Characterization of the *Colletotrichum* Species Causing Anthracnose in Andean Blackberry in Colombia

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Abstract

Afanador-Kafuri, L., González, A., Gañán, L., Mejía, J. F., Cardona, N., and Alvarez, E. 2014. Characterization of the *Colletotrichum* species causing anthracnose in Andean blackberry in Colombia. *Plant Dis.* 98:1503-1513.

Since 1992, anthracnose of Andean blackberry (*Rubus glaucus*) has generated losses as high as 40% for farmers in Colombia. In this study, our goal was to characterize 240 *Colletotrichum* isolates from Andean blackberry in eight areas of Colombia. These isolates were evaluated according to morphological characteristics, sensitivity to benomyl, pathogenicity, and genetic variability. Identification of the genus *Colletotrichum* was achieved by using species complex-specific polymerase chain reaction primers. A multilocus phylogeny approach was used to identify isolates to the species level with sequences from the ribosomal internal transcribed spacer region and partial sequences of the actin, β -tubulin 2, calmodulin, chitin synthase 1, glutamine synthetase, and

glyceraldehyde-3-phosphate dehydrogenase genes. Most of the isolates were identified as *Colletotrichum gloeosporioides* sensu lato, were associated with the Castilla ecotype, showed high sensitivity to benomyl, and were highly aggressive. Isolates identified as *C. acutatum* sensu lato were found mainly on the Thornless ecotype, were highly resistant to benomyl, and showed intermediate aggressiveness. Only three isolates were identified as *C. boninense* sensu lato. The species identified included *C. fructicola*, *C. kahawae* subsp. *ciggaro*, *C. gode-tiae*, *C. karstii*, *C. brassicicola*, and undetermined *Colletotrichum* spp. This study is the first report of these species associated with anthracnose in Andean blackberry.

Andean blackberry (*Rubus glaucus* Benth.), also known as *mora de Castilla* in Spanish, is a tropical fruit native to the northern Andes of South America and is now a crop of economic importance in Latin American countries such as Colombia, Costa Rica, Ecuador, Guatemala, Honduras, Mexico, and Panama (19,23). In Colombia, the Andean blackberry is cultivated mainly by small- and medium-scale farmers, and is a major source of income for more than 12,000 rural families in midaltitude regions (1,500 to 2,500 m above sea level) (23,27). However, the production potential of this crop is constrained by poor agronomic practices, such as lack of fertilizer application and high plant densities (4), dependence on a few varieties that are of poor genetic quality for planting materials (4), and susceptibility of the current planted varieties to diseases, particularly anthracnose (*Colletotrichum* spp.), fruit rot or gray mold (*Botrytis cinerea*), and downy mildew (*Peronospora* sp.) (6,42,43).

Since 1992, when records first became available, anthracnose of Andean blackberry has generated losses as high as 40% for farmers in Colombia (16,42,43). The disease is prevalent in areas with temperatures ranging from 13 to 22°C and relative humidity above 92%. The fungus attacks fruit, inducing rot and mummification. It also attacks stems and branches, causing symptoms such as black lesions and dieback (Fig. 1). Disease incidence and severity depend on locality and weather conditions and, therefore, vary over sites from year to year.

Previous morphological, molecular, and phylogenetic studies of *Colletotrichum* populations attacking Andean blackberry suggest that *Colletotrichum gloeosporioides* and *C. acutatum* are causal agents of anthracnose in this crop (5,35,36). Recently, however, *C.*

gloeosporioides and *C. acutatum* were determined to be species complexes, each with several species that share morphological and even genetic similarities (14,47). Multilocus phylogenetic studies are used to delimit *Colletotrichum* spp. within these two (2,14,15,29,30,33,47) and other *Colletotrichum* complexes, such as the *C. boninense* species complex, which encompasses about 17 species and affects a wide range of hosts (15), and the *C. graminicola* species complex, which is associated with anthracnose in grass species (9,12,13).

For example, Prihastuti et al. (33) elucidated the genetic relationship existing between the species *C. asianum*, *C. siamense*, and *C. fructicola* within the *C. gloeosporioides* complex on the basis of analyzing data on sequences from the ribosomal internal transcribed spacer (ITS) region and the following genes: actin (ACT; partial), β -tubulin 2 (TUB 2), calmodulin (CAL), glutamine synthetase (GS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Cai et al. (8) recommend a polyphasic strategy for recognizing and identifying species within the *Colletotrichum* genus. This strategy must include morphological, physiological, biochemical, and pathogenicity studies, together with phylogenetic analysis of multiple genes, using sequencing data from type specimens.

Given the importance of anthracnose in Colombia's main blackberry-producing areas, additional studies are needed to better understand the disease and its causal agents. Such information is required for planning appropriate pest management and control. Crucial to understanding this disease's epidemiology is not only the correct and timely identification of the pathogens responsible for anthracnose in Andean blackberry but also the determination of the pathogen populations' variability. Such understanding would contribute toward developing genetic improvement programs for this crop.

To meet these needs, our study aimed to identify the *Colletotrichum* spp. affecting Andean blackberry in Colombia, and to characterize a large set of *Colletotrichum* isolates according to specific morphological and cultural characteristics, pathogenicity, and genetic diversity.

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Accepted for publication 21 April 2014.

Materials and Methods

Sampling, isolation, and conservation of the fungus. From 2004 to 2008, samples of symptomatic plant tissues from Andean blackberry were collected at several sites from 38 municipalities of eight departments in Colombia (i.e., Antioquia, Caldas, Cundinamarca, Huila, Quindío, Risaralda, Santander, and Valle del Cauca; Fig. 2). At each site, samples were categorized and collected according to symptom type (necrosis around the spines, stem necrosis, dieback in branches, necrosis in fruit or floral buds, and mummification of fruit; Fig. 1) and phenotypic diversity of the crop (blackberry ecotype).

