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Species from the *Colletotrichum acutatum*, *Colletotrichum boninense* and *Colletotrichum gloeosporioides* species complexes associated with tree tomato and mango crops in Colombia

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Colletotrichum species cause typical anthracnose symptoms in tree tomato and mango. To characterize species of *Colletotrichum* in these two crops in Colombia, 91 isolates were collected from several localities. Phylogenetic analyses using nuclear gene sequencing of the ITS region and the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene allowed the identification of three groups: acutatum, gloeosporioides and boninense. These three groups were further confirmed using two additional genomic regions (*chitin synthase 1* and *actin*) for 30 isolates representative of the three previously identified complexes and one genomic region (*ApMat*) for the *Colletotrichum gloeosporioides* complex strains. The entire approach permitted a robust strain identification that allowed phylogenetic species recognition (PSR) based on the identification of well-supported monophyletic clades and concordance between individual and multilocus phylogenies. Morphological and physiological assays were also conducted. Isolates that were morphologically identified as *C. gloeosporioides* showed high phenotypic diversity. Pathogenicity data revealed a considerable degree of host preference.

Keywords: acutatum, boninense, *Colletotrichum*, gloeosporioides, mango, tree tomato

Introduction

The genus *Colletotrichum* includes an important number of plant pathogens of vegetables, legumes, cereals and many tropical crops worldwide (Sutton, 1992). *Colletotrichum* is recognized as the asexual state of several species of the ascomycete *Glomerella* (e.g. *G. acutata* and *G. cingulata*) (Sutton, 1992; Armstrong-Cho & Baniza, 2006; Damm *et al.*, 2010). In Colombia, *Colletotrichum* species affect a wide range of plants, particularly tree tomato (*Solanum betaceum*) and mango (*Mangifera indica*) (Aranzazu & Rondón, 1999). Tree tomatoes are produced mainly in the states of Cundinamarca, Antioquia, Caldas and Nariño, with a total cultivated area of 4500 hectares. Up to 50% of the harvest in these states, is lost because of this fungus (Aranzazu & Rondón, 1999). Mangoes are primarily grown in the state of Tolima and *Colletotrichum* spp. account for losses of up to 60% of the total mango production in this region. Anthracnose is the most frequent disease symptom found in both crops, although other symptoms are also reported, such as leaf spots and the mummification of both young and immature fruits (Aranzazu & Rondón, 1999). Anthracnose disease symptoms include localized sunken necrotic lesions with concentric orange rings.

The taxonomy and nomenclature of *Colletotrichum* species have proven to be challenging. Several authors, including Cai *et al.* (2009) and Freeman (2000), have recognized four problems in the classification of this genus: (i) the paucity and variability of morphological characters as well as the difficulty in standardizing their measurement; (ii) their variable host range and pathogenicity; (iii) the fact that type specimens are often missing and cannot be used for molecular studies; and (iv) the incomplete information and numerous erroneous sequence names of *Colletotrichum* strains in public databases (Damm *et al.*, 2012a). Several efforts have been undertaken to resolve the ambiguous identification of *Colletotrichum* species. Traditional morphological approaches have taken into account the size and shape of colonies, conidia and appressoria. At a physiological level, the growth rate in various cultivation media and the assimilation of various carbon sources have been employed for identification. At a molecular level, AFLP, RFLP (Brown *et al.*, 1996; Freeman *et al.*, 2001; Guerber *et al.*, 2003; Cai *et al.*, 2009), and, more recently, multilocus genotyping of nuclear genes (Crouch *et al.*,

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2009; Cannon *et al.*, 2012; Damm *et al.*, 2012a,b; Weir *et al.*, 2012) have been used. The latest revisions of the genus made by Cannon *et al.* (2012), Damm *et al.* (2012a,b) and Weir *et al.* (2012) recognize nine *Colletotrichum* clades: the Acutatum, Boninense, Dematium, Destructivum, Gloeosporioides, Graminicola, Orbiculare, Spaethianum and Truncatum clades. The Acutatum and Gloeosporioides clades comprise the largest number of species, including 29 and 22 species, respectively. Recently, Lima *et al.* (2013) identified five *Colletotrichum* species responsible for mango anthracnose in Brazil. Surprisingly, none corresponded to *Colletotrichum gloeosporioides sensu stricto*.

Studies to determine the composition of *Colletotrichum* species on tree tomatoes and mangoes in Colombia are limited. One study, conducted more than 10 years ago in the state of Antioquia, identified *Colletotrichum acutatum* and *C. gloeosporioides* as the causal agents of tree tomato and mango anthracnose, respectively (Afanador-Kafuri *et al.*, 2003). Due to the economic relevance of this pathogen in Colombia, it was decided to undertake a comprehensive approach to determine the species composition of the *Colletotrichum* population attacking tree tomatoes and mangoes in the states of Cundinamarca and Tolima, the main producing regions for tree tomatoes and mangoes, respectively. *Colletotrichum* isolates were identified phylogenetically, using individual and multilocus data sets, morphologically and physiologically, and the pathogenicity and aggressiveness of the isolates on both tree tomatoes and mangoes was evaluated.

Materials and methods

Colletotrichum isolates

A total of 91 isolates of *Colletotrichum* spp. were collected from mango and tree tomato crops in 16 localities in the states of Tolima and Cundinamarca, Colombia from December 2010 to October 2011 (Tables S1 & S2). Samples were isolated from typical anthracnose lesions on fruits or leaves, mummified fruits, and leaf spots. Mango trees were sampled at six different collection sites and a total of 32 isolates were obtained; of these, 28 were isolated from fruits, and four from leaves. The largest number of mango samples (14 samples) was collected from El Espinal (Tolima), one of the main mango-producing areas in Colombia (Table S1). Tree tomatoes were sampled from 10 different collection sites and a total of 59 samples were collected; of these, 42 samples corresponded to tree tomato fruits, and 17 were obtained from leaves. The largest number of isolates came from Silvania (Cundinamarca), the main tree tomato production area in the state of Cundinamarca (Table S1).

