	14	0	9	5	0
Pierson, FL, USA (FJ)	5	0	1	4	0
Pierson, FL, USA (VP)	5	0	0	5	0
Seville, FL, USA	10	0	0	0	10
Florida combined	34	0	10	14	10
Fraijanes, Alajuela, Costa Rica	3	0	3	0	0
San Pablo, Alajuela, Costa Rica	3	0	3	0	0
Cervantes, Cartago, Costa Rica	2	0	2	0	0
Tejar, Cartago, Costa Rica	4	0	4	0	0
Tres Ríos, Cartago, Costa Rica	5	0	5	0	0
Costa Rica combined	17	0	17	0	0
All sites combined	51	0	27	14	10

have identified a large proportion of the variance observed in the prior study. In addition, in the prior study, the genetic distances between leatherleaf fern isolates were much less than the distances between leatherleaf fern isolates and a group J citrus isolate (30), whereas the distances between leatherleaf fern isolates in Florida based on *G3PD* and *GS* sequences were as large as the distances between PFD and leatherleaf fern isolates. Together, these observations suggest that it is unlikely that the described variation in UP-PCR fingerprints correlates with variation in the *G3PD* and *GS* introns observed among Florida isolates. A second possible explanation for the unique genotypes in Florida is that they resulted from two hybridization events with another strain after importation. If this were true, then Costa Rican isolates should occupy the same position within the trees constructed from both *G3PD* and *GS* regions, but this was not the case because only GD-*RcaI*(-)/GS-*Bst*UI(-) genotype isolates from Florida occupied a consistent position in both trees. A third explanation, consistent with the data, is that anthracnose on leatherleaf fern in Costa Rica was imported from Florida, or a third location was the source of strains present in Florida and Costa Rica. It is also worth noting that the *ITS* sequence from fern isolates was identical to that of *Colletotrichum* spp. isolated from wax apple, papaya, mango, and pepper in Taiwan and Thailand (39; deposited without published reference) (GenBank accession nos. DQ454012, EF608061, EF608055, and EF608052). This suggests that *C. acutatum* strains from leatherleaf fern might cause disease on a more taxonomically diverse group of plants, but none of these plant species were included in the present study.

In conclusion, *C. acutatum* isolates from anthracnose-affected Key lime, strawberry, and leatherleaf fern; citrus with PFD symptoms; and ripe-rot-affected blueberry in Florida were genetically distinct from one another and isolates were more pathogenic or the only pathogen on hosts or tissue from which they were isolated. The group designations of the isolates were consistent with those for isolates from these hosts described in other studies, indicating that the group designations provide information that could be used to predict the pathogenicity of isolates. However, within genetic groups, strains may still display host preference as demonstrated for group J isolates from leatherleaf fern, PFD-affected citrus, and Key lime. Results of pathogenicity tests also suggest that senescing tissues, such as flower petals and fruit, are more likely to be infected by genetically divergent strains than physiologically active leaf tissue. Although no teleomorph has been reported for the *C. acutatum* strains isolated from fern, strawberry, and citrus in the state, there was evidence for historical recombination among these isolates. Although there is no clear mechanism by which this recombination might occur, it suggests a way in which novel *C. acutatum* genotypes can be produced from lineages with no apparent sexual stage.

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