Comparison of *Colletotrichum orbiculare* and Several Allied *Colletotrichum* spp. for mtDNA RFLPs, Intron RFLP and Sequence Variation, Vegetative Compatibility, and Host Specificity

B. Liu, L. A. Wasilwa, T. E. Morelock, N. R. O'Neill, and J. C. Correll

First, second, and fifth authors: Department of Plant Pathology, and third author, Horticulture Department, University of Arkansas, Fayetteville 72701; and fourth author: U.S. Department of Agriculture–Agricultural Research Service, Beltsville, MD 20705.
Accepted for publication 16 May 2007.

ABSTRACT

Liu, B., Wasilwa, L. A., Morelock, T. E., O'Neill, N. R., and Correll, J. C. 2007. Comparison of *Colletotrichum orbiculare* and several allied *Colletotrichum* spp. for mtDNA RFLPs, intron RFLP and sequence variation, vegetative compatibility, and host specificity. *Phytopathology* 97:1305–1314.

Based on spore morphology, appressorium development, sequence similarities of the rDNA, and similarities in amplified restriction fragment length polymorphism (AFLP), it has been proposed that *Colletotrichum orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* represent a single phylogenetic species, *C. orbiculare*. In the current study, the phylogenetic relationship among isolates in the *C. orbiculare* species complex was reassessed. In all, 72 isolates of *C. orbiculare* from cultivated cucurbit or weed hosts, *C. trifolii* from alfalfa, *C. lindemuthianum* from green bean, and *C. malvarum* from prickly sida (*Sida spinosa*) were examined for mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs), RFLPs and sequence variation of a 900-bp intron of the glutamine synthetase gene and a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase gene, and vegetative compatibility. In addition, host specificity was examined in foliar inoculations on cucurbit, bean, and alfalfa hosts. Inoculations also were conducted on cucumber fruit. Genetically distinct isolates, based on vegetative compatibility, within the species complex (*C. orbiculare*, *C. trifolii*, and

C. malvarum) had an identical mtDNA haplotype (haplotype A) when examined with each of three different restriction enzymes. Isolates of *C. lindemuthianum* had a very similar mtDNA haplotype to haplotype A, with a single polymorphism detected with the enzyme *Hae*III. The four species represent a phylogenetically closely related group based on a statistical analysis of the 900- and 200-bp intron sequences. However, distinct RFLPs in the 900-bp intron were consistently associated with each species and could be used to qualitatively and quantitatively distinguish each species. Furthermore, each of the species showed distinct host specificity, with isolates of *C. orbiculare* (from cucurbits), *C. lindemuthianum*, and *C. trifolii* being pathogenic only on cucurbits, green bean, and alfalfa, respectively. Consequently, distinct and fixed nucleotide, or genotypic (intron sequences and RFLPs) and phenotypic (host specificity) characteristics can be used to distinguish *C. orbiculare*, *C. lindemuthianum*, and *C. trifolii* from one another; therefore, they should be recognized as distinct species. This species delineation is consistent with the most current species concepts in fungi. More isolates and further characterization is needed to determine whether *C. orbiculare* from cocklebur and *C. malvarum* represent distinct species. RFLPs of the 900-bp intron may represent a relatively inexpensive, reliable, and useful diagnostic tool for general species differentiation in the genus *Colletotrichum*.

Additional keywords: *C. lagenarium*.

Colletotrichum spp. cause anthracnose diseases of many economically important crops worldwide (40). The classification of species within this genus traditionally has been based on conidial shape and size, presence of sclerotia, appressoria production, and, often, on host origin. Therefore, the species concept for the genus *Colletotrichum* is based on both morphological characteristics and host specificity (40). Von Arx (47,48) recognized 13 species in the genus *Colletotrichum* based on conidial shape and size, host range, and type of appressorium produced. Within the species *Colletotrichum gloeosporioides*, nine specialized forms were recognized (47). Sutton (40) later elevated each of these nine forms to a distinct species, which included *C. orbiculare* (Berk. & Mont.) Arx (= *C. lagenarium* (Pass.) Ellis & Halst.) from cucurbits, *C. trifolii* Bain from alfalfa (*Medicago sativa*), *C. lindemuthianum* (Sacc. & Magnus) Brioso & Cavara from green bean (*Phaseolus vulgaris* L.), *C. malvarum* (A. Braun & Casp.) Southw. from prickly sida (*Sida spinosa* L.), *C. gnaphalii* Syd. (no host specified), *C. helichrysi* (G. Winter) Arx (no host specified), *C. miyabeana* (no host specified), *C. musae* (Berk. & M. A.

Curtis) Arx from banana (*Musa* spp. L.), and *C. psoraleae* (Peck) Arx (no host specified). In 1992, 37 species of *Colletotrichum* were recognized, many of which were distinguished based on their host origin (41).

Although initially recognized as “special forms” of *C. gloeosporioides*, *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* are distinct from *C. gloeosporioides* in that they generally have ovoid conidia (38), no septum in germinated conidia (2,3,27,38), and produce appressoria of similar shapes and dimension (38). Thus, although these species can be distinguished from *C. gloeosporioides* based on morphology, their distinction from one another has been based largely on host origin.

Molecular technologies based on the analysis of DNA have been used to examine the relationship of *Colletotrichum* spp. (2,9,15,20,26,32,38). Based on molecular and morphological data, a close relationship between *C. orbiculare* from cucumber, *C. trifolii* from alfalfa, *C. malvarum* from prickly sida, and *C. lindemuthianum* from bean has been observed (2,27,34,38). Based on spore morphology, appressorium development, and sequence similarities of the rDNA, it was proposed that *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* should be considered a single species (38). It also was suggested that, within the broader species concept of *C. orbiculare*, isolates which showed host specialization should be classified as distinct formae speciales

Corresponding author: J. C. Correll; E-mail address: jcorrell@uark.edu

doi:10.1094/PHYTO-97-10-1305

© 2007 The American Phytopathological Society

of *C. orbiculare*. Current species concepts in fungi, however, suggest that host specificity is useful at the species rank rather than the informal rank of formae speciales (18). Thus, host specificity, along with other fixed genotypic characters, could provide justification for species delineation within the genus *Colletotrichum* more specifically within the *C. orbiculare* species complex.

Analysis of DNA sequences continues to be a valuable tool to help resolve relationships among and within species and species complexes of *Colletotrichum* (5,12,13,15–17,20,24–26). DNA sequence analysis, along with morphology and host range, were used to delineate species within the broader *C. graminicola* complex on sorghum, maize, wheat, oat, forage, turf, and amenity grasses (12). The phylogenetic analysis demonstrated the relatively low level of phylogenetic resolution of the internal transcribed spacer (ITS) region compared with the MAT1-2 mating type (HMG) and manganese-type superoxide dismutase gene (SOD2) sequences (12,13). Based on the sequences of HMG and SOD2 deposited in GenBank, it was found that the most phylogenetically informative characters were present in the intron portion of the genes examined (13). In addition, earlier work to define the phylogenetic origin of the panama disease pathogen *Fusarium oxysporum* f. sp. *cubense* also showed that intron sequences within the EF-1 α gene were highly informative (28,29). Single-copy nuclear genes are often favorable for use in phylogenetic analyses because they are biparentally inherited and follow patterns of concerted evolution, and the sequence alignment based on single-copy genes limits the alignment ambiguity (1). In contrast, ITS data typically yield lower consistency index (CI)

and retention index (RI) values compared with other loci, indicating a higher level of homoplasy (1). Moreover, examination of the ITS gene in *Colletotrichum* spp. typically has resulted in low bootstrap value support, indicating that the ITS gene is less phylogenetically informative (13). A recent study of inter- and intra-specific variation within the species complex *C. acutatum* confirmed that sequence variation in two introns from the single-copy genes (glutamine synthase and glyceraldehyde-3-phosphate dehydrogenase) were phylogenetically informative, whereby the concordance of both intron phylogenetic tree topologies revealed several distinct clades that were supported by very high bootstrap values and consistency indices (17). Based on these data, it was hypothesized that a similar approach could be used for a robust analysis of the relationship between *C. orbiculare* and several allied species.