All samples were surface sterilized using 1% sodium hypochlorite for 1 min and 70% ethanol for 1 min and then washed three times using sterile distilled water. Six fragments of each sample were cut and placed on Petri dishes containing potato dextrose agar (PDA). All plates were incubated at 25°C for 4 days. Fungi showing features characteristic of *Colletotrichum* were then isolated on PDA and incubated at 25°C for 7 days (when conidia formation was observed). Conidia were spread on water agar, and a single conidium was subcultured on PDA to obtain a monoconidial culture. Pure cultures were stored in vials containing sterile water, malt agar (MA) and paper, and were deposited in the culture collection of the Mycology and Plant Pathology Laboratory of Universidad de los Andes (LAMFU). Four reference strains were purchased from the CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands): *C. gloeosporioides* CBS 119298, *C. acutatum* CBS 124958, *Colletotrichum fragariae* CBS 14231 (type strain) and *Colletotrichum lupini* CBS 112746.

Molecular identification

Isolates were grown in Sabouraud's dextrose with yeast (SDY) medium (glucose 2%, yeast extract 1% and peptone 1%) and agitated for 7 days at 25°C. The fungus was then filtered through a 25 μm pore filter paper, and samples were lyophilized for 24 h. DNA was extracted according to Goodwin *et al.* (1992). DNA quality was verified using 1% agarose gel electrophoresis, and DNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific). Molecular characterization was achieved using the ITS region and the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene. The ITS region was amplified using ITS1F and ITS4 primers (Frisvad & Samson, 2008). The PCR parameters for the ITS region were as follows: an initial 3 min denaturation step at 95°C; 34 cycles of 95°C for 1 min, 52°C for 30 s and 72°C for 1 min; and a final extension step of 10 min at 72°C. The *GAPDH* gene was amplified using GDF1 and GDR1 primers (Peres *et al.*, 2008). The cycling parameters were as follows: denaturation at 94°C for 4 min; 34 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min; and a final extension step of 10 min at 72°C.

Thirty isolates, representative of complexes identified using the ITS region and the *GAPDH* gene, were further characterized using two additional genomic regions, the *chitin synthase 1* (*CHS-1*) gene and the *actin* (*ACT*) gene. The *CHS-1* gene was amplified using CHS-79F and CHS-345R primers (Weir *et al.*, 2012). The *ACT* gene was amplified using ACT-512F and ACT-783R primers (Weir *et al.*, 2012). Cycling parameters for *CHS-1* and *ACT* were as follows: denaturation at 94°C for 4 min; 34 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min; and a final extension step of 10 min at 72°C. Fourteen isolates were further characterized using the genomic region *ApMat* and primers reported by Silva *et al.* (2012). Conditions were the same as those used for the other three genomic regions except for the annealing temperature, which was 62°C. The PCR mixture for all primer combinations were: 2·5 μL buffer (10×), 2 μL $MgCl_2$ (25 mM), 0·5 μL dNTPs (10 mM), 0·5 μL each primer (10 μM), 0·25 μL *Taq* DNA polymerase (5 U μL^{-1}) and 1 μL DNA made up to a final volume of 25 μL with sterile distilled water.

Sequencing was performed using Applied Biosystems ABI3730XL (at Macrogen, Korea) or ABI3500 (at Universidad de Los Andes). Sequences were deposited in GenBank under the accession numbers ITS: KJ846760–KJ847044; *GAPDH*: KJ846950–KJ847044; *ACT*: KP260563–KP260592; *CHS-1*: KP260593–KP260622; and *ApMat*: KR134288–KR134301.

Phylogenetic reconstructions

Sequences were cleaned and assembled using GENEIOUS v. 5.4 [http://www.geneious.com]. Multiple sequence alignments were performed using the MAFFT (Katoh & Standley, 2013) with a PAM200 scoring matrix for nucleotide sequences. Due to the high number of hypervariable sites in all molecular markers sequenced, the gap open penalty was slightly increased (1·54–1·8). All multiple sequence alignments were then adjusted

manually in JALVIEW (Waterhouse *et al.*, 2009). Nucleotide substitution models were established using JMODELTEST software (Darriba *et al.*, 2012). A tree reconstruction was performed using maximum likelihood and Bayesian inference analysis for each gene, and for a partitioned supermatrix of the ITS and *GAPDH* loci. Maximum likelihood was performed using RAXML (Stamatakis, 2006) with 5000 bootstrap replicates and a GTRC+I+G model of nucleotide substitution. MRBAYES v. 3.1.2 (Ronquist & Huelsenbeck, 2003) was used for Bayesian inference with the following parameters: 20 million generations of Markov chains (MCMCMC), burning 10% of the total run and using the models of nucleotide substitution as priors per gene and for the partitioned matrix.

Additional phylogenetic analyses were carried out, as described above, for the *ACT* and *CHS-1* genes and for a concatenated matrix of the four genes (ITS, *GADPH*, *ACT* and *CHS-1*). These analyses were performed for a subset of 30 isolates, representative of the species complexes identified using the ITS region and the *GAPDH* gene. The genomic region *ApMat* was used to refine the classification of 14 isolates belonging to the gloeosporioides complex.

Sequences retrieved from public databases, representative of species identified within the *C. gloeosporioides*, *Colletotrichum boninense*, *Colletotrichum truncatum* and *C. acutatum* species complexes (Table S3), were included in the phylogenetic analyses to define the taxonomic status of the mango and tree tomato isolates. Phylogenetic species recognition was achieved following the phylogenetic concordance criteria. Monophyletic groups that were concordantly supported by the majority of loci, or that were well supported (ML bootstrap values greater than 70% or values higher than 0·95 for Bayesian posterior probabilities as defined by Weir *et al.* (2012)) by at least one locus but not contradicted by the other loci, were considered a species (Dettman *et al.*, 2003). Sequences that corresponded to reference isolates included in those groups were used to assign isolates to species. Certain isolates could not be assigned to species if they were included in clades that were not in agreement with the concordance criteria or if, for example, they were clustered in a clade that included sequences from two or more reference strains. In the latter case, an unambiguous assignment could not be achieved.

Haplotype diversity and polymorphic sites were calculated using the program DNASP v. 4.90.1 (Librado & Rozas, 2009).

Morphological characterization

Each isolate was morphologically identified using parameters described in dichotomic keys (Sutton, 1980). The morphological parameters evaluated were colony colour, reverse colony colour, appressoria formation, and the shape and size of conidia on PDA and MA media. Colony colour was recorded 7 days after incubation at 25°C according to previously described colour tables (Kuppers, 1996). To measure the size of the conidia produced by each isolate, 5 mL sterile distilled water were added to a 7-day-old culture. Colonies were scraped with a round loop, and conidial suspensions were collected. Pictures of 35 conidia were taken and the length and width of each conidium was determined using IMAGEJ v. 1.45 software (National Institutes of Health). Three independent cultures were used and a total of 100 conidia were measured per isolate.

