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Microsatellite isolation and characterization for *Colletotrichum* spp, causal agent of anthracnose in Andean blackberry

M.L. Marulanda, A.M. López, L. Isaza and P. López

Facultad de Ciencias Ambientales, Universidad Tecnológica de Pereira, Pereira, Colombia

Corresponding author: M.L. Marulanda
E-mail: ubioteve@utp.edu.co

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ABSTRACT. The genus *Colletotrichum*, comprised of pathogenic fungi that affect plants grown worldwide, causes the disease known as anthracnose in several fruit and vegetable species. Several studies conducted on plants have shown that the disease is characterized by the presence of one or several species of the fungus attacking the fruit or other organs of the same host. To develop and implement effective control strategies, it is vital to understand the genetic structure of the fungus in agricultural systems, identify associated *Colletotrichum* species, and define the subpopulations responsible for the disease. Molecular tools were accordingly developed to characterize genotypic populations of *Colletotrichum* spp, causal agent of anthracnose in commercial crops of Andean blackberry (*Rubus glaucus* Benth.). A microsatellite-enriched library for *Colletotrichum gloeosporioides* was developed to identify and characterize microsatellite loci among isolates collected in *R. glaucus* plantations. Thirty microsatellites were developed and tested in 36 isolates gathered from eight different blackberry-production areas of Colombia. Ten pairs of microsatellites were polymorphic.

Key words: Simple sequence repeats; *Colletotrichum acutatum*; Anthracnose; *Colletotrichum gloeosporioides*; *Rubus glaucus*; Colombia

INTRODUCTION

The genus *Colletotrichum* contains some of the world's most economically important phytopathogenic fungi, which together with the teleomorph *Glomerella cingulata*, are among the pathogens most studied by scientists, because each year they are responsible for significant production losses in many tropical, subtropical, and temperate crops worldwide, particularly cereal, vegetable, and fruit crops, as well as ornamental species (Freeman, 2000). Because of their high genetic plasticity and their dependency on environmental factors, these fungi constantly undergo physiological and morphological changes that affect their pathogenicity (Michereff, 2000). The fruit production of high-value crops in temperate markets, such as strawberry, mango, citrus, and avocado, as well as that of staple crops such as banana, is seriously affected. *Colletotrichum* species are responsible for causing a devastating disease that affects coffee berries in Africa, and also seriously affect maize, sugarcane, and sorghum. The genus was recently voted the eighth most important group of phytopathogenic fungi in the world, based on perceived scientific and economic importance (Dean et al., 2012).

Traditionally, *Colletotrichum* species have been identified and characterized on the basis of morphological characteristics, such as colony color and conidial size and shape (Gunnell and Gubler, 1992). However, these criteria are not sufficient to differentiate between *Colletotrichum* species because of the overlapping of morphological and phenotypic characters between species. The molecular techniques available nowadays offer more precise methods to conduct taxonomic studies, define species, study plant-pathogen relationships and, more recently, understand the structure of pathogen populations at an intraspecific level (MacLean et al., 1993).

Molecular techniques based on specific primers, such as the internal transcribed spacer (ITS), have become a very valuable tool for identifying *Colletotrichum* species (Brown et al., 1996). Disease control and management programs need to consider the molecular basis of the action of plant pathogens, including the structure and evolution of genes related to pathogenicity (Medeiros et al., 2010).

DNA techniques have become the most precise method to determine the genetic diversity and phylogeny of different fungal species (Bogale et al., 2009). Amplified fragment length polymorphism (AFLP) markers have been used in studies of fungal populations to distinguish species and phylogenetic groups. Microsatellites or simple sequence repeats (SSRs) have also been used to differentiate between fungal populations because of their high capacity to differentiate at the intraspecific level (Bogale et al., 2009).

Several studies have been conducted on microsatellite-enriched genomic libraries in phytopathogenic fungal species and their use in population analyses and genetic characterization, particularly in *Colletotrichum* species. One such study was that conducted by Ranathunge et al. (2009) to obtain SSRs of the causal agent of anthracnose in red pepper (*Capsicum* spp), using 27 microsatellites to characterize 52 isolates from different regions of India, Sri Lanka, and Thailand. Recently, microsatellite markers were also developed and applied to study isolates and populations of several phytopathogenic fungi, such as *Rhizoctonia solani*, *Botrytis cinerea*, *Phytophthora infestans*, *Magnaporthe grisea*, and *Ascochyta rabiei* (Ciampi et al., 2008; Ranathunge et al., 2009).