To isolate the fungus, 0.5-cm fragments were cut from both healthy and diseased tissues. The tissues were then disinfected (first in 70% ethanol for 1 min and then in 1% sodium hypochlorite for 2 min), washed in sterilized distilled water for 1 min, and dried on sterilized paper towels. Five fragments of disinfected blackberry tissue were then placed on potato dextrose agar (PDA; Difco Laboratories) medium amended with 1 ml of 25% lactic acid per liter (Merck & Co., Inc.) and incubated at room temperature (24°C) under continuous light.

The cultures were evaluated at 2-day intervals until fungal colonies began to develop. Portions of mycelium obtained from colony edges were then transferred to fresh, acidified PDA (APDA) medium for purification and to obtain mono-conidial isolates.

Pieces of sterilized filter paper, colonized with mycelium and conidia from monoconidial isolates of the fungus, were stored at 4°C. For this study, 240 isolates were selected according to their geographical origin, blackberry ecotype ('Castilla' or 'Thornless'), diseased tissue, and colony morphology of each fungal strain on APDA medium (Table 1).

Morphological characterization. All the *Colletotrichum* isolates were examined for their cultural and morphological traits. Mycelial plugs (0.5 mm in diameter) were taken from the edges of 12-day-old colonies, transferred to the middle of 9-cm-diameter petri dishes containing APDA medium, and incubated at $22 \pm 1^\circ\text{C}$ and a light-and-dark cycle at 12 and 12 h, respectively, using a cool, white, fluorescent light. Colony characteristics (i.e., texture, density, mycelial color on the upper and lower surfaces, presence or absence of sectors, type of conidiation, and fungal mat edges) were determined visually after 15 days of incubation. Colony color was rated according to Munsell Color Charts (25). Growth rate was calculated as the 12-day average of mean daily growth (millimeters per day).

Conidia were harvested from 15-day-old cultures incubated under the same conditions mentioned above. In most cases, sporulation was induced by cutting portions of the colony with a scalpel, thus mechanically injuring the mycelium.

Appressorium formation was induced by germinating spores in drops of deionized water in the wells of single, concave, microscope slides (Parco Scientific Co.) and incubating them in a humid chamber overnight (11). Each well contained 30 μl of conidial suspension.

The size and shape of conidia and appressoria were observed under an Olympus CX41 light microscope (Olympus Deutschland GmbH), photographed with an Olympus digital camera (Olympus C5060ADU), and then measured with the software program Scion Image for Windows (Scion Corporation). For each isolate, 60 conidia and 21 appressoria from each replicate were analyzed.

Additionally, the presence or absence of the sexual phase was evaluated based on micropreparations of fungal structures that consisted of round, compact, hard, dark-brown- or black-colored masses which were immersed in the medium. These masses were indicative of sexually derived perithecia or primordia.

Morphological studies were conducted with three replicates, using a randomized complete block design. To establish differences

Fig. 2. Andean blackberry-producing areas in Colombia where *Colletotrichum* isolates were collected. Georeference points of each collection site (38 in all) are marked with a circle. Numbers refer to Colombian departments: 1, Antioquia; 2, Santander; 3, Caldas; 4, Cundinamarca; 5, Risaralda; 6, Quindío; 7, Valle del Cauca; 8, Huila.

Fig. 1. Anthracnose symptoms in Andean blackberry. **A**, Dieback and floral bud rot. **B**, Fruit rot and mummification. **C**, Stem lesions.

between isolates, morphological data were subjected to multiple correspondence and principal components analyses, and growth rate values to analysis of variance (ANOVA). Separation of means was conducted using the Ryan-Einot-Gabriel-Welsch (REGW) multiple step-down range test ($\alpha \leq 0.05$) (37).

Evaluating sensitivity to benomyl. Each isolate was placed on PDA plates supplemented with benomyl (Benlate 50 WP; Dupont) at 5 $\mu\text{g a.i. ml}^{-1}$. Sensitivity or resistance of isolates to benomyl was evaluated by measuring colony radius, which was then used to calculate colony area. Resulting data were expressed as percentages of inhibition (%I) of fungal growth in relation to the fungal growth on unamended PDA medium (control). A randomized complete block design was used, with three replicates. Data of the %I of growth were arcsine-square root transformed and then analyzed using an ANOVA to establish differences between isolates, and separation of means was carried out with a REGW test ($\alpha \leq 0.05$) (37). Isolates were termed highly sensitive if percentage of growth inhibition was greater than 90%, sensitive at >75 to 90% inhibition, moderately resistant at >60 to 75%, resistant at >40 to 60%, and highly resistant if growth inhibition was lower than 40%.

Pathogenicity tests. The aggressiveness of 240 *Colletotrichum* isolates was evaluated by artificially inoculating three replicates of Castilla blackberry stems (20-cm-long cuttings, averaging 1 cm in diameter) per isolate, following the method of Stewart et al. (40). The stems had previously been disinfected with a solution of 1.5% sodium hypochlorite, washed with sterilized distilled water, and dried at room temperature. Each stem was inoculated with a 0.5-cm disc of APDA medium carrying fungal mycelium and then sealed with a Parafilm M strip (Bemis Flexible Packaging Com-

pany). Three stems inoculated with fungus-free APDA discs were used as controls for each treatment (isolate tested).

Inoculated stems were then placed in transparent covered plastic trays, the bottoms of which contained water (1 cm deep) to maintain humidity, and incubated at 22°C, with 8 h of daylight.

The width and length of each resulting lesion were measured from day 5 to day 20 after inoculation. Lesion area was estimated by assuming an elliptical shape.