The objective of this study were to test the hypothesis that distinct and fixed phenotypic (host specificity) and genotypic (sequence) characteristics exist among the various species in the *C. orbiculare* complex. To test this hypothesis, representative isolates of *C. orbiculare*, *C. lindemuthianum*, *C. trifolii*, and *C. malvarum* were compared for mitochondrial (mt)DNA restriction fragment length polymorphisms (RFLPs), intron RFLPs and sequence variation, vegetative compatibility, and host specificity on cucurbits, green bean, and alfalfa.

MATERIALS AND METHODS

Isolates. The isolates used in this study were either recovered from symptomatic tissue by the authors, received from other

TABLE 1. Isolates of *Colletotrichum orbiculare*, *C. lindemuthianum*, *C. trifolii*, and *C. malvarum*, their host specificity, and their 900-bp intron restriction fragment length polymorphism (RFLP) haplotype

Species	Isolate	VCG ^w	Host	Host reaction ^x			mtDNA ^y	Intron haplotype ^z						
				A	B	C		HHH	HHM	PstI	MspI	HhaI	HaeIII	P+M
<i>C. orbiculare</i>	JC1	1001	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	NC3	1001	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	AK9	1001	Pumpkin	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	CP12	1002	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	LB4	1001	Cantaloupe	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	MMB	1001	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	JH3	1001	Cucumber	+	–	...	...	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	LD1	1001	Cucumber	+	–	–	...	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	AK6	1002	Cantaloupe	+	–	...	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	BB17	1002	Cantaloupe	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	CP6	1002	Watermelon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	CP7	1002	Watermelon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	JX13	1002	Watermelon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	LB2	1002	Cucuzzi gourd	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	MME	1002	Watermelon	+	–	–	...	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	RL1	1002	Watermelon	+	–	–	...	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	RL2	1002	Watermelon	+	...	...	...	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	CR3	1002	Watermelon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	JX11	1002	Watermelon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	MH2	1003	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	MH5	1003	Cucumber	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	DAR61396	1003	Cucumber	+	–	...	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	JX10A	1004	Melon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. orbiculare</i>	JX10B	1004	Melon	+	–	–	A	C1	C1	C	C	C	D	C
<i>C. lindemuthianum</i>	14-2-39	CL-1	Bean	–	...	–	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	14-2-45	CL-1	Bean	–	+	–	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JZ1	CL-1	Bean	–	...	...	...	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JZ2	CL-1	Bean	–	+	–	...	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK1		Bean	–	+	–	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK2		Bean	–	+	–	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK3		Bean	–	+	–	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK4		Bean	–	+	–	A2	D	D	C	D	C	D	D

(continued on next page)

^w VCG = vegetative compatibility group.

^x A = cucurbits, B = green bean, C = alfalfa, and ... = missing data. + = disease symptoms and – = no disease symptoms.

^y Mitochondrial (mt)DNA RFLP haplotype of total DNA cut with *PvuII* and probed with mtDNA clones from *C. orbiculare* isolate JC1.

^z Haplotypes were determined based on the RFLP pattern of the 900-bp intron cut with individual enzymes or in combination; HHH = *HindIII* + *HinfI* + *HaeIII*; HHM = *HindIII* + *HinfI* + *MspI*; P+M = *PstI* + *MspI*. The same letter, within a column, denotes that the isolates have an identical RFLP haplotype.

researchers, obtained from the American Type Tissue Culture Collection (ATCC, Rockville, MD), or obtained from the *Colletotrichum* collection, University of Arkansas (Table 1). Single-spore isolates were stored on desiccated filter paper at 4°C. Isolates of *C. orbiculare* from cucumber (*Cucumis sativus* L.), cantaloupe (*C. melo* L. (Cantaloupensis group)), watermelon (*Citrullus lanatus* (Thunb.) Matsumura & Nakai), melon [*Cucumis melo* L. (Reticulatus group)], gooseberry gourd (*C. myriocarpus*), and cocklebur (*Xanthium spinosum* L.) were used in this study. The isolates of *Colletotrichum orbiculare* used were representative of the genetic diversity previously identified within a worldwide collection of *C. orbiculare* (51,52). The isolates belonged to four different vegetative compatibility groups (VCGs) (52) (Table 1). Two of the isolates of *C. orbiculare* (LW1 and LW6) were from cocklebur (49,51). The isolates were collected from throughout the United States or from Australia and Taiwan from 1990 to 1997.

In all, 23 isolates of *C. lindemuthianum* recovered from green bean (*P. vulgaris*), 20 isolates of *C. trifolii* from alfalfa (*M. sativa*) (4,23), and three isolates of *C. malvarum* from prickly sida (*S. spinosa*) (22) were examined (Table 1). The isolates were obtained from the *Colletotrichum* culture collection of the University of Arkansas between 1996 and 1997, or from other researchers. The isolates of *C. trifolii* originated from Canada or the United States. The isolates of *C. lindemuthianum* originated from the United States, Canada, Mexico, Costa Rica, the Dominican Republic, Honduras, Ecuador, Argentina, and Columbia.

DNA isolation. The isolates of *C. orbiculare*, *C. lindemuthianum*, *C. trifolii*, and *C. malvarum* were examined for mtDNA

RFLPs (Table 1). All isolates were grown in 0.5-liter flasks containing 200 ml of complete medium broth (7). The flasks were incubated at room temperature on an orbital shaker at 120 rpm for 4 to 5 days. Mycelium was harvested by vacuum filtration through miracloth, rinsed with 300 ml of deionized water, and frozen. The mycelium was lyophilized and ground into a powder using liquid nitrogen. Total DNA was extracted using a modified miniprep protocol (7).

RFLP analysis. Three restriction enzymes were used individually to digest 1.5 µg of DNA by incubating with 10 units of endonucleases (*Eco*RI, *Hae*III, and *Pvu*II) for 16 h at 37°C following the manufacturer's recommendations (New England Biolabs, Inc., MA). Electrophoresis was conducted in 0.8% agarose gels (25 by 20 cm) for 2 h at 20 V and 18 h at 54 V. Each gel was photographed on a UV transilluminator. DNA was blotted onto a nylon membrane (Hybond N⁺; Amersham, Arlington Heights, IL) by Southern blotting. The membrane was then heated 80°C for 2 h.

Two large non-overlapping mtDNA clones, 4U40 (13.7 kb) and 2U18 (10.1 kb), of mtDNA of *C. orbiculare* (representing 65% of the mitochondrial genome) were combined in equimolar concentrations and used for hybridization (11). The enhanced chemiluminescent (ECL) nonradioactive labeling and detection kit was used for hybridization following the manufacturer's instructions (Amersham). Prehybridization (1 h) and hybridization (12 to 16 h) were done at 42°C in a shaker according to the manufacturer's instructions.