Physiological tests

For the physiological tests, rate of colony growth, ammonium tartrate assimilation, sporulation start time, and number of conidia produced after 7 days' incubation were evaluated for all isolates included in this study. Rate of colony growth was assessed on PDA, MA and Medium B media (Lynch *et al.*, 1981) supplemented with tartrate (MB+T). Mycelial discs (4 mm) were deposited on these media and incubated at 25°C. Colony diameter was measured using IMAGEJ v. 1.45 software every 2 days starting from day 1 until day 15. Growth rate was calculated as the mean daily growth (cm per day). The use of ammonium tartrate as a carbon source was tested according to a method previously described by Bridge *et al.* (2008). Briefly, Medium B containing 1·2% (w/v) agar was supplemented with 1% (w/v) ammonium tartrate and 0·005% (w/v) bromocresol purple (Waller *et al.*, 1993; Bridge *et al.*, 2008). A positive control containing dextrose, and a negative control lacking a carbon source were used. Media were inoculated with a 4-mm diameter plug of a *Colletotrichum* isolate. The ability to use the offered carbon source was determined by direct observation of pathogen growth and by the production of a purple colour on the media after 15 days' incubation at 25°C. To establish the sporulation start time, mycelia were observed and conidia formation was monitored under the microscope for 7 days from being subcultured. To determine the number of conidia produced 7 days after incubation, 5 mL sterile distilled water were added to a 7-day-old PDA culture, colonies were scraped with a round loop, and conidial suspensions were collected. The number of conidia was determined using a haemocytometer.

Pathogenicity tests

All 91 isolates were grown on PDA for 7 days at 25°C. A conidial suspension was obtained by adding 10 mL sterile distilled water to the culture, and subsequently filtering the suspension through gauze. The spore concentration was adjusted to 10^6 conidia mL^{-1} using a haemocytometer (Than *et al.*, 2008b). Leaves and fruits from two tree tomato varieties (red and yellow or common) and from the mango cultivar Tommy Atkins were surface sterilized by immersing them in 70% ethanol for 2 min, then in a 1% sodium hypochlorite solution for 1 min and subsequently washing them three times in sterile distilled water for 2 min (Sanders & Korsten, 2003; Montri *et al.*, 2009); after sterilization, leaves and fruits were inoculated. Leaves were placed inside Petri plates containing a plastic mesh, sterile paper, and 1 mL sterile distilled water. Fruits were placed inside a plastic box (one fruit per box) that contained sterile paper soaked in sterile distilled water to maintain humidity (Montri *et al.*, 2009). Leaves, placed inside Petri plates, were inoculated with 10 μL of the conidial suspension at six different spots, and fruits were inoculated with 100 μL of the conidial suspension on a wound of approximately 1 mm in diameter; controls were inoculated with the corresponding volume of sterile distilled water (Than *et al.*, 2008a). Each isolate was evaluated on its original host and on the alternate host. Inoculated samples were incubated at room temperature with alternate light and dark periods of 12 h each. Photographs of the symptoms were taken every other day for 15 days and the percentage of the sample showing a lesion was determined using IMAGEJ v. 1.45 software. Three leaves and three fruits were inoculated per isolate, and the entire experiment was repeated three times.

The significance of the interaction between isolates and inoculated samples was tested using an analysis of variance (ANOVA). The sources of variance analysed were the inoculated sample (mango fruits (infection was never observed on mango leaves), tree tomato red variety fruits and leaves, and tree tomato yellow variety fruits and leaves), the original host of the isolate (mango

or tree tomato), the combination original host × inoculated sample and the combination original host × days after inoculation (7 or 15 days). To determine host specificity of the isolates, a Welch's two-sample *t*-test was performed to compare the percentage of the sample showing a lesion for the isolates obtained from mango versus the isolates from tree tomato. All statistical analyses were conducted using R (R Development Core Team, http://www.r-project.org/).

Results

Phylogenetic analyses of gene trees

Phylogenetic relationships among all isolates collected and reference strains were determined using maximum likelihood and Bayesian inference for the ITS region and *GAPDH* gene sequence. Both approaches showed similar results. Gene trees for the ITS region and *GAPDH* gene, constructed using maximum likelihood and Bayesian inference analyses, showed similar results and identified three different groups. The first group contained the *C. gloeosporioides* and *C. fragariae* reference strains, the second group contained the *C. acutatum* and *C. lupini* reference strains, and the third group contained sequences of the boninense complex (Figs S1 & S2).

Three groups were also identified in a concatenated tree using the ITS region and *GAPDH* gene sequences (Fig. 1). These three groups were further confirmed in the gene trees constructed using *ACT* and *CHS-1* gene sequences (Figs S3 & S4). The first group was termed the acutatum complex and included the *C. acutatum* and *C. lupini* reference strains as well as 50 isolates from this study, which were principally isolated from tree tomato. The second group was termed the gloeosporioides complex. This group included the *C. gloeosporioides* and