Lesion area data, recorded on alternate days, were used to estimate the area under the disease progress curve (AUDPC). For statistical analysis, AUDPC data were log transformed, then analyzed, assuming a randomized complete block experimental design and using SAS (version 9.2; SAS Institute Inc.) (37). ANOVA was performed to establish differences between isolates and the REGW test was run to evaluate statistically significant differences at $\alpha \leq 0.05$ (37).

Molecular characterization. *DNA isolation and purification.* Each isolate was cultured for 10 days in an Erlenmeyer flask containing 100 ml of liquid potato dextrose medium (potato at 200 g/liter, dextrose at 20 g/liter, and 1 liter of distilled water) plus 60 μl of ampicillin (20 mg/liter). Cultures were incubated at room temperature ($\pm 24^\circ\text{C}$) under constant artificial light and no agitation.

DNA extraction and purification were performed according to Mahuku (22), as modified by Alvarez et al. (3). The DNA pellet was dissolved in 100 μl of Tri-EDTA buffer (10 mM Tri-HCL and 1 mM EDTA, pH 8.0). Quality was determined by electrophoresis in 0.8% agarose gel and quantified by fluorometry, using a DyNA Quant 200 Fluorometer (Hoefer, Inc.). The DNA was subsequently diluted to 5 $\text{ng } \mu\text{l}^{-1}$, as a working solution for polymerase chain reaction (PCR) tests.

Table 1. Morphological, cultural, and pathogenic characteristics of the 240 *Colletotrichum* isolates obtained from Andean blackberry and grouped according to species complex and genetic diversity cluster, as identified through intersimple sequence repeat (ISSR) analysis^a

Species complex, cluster ^v	Infected tissue	Colony type ^w	Conidium			Appressorium			%I ^x	Growth (mm) ^y	AUDPC ^z	Identified species
			Shape	Length (μm)	Width (μm)	Shape	Length (μm)	Width (μm)				
<i>Colletotrichum gloeosporioides</i> sensu lato cluster I (138)	Fruit, leaf, stem	A, B, C, E	Oblong-elliptical to fusiform	10.8 a	3.0 a	Globose or ovoid	6.6 ab	2.7 a	78.3 ab	5.8 ab	151.8 a	<i>C. kahawae</i> subsp. <i>ciggaro</i> (isolates C022-1, CD024t, H043, Q025, Q041, R019, VC097)
<i>C. gloeosporioides</i> sensu lato cluster II (21)	Stem	A, B	Elliptical or oblong-elliptical	10.2 a	2.9 a	Globose or ovoid	6.5 ab	2.7 a	92.0 a	4.6 abc	167.0 a	<i>C. fruticicola</i> (isolates CD009h, R002b)
<i>C. gloeosporioides</i> sensu lato cluster III (2)	Fruit, stem	A, G	Oblong-elliptical	10.7 a	3.2 a	Ovoid	6.2 b	2.7 a	85.2 a	6.0 a	26.9 ab	<i>C. kahawae</i> subsp. <i>ciggaro</i> (isolates S007-3, R046)
<i>C. gloeosporioides</i> sensu lato cluster VI (4)	Fruit	A	Oblong-elliptical	10.4 a	2.9 a	Ovoid	6.4 ab	2.7 a	68 abc	4.6 abc	3.0 bc	<i>Colletotrichum</i> sp. (isolate Q026)
<i>C. acutatum</i> sensu lato cluster IV (35)	Fruit, inflorescence, stem	B, C, E, H	Oblong-elliptical to fusiform	10.5 a	2.9 a	Globose or ovoid	6.5 ab	2.7 a	39.3 d	4.2 c	48.0 ac	<i>C. godetiae</i> (isolates AN014, S025-2, VC017-1)
<i>C. acutatum</i> sensu lato cluster V (37)	Fruit, inflorescence, stem	A, B, C, D, E, F	Oblong-elliptical to fusiform	9.9 a	2.8 a	Globose or ovoid	6.5 ab	2.7 a	36.1 d	3.9 c	78.5 ac	<i>Colletotrichum</i> sp. (isolates AN035a, Q043, Q030, Q003-1)
<i>C. boninense</i> sensu lato cluster VI (3)	Fruit, stem	B	Elliptical or oblong-elliptical	9.5 a	2.7 a	Ovoid	5.9 b	2.6 a	66.8 abc	3.1 c	1.8 c	<i>C. brassicicola</i> (isolate CD015-1), <i>C. karstii</i> (isolate R001), <i>Colletotrichum</i> sp. (isolate R033)

^a Mean difference between each group is significant at the 0.05 level; values with same letters based on REGW multiple range test in a column do not differ significantly.

^v ISSR and M13 clusters. Values in parentheses show the number of isolates in each group.

^w A = gray colony, cottony texture, high density, sectors absent, irregular edges; B = gray to olive-gray colony, cottony texture, high density, sectors absent, uniform edges; C = pinkish- to grayish-white colony, fructification present in colony center, cottony texture, sectors absent, high density, uniform edges; D = gray colony, fructification present in colony center, velvety texture, high density, sectors absent, uniform edges; E = gray colony, fructification present in colony center, cottony texture, high density, sectors absent, uniform edges; F = gray colony, abundant fructification rings throughout colony, velvety texture, intermediate density, sectors absent, uniform edges; G = pinkish-white colony, reddish-yellow fructification rings throughout colony, velvety texture, intermediate density, sectors present, uniform edges; H = olive-gray colony, cottony texture, high density, sectors absent, uniform edges.

^x Sensitivity to benomyl is indicated as percentage of inhibition (%I) of colony growth in relation to the control.

^y Growth rate on potato dextrose agar (mm day⁻¹).

^z AUDPC = area under disease progress curve.