VCGs. All isolates of *C. orbiculare* were characterized for vegetative compatibility using nitrate-nonutilizing (*nit*) mutants as

TABLE 1. (continued from preceding page)

Species	Isolate	VCG ^w	Host	Host reaction ^x			mtDNA ^y	Intron haplotype ^z						
				A	B	C		HHH	HHM	PstI	MspI	HhaI	HaeIII	P+M
<i>C. lindemuthianum</i>	JK5		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK6		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK7		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK8		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK9		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK10		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK11		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK12		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK14		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK15		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK16		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK17		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK18		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK19		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. lindemuthianum</i>	JK20		Bean	—	+	—	A2	D	D	C	D	C	D	D
<i>C. trifolii</i>	14-2-63	CT-1	Alfalfa	...	...	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON2		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON3		Alfalfa	—	—	—	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON7		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON9		Alfalfa	—	—	...	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON10		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON12		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON15		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON16		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON1		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON4		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON5		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON6		Red clover	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON8		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON11		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON13		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON14		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON17		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON18		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. trifolii</i>	ON19		Alfalfa	—	—	+	A	E	E	C	C	C	D	C
<i>C. orbiculare</i>	LW1	1051	Cocklebur	...	...	—	A1	G	G	D	E	D	D	C
<i>C. orbiculare</i>	LW6	1051	Cocklebur	...	...	—	A1	G	G	D	E	D	D	C
<i>C. malvarum</i>	3/7/2011	CM-1	Prickly sida	...	—	—	A	F	F	D	E	D	E	C
<i>C. malvarum</i>	4/3/2012	CM-1	Prickly sida	...	—	—	A	F	F	D	E	D	E	C
<i>C. malvarum</i>	4/3/2018	CM-1	Prickly sida	...	—	—	A	F	F	D	E	D	E	C

described in Puhalla (36) and Correll et al. (10). Nitrate non-utilizing mutants were recovered on minimal medium containing 1.5% potassium chlorate (10) as previously described (52). The mutants were characterized into two phenotypes (*nit1* and NitM), whereby *nit1* mutants grew as thin colonies on MM amended with nitrate as the sole nitrogen source and, NitM mutants grew as thin colonies on MM with hypoxanthine as the sole nitrogen source (10). Vegetative compatibility tests were performed by pairing NitM mutants with *nit1* mutants in all possible combinations. Vegetative compatibility tests were conducted at least twice. Pairing among isolates where a distinct heterokaryon developed as a result of complementation were vegetatively compatible and were placed into the same VCG.

All isolates of *C. lindemuthianum*, *C. trifolii*, and *C. malvarum* were tested for their vegetative compatibility with the reference VCGs of *C. orbiculare* (51,52). In addition, a subset of isolates of each of these species were characterized for vegetative compatibility with one another.

Pathogenicity tests. All isolates were tested for pathogenicity on several cucurbit hosts, green bean, and alfalfa. Emerson medium (YPSS) was used for producing inoculums (46). Cultures were grown on YPSS and incubated at room temperature ($\approx 23^{\circ}\text{C}$) under a 16-and-8-h light-and-dark cycle, where light was provided by six fluorescent lights (34 W). Inoculum was prepared from 7- to 10-day-old cultures by washing conidia off the agar surface using cold, sterile, deionized water and straining the suspensions through two layers of cheese cloth. The conidial suspensions were adjusted to a spore concentration of 8×10^4 conidia/ml using a hemacytometer.

Pathogenicity tests on cucurbits were conducted as previously described (52). Four differential cultivars—cucumber cvs. Marketer (Harris Moran Co.) and Arkansas Little Leaf (H19; Peto Seed) and watermelon cvs. Black Diamond (Northrup King Co.) and Charleston Gray (Northrup King Co.)—that have been shown to distinguish races 1, 2, and 2B in cotyledon inoculations were

used (51,52). To ensure that all plants were of uniform age and size at the time of inoculation, seed were pregerminated on moist tissue paper in the dark at room temperature (23°C). Germinated seed were planted in a soilless mixture of 1:1 peat and perlite (Sunshine mix no. 1). Fully expanded cotyledons (4-day-old seedlings) were sprayed with the conidial suspension to run-off using an air brush. Inoculated plants were incubated in a dew chamber at a relative humidity (RH) of 100% (23 to 28°C) for 24 h. After incubation, the plants were returned to the greenhouse and maintained at 23 to 35°C .

Evidence of disease symptoms and severity ratings were obtained by visually assessing the cotyledon area showing symptoms (chlorosis and necrosis) of infection as previously described (52). A disease rating scale of 0 to 7 was used, where 0 = no infection, 1 = 1 to 10, 2 = 11 to 25, 3 = 26 to 50, 4 = 51 to 75, 5 = 76 to 89, 6 = >90 to 98, and 7 = 100% necrosis or chlorosis. The mean disease ratings on the eighth day were used to evaluate pathogenicity. Isolates with mean disease ratings of 0.5 on the susceptible cvs. Marketer and Black Diamond were considered nonpathogenic. All cotyledon inoculation tests consisted of three replicates (pot) and three plants per replicate. Disease ratings per replicate were averaged from six cotyledons. The inoculation experiments were conducted three times.

A subset of isolates representing the various taxa was used in an inoculation test on cucumber fruit (Table 2). Cucumber fruit from an unknown slicing cucumber type cultivar were purchased at a grocery store. Fruit were washed with a commercial dishwashing detergent and surface sterilized with 10% (vol/vol) solution of commercial bleach for 2 min. The fruit then were rinsed in deionized water and air dried. Fruit were wounded with a cork borer, making a hole 8 mm in diameter and ≈ 5 mm deep. The holes then were inoculated by placing a colonized green bean agar plug (8 mm in diameter) into the wounds. Uncolonized plugs were used as controls. Fruit inoculation sites were a minimum of 4 cm apart. Following inoculation, fruit were incubated for 7 days

TABLE 2. Virulence of *Colletotrichum orbiculare* and allied *Colletotrichum* spp. on fruit of cucumber, *Cucumis sativus*

Isolate	VCG ^w	Race ^x	mtDNA haplotype ^y	Fruit lesion		Host origin
				Diameter (mm) ^z	Volume (cm ³)	
<i>Colletotrichum orbiculare</i>						
JC1	1001	1	A	30.1 a	4.06 ab	<i>Cucumis sativus</i>
BB18	1002	2	A	31.4 a	4.78 a	<i>C. melo</i>
CR2	1002	2B	A	31.6 a	4.36 ab	<i>Citrullus lanatus</i>
MH2	1003	1	A	25.7 ab	2.47 bc	<i>Cucumis sativus</i>
JX10	1004	2B	A	29.3 a	3.74 ab	<i>C. melo</i>
LW1	1050	av	A1	11.3 d	0.32 d	<i>Xanthium spinosum</i>
LW6	1050	av	A1	11.0 d	0.29 d	<i>X. spinosum</i>
<i>Colletotrichum trifolii</i>						
4-1-32	CT-1	1	A	11.2 d	0.26 d	<i>Medicago sativa</i>
14-2-63	CT-1	2	A	11.5 d	0.49 d	<i>M. sativa</i>
14-3-1	CT-1	2	A	10.1 d	0.20 d	<i>M. sativa</i>
14-3-3	CT-1	1	A	14.4 cd	0.52 d	<i>M. sativa</i>
<i>C. malvarum</i>						
3-7-11	CM-1	—	A	10.4 d	0.26 d	<i>Sida spinosa</i>
4-3-12	CM-1	—	A	11.0 d	0.27 d	<i>S. spinosa</i>
4-3-18	CM-1	—	A	11.1 d	0.29 d	<i>S. spinosa</i>
<i>C. lindemuthianum</i>						
14-2-37	CL-1	Beta	A2	10.7 d	0.25 d	<i>Phaseolus</i> spp.
14-2-39	CL-1	Sigma	A2	12.8 d	0.39 d	<i>Phaseolus</i> spp.
14-2-45	CL-1	Lambda	A2	19.6 bc	2.67 c	<i>Phaseolus</i> spp.
JZ1	CL-1	Alpha	—	11.6 d	0.26 d	<i>Phaseolus</i> spp.
JZ2	CL-1	Beta	—	13.3 cd	0.51 d	<i>Phaseolus</i> spp.
14-2-41	CL-X	Epsilon	A2	11.7 d	0.31 d	<i>Phaseolus</i> spp.