Anthracnose, a disease caused by the fungus *Colletotrichum* spp, mainly *C. acutatum* and *C. gloeosporioides*, occurs in all regions of Colombia where the Andean blackberry (*Rubus glaucus*) is grown. Disease incidence ranges between 50 and 73%, but can reach 100% in some cases (Tamayo, 2003). The incidence of *C. gloeosporioides* is higher in blackberry

production areas in central-western Colombia. However, several *Colletotrichum* species that are morphologically indistinguishable are found to be related to a single host. Hence, the host-pathogen combination involved in the incidence of anthracnose is often insufficient to serve as a diagnostic indicator of disease etiology (Abang et al., 2003).

Studies carried out in strawberry indicate that anthracnose can be associated with more than one fungal species attacking the fruits or other organs of a single host plant (Freeman, 2000). In strawberry, anthracnose is caused by three different fungi: *C. gloeosporioides*, *C. fragariae*, and *C. acutatum* (Denoyes-Rothan et al., 2003; Mackenzie et al., 2006). Studies have also demonstrated that the fungal species *C. fragariae* and *C. gloeosporioides* are highly interrelated regarding morphological and pathological aspects. Both produce a reddish necrosis, which eventually causes plant death (Smith and Black, 1990).

The filamentous fungus *C. gloeosporioides* has been widely studied in several crops of temperate, tropical, and subtropical areas worldwide (Alahakoon et al., 1994). In Latin America, studies have been conducted on anthracnose caused by *G. cingulata* in *R. glaucus* in Venezuela (Cedeño and Palacios, 1992).

In Colombia, studies carried out by Afanador et al. (2006) and Marulanda et al. (2007) described *Colletotrichum* spp to be the causal agent of anthracnose, in particular *C. gloeosporioides* and *C. acutatum* and, in some cases, a combination of both fungi. In both studies, the isolates were submitted to molecular and morphological analyses using ITS. A preliminary conclusion of the research conducted by Afanador et al. (2006) was that the species *C. acutatum* was the causal agent of anthracnose in Andean blackberry; however, Marulanda et al. (2007) reported the presence of both *C. gloeosporioides* and *C. acutatum* associated with anthracnose in blackberry crops in Colombia's coffee-growing region, with *C. gloeosporioides* being the predominant species.

In the case of this group of cosmopolitan pathogens, information on populations may prove to be useful to help better understand the population structure of pathogens in crops, and it could explain the differences in pathogenicity between isolates of the same fungus (McDonald and Linde, 2002). Gene flow patterns can be identified by analyzing the distribution of genetic diversity within and between populations. A high degree of shared similarity or the detection of rare alleles present between geographically isolated populations is evidence of the gene flow between these populations, with possible consequences for the level of effectiveness of control strategies, in addition to demonstrating the existence of structured subpopulations (McDonald and Linde, 2002). Understanding the genetic structure of pathogen populations offers information about the pathogen's future evolutionary potential, which could prove to be useful not only in the search for resistance genes, but also in defining the appropriate use of fungicides in agriculture (McDonald and Linde, 2002; Ciampi et al., 2008). Temporal and spatial information on genetic diversity and population structure is highly important to understand evolutionary adaptability and the pathogen's potential to overcome the potential resistance of the host plant (Ciampi et al., 2008).

In this study, SSR molecular markers were developed to characterize genetic populations of *Colletotrichum* spp, the causal agent of anthracnose in commercial crops of Andean blackberry. This pioneer research on the population structure of *C. gloeosporioides* will also serve as a model to conduct research on *C. gloeosporioides* in other species. It is important to emphasize that *C. gloeosporioides* is a broad-range pathogen, also affecting soursop (*Annona muricata*), tree tomato (*Cyphomandra betaceae*), and mango (*Mangifera indica*), among other species (Cannon et al., 2012).

MATERIAL AND METHODS

This study aimed to build a microsatellite-enriched genomic library and develop primers to characterize different populations of *Colletotrichum* spp, the causal agent of anthracnose in cultivars of *R. glaucus*.