Identification of *Colletotrichum* complexes using PCR. The amplification of complex-specific regions of the ITS region was performed using the universal primer ITS4, together with the primers specific for *C. acutatum* sensu lato (CaInt2), *C. gloeosporioides* sensu lato (CgInt), and *C. boninense* sensu lato (Coll) (1,24,31,38). The amplification of the regions associated with the TUB 2 gene was performed with the TBCA primer (specific for *C. acutatum* sensu lato) and the primer TBCG (specific for *C. gloeosporioides* sensu lato), in combination with the conserved primer TB5, which is useful for both complexes (1,41).

The DNAs of strains GND-1, C339, Tom 10, Tom 21, Pass 063, and Pass 52 were used as positive controls for amplification. The negative control was high-performance liquid chromatography (HPLC)-grade ultrapure water.

Each reaction comprised 1× enzyme buffer, 0.2 mM each dNTP, 0.5 μM each primer, 1.5 mM MgCl₂, DNA at 0.2 ng μl⁻¹, HPLC-grade ultrapure water to complete a final volume of 25 μl, and Bioline *Taq* DNA polymerase (Bioline Inc.) at 0.1 U μl⁻¹. DNA amplification was performed using a PTC-100 thermal cycler (MJ Research), with an initial denaturing step of 95°C for 5 min; 40 cycles at 95°C for 30 s and 30 s at 61°C for *Coll*, 62°C for *CgInt*, and 60°C for *CaInt2*; plus extension for 90 s at 72°C. A final extension for 4 min at 72°C was used post-cycling. Conditions for the TBCA and TBCG primers were published by Talhinas et al. (41).

Amplification products (6 μl) were separated by electrophoresis in a 1.5% agarose gel in 0.5× Tris-borate EDTA buffer, stained with 1.3 μM ethidium bromide at 5 V cm⁻¹ for 4 h, and visualized using UV light. Each gel had two lanes loaded with 2 μl of the molecular weight marker Hyperladder II to facilitate comparisons of sample band sizes. Images were captured using the gel reader software Gel Doc 2000 (Bio-Rad Laboratories, Inc.).

Determining genetic diversity with intersimple sequence repeat and M13 PCR markers. The 240 *Colletotrichum* isolates were tested using five different primers of the type intersimple sequence repeat (ISSR)—(CAG)₅, (AGG)₅, (GACA)₄, (GACAC)₃, and (TCC)₅—and the MR primer (5′ GAGGGTGGCGTTCT 3′, core sequence of phage M13), which were previously reported to produce polymorphic and reproducible DNA fingerprint patterns from *C. acutatum* sensu lato and *C. gloeosporioides* sensu lato (41). Each PCR reaction (25 μl) consisted of 1× enzyme buffer, 0.2 mM each dNTP, 0.5 μM primer, 1.5 mM MgCl₂, DNA at 0.2 ng μl⁻¹,

HPLC-grade ultrapure water to a final volume of 25 μl, and Bioline *Taq* DNA polymerase (Bioline Inc.) at 0.1 U μl⁻¹. The PCR conditions for ISSR and M13 amplifications consisted of an initial denaturation carried out with a preliminary cycle of 5 min at 95°C; followed by 30 cycles of denaturation for 30 s at 95°C; annealing for 30 s at 60°C for primers (CAG)₅, (AGG)₅, MR, and (TCC)₅ or 48°C for primers (GACA)₄ and (GACAC)₃; and extension for 1.5-min at 72°C. The amplification products were separated and visualized, as mentioned above.

Genetic diversity was interpreted according to general band patterns, assigning a value of one (1) if present and zero (0) if absent and generating a binary data matrix. Genetic relationships among isolates were ascertained using multiple correspondence analyses (MCA) and constructing a three-dimensional scatter plot. The row-coordinates profile was obtained from the MCA, while group assignment was obtained from cluster analysis and average nearest neighbor distance calculations. All data were analyzed with SAS, version 9.2 for Windows (37).

Multilocus sequencing and phylogenetic analysis. *Specimen selection.* A subset of 22 isolates was selected to represent the *Colletotrichum* complexes identified and the range of genetic diversity found according to the M13 and ISSR-PCR marker analyses.

PCR amplification and sequencing. Nucleotide sequences were determined for seven PCR-amplified nuclear amplicons, including the complete ribosomal ITS region, ACT, TUB2, GAPDH, chitin synthase 1 (CHS-1), CAL, and GS genes, which were amplified and sequenced using the primer pairs ITS-5 + ITS-4 (48), ACT-512F + ACT-783R (10), Bt2a + Bt2b (17), GDF1 + GDR1 (46), CHS-79F + CHS-354R (10), CL1 + CL2A (26), and GSF1 + GSR1 (39), respectively.

The PCR amplifications were performed in a 25-μl reaction volume, consisting of 10 ng of DNA template, a 1× final concentration of Fermentas DreamTaq Green PCR Master Mix (Applied Biosystems Inc.), and 0.5 μM each primer. The reactions were performed with a thermal cycler (PTC-100; MJ Research Inc.), using the thermal programs as previously described for ITS, ACT, TUB 2, GAPDH, and GS (33), and for CHS-1 and CAL (47).