^w VCG = vegetative compatibility group.

^x Races of *C. orbiculare* identified by Wasilwa et al. (51,52); races of *C. trifolii* identified by Ostazeski and Elgin (33) and Welty and Mueller (53); and races of *C. lindemuthianum* described by Zaumeyer and Meiners (55).

^y Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) haplotypes based on patterns observed with *Hae*III. The same letter within a column indicates a common mtDNA RFLP haplotype; — = information not available.

^z Each disease parameter is a mean of three replications from each of three independent experiments. Numbers followed by the same letter within a column are not significantly different from one another (least significant difference = 0.05%).

at 23°C at 100% RH. After 7 days, lesion diameters were recorded by measuring two perpendicular axes of the lesion surface. A cross section through the center of the lesion was made and lesion depth and width were measured. The lesions formed in a cone shape from the point of inoculation and, as a result, the lesion volume was estimated using the mathematical formula for the volume of a cone. Visible sporulation on the fruit surface also was recorded as either present or absent. All fruit inoculation tests were conducted three times and each inoculation was treated as a block. Data were analyzed as a randomized complete block by analysis of variance with block-treatment used as the error term. The least significant difference at the 5% probability level was used for mean separation.

Green bean inoculation tests were performed similarly to those previously described (21). Seedlings (10 to 14 days old) of the cv. Cardinal were spray inoculated until run-off with a conidial suspension (1×10^6 conidia/ml). Plants were incubated at $\approx 24^\circ\text{C}$ for 24 h at 100% RH before being moved to the greenhouse. Seven days after inoculation, plants were scored for the severity of anthracnose symptoms on leaves and stems. For the purpose of this study, isolates were evaluated simply as pathogenic, causing severe anthracnose symptoms; weakly pathogenic, showing only a few anthracnose lesions; or nonpathogenic, showing no symptoms of infection. The inoculations were performed in two independent inoculation tests.

Alfalfa inoculation tests were performed similarly to those previously described (4,30,31,33,53). Twenty-five seed of cv. Saranac were grown per pot. Fourteen-day-old seedlings were inoculated by spraying until run-off with a conidial suspension of each isolate (2×10^6 conidia/ml in sterile distilled water containing two drops of Tween 20 per liter). Plants were placed in a mist chamber maintained at 23°C for 48 h and then moved to a growth chamber maintained at 23°C and a 16-h photoperiod. Eight days after inoculation, the plants were evaluated for disease reactions using a Horsfall-Barret scale based on the degree of necrosis, where 0 = no disease and 9 = a dead plant. Isolates were considered nonpathogenic if plants were scored as 0 or 1. Isolates were considered pathogenic if plants scored as 7 to 9. No plants received intermediate scores of 2 to 6. Four replicate pots were used per experiment per isolate and the entire experiment was conducted twice.

Intron amplification. The forward primer GSF1 (5'-ATGGCCGAGTACATCTGG-3') and the reverse primer GSR1 (5'-GAACCGTCGAAGTTCCAC-3') were used to amplify an ≈ 900 -bp intron region of the glutamine synthetase (GS) gene (17,24,

25,39,45). The forward primer GDF1 (5'-GCCGTCAACGAC-CCCTTCATTGA-3') and the reverse primer GDR1 (5'-GGGTG-GAGTCGTAAGTTCGATGT-3') were used to amplify a 200-bp intron region of the glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene (17,24,25,44). A Hybaid DNA thermocycler was used to perform the polymerase chain reaction amplification of the introns using 35 cycles of denaturation at 94°C and annealing at 60°C for 1 min, with final extension at 72°C for 3 min.

Amplified DNA was digested with the following enzyme combinations: A, *Hind*III + *Hinf*I + *Hae*III; and B, *Hind*III + *Hinf*I + *Msp*I. The restriction fragments were electrophoretically separated in a 3.0% Agarose 1000 (Invitrogen Corp., Carlsbad, CA) gel in 0.5 Tris-borate-EDTA buffer for 3.5 h at 140 V. Band fragments between 0.05 and 1.0 kb (Table 3) were scored for their presence or absence for a given enzyme combination and the data converted into a binary character matrix. The data then were analyzed using a cluster analysis of the similarity coefficients with the unweighted pair-grouping method with arithmetic averages (UPGMA) in NTSYS-pc software (Department of Ecology and Evolution, State University of New York, Stony Brook, NY), to determine the relative relatedness.

In addition, the software Quantitative One (Bio-Rad, Richmond, CA) was used to determine the approximate size of the various fragments based on the relative distance from the top comparing with a 100-bp molecular marker (Invitrogen).

DNA sequencing. The 900- and the 200-bp intron fragments were purified with the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA), and used as templates for sequencing reactions using the ABI Prism Dye Terminator cycle sequencing system (Applied Biosystems Inc., Foster City, CA). Sequencing reactions were performed as previously described (17).

Sequence alignment and phylogenetic analysis. The sequence of the 900- and 200-bp introns of 19 isolates used for phylogenetic analyses were performed using four isolates as an outgroup; the four isolates, representing other *Colletotrichum* spp., included *C. dematium* (JG13) from spinach, *C. acutatum* (A38) from apple, *C. gloeosporioides* (NC329) from apple, and *C. magna* (AK7) from pumpkin (17).

Intron sequences were entered into the Seqpup DNA sequence editor as previously described (14). The combined data were aligned using ClustalX (43), and the phylogenetic analyses and partition homogeneity test were performed using PAUP (42). Three methods of tree building were used: maximum-parsimony (MP), maximum-likelihood (ML), and neighbor-joining (NJ). For each method, alignment gaps were treated as missing data in the

TABLE 3. Approximate base-pair fragment size of the intron polymerase chain reaction products digested by the restriction enzyme combination *Hind*III + *Hinf*I + *Hae*III (HHH) or *Hind*III + *Hinf*I + *Msp*I (HHM)

<i>Colletotrichum orbiculare</i>	<i>C. lindemuthianum</i>	<i>C. trifolii</i>	<i>C. orbiculare</i> (<i>Xanthium</i> sp.)	<i>C. malvarum</i>	<i>C. dematium</i>	<i>C. acutatum</i>	<i>C. gloeosporioides</i>	<i>C. magna</i>
HHH RFLPs ^y								
240	260	240 × 2 ^z	240	260	190	230	230	250
210	220	140	190	170	140 × 2 ^z	190	190	135
140	140	110	140	130	135	140	150	120
120	120	95	110	105	110	120	135	90
95	95	70	95	90	90	105	110	80
80	90	60	70	80	40 × 2 ^z	90	90	60
70	60	45	60	60		60	60	50
45	45		45	55		50	55	30
HHM RFLPs								
430	500	430	430	500	270	230	270	200
250	230	250	250	250	180	190	250	180
145	170	170	130 × 2 ^z	130	130	170	220	170
75	70	75	45	45	120	130	190	140
70	45	70			85	65	150	130
45		45			30		140	90
							130	

^y RFLPs = restriction fragment length polymorphisms.

^z Two fragments with equal molecular weights existed in the digested band patterns.

phylogenetic analyses. The tree topology was evaluated by statistical confidence using bootstrap analysis; 1,000 replicates were performed to examine the relative bootstrap support for each group in the resultant topologies (14).

For MP and ML analyses, a heuristic search was employed and starting trees always were obtained by random sequence addition. Tree visualization was done using TreeView (Win32) version 1.5.2.