Developing microsatellite markers

Microsatellite markers were developed at the Center for Molecular Biology and Genetic Engineering, Universidade Estadual de Campinas (UNICAMP) in Campinas, São Paulo, Brazil.

A microsatellite-enriched genomic library for *C. gloeosporioides* was constructed according to the methodology developed by Billotte et al. (1999) to identify and characterize microsatellite loci. Genomic DNA was extracted from single-spore mycelium of the fungus using the protocol of Lee and Taylor (1990) and 5 µg DNA were digested with *Afa*1 and ligated to the double-strand adaptors RSA21 (5'-CTCTTGCTTACGCGTGGACTA-3') and RSA25 (5'-TAGTCCACGCGTAAGCAAGAGCACA-3'). Enrichment was based on hybridization-based capture with $(GT)_8$ and $(CT)_8$ biotin-linked probes and streptavidin-coated magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega). Selected fragments were then cloned into pGEM-T Easy vectors (Promega) and inserted into the *Escherichia coli* DH5-α strain. Positive colonies were randomly selected by the blue/white screening protocol, and 72 clones were double sequenced on an ABI PRISM 377 automated sequencer (Applied Biosystems) using T7 and SP6 primers and the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Raw sequences were identified using the SAT software (SSR Analysis Tool; Dereeper et al., 2007) and microsatellites showing di-, tri-, tetra-, penta-, or hexanucleotide motifs were edited, aligned, and, if redundant, eliminated using the SeqMan software (DNAStar). Primer pairs were designed with Gene Runner v3.05.

Characterizing *Colletotrichum* populations

Collection of material

Thirty-six isolates obtained from Andean blackberry crops located in eight blackberry-producing areas of the departments of Risaralda, Quindío, Caldas, Antioquia, Cundinamarca, Santander, Valle del Cauca, and Tolima, located in Colombia's Andean region, were selected for this study (Table 1). Samples were collected from leaves and branches exhibiting symptoms of anthracnose.

Isolating the fungus

Single-spore cultures isolated from samples collected in the field in the eight abovementioned departments of Colombia were placed in potato dextrose agar (PDA) culture medium, acidified with lactic acid. Spores were taken from pure isolates and placed in test tubes by depletion.

Table 1. Collection sites of *Colletotrichum* spp in commercial crops of Andean blackberry (*Rubus glaucus*) in central-western Colombia.

Sample	Species identified by specific primers	Collection site	Department
6	*C. gloeosporioides*	4°4'99.2"N;75°41'86"W	Risaralda
SP	*C. gloeosporioides*	NA	Risaralda
SCP	NA	NA	Risaralda
SJM	*C. gloeosporioides*	NA	Risaralda
SCJM	NA	NA	Risaralda
7D1	*C. gloeosporioides*	4°86'97.5"N; 5°66'50.5"W	Risaralda
7D2	*C. gloeosporioides*	4°86'91.1"N; 75°66'52.7"W	Risaralda
2B	*C. gloeosporioides*	4°45'520"N; 75°38'16"W	Risaralda
3S1	*C. gloeosporioides*	5°12'55.1"N; 75°00'20.0"W	Risaralda
3S2	*C. gloeosporioides*	5°12'18.2"N; 75°00'28.2"W	Risaralda
3S3	*C. gloeosporioides*	5°12'29.5"N;75°00'14.2"W	Risaralda
4S1	*C. gloeosporioides*	4°63'58.9"N; 75°56'94.2"W	Quindío
4S2	*C. gloeosporioides*	4°63'49.6"N; 75°56'98.3"W	Quindío
4S3	*C. acutatum*	4°59'97.1"N; 75°57'86.7"W	Quindío
4S4	*C. acutatum*	NA	Quindío
1M	*C. gloeosporioides*	5°22'9.7"N; 75°29'00.4"W	Caldas
5V1	*C. gloeosporioides*	4°57'49.9"N; 75°30'15.4"W	Caldas
5V2	*C. gloeosporioides*	4°57'48"N; 75°30'14.1"W	Caldas
5V3	*C. gloeosporioides*	4°57'46.7"N; 75°30'16.3"W	Caldas
5V4	*C. gloeosporioides*	4°57'44.7"N; 75°30'18.2"W	Caldas
A1	*C. acutatum*	6°16'38"N; 75°24'43"W	Antioquia
A3	*C. gloeosporioides*	6°16'49"N; 75°24'12"W	Antioquia
C11	NA	4°24'13"N; 74°19'24"W	Cundinamarca
C14	NA	4°24'28"N; 74°19'46"W	Cundinamarca
C18	*C. gloeosporioides*	4°24'55"N; 74°19'12"W	Cundinamarca
S24	*C. gloeosporioides*	6°59'39"N; 72°59'17"W	Santander
S31	NA	6°59'43"N; 72°59'28"W	Santander
S34	NA	6°59'56"N; 72°59'27"W	Santander
S36	*C. gloeosporioides*	6°59'58"N; 72°59'34"W	Santander
V38	*C. gloeosporioides*	3°46'50"N; 76°11'48"W	Valle del Cauca
V42	*C. gloeosporioides*	3°46'36"N; 76°11'51"W	Valle del Cauca
V46	NA	3°46'31"N; 76°11'47"W	Valle del Cauca
V54	NA	3°46'14"N; 76°11'25"W	Valle del Cauca
T59	*C. gloeosporioides*	4°17'77"N; 74°53'29"W	Tolima
T63	*C. gloeosporioides*	4°17'86"N; 74°53'55"W	Tolima
T82	*C. gloeosporioides*	4°24'38"N; 74°34'46"W	Tolima