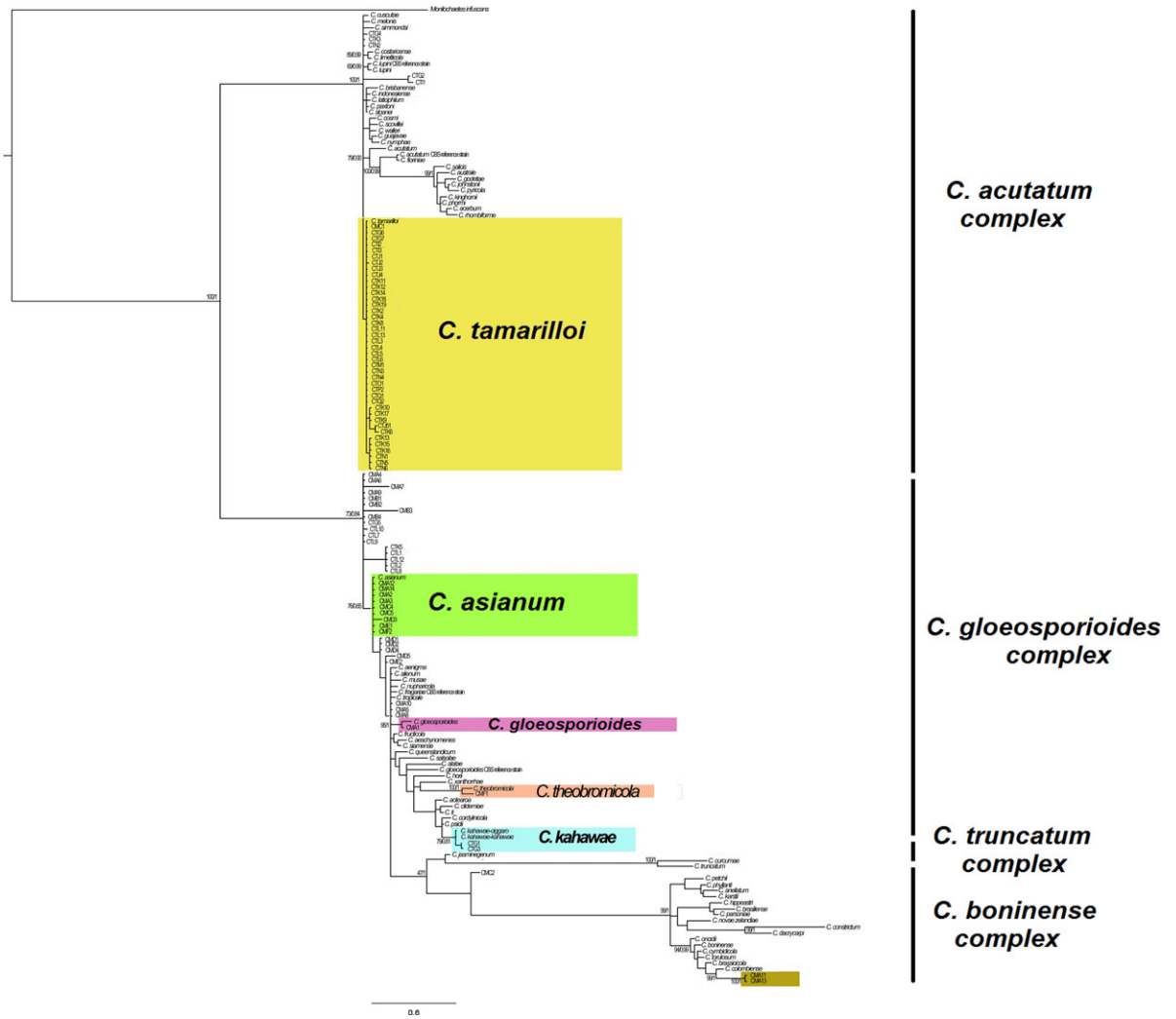

Figure 1 Concatenated tree constructed using the internal transcribed spacer region and the *glyceraldehyde 3-phosphate dehydrogenase* gene. Sequences from reference strains that are recommended for use in phylogenetic studies by the CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) are included. Highlighted taxa correspond to isolates that were assigned to species following the phylogenetic concordance criteria. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

C. fragariae reference strains as well as 38 isolates from this study, principally isolated from mango, with the exception of two that were isolated from tree tomato. The third group was termed the boninense complex. This third group included isolates CMA11, CMA13 and CMC2 isolated from mango (Fig. 1).

The information obtained from all gene trees as well as the phylogenetic concordance criteria allowed the identification to species level of the majority of isolates included in this study (Table 1). In total, six species were identified. In mango, the predominant species complex was gloeosporioides, containing the species *C. gloeosporioides*, *Colletotrichum asianum*, *Colletotrichum siamense* and *Colletotrichum theobromicola*. Additionally, three isolates (CMA11, CMA13 and CMC2) were grouped in the boninense species complex. In tree tomato, the predominant species complex was acutatum, with *Colletotrichum tamarilloi* being the most representative species. One isolate, CTG1, was from the gloeosporioides complex and was identified as *Colletotrichum kahawae*.

Isolates that clustered with either *C. gloeosporioides* or *C. theobromicola* were well supported in the concatenated gene trees, ITS and *GADPH* (Fig. 1) and ITS, *GADPH*, *ACT* and *CHS-1* (Fig. 2a), and no discrepancies were observed in any of the other phylogenies that contained those sequences. Isolates clustering with the *C. tamarilloi* reference sequence were well supported in the concatenated tree of the ITS region and the *GAPDH* gene (Fig. 1), and the topology observed was consistent across all phylogenies that contained sequences from this clade (Figs S1–S4). The same was true for isolates identified as *C. asianum*, as this clade was well supported when using the concatenated tree of the ITS region and the *GAPDH* gene as well as when using the four-loci gene tree (Fig. 2a). However, this was not the case for isolate CTG3, which appears in a well-supported clade with *C. kahawae* in the concatenated tree of the ITS region and *GAPDH* gene (Fig. 1), but also clustered with *C. tamarilloi* in the *ACT* gene tree (Fig. S3). This isolate could not be assigned to species due to the discordance between phylogenies. Additionally, the *ApMat* phylogeny allowed refinement of species assignations within the *C. gloeosporioides* species complex (Fig. 2b). This region alone was able to discriminate all 14 isolates sequenced, demonstrating the resolution power reported by Silva *et al.* (2012) and Sharma *et al.* (2013) for this genus.

Haplotype diversity was estimated for the two predominant groups, gloeosporioides and acutatum. The gloeosporioides group exhibited greater haplotype diversity than the acutatum group, with 0·77 for the ITS region and 0·86 for the *GAPDH* gene in comparison to the acutatum complex, with haplotype diversity of 0·26 for the ITS region and 0·39 for the *GAPDH* gene.