The Hasegawa-Kishino-Yano model (HKY85) was used for NJ tree construction (19). Each indel (insertion or deletion), regardless of its length, was treated as a single nucleotide substitution. For the MP analysis, the heuristic search procedure was used with the following parameters: transition/transversion ratio = 2; starting branch length was obtained using the Rogers-Swofford approximation method; the substitution rates were set to conform to a gamma distribution; and the molecular clock was not enforced. For the ML analysis, the heuristic algorithm with TBR of PAUP was used because the data set was too large to be used with the exhaustive or branch-and-bound algorithms. ML settings were as follows: number of substitution types = 2; transition/transversion ratio = 2; kappa = 4.027. The assumed nucleotide frequency (empirical frequencies) A = 0.25788, C = 0.31081, G = 0.20218, and T = 0.22914. The assumed proportion of invariable sites = none; distribution rates at variable sites = equal.

The tree length CI, the CI excluding uninformative characters, the homoplasy index (HI), the HI excluding uninformative characters, the RI, and the rescaled consistency index (RC) for all of the MP trees were recorded. The Kishino-Hasegawa tests were performed to determine whether the trees stored in memory were significantly different. The NJ tree distance matrix was calculated based on the HKY85 model.

GenBank accession numbers for two intron sequences. The 900-bp intron sequences were deposited as follows: DQ792871 (isolate A38), DQ792872 (NC329), DQ792873 (AK7), DQ792874 (JG13), DQ792875 (ON12), DQ792876 (ON7), DQ792877 (14-2-63), DQ792878 (JK10), DQ792879 (14-2-39), DQ792880 (JK7), DQ792881 (JK12), DQ792882 (LW6), DQ792883 (LW1), DQ792884 (AK9), DQ792885 (DAR61396), DQ792886 (MH2), DQ792887 (JX10), DQ792888 (JX13), DQ792889 (JC1), DQ792890 (CP6), DQ792891 (RL1), DQ792892 (3-7-11), and DQ792893 (4-3-12); the 200 bp intron sequences deposited were as follows: DQ792848 (A38), DQ792849 (NC329), DQ792850 (AK7), DQ792851 (JG13), DQ792852 (ON12), DQ792853 (ON7), DQ792854 (14-2-63),

DQ792855 (JK10), DQ792856 (14-2-39), DQ792857 (JK7), DQ792858 (JK12), DQ792859 (LW6), DQ792860 (LW1), DQ792861 (AK9), DQ792862 (DAR61396), DQ792863 (MH2), DQ792864 (JX10), DQ792865 (JX13), DQ792866 (JC1), DQ792867 (CP6), DQ792868 (RL1), DQ792869 (3-7-11), and DQ792870 (4-3-12).

RESULTS

mtDNA RFLP. The geographically diverse isolates of *C. orbiculare* from various cucurbit hosts representing the four known VCGs of *C. orbiculare* (VCGs 1001, 1002, 1003, and 1004) and including races 1, 2, and 2B, had a common mtDNA RFLP haplotype (haplotype A) when examined with three restriction enzymes (*EcoRI*, *PvuII*, and *HaeIII*) and probed with two mtDNA clones of *C. orbiculare* (404U [13.7 kb] and 2U18 [10.1 kb]) (Table 1; Fig. 1). Also, two representative isolates of *C. orbiculare* from cocklebur from Australia had a similar mtDNA haplotype A, with one polymorphism detected with the enzyme *PvuII* (Table 1). This polymorphism previously was shown to be due to the occurrence of one additional restriction site in the mitochondrial genome among isolates from cocklebur (11).

The isolates of *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* had an mtDNA haplotype identical to haplotype A of *C. orbiculare* when examined with two (*EcoRI* and *PvuII*) different restriction enzymes. The isolates of *C. orbiculare*, *C. trifolii*, and *C. malvarum* also had an identical mtDNA haplotype (haplotype A) when examined with a third restriction enzyme (*HaeIII*). However, a single mtDNA RFLP polymorphism (1.7 kb) was detected among all of the isolates of *C. lindemuthianum* with the enzyme *HaeIII* (Fig. 1).

Vegetative compatibility. *nit* Mutants were recovered from isolates of all four taxa (*C. orbiculare*, *C. trifolii*, *C. malvarum*, and *C. lindemuthianum*) on minimal medium amended with potassium chlorate after 10 to 40 days (47,52). All isolates of *C. orbiculare* that originated from cucurbit hosts belonged to one of four distinct VCGs (VCGs 1001, 1002, 1003, and 1004) (Table 1). Isolates of *C. orbiculare* from cocklebur (LW1 and LW6) were vegetatively compatible with each other but vegetatively incompatible with isolates in VCGs 1001, 1002, 1003, and 1004. In addition, all isolates of *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* were vegetatively incompatible with isolates of all four VCGs of *C. orbiculare* and with each other (Table 1). Four isolates of *C. trifolii* tested were in the same VCG and designated VCG CT-1. The three isolates of *C. malvarum*, also in mtDNA haplotype A, belonged to a single VCG designated VCG CM-1. Five of the seven isolates of *C. lindemuthianum* examined belonged to a single VCG (CL-1).

Foliar pathogenicity tests. Overall, the cucurbit isolates of *C. orbiculare*, the green bean isolates of *C. lindemuthianum*, and the alfalfa isolates of *C. trifolii* were pathogenic on cucurbits, green bean, and alfalfa, respectively (Table 1). The cocklebur isolates of *C. orbiculare* and the *C. malvarum* isolates from prickly sida were not pathogenic on any of these three hosts.

All isolates of *C. orbiculare* that originated from cucurbit hosts were pathogenic on the susceptible cucumber (Marketer) and watermelon (Black Diamond) differentials (disease ratings 5.0) (Table 1). H19 was resistant (disease ratings 2.5) to races 2 and 2B and Charleston Gray was resistant to races 1 and 2B (52). The mean disease ratings of isolates of *C. orbiculare* from cocklebur and isolates of *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* all were <0.5 and no evidence of infection was observed for the isolates examined; thus, were all considered nonpathogenic on the susceptible cucurbit hosts (Table 1). Furthermore, several isolates of all of the species were tested in additional pathogenicity tests whereby inoculum concentrations were increased to 4×10^6 conidia/ml and, again, no infections were observed on the cotyledons of any of the cucurbit differentials (data not shown). All

Fig. 1. Mitochondrial (mt)DNA restriction fragment length polymorphisms of the various *Colletotrichum* spp. Total DNA was digested with *HaeIII* and probed with two mtDNA clones (4u40 and 2u18) of *Colletotrichum orbiculare* (11).

isolates of *C. lindemuthianum* tested were pathogenic on green bean whereas no other isolates were observed to cause disease on green bean. Similarly, only isolates of *C. trifolii* caused disease on alfalfa; one isolate from alfalfa (ON3) did not cause disease on alfalfa.

Fruit pathogenicity tests. Although all taxa were able to colonize wounded cucumber fruit, only isolates of *C. orbiculare* from cucurbits (except MH2) had significantly larger lesion diameters and lesion volumes than isolates of *C. orbiculare* from cocklebur, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* (Table 2). There were no significant differences in lesion diameter or lesion volume among isolates representing VCGs 1001, 1002, 1003, and 1004 of *C. orbiculare* (Table 2). Conidial production was evident on the cucumber fruit surface after 7 days for all cucurbit isolates of *C. orbiculare*. No sporulation was visible on the lesion surface for isolates of *C. trifolii*, *C. lindemuthianum*, *C. malvarum*, or *C. orbiculare* from cocklebur.

Intron RFLPs and sequence. An intron of the GS gene and an intron of the GPDH gene were amplified successfully from 19 representative isolates of *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* (Table 1). Although some variation in size was observed, the fragment from the various species was ~900 bp (864 to 912 bp) for GS intron and ~200 bp (97 to 193) for GPDH intron. The lower size range for the GPDH intron among the isolates of *C. lindemuthianum* was due to a series of relatively large deletions near the 3' end of the intron.