NA = data not available.

DNA extraction and species identification

Mycelium had to be generated to be able to extract the DNA. Therefore, the 36 isolates were placed in liquid medium based on peptone, glucose, and yeast extract, subsequently filtered, cultured on PDA, and then left to develop at 35°C for three days. After incubation, the mycelium was collected and the DNA was extracted using the protocol described by Lee and Taylor (1990). To determine the species, DNA was amplified using universal primers from the rDNA conserved region as described by White et al. (1990) and the specific primers described by Brown et al. (1996): CgInt: 5'-GGCCTCCCGCCTCCGGGCGG-3' for *C. gloeosporioides* and CaInt2: 5'-GGCGCCGGCCCCGTCACGGGGG-3' for *C. acutatum*. Amplification reactions were performed in a final volume of 25 µL with 0.01 mM oligonucleotide CgInt, CaInt2 or ITS4, 250 mM each dNTP, 1X reaction buffer, 1 U Taq polymerase, 1.5 mM $MgCl_2$ and 10 ng/µL DNA. The amplification profile consisted of 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Fourteen isolates had been previously identified (Marulanda et al., 2007), 14 isolates were identified in this study, and eight isolates were not identified (Table 1).

Microsatellite characterization

The microsatellite fragments were amplified by polymerase chain reaction (PCR) in 10-µL reaction volumes, consisting of 10 ng template DNA, 20 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.3 µM of each primer, and 0.5 U Taq polymerase per reaction. PCR was performed according to the following parameters: 94°C for 4 min; 10 cycles at 94°C for 30 s, 65°C (-1°C/cycle) for 30 s, and 72°C for 1 min; 35 cycles at 94°C for 15 s, annealing temperature (°C) (Table 2) for 30 s and 72°C for 1 min; and 72°C for 5 min. Amplification products were visualized in polyacrylamide-bisacrylamide denaturing gels stained with silver nitrate (Benbouza et al., 2006).

Statistical analysis

Statistical analyses were carried out using the GenAlex v6.2 (Peakall and Smouse, 2006). Nei's genetic distance (1978) was calculated and a principal coordinates analysis performed. The polymorphism information content (PIC) was calculated for each SSR primer pair according to Cordeiro et al. (2003).

Using the same software, analysis of molecular variance (AMOVA) was used to determine the partitioning of genetic variation within and between groups by the SSR marker system.

RESULTS AND DISCUSSION

Developing microsatellite markers

The microsatellite-enriched library consisted of 96 clones, from which 53 (55.2%) high-quality sequences were obtained. Of these 53 sequenced clones with microsatellite motifs, 30 primer pairs were complementary to sequences flanking the repeat motifs (Table 2). Of these 30 microsatellites, 10 showed polymorphisms in the populations analyzed, and 10 amplified samples of *C. gloeosporioides* and *C. acutatum*, demonstrating transferability of the microsatellite primers developed (Table 2).