Morphological and physiological variation

Morphological and physiological variation classified all isolates into two main groups: each group included one

Table 1 Summary of the molecular identification of *Colletotrichum* isolates collected from mango and tree tomato crops in Colombia

Isolate/species	Host (or host % if mixed)	Phylogenetic species complex	Phylogenetic species assignation
CBS 112746/*C. lupini*		AC	*C. lupini*
CBS 119298/*C. gloeosporioides*		GC	*C. gloeosporioides*
CBS 124958/*C. acutatum*		AC	*C. acutatum*
CBS 14231/*C. fragariae*		GC	*C. fragariae*
CMA1	Mango	GC	*C. gloeosporioides*
CMA2, CMA3, CMA8, CMA9, CMA12, CMA14, CMB3, CMB4, CMC3, CMC4, CMC5, CMD2, CMD3, CME1, CMF2	Mango	GC	*C. asianum*
CMC2, CMA11, CMA13	Mango	BC	NA
CME2, CMF1	Mango	GC	*C. theobromicola* (syn. *C. fragariae*)
CTG3	Mango	AC	NA
CMA4, CMA6, CMA7, CMA10, CMB1, CMB2, CMD1, CMD4, CMD5, CTG5, CTK5, CTL1, CTL7, CTL8, CTL9, CTL10, CTL12	Mango (53%), tree tomato (47%)	GC	NA
CMA5, CTL2	Mango (50%), tree tomato (50%)	GC	*C. siamense*
CTG1	Tree tomato	GC	*C. kahawae*
CTG2, CTG4, CTG6, CTG7, CTI1, CTI2, CTI3, CTJ1, CTJ2, CTJ3, CTJ4, CTJ5, CTK2, CTK3, CTK4, CTK6, CTK8, CTK9, CTK10, CTK11, CTK12, CTK13, CTK14, CTK15, CTK16, CTK17, CTK18, CTK19, CTL3, CTL4, CTL5, CTL6, CTL11, CTL13, CTM1, CTN1, CTN2, CTN3, CTN4, CTN5, CTN6, CTO1, CTP1, CTP2, CTQ1, CTQ2	Tree tomato	AC	*C. tamarilloi*

Phylogenetic analyses using individual or concatenated sequences permitted the identification of species based on the clustering of the isolates' sequences relative to the reference sequences from previous studies.

The first four strains in the table are reference strains recommended for use in phylogenetic studies by the CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands).

AC: acutatum species complex; GC: gloeosporioides species complex; BC: boninense species complex; NA: not assigned.

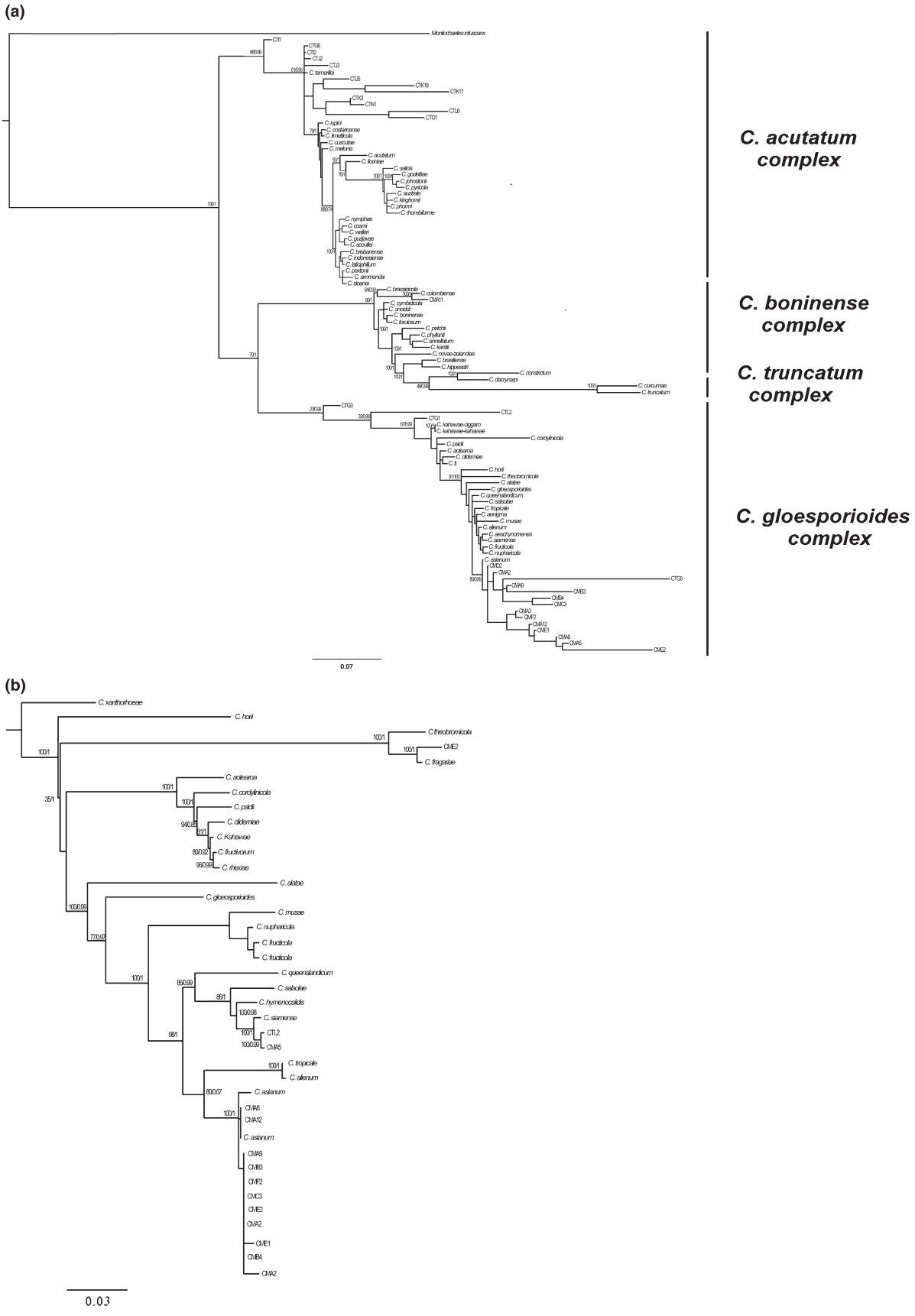
(a)
Monilochaetes infuscans
CTT1
CTG6
CTG
CTL2
CTL3
C. torrendiae
CTL5
CTK18
CTK17
CTK3
CTN1
CTL6
CTO1
C. lupini
C. costaricense
C. limetticola
C. cuscutae
C. melonis
C. acutatum
C. lilomiae
C. salicis
C. godetiae
C. johnstonii
C. pyricola
C. australe
C. kinghornii
C. phormi
C. mombasiense
C. nymphaeae
C. cosmi
C. walleri
C. guajavae
C. acutilet
C. brasiliense
C. indonesiense
C. bilophilum
C. pandanii
C. simmondsii
C. sloanei
C. brassicicola
C. colombiense
CMA11
C. cymbidicola
C. orchidii
C. boninense
C. torulosum
C. petchii
C. phyllanthi
C. annellatum
C. karsii
C. novae-zelandiae
C. brasiliense
C. hippeastri
C. constrictum
C. dracycarpi
C. curcumae
C. truncatum
CTG3
CTL2
CTG1
C. kahawae-ciggaro
C. kahawae-kahawae
C. cordylinicola
C. psidii
C. actinatum
C. aldemiae
C. ti
C. horii
C. theobromicola
C. alatae
C. gloeosporioides
C. queenslandicum
C. salsolae
C. tropicale
C. aenigma
C. musae
C. alienum
C. aeschynomenes
C. siamense
C. fructicola
C. nupharicola
C. asianum
CMD2
CMA2
CTG5
CMA9
CMB3
CMB4
CMC3
CMA3
CMF2
CMA12
CME1
CMA8
CMA5
CME2
C. acutatum complex
C. boninense complex
C. truncatum complex
C. gloesporioides complex
0.07
(b)
C. xanthorrhoeae
C. horii
C.theobromicola
CME2
C. fragariae
C. actinatum
C. cordylinicola
C. psidii
C. aldemiae
C. Kahawae
C. fructicorum
C. rhexiae
C. alatae
C. gloeosporioides
C. musae
C. nupharicola
C. fructicola
C. fructicola
C. queenslandicum
C. salsolae
C. hymenocallidis
C. siamense
CTL2
CMA5
C. tropicale
C. alienum
C. asianum
CMA8
CMA12
C. asianum
CMA9
CMB3
CMF2
CMC3
CME2
CMA2
CME1
CMB4
CMA2
0.03