Various restriction enzymes were able to cut the 900-bp GS intron, but a combination of enzymes, particularly *Hind*III + *Hinf*I + *Hae*III and *Hind*III + *Hinf*I + *Msp*I, produced highly polymorphic

profiles with seven to nine polymorphic bands and provided the highest level of resolution for qualitatively distinguishing each species (Fig. 2; Table 3). In addition, isolates of *C. orbiculare* from cucurbit hosts could be distinguished from those from cocklebur (Fig. 2). UPGMA analysis based on the RFLP data from the 900-bp GS intron clearly distinguished the various taxa (Fig. 3).

The 900-bp intron of the GS gene and the 200-bp GPDH intron of the GPDH gene from 19 isolates of *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* also were sequenced and compared. The overall sequence similarity among isolates of *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* was >90% for the 900-bp intron and >86% for the 200-bp intron (except for *C. lindemuthianum*, which was 50 to 53% due to large deletions near the 3' end of the intron sequence). The overall sequence similarity of the 900- and 200-bp intron among isolates within each species was >97%.

Interestingly, isolates of *C. orbiculare* described from *Xanthium* spp. in Australia (41) had a 900-bp intron sequence (>99.9%) very similar to isolates of *C. malvarum* from the United States. In addition, the 200-bp intron sequence of *Xanthium* isolates was 95% similar to those of *C. malvarum* and 98% similar to isolates of *C. orbiculare*.

Various isolates representing *C. dematium*, *C. acutatum*, *C. gloeosporioides*, and *C. magna* as outgroups showed little sequence similarity to isolates in the *C. orbiculare* complex and were <64 and <30% for the 900- and 200-bp introns, respectively.

Overall, the statistical clustering of isolates based on all three tree topologies were very similar for both the 900- and 200-bp intron as well as the combined data set of both introns (only MP trees shown) (Figs. 4, 5, and 6). For the 900-bp intron, all three tree methods grouped all of the taxa examined (excluding the outgroups) into a single clade with 100% bootstrap values for both

Fig. 2. Restriction fragment length polymorphisms of the 900-bp intron of the glutamine synthetase gene of various *Colletotrichum* spp. The amplified product (~900 bp) was digested with a combination of three enzymes (**A**, *Hind*III + *Hinf*I + *Hae*III and **B**, *Hind*III + *Hinf*I + *Msp*I) and the fragments then separated in a 3% agarose 1000 gel.

Fig. 3. Cluster analysis of the 900-bp intron based on the presence or absence of the bands of the glutamine synthetase intron restriction fragment length polymorphisms digested with enzyme combination *Hind*III + *Hinf*I + *Hae*III. The dendrogram was generated using the unweighted pair-grouping method with arithmetic averages of NTSYS-PC.

introns. The high CI and low HI values clearly indicated the phylogenetic relationship with a high degree of confidence with intron sequence data.

For the 900-bp intron, tree topologies of the sequence data for all three methods (MP, ML, and NJ) consistently grouped *C. orbiculare*, *C. trifolii*, and *C. malvarum* together and separate from *C. lindemuthianum* (Fig. 4, MP tree shown). However, with the 200-bp intron, *C. lindemuthianum* was nested with *C. trifolii* (Fig. 5). This may be due to a smaller number of bases compared (only 97) due to the deletions in the 200-bp intron among the isolates of *C. lindemuthianum*, and these data may not be as robust as that from the 900-bp intron.

Of the total 1,313 characters in the 900-bp GS intron sequence, 172 were phylogenetically informative in the MP analysis. The tree length was 491 steps, the CI was 0.8916, the CI excluding uninformative characters was 0.8083, the HI was 0.1039, the HI excluding uninformative characters was 0.1917, the RI was 0.948, and the RC was 0.8019 (Fig. 4).

Of the total 303 characters in the 200-bp intron sequence, 55 were phylogenetically informative in the MP analysis. The tree length was 152 steps, the CI was 0.8487, the CI excluding uninformative characters was 0.7500, the HI was 0.1513, the HI excluding uninformative characters was 0.2500, the RI was 0.8631, and the RC was 0.7325 (Fig. 5).

For the combined 900- and 200-bp intron sequences in the MP analysis, there were 1,610 characters, of which 229 were phylogenetically informative. The tree length was 660 steps, the CI was 0.9045, the CI excluding uninformative characters was 0.8147, the HI was 0.0955, the HI excluding uninformative characters was 0.1826, the RI was 0.9023, and the RC was 0.8162 (Fig. 6).

Before the two intron sequences were combined, the partition homogeneity test was used to compare the sequences. The *P* value of the partition homogeneity test for the two intron sequence data sets were calculated using PAUP. The high *P* value (*P* = 0.79) from the two sets of intron sequences in this study indicated that there is no significant difference between the two sequence data sets and, thus, two intron sequences can combine to infer phylogenetic relationship. Moreover, the similar tree topologies based on two intron sequences and the combined sequences indicate that the considerable concordance existed between the two intron sequences.

DISCUSSION

There is considerable uncertainty in the literature regarding the demarcation of taxa of many *Colletotrichum* spp., including the species complex *C. orbiculare*, which includes *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum*. Spore morphology, appressorium development, and sequence similarities of the rDNA have been used to infer that *C. orbiculare* from cucurbits, *C. trifolii* from alfalfa, *C. lindemuthianum* from bean, and *C. malvarum* from prickly sida represent a single phylogenetic species and should be collectively recognized as *C. orbiculare* (2,27, 32,38). In the current study, a common mtDNA RFLP haplotype, haplotype A, was observed among representative isolates of *C. orbiculare*, *C. trifolii*, and *C. malvarum*. Isolates of *C. lindemuthianum* had an mtDNA haplotype very similar to haplotype A, with a single mtDNA RFLP detected with the enzyme *Hae*III. These data provide supporting evidence that these taxa clearly have a relatively recent common ancestry. The fact that these taxa have such a similar mtDNA haplotype may indicate that they may

Fig. 4. Maximum-parsimony (MP) tree based on the glutamine synthetase intron sequence showing the relationship among species in the *Colletotrichum orbiculare* complex. MP tree scores were as follows: 1,313 total characters, tree length = 491, consistency index = 0.8961, homoplasy index = 0.1039, retention index = 0.8948, and rescaled consistency index = 0.8019. Bootstrap values are labeled on the branch of the tree. Scale bar represents the number of transformations from one character to another.

Fig. 5. Maximum-parsimony (MP) tree based on the glyceraldehyde-3-phosphate dehydrogenase intron sequence showing the relationship of *Colletotrichum orbiculare* complex. MP tree scores were as follows: 303 total characters, tree length = 152, consistency index = 0.8487, homoplasy index = 0.1513, retention index = 0.8631, and rescaled consistency index = 0.7325. Bootstrap values are labeled on the branch of the tree. Scale bar represents the number of transformations from one character to another.

even belong to a common ancestral mating population. Isolates of *C. lindemuthianum* have been shown to be heterothallic and produce the *Glomerella lindemuthiana* sexual stage (37). However, little is known about mating abilities between these different taxa.

Sequence analysis of two independent introns in the current study were phylogenetically informative and clearly show that isolates of *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* represent a single well-supported clade. Although similar, each of the taxa, including isolates of *C. orbiculare* that were pathogenic and nonpathogenic to cucurbits, could consistently be demarcated based on the qualitative (RFLPs) and quantitative (sequence) analyses of the two introns. Similarly, sequence information on both of these introns also has been phylogenetically informative in *C. acutatum*, whereby distinct clades could be demarcated within the broader species complex (17). Thus, sequence information of these two introns had a very high level of resolution relative to other target DNA sequences such as ITS regions, was statistically robust, and, consequently, can be phylogenetically informative at both the inter- and intraspecific levels for the genus *Colletotrichum*.