Total PIC of the 10 microsatellite primer pairs showing polymorphism were quantified by amplifying *Colletotrichum* isolates (Table 2). Primers with the highest PIC values were CgE6-1 (0.7413), CgC12-1 (0.7066), and CgG2 (0.7003), and those showing the lowest PIC values were CgF4 (0.4553) and CgD10-3 (0.4582), with the latter being referred to as less informative primers (Table 2). All markers displayed differences in terms of observed heterozygosity (H_O), with marker CgC4-3 showing the highest H_O at 0.958 and marker CgF5-2 the lowest value at 0.188 (Table 2).

The mean H_E value was 0.906 and the mean H_O value was 0.500. According to Feres et al. (2009), the differences between H_E and H_O values can be attributed to the presence of null alleles, which mask heterozygous individuals as a single band. These results demonstrate that homozygosity was low. According to Montero et al. (1998), the genetic variability of *C. gloeosporioides* is high because this pathogen has a short life cycle and exhibits both sexual and asexual reproduction, with a resulting high rate of mutation and recombination by crossing. In summary, genetic diversity parameters serve to identify multiallelic markers and H_O values higher than H_E values.

Table 2. Description of primers developed for *Colletotrichum gloeosporioides*, causal agent of anthracnose in Andean blackberry.

Name	GenBank accession No.	Motif	No. of loci	Expected size (bp)	Annealing temperature (°C)	Primer pair sequence (5'-3')	H_E	H_O	PIC
CgA2	JQ862554	$(GT)_8$	2	269-284	57.0	AGAAAGAACGTCCGCAATAG	-	-	-
						ATCTCACCCTCTCTCTCTCAC			
CgA4	JQ862556	$(GT)_7$	2	305-318	57.0	GACCAGACCGTAGCAATAG	0.818 ± 0.123	0.481 ± 0.071	0.6863
						CGTGGACTAATGTATCATCTAC			
CgA5[a]	JQ862557	$(CA)_{14}$	2	187-214	53.0	TCAAGAGTGATGCTCGTG	-	-	-
						TGATGGATGGATGGGATG			
CgA9[a]	JQ862560	$(GT)_{13}$	2	322-347	55.0	CATTGTATCCGTAGGGTTC	-	-	-
						GTGCTCTTACCAAACACAC			
CgC4-3	JQ862570	$(AG)_6$	1	548-559	55.0	ACAGACAGAGAGAGAGAAAC	0.534 ± 0.025	0.958 ± 0.042	0.5831
						TCTGCAGCTGAGGTACTG			
CgC4-5[a]	JQ862570	$(TG)_{11}$	1	779-800	53.0	TGACAGAGAAAGAGCCAG	-	-	-
						CCCTTCTGTCTCTTTATCTC			
CgC5	JQ862571	$(AC)_{18}$	1	774-809	55.0	GTTTCACCCGGAGAAGTG	-	-	-
						GTGGACTAGCAGCAGTGTG			
CgC11	JQ862572	$(AT)_5$	1	148-157	53.0	ACTAGCTGAAGTGTAAGGG	-	-	-
						TGCACATTAAACAAGC			
CgC12-1	JQ862573	$(CA)_9$	2	373-390	57.0	TACACCACACCACCAATCGAC	1.000 ± 0.000	0.553 ± 0.032	0.7066
						CGAACTGGGAAAGGACGTG			
CgC12-2	JQ862573	$(CTG)_5$	1	670-684	59.0	CCCATCATCCACCACACATC	-	-	-
						CAGCAGCAGCAGCAGTAGTTAG			
CgD4-2[a]	JQ862575	$(TG)_5$	1	204-213	57.0	TCGACCCGGTGAGATTCAAC	1.000 ± 0.000	0.603 ± 0.006	0.5717
						GCACGCACACACACAGGATAC			
CgD7-1[a]	JQ862576	$(CT)_6$	1	15-26	57.0	GTTTCGTCCTCTCTCTCTCTCG	-	-	-
						AATGCGGCATACACAAACGTAC			
CgD7-2[a]	JQ862576	$(TG)_5$	2	221-230	57.0	GTGTGATGTGTGCGTTTGC	-	-	-
						AGCGAAATGTTGCTCCAAAC			
CgD10-2	JQ862577	$(CA)_7$	2	172-185	55.0	CACACACGTAGCTGTTTCCC	-	-	-
						AAAGGGACACAGCTGCTTG			
CgD10-3	JQ862577	$(AC)_5$	1	259-268	57.0	CAGAAACACAGGTTGGTATGGC	1.000 ± 0.000	0.528 ± 0.019	0.4582
						GTGTGTGATTTGATGGAAATGG			

Continued on next page

Table 2. Continued.