The PCR products were purified with the PEG-NaCl method. Briefly, the sample was mixed with 1× volume of 20% PEG-NaCl [PEG, MW 6,000, and 2.5 M NaCl] and incubated for 15 min at

Table 2. GenBank accession numbers of *Colletotrichum* isolates from Andean blackberry in Colombia as identified by multilocus phylogenetic analysis

<i>Colletotrichum</i> spp.	Isolate code	GenBank accession numbers ^z						
		ACT	CAL	CHS-1	GAPDH	GS	ITS	TUB 2
<i>Colletotrichum brassicicola</i>	CD015-1	KC859967	...	KC859992	KC860010	...	JN715844	KC860036
<i>C. fruticola</i>	CD009h	KC859959	KC859977	KC859984	KC860002	...	JN715843	KC860028
	R002b	KC859960	KC859978	KC859985	KC860003	...	KC860041	KC860029
<i>C. godetiae</i>	AN014	KC859961	...	KC859986	KC860004	...	JN715834	KC860030
	S025-2	KC859962	...	KC859987	KC860005	...	KC860043	KC860031
	VC017-1	KJ001105	...	KJ001112	KJ001116	...	KJ001123	KJ001127
<i>C. kahawae</i> subsp. <i>ciggaro</i>	C022-1	KJ001102	KJ001106	KJ001109	KJ001113	KJ001117	KJ001120	KJ001124
	CD024t	KC859955	KC859973	KC859981	KC859998	KC860019	JN715845	KC860024
	H043	KC859953	KC859971	KC859979	KC859996	KC860015	JN715838	KC860022
	Q025	KJ001103	KJ001107	KJ001110	KJ001114	KJ001118	KJ001121	KJ001125
	Q041	KJ001104	KJ001108	KJ001111	KJ001115	KJ001119	KJ001122	KJ001126
	R019	KC859954	KC859972	KC859980	KC859997	KC860017	JN715847	KC860023
	R046	KC859957	KC859975	KC859982	KC860000	KC860018	JN715848	KC860026
	S007-3	KC859958	KC859976	KC859983	KC860001	KC860014	JN715836	KC860027
	VC097	KC859956	KC859974	KF312208	KC859999	KC860021	JN715837	KC860025
<i>C. karstii</i>	R001	KC859968	...	KC859993	KC860011	...	KC860040	KC860037
<i>Colletotrichum</i> sp.	AN035a	KC859963	...	KC859988	KC860006	...	JN715835	KC860032
	Q043	KC859964	...	KC859989	KC860007	...	JN715840	KC860033
	Q030	KC859965	...	KC859990	KC860008	...	JN715841	KC860034
	Q003-1	KC859966	...	KC859991	KC860009	...	JN715842	KC860035
	R033	KC859969	...	KC859994	KC860012	...	KC860042	KC860038
	Q026	KC859970	...	KC859995	KC860013	...	JN715839	KC860039

^z Abbreviations: actin (ACT; partial), calmodulin (CAL), chitin synthase 1 (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamine synthetase (GS), internal transcribed spacer (ITS), and β-tubulin 2 (TUB 2).

room temperature. The precipitate was collected by centrifuging at 13,000 rpm for 15 min. The pellet was washed with 70% ethanol, air dried, and dissolved in 20 µl of sterilized distilled water.

The DNA sequences were obtained in both directions on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems Inc.). All sequencing reactions were performed at the Biotechnology Unit of Iowa State University, Ames.

Phylogenetic analysis. The nucleotide sequences were assembled into contigs, edited with ChromasPro version 1.5 (Technelysium Pty. Ltd.), and aligned, using ClustalW as implemented in MEGA version 5.1 (44). Individual gene alignments were concatenated, using FASconCAT version 1.0 (20).

Bayesian Metropolis-coupled Markov chain Monte Carlo (MCMC) analysis was performed on the dataset using MrBayes version 3.2.1 (34), with substitution models determined separately for each partition, according to the corrected Akaike information criterion (AICc) as determined by jModelTest version 0.1.1 (32). For the Bayesian inference analysis, two MCMC chains were run twice for 10,000,000 generations, with trees sampled every 1,000 generations. After omitting the first 25% of saved trees (burn-in), the remaining sampled trees were used to estimate a 50% majority rule consensus tree.

Sequences derived in this study have been lodged at GenBank (Table 2) and sequence alignments in TreeBASE (<http://treebase.org/treebase-web/home.html>) under the accession number S14027. Sequences corresponding to the target genes of ex-type and authentic *Colletotrichum* isolates described by Damm et al. (14,15) and Weir et al. (47) were obtained from the GenBank and included in the phylogenetic analysis.

Results

Strain isolation. In the eight production areas where samples were gathered (Fig. 1), anthracnose was detected in the Castilla and Thornless ecotypes. In the Castilla ecotype, anthracnose was observed attacking mostly stems and causing black lesions and dieback whereas, in the Thornless ecotype, the disease mostly attacked floral buds and fruit, causing necrosis and mummification. In total, 240 single-spore isolates of *Colletotrichum* were included in this study.

Morphological characterization of *Colletotrichum* isolates. Most of the *Colletotrichum* isolates produced colonies with uniform or irregular edges, composed of mycelium that were light to dark gray to olive gray on both the upper and lower surfaces (Fig. 3), with cottony texture, high density, and low presence of sectors. In cases in which the sporulation occurred normally without injury, spore masses appeared on the colony surface, usually toward the middle of the colony. Spore masses were reddish yellow to salmon pink. In 63% of isolates, the conidia were oblong-elliptical; other shapes observed were elliptical-fusiform and elliptical (Fig. 3). In 86% of isolates, appressoria were oval.

On APDA medium, in colonies more than 20 days old, perithecia appeared as hard, dark-brown, or black structures, resistant to rupture. These were either distributed irregularly within the colony or formed aggregates in bands or rows where they met. Microscopy corroborated the presence of unitunicate perithecial asci with thin walls and eight ascospores per ascus. The ascospores were unicellular, hyaline, ellipsoidal, and slightly curved. The sexual state (*Glomerella* spp.) was observed in 116 isolates from the *C. gloeosporioides* complex and 5 from the *C. acutatum* complex.

Multiple correspondence analyses of qualitative variables indicated phenotypic variability among isolates, grouping them into eight morphological groups. The highest morphological variability was found in the *C. acutatum* complex composed of eight morphological groups, while the least variability was found in the *C. gloeosporioides* complex with four groups and the *C. boninense* complex with only one group (Table 1).