The intron sequences that have been examined appear universal in *Colletotrichum* spp. in that they have been recovered from a wide array of taxa, including *C. acutatum*, *C. gloeosporioides*, *C. orbiculare*, *C. lindemuthianum*, *C. trifolii*, *C. malva*, *C. magna*, *C. dematium*, *C. graminicola*, *C. sublineolum*, and several *Colletotrichum* taxa from turfgrass (8,16,17,24,25,50). Because of the RFLP diversity observed between the taxa, it is anticipated that the RFLP haplotypes identified from the restriction digestion of the 900-bp intron could be a valuable diagnostic tool for species identification, particularly where time, expertise, and expense may preclude sequencing efforts from being conducted.

Fig. 6. Maximum-parsimony (MP) tree based on the combined glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase intron sequences showing the relationship of *Colletotrichum orbiculare* complex. MP tree scores were as follow: 1,610 total characters, tree length = 660, consistency index = 0.9045, homoplasy index = 0.0955, retention index = 0.9023, and re-scaled consistency index = 0.8162. Bootstrap values are labeled on the branch of the tree. Scale bar represents the number of transformations from one character to another. $P = 0.79$ (partition homogeneity test) indicating, considerable concordance between the evolutionary ratio of the two intron sequences.

Although a limited number of isolates were examined for vegetative compatibility, none of the isolates representing *C. orbiculare*, *C. trifolii*, *C. lindemuthianum*, and *C. malvarum* were vegetatively compatible with each other. Among isolates of *C. orbiculare* from cucurbits, the four VCGs 1001, 1002, 1003, and 1004 were identified (9,51,52). There are a limited number of studies that have used vegetative compatibility (*nit* mutants) to study population diversity of *C. trifolii* (6). In the current study, four isolates of *C. trifolii* representing two races (races 1 and 2) belonged to a single VCG (CT-1). Isolates of *C. malvarum* from prickly sida (*S. spinosa*) from Arkansas, Mississippi, and Tennessee also belonged to a single VCG (CM-1), indicating that this VCG has a wide geographical distribution in the United States.

Data from the current study support the hypothesis that *C. orbiculare*, *C. lindemuthianum*, *C. trifolii*, and *C. malvarum* represent a closely aligned phylogenetic species (43). However, isolates of each taxa showed a distinct host specificity whereby only isolates of *C. orbiculare*, *C. trifolii*, and *C. lindemuthianum* were pathogenic on cucurbits, alfalfa, and bean, respectively. These data support the host specificity of *C. lindemuthianum* to legumes (21,55), *C. trifolii* to alfalfa and red clover (*Trifolium pratense* L.) (4,33), and *C. malvarum* to hosts in the Malvaceae (prickly sida and hollyhock [*Althaea rosea* L.]) (20). Kirkpatrick et al. (22) showed that isolates of *C. malvarum* were unable to infect 38 plant species, including cucurbits (*Citrullus lanatus* (Thunb.) Matsum and Nakai (watermelon), *Cucumis sativus* L. (cucumber), *C. melo* L. (muskmelon), *Cucurbita pepo* (pumpkin), or *P. vulgaris* L. (bean). Because isolates within the broader species concept of *Colletotrichum orbiculare* (which includes *C. lindemuthianum*, *C. trifolii*, and *C. malvarum*) clearly show host specificity to various hosts, it is proposed that this fixed phenotypic characteristic can be used to distinguish the species. Recent recommendations support the use of phenotypic characteristics, such as host specialization, to be useful at the species rank rather than as the informal rank of formae speciales (18). Consequently, because *C. orbiculare* from cucurbit hosts, *C. orbiculare* from noncucurbit hosts, *C. lindemuthianum*, *C. trifolii*, and *C. malvarum* can be distinguished based on apparently fixed genotypic (intron nucleotide sequence differences) and phenotypic (host specificity) characters, they should be recognized as distinct but closely related species.

Molecular studies to examine species boundaries in *Colletotrichum* will continue to provide a better understanding of inter- and intraspecific variation within the genus, particularly within the broader species complexes such as *C. acutatum* sensu lato, *C. gloeosporioides* sensu lato, and *C. graminicola* sensu lato. However, it is important, as we continue to demarcate individual taxa within the genus, that we have a concomitant effort on developing a better understanding of the lifestyles (35,54), the ecology, and overall biology of these individual taxa and their impact and control in agricultural ecosystems.

LITERATURE CITED

- Alvarez, I., and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetics inference. *Mol. Phylogenet. Evol.* 29:417-434.
- Bailey, J. A., Nash, C., Morgan, L. W., O'Connell, R. J., and TeBeest, D. O. 1996. Molecular taxonomy of *Colletotrichum* species causing anthracnose of Malvaceae. *Phytopathology* 86:1076-1083.
- Bailey, J. A., Sherriff, C., and O'Connell, R. J. 1995. Identification of specific and intraspecific diversity in *Colletotrichum*. Pages 197-211 in: *Disease Analysis Through Genetics and Biotechnology—Interdisciplinary Bridges to Improved Sorghum and Millet Crops*. J. H. Leslie and R. A. Frederickson, eds. Iowa State University Press, Ames.
- Bain, S. M., and Essary, S. H. 1906. A new anthracnose of alfalfa and red clover. *J. Mycol.* 12:192-193.
- Balardin, R. S., Smith, J. J., and Kelly, J. D. 1999. Ribosomal DNA polymorphism in *Colletotrichum lindemuthianum*. *Mycol. Res.* 103:841-848.
- Brooker, N. L., Leslie, J. F., and Dickman, M. B. 1991. Nitrate non-utilizing mutants of *Colletotrichum* and their use in studies of vegetative compatibility and genetic relatedness. *Phytopathology* 81:672-677.