Name	GenBank accession No.	Motif	No. of loci	Expected size (bp)	Annealing temperature (°C)	Primer pair sequence (5'-3')	H_E	H_O	PIC
CgD10-4	JQ862577	$(CA)_5$	1	304-313	57.0	TGTGTCCCTTTGCATAACATTC CTCCCTGTGTTTCGTTTCGTG	-	-	-
CgE6-1[a]	JQ862580	$(GT)_7$	2	101-114	57.0	GTGCGGGCCACATTGTGAAC TGATTGCTCTTTCGATTGCTTC	0.878 ± 0.073	0.550 ± 0.052	0.7413
CgE6-2[a]	JQ862580	$(CAA)_5$	1	641-655	57.0	TGGCAGAGAGAAACAGACAAAC CAACATCGGCATCAATTCC	-	-	-
CgE7-1[a]	JQ862580	$(CT)_5$	1	258-267	57.0	TCTTTGAGCGTTCACAGCAG GTGTGTGTTGCAGTTCAGGC	-	-	-
CgE11-1[a]	JQ862580	$(CA)_7$	1	213-226	61.0	CAAGTACCTCGAATCGAGTACG GCCAGCAAGCAGTACCAAG	0.944 ± 0.037	0.598 ± 0.023	0.5625
CgE11-2	JQ862580	$(GCT)_6$	2	364-381	61.0	GCTACCTACCTTGCGCTAAGC GTCTGATCGCCACCTCTTTG	-	-	-
CgE12-1	JQ862580	$(TG)_{17}$	1	107-140	59.0	CATTGAGGAGTCTGCAGTGTG AGCGCTGCTGTGAACACAC	-	-	-
CgF4	JQ862588	$(CT)_7$	1	864-877	61.0	GCGCGGAAACTCCTTCTAG TGTGCGAGAGAGAGAGAGAGTG	0.926 ± 0.062	0.508 ± 0.024	0.4553
CgF5-1	JQ862589	$(CA)_7$	2	170-183	57.0	TCTCTGCAGGTCCTTCATGC TATGTGTGTGTGTGTGCGTGAG	-	-	-
CgF5-2[a]	JQ862589	$(AC)_{12}$	2	185-208	61.0	TCACGCACACACACACACATAC AAGCCTGCTGGAGGTCTGAG	0.185 ± 0.093	0.188 ± 0.132	0.6076
CgF5-3	JQ862589	$(TG)_7$	2	542-555	57.0	TTCAGGAGAGTGGAGTGTGGG GGAATGTCGATCTGCTTCACG	-	-	-
CgF6-1	JQ862590	$(TG)_9$	1	157-174	59.0	TATTCGAGTCCCATTGCTTCAG TAGGCAGTCTGTTCGTTCACAC	-	-	-
CgF10-1[a]	JQ862593	$(CA)_5$	1	60-69	59.0	CTTTACACTCTCAAGCTCTGCC TGTGTTCTTCCACCTGTTC	-	-	-
CgF10-2	JQ862593	$(AC)_5$	3	74-83	59.0	CACACCAAACACACACACATGC AAGCAAACACTACCAGACCACC	-	-	-
CgG2	JQ862594	$(CA)_{20}$	3	477-516	59.0	TCTCCAAGGTCGATCACGG CGCTGATACTCGGATTCTTGTG	0.636 ± 0.101	0.525 ± 0.073	0.7003

[a]Pairs of primers amplifying *Colletotrichum acutatum* isolates. H_E = expected heterozygosity; H_O = observed heterozygosity; PIC = polymorphism information content.