Figure 2 (a) Concatenated tree from a multilocus analysis using internal transcriber spacer, *glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*) gene, *actin* (*ACT*) gene and *chitin synthase 1* (*CHS-1*) gene for a subset of 30 isolates. (b) Phylogenetic tree constructed using the *ApMat* intergenic region for a subset of 14 isolates belonging to the *Colletotrichum gloeosporioides* complex. The trees shown are the result of the Bayesian inference analysis. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

of the reference strains, *C. acutatum* or *C. gloeosporioides*. Among the isolates collected from the two hosts, only two morphological groups were identified according to the parameters used in the taxonomic keys (Sutton, 1980). These groups corresponded to *C. acutatum* and *C. gloeosporioides* species complexes (Table S2). The *C. fragariae* and *C. lupini* reference strains exhibited similarities with isolates in the *C. gloeosporioides* and *C. acutatum* species complexes, respectively. Isolates that were morphologically identified as *C. gloeosporioides* from both hosts showed the highest variability at colony level, with colonies displaying white, black or grey colours (Fig. S5a; Table S2). In addition, variability in conidia size was observed; conidia ranged from 10 to 16 μm in length and from 1 to 13 μm in width (Table S2).

More than 20 of the 59 isolates from tree tomato were morphologically identified as *C. acutatum*. *Colletotrichum acutatum* isolates presented white to pink mycelia, pink/salmon reverse colony colour (Fig. S5b; Table S2), and did not produce sclerotia. Conidia ranged from 8 to 15 μm in length and from 1 to 4 μm in width.

Appressoria could only be observed for a limited number of isolates immediately after isolation. After subsequent transfers through media, appressoria were not observed. Thus, this morphological trait was not included in the analysis.

Average colony growth on PDA, MA and MB+T media 7 and 15 days after incubation are shown on Table S4. Growth trends on the three different media were similar for each isolate. Thus, the MA data will be described to illustrate the growth trends. Two main groups were observed (Table S4). The first group included the *C. acutatum* and *C. lupini* reference strains and exhibited growth rates ranging from 0·02 to 0·5 cm per day and a mean growth rate of $0{\cdot}35 \pm 0{\cdot}14$ cm per day. The second group included the *C. gloeosporioides* and *C. fragariae* reference strains and exhibited growth rates ranging from 0·5 to 1·3 cm per day and a mean growth rate of $1{\cdot}02 \pm 0{\cdot}12$ cm per day.

All isolates were tested for their ability to utilize tartrate as the sole source of carbon. Even though all isolates were able to grow in the medium, 35 isolates changed the colour of the medium to a dark blue to purple colour, whereas 56 were unable to change the colour or exhibited a yellow to orange colour. However, the test could not discriminate the reference strains according to previous taxonomic classifications; *C. gloeosporioides*, *C. acutatum* and *C. fragariae* exhibited a yellow to orange colour, and *C. lupini* exhibited a blue to purple colour change (Table S5).

Pathogenicity analysis

Pathogenicity analyses showed that isolates did exhibit host preference. In general, the reference strains of *C. acutatum*, *C. lupini* and *C. fragariae* infected the various tissues evaluated from both hosts, with the exception of mango leaves, where infection was never observed. The reference strain of *C. gloeosporioides* did not infect tree tomato fruits, although it did infect tree tomato leaves. Overall, 43·8% of the isolates were able to infect the various tissues evaluated from both hosts (except mango leaves), 18·7% of the isolates only infected mango fruits, 19·8% of the isolates infected both mango fruits and one of the two tree tomato varieties, 10·4% of isolates infected both tree tomato varieties, 3·1% of isolates only infected one tree tomato variety and 4·2% of the isolates did not infect any tissue.

The aggressiveness of the isolates from tree tomato ($n = 59$) and from mango ($n = 32$) were compared on their original hosts and on the alternate host. Aggressiveness was determined from the percentage of the sample affected. The results from the cross-inoculations showed a significant interaction among factors host of origin × inoculated sample (ANOVA analysis; $F = 8{\cdot}79$, $P < 0{\cdot}01$). The data also revealed some degree of host preference, as determined by the *t*-test analyses (Fig. 3). These results indicated that, in general, isolates from mango and isolates from tree tomato were more aggressive on their host of origin (Fig. 3). Tree tomato red variety could not differentiate the isolates according to their host of origin (Fig. 3) and could be infected similarly by isolates collected from mango or tree tomato.