7. Correll, J. C., Gordon, T. R., and McCain, A. H. 1992. Genetic diversity in California and Florida populations of pitch canker fungus *Fusarium subglutinans* f. sp. *pini*. *Phytopathology* 82:415-420.
8. Correll, J. C., Guerber, J. C., Bentley, T., and Uddin, W. 2003. Characterization of *Colletotrichum graminicola* from turfgrass using mtDNA and intron RFLPs, vegetative compatibility, colony and spore morphology, and intron sequence analysis. (Abstr.) *Phytopathology* 93:S112.
9. Correll, J. C., Guerber, J. C., Wasilwa, L. A., Sherrill, J. F., and Morelock, T. E. 2000. Inter- and intra-species variation in *Colletotrichum* and mechanisms which affect population structure. Pages 145-179 in: *Colletotrichum: Host Specificity, Pathogenicity, and Host-Pathogen Interactions*. D. Prusky, M. B. Dickman, and S. Freeman, eds. American Phytopathological Society, St. Paul, MN.
10. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility. *Phytopathology* 77:1640-1645.
11. Correll, J. C., Rhoads, D. D., and Guerber, J. C. 1993. Examination of mitochondrial DNA restriction fragment length polymorphisms, DNA fingerprints, and randomly amplified polymorphic DNA of *Colletotrichum orbiculare*. *Phytopathology* 83:1199-1204.
12. Crouch, J. A., Clarke, B. B., and Hillman, B. I. 2006. Unraveling evolutionary relationships among the divergent lineages of *Colletotrichum* causing anthracnose disease in turfgrass and corn. *Phytopathology* 96:46-60.
13. Du, M. Z., Schardl, C. L., Nuckles, E. M., and Vaillancourt, E. M. 2005. Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. *Mycologia* 97:641-658.
14. Felsenstein, J. 1985. Confidence limits on phylogenies. An approach using the bootstrap. *Evolution* 39:783-791.
15. Freeman, S., Katan, T., and Shabi, E. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Dis.* 82:596-605.
16. Gonzalez, E., Sutton, T. B., and Correll, J. C. 2006. Clarification of the etiology of Glomerella leaf spot and bitter rot of apple caused by *Colletotrichum* spp. based on morphology and genetic, molecular, and pathogenicity tests. *Phytopathology* 96:982-992.
17. Guerber, J. C., Liu, B., Correll, J. C., and Johnston, P. R. 2003. Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* 95:872-895.
18. Harrington, T. C., and Rizzo, D. M., 1999. Defining species in the fungi. Pages 43-71 in: *Structure and Dynamics of Fungal Populations*. J. J. Worrall, ed. Kluwer Press, Dordrecht, The Netherlands.
19. Hasegawa, M., Kishino, J., and Yano, T. 1985. Dating of the human - ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 21:160-174.
20. Johnston, P. R., and Jones, D. 1997. Relationships among *Colletotrichum* isolates from fruits-rots assessed using rDNA sequences. *Mycologia* 89:420-430.
21. Kelly, J. D., Afanador, L., and Cameron, L. S. 1994. New races of *Colletotrichum lindemuthianum* in Michigan and implication in dry bean resistance breeding. *Plant Dis.* 78:892-894.
22. Kirkpatrick, T. L., Templeton, G. E., and TeBeest, D. O. 1982. Potential of *Colletotrichum malvarum* for biological control of prickly sida. *Plant Dis.* 66:323-325.
23. Lenné, J. M. 1992. *Colletotrichum* diseases of legumes. Pages 134-166 in: *Colletotrichum: Biology, Pathology and Control*. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, UK.
24. Liu, B., and Correll, J. C. 2000. Characterization of *Colletotrichum* species by analysis of a 1 kb intron of the glutamine synthetase gene. (Abstr.) *Phytopathology* 90:S8.
25. Liu, B., Guerber, J. C., and Correll, J. C. 2001. Genetic diversity within and between subgroups of *Colletotrichum acutatum* based on RFLP and sequence analysis of introns of the glutamine synthetase and glyceraldehyde phosphate dehydrogenase genes. (Abstr.) *Phytopathology* 91:S55.
26. Mesquita, A. G., Paula, T. J., Moreira, M. A., and de Barros, E. G. 1998. Identification of races of *Colletotrichum lindemuthianum* with aid of PCR-based molecular markers. *Plant Dis.* 82:1084-1087.
27. O'Connell, R. J., Nash, C., and Bailey, J. A. 1992. Lectin cytochemistry: A new approach to understanding cell differentiation, pathogenesis and taxonomy in *Colletotrichum*. Pages 67-87 in: *Colletotrichum: Biology, Pathology and Control*. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, UK.
28. O'Donnell, K., Kistler, H. C., Cigelnik, E., and Plötz, R. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. USA* 95:2044-2049.
29. O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C., and Takayuki, A. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genet. Biol.* 41:600-623.
30. O'Neill, N. R., and Baughan, G. R. 2000. Sources of resistance to anthracnose in the annual Medicago core collection. *Plant Dis.* 84:261-267.
31. O'Neill, N. R., Elgin, J. H., and Baker, C. J. 1989. Characterization of induced resistance to anthracnose in alfalfa by races, isolates, and species of *Colletotrichum*. *Phytopathology* 79:750-756.
32. O'Neill, N. R., van Berkum, P., Lin, J., Kuo, J., Ude, G. N., Kenworthy, W., and Saunders, J. A. 1997. Application of amplified restriction fragment polymorphism for genetic characterization of *Colletotrichum* pathogens of alfalfa. *Phytopathology* 87:745-750.
33. Ostazeski, S. A., and Elgin, J. H., Jr. 1980. Physiologic races of *Colletotrichum trifolii*. (Abstr.) *Phytopathology* 70:691.
34. Pain, N. A., O'Connell, R. J., Bailey, J. A., and Green, J. R. 1992. Monoclonal antibodies which restricted binding of four *Colletotrichum* species: *C. lindemuthianum*, *C. malvarum*, *C. orbiculare*, and *C. trifolii*. *Physiol. Mol. Plant Pathol.* 40:111-126.
35. Perez, N. A., Timmer, L. W., Adaskaveg, J. E., and Correll, J. C. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Dis.* 89:784-796.
36. Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Can. J. Bot.* 63:179-183.
37. Rodriguez-Guerra, R., Ramirez-Rueda, M. T., Cabral-Enciso, M., Garcia-Serrano, M., Lira-Maldonado, Z., Gonzalez-Chavira, M., and Simpson, J. 2005. Heterothallic mating observed between Mexican isolates of *Glomerella lindemuthiana*. *Mycologia* 97:793-803.
38. Sherrill, C., Whelan, M. J., Arnold, G. M., Lafay, J., Brygoo, Y., and Bailey, J. A. 1994. Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Exp. Mycol.* 18:121-138.
39. Stevenson, S. A., Green, J. R., Manners, J. M., and Maclean, D. J. 1997. Cloning and characterization of glutamine synthetase from *Colletotrichum gloeosporioides* and demonstration of elevated expression during pathogenesis on *Stylosanthes guianensis*. *Curr. Genet.* 31:447-454.
40. Sutton, B. C. 1980. The Coelomycetes—Fungi Imperfecti with Pycnidia Acervuli and Stromata. Commonwealth Mycological Institute, Kew, Surrey, England.
41. Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 523-537 in: *Colletotrichum: Biology, Pathology and Control*. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, UK.
42. Swofford, D. L. 2002. PAUP. Phylogenetic Analysis Using Parsimony (and Other Methods), version 4. Sinauer Associates, Sunderland, MA.
43. Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genet. Biol.* 31:21-32.
44. Templeton, M. D., Rikkersink, E. H., Solon, S. L., and Crowhurst, R. N. 1992. Cloning and molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase gene-encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. *Gene* 122:225-230.
45. Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
46. Tuite, J. 1969. *Plant Pathological Methods*. Burgess Publ. Co., Minneapolis, MN.
47. Von Arx, J. A. 1957. Die arten der gattung *Colletotrichum* cda. *Phytopathol. Z.* 29:413-468.
48. Von Arx, J. A. 1970. A revision of the fungi classified as *Gloeosporium*. *Bibl. Mycol.* 24:1-203.
49. Walker, J., Nikandrow, A., and Millar, G. D. 1991. Species of *Colletotrichum* on *Xanthium* (Asteraceae) with comments on some taxonomic and nomenclatural problems in *Colletotrichum*. *Mycol. Res.* 95:1175-1193.
50. Ware, S. B. 2000. Genetic diversity of *Colletotrichum sublineolum* based on vegetative compatibility, mtDNA and nuDNA RFLPs, and intron sequences. M.S. thesis, University of Arkansas, Fayetteville.
51. Wasilwa, L. A. 1997. Characterization of *Colletotrichum orbiculare* and allied *Colletotrichum* species using mtDNA RFLPs, vegetative compatibility, and virulence on cucurbits, and screening cucumber and watermelon for anthracnose resistance. Ph.D. diss., University of Arkansas, Fayetteville.
52. Wasilwa, L. A., Correll, J. C., Morelock, T. E., and McNew, R. 1993. Re-examination of races of the cucurbit anthracnose pathogen, *Colletotrichum orbiculare*. *Phytopathology* 83:1190-1198.
53. Welty, R. E., and Mueller, J. P. 1979. Occurrence of a highly virulent isolate of *Colletotrichum trifolii* on alfalfa in North Carolina. *Plant Dis. Rep.* 63:666-670.
54. Wharton, P. S., and Dieguez-Urbeondo, J. 2004. The biology of *Colletotrichum acutatum*. *Anal. Bot.* 61:3-22.
55. Zaumeyer, W. J., and Meiners, J. P. 1975. Disease resistance in beans. *Annu. Rev. Phytopathol.* 13:313-334.