Characterizing *Colletotrichum* populations

Most primer pairs amplified one or two loci, with only primers CgG2 and CgF10-2 amplifying three loci. CgG2, CgC12-1, CgE6-1, and CgA9 were multiallelic microsatellites. The locus CgA9 had the highest number of alleles (11 in total), followed by loci CgG2, CgG12-1, and CgE6-1 with 10 alleles each. Overall, 153 alleles were detected in the 46 loci, and the average of polymorphic loci was 95.11%. Microsatellites CgD7-2, CgF5-2, CgC4-5, CgE6-1, CgE12-1, CgC12-1, CgA4, CgE11-1, and CgC12-1 produced exclusive alleles that helped differentiate the isolates from different geographical regions such as Risaralda, Caldas, Cundinamarca, Santander, and Tolima.

In studies conducted by Ranathunge et al. (2009), the number of alleles was higher in 27 primer pairs developed and evaluated for another species of the same genus, *C. capsici*. Locus CCSSR1 showed 18 alleles detected across the 52 isolates and several common alleles within groups of isolates from India, Sri Lanka, and Thailand. Similarly, locus CCSSR53 revealed 11 different alleles and locus CCSSR59 nine alleles, with 141 alleles in 25 loci.

AMOVA results indicated that the highest percentage of variation (80%) was within populations, followed by variation between populations (20%) (Table 3). Masel et al. (1990) indicated that the genome of *C. gloeosporioides* populations is highly variable under field conditions, but the reason behind this polymorphism is unknown. Their results suggest, however, that chromosomal rearrangements can be considered a means to generate variability in this fungus.

Table 3. Data resulting from the analysis of molecular variance (AMOVA).

Source	d.f.	Estimated variation	Variation (%)
Between populations	7	4.880	20
Within populations	28	18.937	80
Total	35	23.817	100

d.f. = degrees of freedom.

McDonald and Linde (2002) also found that pathogens showing sexual reproduction or open growth pose a greater risk than when the reproductive pattern leads to inbreeding, because new genotypes emerge during the sexual cycle. Furthermore, the main mechanisms by which genetic diversity can be generated in populations of pathogenic microorganisms are mutation, population gene flow, and sexual and asexual recombination (Agrios, 1996).

Principal coordinate analysis was performed on the basis of the genetic distance between individuals (Figure 1). *C. gloeosporioides* isolates were observed to separate from *C. acutatum* isolates, which agrees with the results of Marulanda et al. (2007). In addition, genetic distance measurements showed some clustering of isolates regarding their geographical origin.

The clusters obtained in the genetic distance dendrogram (Figure 2) tended to form groups according to the place of origin of each isolate. Similar results have been reported in other host-pathogen interactions (González et al., 1998; Casarrubias-Carrillo et al., 2003). There was also a clear separation of samples corresponding to *C. acutatum* (4S3 and 4S4) from samples corresponding to *C. gloeosporioides*.

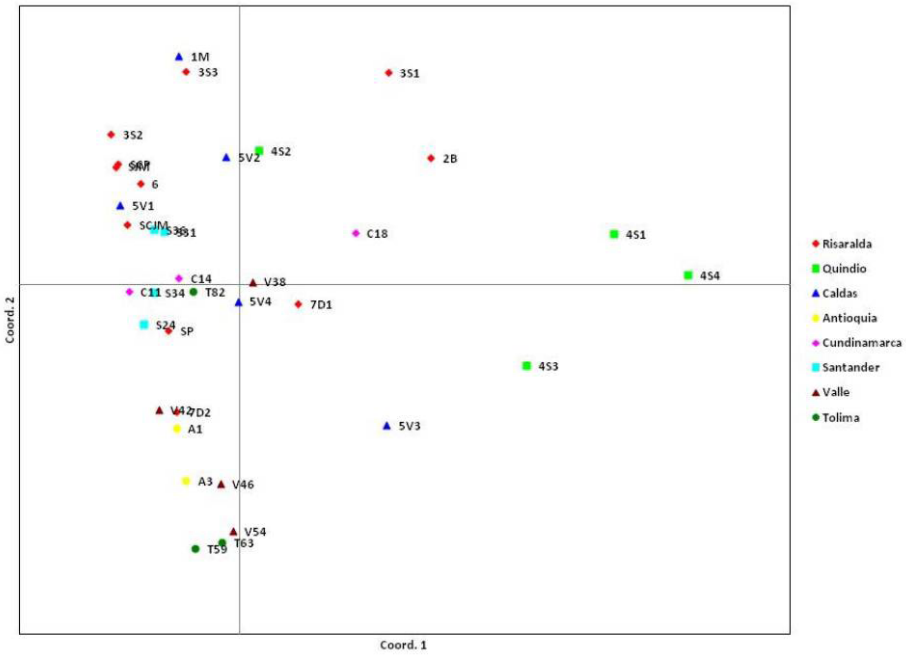

Figure 1. Principal coordinates analysis of *Colletotrichum* isolates.