Discussion

The main objective of this study was to characterize *Colletotrichum* isolates from mangoes and tree tomatoes in Cundinamarca and Tolima, two important production regions in Colombia. The *Colletotrichum* species, like other fungal species within genera such as *Fusarium*, *Aspergillus* and *Penicillium*, are very difficult to unambiguously describe due to their wide molecular and morphological variability. Therefore, the molecular, morphological and physiological characteristics of *Colletotrichum* spp. were all evaluated and the data all analysed to establish the taxonomic status of 91 isolates obtained from tree tomatoes and mangoes in the Colombian states of Cundinamarca and Tolima. The molecular analyses were better able to resolve the complexes and species within this population than the morphological, physiological and pathogenicity assays.

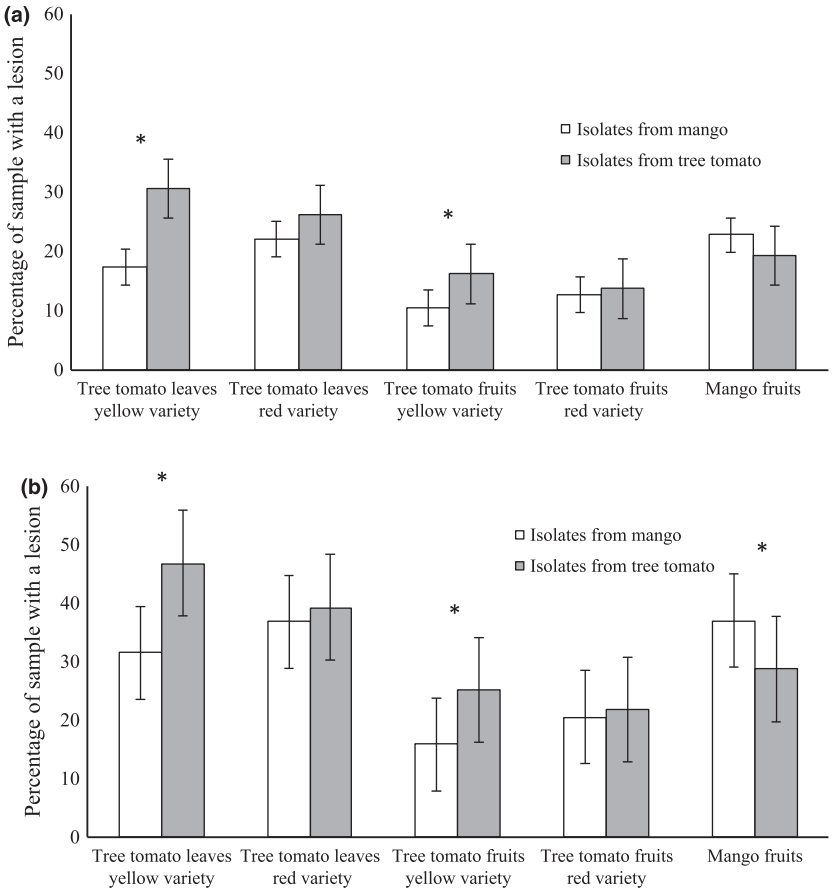

Figure 3 Occurrence of lesions produced 7 (a) and 15 (b) days after inoculation with *Colletotrichum* spp. All isolates were inoculated on mango fruits, and on leaves and fruits of two different tree tomato varieties (yellow and red). Error bars indicate ± standard error of the means. Asterisks denote significant differences for each inoculated sample between isolates collected from tree tomato and mango. Significance was tested using a Welch's two-sample *t*-test ($P < 0{\cdot}05$).

Phylogenetic concordance criteria have been previously used to identify species in other fungal groups that also exhibit hard-to-resolve species complexes (Dettman *et al.*, 2003). Moreover, it has been shown that phylogenetic species recognition also coincides with biological species delimitations (Avise & Wollenberg, 1997), thus being a powerful tool for the identification of species when biological assays are difficult to perform. In the present analysis, the ITS and *GAPDH* genomic regions allowed the separation of three groups or species complexes. The first group contained the *C. gloeosporioides* and *C. fragariae* reference strains, the second group contained the *C. acutatum* and *C. lupini* reference strains, and the third group contained sequences of the boninense complex. Authors such as Cai *et al.* (2009), Hyde *et al.* (2009) and others (Damm *et al.*, 2010, 2012a; Cannon *et al.*, 2012; Weir *et al.*, 2012) have recognized that the ITS region is insufficient to properly identify the *Colletotrichum* species and have suggested that it is necessary to use more genes to clarify the phylogenetic relationships in this genus. In fact, the current *Colletotrichum* classification is defined using eight loci (Cannon *et al.*, 2012). In the present study, it was possible to assign all isolates to species complexes and some isolates were further assigned to individual species by using two genomic regions, ITS and *GAPDH*. Given that the phylogenetic concordance criteria suggest that contradictions between individual phylogenies may be missed if locus sampling is poor, it was decided to characterize two additional genomic regions (*ACT* and *CHS-1*) for a subset of 30 isolates representative of the three major complexes identified using the ITS and *GAPDH* genomic regions. These additional loci were important for species assignment and discordance findings. For example, the *ACT* phylogeny presented the isolate CTG3 forming a well-supported monophyletic clade with *C. tamarilloi*, meaning that it could not be assigned to *C. kahawae* despite this being indicated by its clustering within ITS, *GAPDH* and ITS-*GAPDH* trees. Moreover, recently developed primers (Silva *et al.*, 2012) have made it possible to sequence the intergenic region between the *Apn2* and the *Mat1-2-1* region, and this marker has been shown to be very powerful in separating species within the *C. gloeosporioides* complex, as demonstrated in the present study. These results should also encourage the development of primers for such regions in the rest of

the species complexes and so dramatically improve the taxonomy of the whole genus.

The phylogenetic species recognition enabled the discrimination of different species in three main complexes. In mango, two of the species identified were *C. asianum* and *C. gloeosporioides*, which are typically isolated from mango (Afanador-Kafuri *et al.*, 2003; Than *et al.*, 2008a; Rojas *et al.*, 2010). Several authors have reported that the genetic and morphological uniformity of these two species makes it difficult to discriminate between them (Sanders & Korsten, 2003). *Colletotrichum theobromicola*, a cosmopolitan fungus that attacks various hosts in tropical countries (Prihastuti *et al.*, 2009; Rojas *et al.*, 2010) was also identified. *Colletotrichum kahawae* was isolated from tree tomato and placed in the gloeosporioides complex. This fungus is typically isolated from coffee plants in various localities worldwide; its relationship with tree tomato can possibly be attributed to host proximity (Waller *et al.*, 1993). All of the above-mentioned species belong to the gloeosporioides complex; these results are concordant with the finding that the greatest haplotypic diversity was found in the gloeosporioides complex compared to the acutatum complex. In tree tomato, *C. tamarilloi* was identified, a species originally described by Damm *et al.* (2012a) using isolates collected from tree tomato in Colombia. Afanador-Kafuri *et al.* (2003) were not able to differentiate *C. tamarilloi* as a separate species from *C. acutatum* with the molecular markers used in their study.