Morphological group A was formed by 167 isolates (68%), mostly from the *C. gloeosporioides* complex (153 isolates), with only 12 isolates from the *C. acutatum* complex. Morphological group B included 41 isolates (17%), mostly from the *C. acutatum*

complex, with 3 isolates from the *C. boninense* complex. Morphological groups A and B were found in all Andean blackberry-producing areas (Table 1).

Conidium length (2.6 to 15.8 µm), conidium width (0.7 to 4.6 µm), and appressorium length (2.1 to 8.2 µm) were significantly different ($P < 0.001$) both among isolates and species complexes (Table 1).

Significant differences ($P < 0.001$) in growth rate were observed between isolates. Three groups were identified: (i) isolates with high growth rates (5.0 to 7.1 mm day⁻¹), (ii) isolates with intermediate growth rates (3.9 to 5.9 mm day⁻¹), and (iii) isolates with low growth rates (2.3 to 5.0 mm day⁻¹). Isolates belonging to the *C. gloeosporioides* complex had intermediate to high growth rates, those of the *C. acutatum* complex had intermediate rates, and those of the *C. boninense* complex had low rates (Table 1).

Benomyl sensitivity. The evaluated isolates showed a variable response to benomyl. Sensitivity to benomyl significantly differed ($P < 0.001$) between complexes and among complexes or genetic clusters (Table 1). Isolates of the *C. gloeosporioides* complex were sensitive; colony-growth was strongly inhibited (mean of 78.4%), with only six isolates inhibited by less than 51%. In contrast, isolates of the *C. acutatum* complex were highly resistant to benomyl. The mean colony-growth inhibition of *C. acutatum* isolates was only 31%, and only 3 of 72 isolates were inhibited by more than 69%. The three isolates of the *C. boninense* complex were immediately sensitive to benomyl; mean colony-growth inhibition was 66.8% (Table 1).

Pathogenicity evaluation. Inoculated stems developed reddish-brown lesions that turned violet black over time. The lesions spread around the inoculation point and joined to form one larger, oval lesion. The fungus sporulated abundantly as the lesions matured between 15 and 25 days after inoculation, especially in the area wrapped with Parafilm. Sometimes, perithecia of the teleomorph *Glomerella* spp. were observed beneath the Parafilm. Control stems developed a small brown area that remained restricted to the inoculation point.

Significant differences ($P < 0.001$) in AUDPC were found both between species complexes, and among isolates within each complex or genetic cluster (Table 1). Isolates of the *C. gloeosporioides* complex (clusters I and II) were the most aggressive, with mean AUDPC scores of 151.8 and 167.0, respectively. Isolates of the *C. acutatum* complex (clusters IV and V) and two isolates of the *C. gloeosporioides* complex (cluster III) were moderately aggressive, with AUDPC means of 26.9 and 78.5, respectively. The least aggressive isolates belonged to the *C. boninense* complex (cluster VI) and *C. gloeosporioides* complex (cluster VI), with mean AUDPC scores of 1.8 and 3.0, respectively (Table 1).

Molecular characterization. *Identifying complexes with specific primers.* Primers *CaInt2* (specific) and ITS4 amplified a 450-bp DNA fragment for 32% of evaluated isolates and the reference isolates of *C. acutatum* sensu lato (Tom 10 and Tom 21). Primers *CgInt* (specific) and ITS4 amplified a 450-bp DNA fragment for 67% of isolates and the reference strains of *C. gloeosporioides* sensu lato (GND-1 and C339). For primers *ColI* (specific) and ITS4, an amplification product of 520 bp was obtained for isolates R001 and R033 from Risaralda, CD015-1 from Caldas, and for the reference strains of *C. boninense* sensu lato (Pass 063 and Pass 52). The species identified for the *C. gloeosporioides* and *C. acutatum* complexes were confirmed using primers TBCG and TBCA combined with TB5, which amplified a fragment of about 330 bp for both complexes.

These results indicate that isolates of the *C. gloeosporioides* complex are the primary causal agents of anthracnose in Andean blackberry in Colombia, followed by isolates of the *C. acutatum* complex and, to a lesser degree, isolates of the *C. boninense* complex (Table 1). A high proportion of isolates assigned to the *C. gloeosporioides* complex were collected in the Andean blackberry ecotype Castilla. In contrast, a greater proportion of *C. acutatum* complex isolates were collected in the Thornless ecotype (Fig. 4).

Fig. 3. A–F, Fourteen-day-old colonies of *Colletotrichum* spp. from Andean blackberry and grown on potato dextrose agar culture medium. Above and reverse views are shown: A, *Colletotrichum fructicola* (isolate CD009h); B, *C. kahawae* subsp. *cigaro* (isolate R019); C, *C. godetiae* (isolate AN014); D, *C. karstii* (isolate R001); E, *C. brassicicola* (isolate CD015-1); and F, *Colletotrichum* sp. (isolate R033). G–I, Shapes of *Colletotrichum* conidia: G, elliptical; H, oblong-elliptical; and I, elliptical-fusiform. Scale bar = 10 μ m.

Determining the genetic variability of isolates using ISSR primers and the M13 primer. The 240 isolates of *Colletotrichum* spp. from Andean blackberry were evaluated using five ISSR primers and the MR primer. On average, 192 polymorphic bands each and 14.7 bands per isolate were amplified. Multiple correspondence analysis identified six genetic clusters (Fig. 5), with cluster I comprising 56% of isolates; cluster II, 9%; cluster III, 2%; cluster IV, 14%; cluster V, 15%; and cluster VI, 4%. The clusters corresponded largely to the *Colletotrichum* complexes identified through specific primers discussed above. Clusters I and II brought together most (96%) of the isolates belonging to the *C. gloeosporioides* complex, including reference strains. The remaining 4% formed part of clusters III and VI (Fig. 5; Table 1).