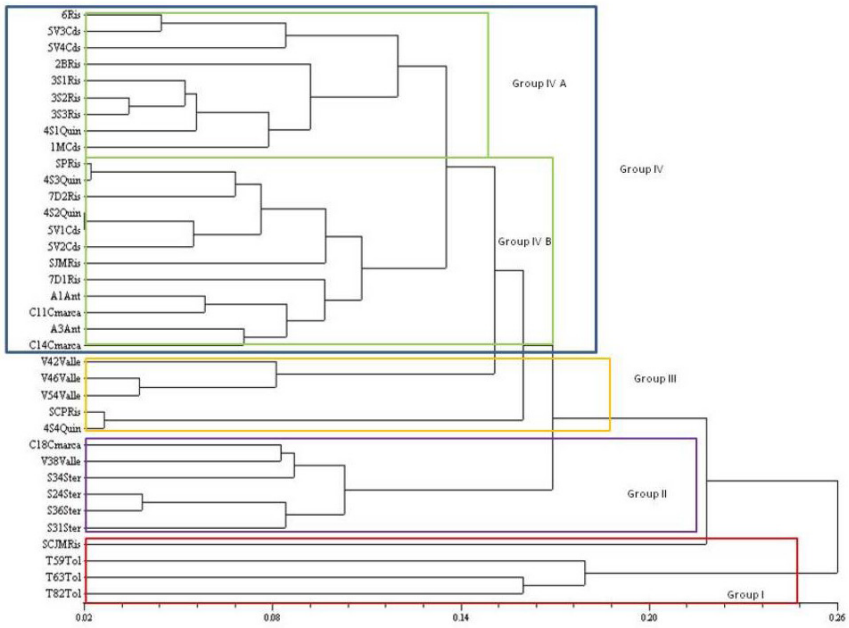

Figure 2. Dendrogram built based on Nei's distance coefficient (1978) and the UPGMA grouping method for SSR analysis of *Colletotrichum* isolates.

In this study, four groups were formed (Figure 2). The first group consisted of *C. gloeosporioides* isolates T59, T63, and T68 from Tolima and exhibited the largest genetic distance regarding the other groups. The second group gathered isolates S34, S24, S36, and S31 from Santander and V38 from Valle del Cauca (mostly *C. gloeosporioides*). Meanwhile, the third group was formed by isolates V42, V46, and V54 from Valle del Cauca, SCP from Risaralda, and S31 from Quindío. The fourth group had two subgroups: 4A and 4B. Both were composed of isolates mostly from Caldas, Risaralda, and Quindío, which indicated that these isolates showed great genetic similarity, possibly because of their geographical proximity. According to Tuskan et al. (1990), more than one gene may be responsible for this behavior, and it is possible that many genes are involved in the varying degrees of adaptation to different climatic conditions attributed to the broad variability of *C. gloeosporioides* and its ability to adapt to different regions with different climatic conditions and different physiological characteristics of the host plant.

CONCLUSIONS

Thirty microsatellite primer pairs were developed, 10 of which showed polymorphisms in the populations analyzed and 10 of which amplified samples of *C. gloeosporioides* and *C. acutatum*, demonstrating transferability of the microsatellite primers developed.

When genetic diversity parameters were assessed, the microsatellite pairs helped identify multiallelic markers and H_O values higher than H_E values. Differentiation was also observed between the species *C. gloeosporioides* and *C. acutatum*. The tendency of isolates to group according to geographical origin evidenced the existence of significant intraspecific genetic variability because of their short life cycle and sexual and asexual reproduction.

The molecular tools developed will serve to identify *C. gloeosporioides* isolates causing anthracnose in other crops, especially tropical fruits.

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