Clusters IV (49%) and V (51%) comprised all of the isolates belonging to the *C. acutatum* complex, including the reference strains. Isolates in the *C. boninense* complex fell into cluster VI, together with some isolates belonging to other complexes.

Phylogenetic analysis. Most of the 22 new *Colletotrichum* isolates fell into three well-defined complexes: *C. acutatum* sensu lato (isolates AN014, AN035a, Q003-1, Q030, Q043, S025-2, and VC017-1), *C. gloeosporioides* sensu lato (isolates C022-1, CD009h, CD024t, H043, Q025, Q041, R002b, R019, R046, S007-3, and VC097), and *C. boninense* sensu lato (isolates CD015-1, R001, and R033). That is, the complex-specific primers confirmed the identification of these isolates in their respective complexes.

A dendrogram constructed from a multilocus phylogenetic analysis of isolates belonging to the *C. gloeosporioides* complex is presented (Fig. 6). Isolates CD009h and R002b and the reference strains clustered in a well-defined clade for *C. fruticola* at a Bayesian posterior probability of 1. The other isolates of the *C. gloeosporioides* complex clustered within the clade for *C. kahawae* at a Bayesian posterior probability of 1. An analysis of the GS gene sequences indicated that these isolates belong to *C. kahawae* subsp. *ciggaro*, as determined by a sequence difference and the absence of a 22-bp deletion (48).

With respect to the isolates belonging to the *C. acutatum* complex, Bayesian inference indicated that isolates AN014, S025-2 and VC017-1 from blackberry clustered in a well-defined subclade within clade 5 of *C. acutatum* sensu lato, together with the reference strains for *C. godetiae*. The other isolates belonging to this complex clustered within clade 1 of *C. acutatum* sensu lato. However, they did not cluster within subclades defined for previously epitypified reference species and, therefore, were considered as undetermined species within this complex (Fig. 7).

Finally, isolates belonging to the *C. boninense* complex were identified at the species level such as *C. karstii* (isolate R001) and *C. brassicicola* (isolate CD015-1). However, isolate R033 was not associated with any species in the complex, and was also considered to be an undetermined species (Fig. 8).

Fig. 4. *Colletotrichum* species complexes distribution of the isolates obtained from two Andean blackberry ecotypes in Colombia.

We emphasize that the results of the ISSR and M13 test to evaluate the genetic diversity of the isolates within each species complex showed that the genetic groups are associated with specific species of each *Colletotrichum* complex. That is, for species in the *C. gloeosporioides* complex, cluster I and cluster III comprised isolates identified as *C. kahawae* subsp. *ciggaro* while cluster II included all the *C. fruticola* isolates. For species in the *C. acutatum* complex, cluster IV was formed by the *C. godetiae* isolates, and those of cluster V corresponded to undetermined *Colletotrichum* sp. isolates in clade 1. Cluster VI comprised all the species of the *C. boninense* complex.

In addition, isolate Q026 did not group with any of the *Colletotrichum* complexes. Analyses of the ITS region showed this isolate to be intermediate between the *C. gloeosporioides* and *C. boninense* complexes (data not shown). This isolate was located at cluster VI, in the MCA analysis (Fig. 5), together with the *Colletotrichum* isolates belonging to the *C. boninense* complex.

Discussion

The PCR amplification of the ribosomal DNA-ITS region and the β -tubulin gene with specific DNA primers has been used in earlier molecular studies to determine the etiology of anthracnose in many crops, especially fruit crops (5,7,21,28,31,35). However, these primers cannot be considered species specific, because of the similarity in sequences of the ITS region and β -tubulin gene of different *Colletotrichum* spp. This has caused misidentification of many strains that were previously identified as either *C. gloeosporioides* or *C. acutatum* at the species level. Nevertheless, as demonstrated by our study and Alvarez et al. (2), these primers can be considered to be particularly important for identifying isolates at the complex level.

Isolates of the *C. gloeosporioides* complex were present in all production areas evaluated, predominating in those regions where the Castilla ecotype was planted (Antioquia, Cundinamarca, Huila, Santander, and Valle del Cauca). In contrast, isolates of the *C. acutatum* complex were found more frequently in the coffee-growing regions (Caldas, Quindío, and Risaralda), where the thornless blackberry ecotype was predominant.

Although minimally present, isolates of the *C. boninense* complex in blackberry-producing areas of Colombia may indicate its high plasticity to adapt to different hosts; it has already been detected in other fruit species such as mango, tree tomato, and passion fruit species (1).

In this study, the use of ISSR and the M13 primers divided *Colletotrichum* populations collected from Colombian Andean blackberry into molecular clusters exclusive to each complex and

Fig. 5. Three-dimensional scatter plot resulting from a multiple correspondence analysis (MCA) shows the relationships among 240 *Colletotrichum* isolates obtained from Andean blackberry and the three *Colletotrichum* species complexes: *Colletotrichum gloeosporioides* complex (clusters I, II, and III), *C. acutatum* complex (clusters IV and V), and *C. boninense* complex (cluster VI). The plot was derived from an analysis of intersimple sequence repeat and M13 primers.

into other clusters shared among complexes. Regardless of the marker used, the two most important complexes (*C. gloeosporioides* and *C. acutatum*) appear as one or two predominant genetic clusters in each of the blackberry-producing regions of Colombia. Such distribution may be attributed to the existence of clonal lineages, which have been disseminated throughout Colombia as a result of vegetative propagation by farmers. Similarly, the blackberry ecotypes used to establish new crops may have favored the presence of a given fungal species or complex in some production areas and in certain plant tissues.

Differences observed in the isolates' aggressiveness on stems of the blackberry ecotype Castilla could be attributed to the infectivity of the three complexes on different plant tissues, as indicated by

symptom type and tissue sampling conducted to obtain the isolates. More specifically, *C. gloeosporioides* sensu lato and *C. boninense* sensu lato were closely associated with stems, black lesions, and dieback symptoms. *C. acutatum* sensu lato isolates from fruit and floral buds produced mummification and necrosis. The simultaneous appearance of isolates from the three complexes was sometimes observed on the same tissues, especially stems, possibly indicating cross-infection under field conditions.

The apparent differences in infection strategies of the three complexes may be attributed to differences in pathogenicity, as found in tests carried out under controlled greenhouse conditions. Peres et al. (28) demonstrated that *C. acutatum* sensu lato has different life forms resulting from colonization strategies according to host,

Fig. 6. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum gloeosporioides* complex of isolates obtained from Andean blackberry. The tree was built using concatenated sequences of the genes ACT, CHS-1, CAL, GAPDH, ITS, and TUB 2, and run for 1×10^7 generations, each with a separate model of DNA evolution. *C. boninense* sensu stricto (ICMP 17904) was used as the outgroup.

Fig. 7. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum acutatum* species complex of *Colletotrichum* isolates obtained from Andean blackberry. The tree was built using concatenated sequences of the genes ACT, CHS-1, GAPDH, ITS, and TUB 2, and run for 1×10^7 generations, each with a separate model of DNA evolution. *C. orchidophilum* (CBS 119291) was used as the outgroup.

ability to move between hosts, and changing genetic relationships between isolates from different hosts. *C. acutatum* sensu lato is a highly specialized pathogen in the way it attacks host tissues, particularly in apple and orange, where leaves may be disease-free but the flowers and fruit are severely infected (28).

Likewise, in blackberry, the *C. acutatum* sensu lato pathogen may preferentially attack flowers and fruit, with stems remaining relatively immune. This phenomenon would explain the results obtained for artificial inoculations of detached stems. However, the pathogenicity of this species complex in blackberry and its preference for specific tissues such as fruit and floral buds cannot be conclusively demonstrated because of the difficulties of inoculating clusters of completely disease-free floral buds. Therefore, a more precise methodology must be developed to study differences in pathogenicity between the various complexes under controlled laboratory or greenhouse conditions.

The status of the *C. acutatum* complex, which is currently identified as having a low level of pathogenicity and low incidence, could change to being more aggressive with the increasing presence of a more susceptible blackberry genotype.

In this study, the multilocus phylogenetic analysis resolved *C. godetiae* (clade 5, molecular group A5) and some indeterminate species (clade 1, group A8) in the *C. acutatum* complex; *C. kahawae* subsp. *ciggaro* and *C. fructicola* in the *C. gloeosporioides* complex; and *C. karstii*, *C. brassicicola*, and one indeterminate species in the *C. boninense* complex. To our knowledge, this is the first report of these species attacking Andean blackberry.

All of the identified taxa have been isolated from other hosts of economic importance, indicating the capacity of these species to cross-infect various host plants (30). For example, in Colombia, *C. karstii* was reported as attacking *Annona muricata*, *Passiflora edulis*, *Musa* AAA, and *Solanum betaceum* (2,15). *C. gode-*

Fig. 8. Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships within the *Colletotrichum boninense* complex of isolates obtained from Andean blackberry. The tree was built using concatenated sequences of the genes ACT, CHS-1, GAPDH, ITS, and TUB 2, and run for 1×10^7 generations, each with a separate model of DNA evolution. *C. gloeosporioides* sensu stricto (CBS 112999) was used as the outgroup.

tiae was also reported as causing anthracnose in *S. betaceum*, and Latin American isolates may occupy a separate subclade from other strains collected outside this region (14). This finding was confirmed by our study, with strains from Andean blackberry being closely related genetically to strains CBS 129816 and CBS 129809, isolated from *S. betaceum* in Colombia. Thus, we suggest that these strains must be reevaluated, possibly considering them a subspecies of *C. godetiae* or a new species specific to the neotropics and subtropics of the western hemisphere.

Likewise, the isolates belonging to an undetermined species within the *C. acutatum* complex clustered with species that were representative of clade 1 of *C. acutatum* sensu lato, the origin of which is restricted to Central and South America or the southern United States (14). Particularly in this clade, it would be necessary to test other markers that would permit us to delimit the unnamed species in some species already recognized or describe them as new taxa.

Only *C. fioriniae* and *C. godetiae* had previously been associated with *Rubus* spp. (14). Our study corroborated the association of *C. godetiae* and identified four other species, both pathogenic to blackberry and highly dispersed geographically.

Using a gene sequence from this study (GS), we found isolates from blackberry that belonged to *C. kahawae* subsp. *ciggaro*, and confirmed that they did not belong to *C. kahawae* subsp. *kahawae* (coffee-berry pathogen), a species that is of high quarantine importance in Colombia and which is found to a limited extent in Africa (45,46,47).

Despite the low incidence in Andean blackberry in Colombia, the *C. boninense* complex was represented by *C. brassicicola*, *C. karstii*, and an undetermined species.

Our findings represent an important contribution to the etiology and epidemiology of anthracnose of Andean blackberry in Colombia. It establishes the existence of a diversity of *Colletotrichum* spp. associated with the disease in this crop and, most probably, in other fruit species. Our study facilitates the development of future epidemiological research in Andean blackberry and other fruit crops, offering relevant information to the design of management strategies such as chemical management, crop rotation, and the evaluation of resistance in *Rubus* germplasm to the different *Colletotrichum* spp. reported here.

Acknowledgments

This work was financially supported by the Regional Fund for Agricultural Technology (FONTAGRO), ID 16 (2007 to 2010). We thank P. Zapata and C. Escobar for field surveys assistance, M. M. Marroquín and J. B. Cuasquer for data analysis, M. W. Blair for reviewing the manuscript, and the reviewers for their useful comments.

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