As previously shown in Colombia, *C. acutatum* was mainly isolated from tree tomato, whereas *C. gloeosporioides* was mainly isolated from mango (Afanador-Kafuri *et al.*, 2003). A third group, the boninense complex, was identified; whether this complex corresponds to the unidentified species noted by Afanador-Kafuri *et al.* (2003) remains to be determined. Therefore the ITS sequences from these unidentified species (isolated from mango and tree tomato) were included in the present analyses and it was confirmed that they belong to the boninense complex and are closely related to the isolates collected for this study (Fig. S1). The acutatum group exhibited lower levels of morphological and molecular variability, most probably caused by recent divergence associated with a novel host (tree tomato) preference. All but one of the *C. acutatum* isolates were from the same host (tree tomato). The isolate CMC1, which was from mango, was identified as *C. tamarilloi*, based on molecular markers, but displayed morphological characteristics typical of *C. gloeosporioides* (Table S2). This could be the first report of *C. tamarilloi* isolated from *Mangifera indica* because *C. gloeosporioides* is usually associated with anthracnose and mummification in this host.

When a set of morphological and physiological (including pathogenicity) characteristics was used, all isolates collected in this study were only differentiated into two complexes: *C. gloeosporioides* and *C. acutatum*. Various authors, including Bridge *et al.* (2008) and Cannon *et al.* (2012), have mentioned the difficulty of morphologically separating *C. gloeosporioides* and *C. acutatum* due to the great variability in their colony and conidia characteristics. Crouch *et al.* (2006) suggest that the observed morphological variations in colony colour and conidial size and shape can be related to physiological components, such as exposure to light, the media types tested, temperature and other variables. Indeed, the molecular identification of the isolates showed the lack of congruence between a morphological and a phylogenetic identification of some isolates (Tables 1 & S2), highlighting the difficulties of achieving a proper identification using only morphological traits.

Bridge *et al.* (2008), Prihastuti *et al.* (2009) and Frisvad & Samson (2008) recognized that the utilization of different carbohydrates helped clarify the classification of certain fungal groups. In the present case, the use of different physiological and biochemical tests simply confirmed the morphological classification. Groups of *Colletotrichum* spp. differ in their ability to utilize carbon sources such as citrate and ammonium tartrate. Thus, differential growth rates on media containing diverse carbon sources can facilitate identification. Overall, it was observed that growth in Medium B was limited for all isolates included in this study. Colony growth on PDA and MA allowed the identification of two groups that corresponded to (i) fast-growing isolates that were morphologically identified as *C. gloeosporioides*, and (ii) isolates exhibiting limited growth that were morphologically identified as *C. acutatum*. This is consistent with the findings of Hyde *et al.* (2009), where *C. acutatum* exhibited slower growth than *C. gloeosporioides* in several culture media. It is possible that these differences in colony growth may be related to the ability of certain strains to better absorb nutrients from the substrate. In addition, colony colour changes are known to occur in certain strains depending on the culture media evaluated (Goodwin *et al.*, 1992; Brown *et al.*, 1996). For example, the isolate CMC5 was white on PDA and dark brown on MA. The colour change may be related to the carbon source present in the medium and the assimilation capability of the growing fungus.

The relative pathogenicity assays for the isolates included in this study on their original host and on the alternate host allowed the gathering of important pathogenic characteristics of the isolates. In general, isolates from the yellow, or common, tree tomato variety were able to grow better on this host than isolates collected from mango. However, isolates from both tree tomatoes and mangoes were able to cause similar symptoms on the red tree tomato variety; this is of concern because this is the most common tree tomato variety grown in the region (Table S1). The reference strains could also infect different inoculated plant tissues; *C. lupini*, *C. acutatum* and *C. fragariae* caused symptoms on tree tomato leaves and fruits as well as on mango fruits, and, interestingly, the reference strain *C. gloeosporioides* infected tree tomato leaves but was unable to infect tree tomato fruits. In the present study, *C. gloeosporioides* was successfully isolated from tree tomatoes, in contrast to a previous

study conducted in Colombia, where *C. gloeosporioides* species were not isolated from tree tomatoes (Afanador-Kafuri *et al.*, 2003). Finally, a total of six isolates, belonging to the gloeosporioides and acutatum species complexes, did not produce lesions in the evaluated hosts; it may be that these strains have an endophytic relationship with the host, as mentioned by Cannon *et al.* (2012), who recognized that microorganisms can change between pathogen and endophyte depending on the host. Alternatively, these isolates may have lost their pathogenicity after several transfers through media. However, because wounded fruits were used in the present study, the results may not reflect the pathogenicity of the isolates; natural inoculations would clarify this.

In conclusion, phylogenetic analyses allowed the identification of most of the *Colletotrichum* isolates at the species level. The traditional morphological, physiological and pathogenicity assays served to gather important information on the isolates; however, it has been shown that the results of such assays should be analysed with great care and further studies should rely on multilocus phylogenetic identification.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Phylogenetic tree constructed using the ITS region for all isolates included in this study. The tree shown is the result of a Bayesian inference analysis. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

Figure S2 Phylogenetic tree constructed using the *GAPDH* gene for all isolates included in this study. The tree shown is the result of a Bayesian inference analysis. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

Figure S3 Phylogenetic tree constructed using the *ACT* gene region for a subset of 30 isolates included in this study. The tree shown is the result of the Bayesian inference analysis. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

Figure S4 Phylogenetic tree constructed using the *CHS-1* gene for a subset of 30 isolates included in this study. The tree shown is the result of the Bayesian inference analysis. Support values are: bootstrap support values (maximum likelihood)/posterior probabilities (from the Bayesian inference).

Figure S5 (A) Morphological variability of *Colletotrichum gloeosporioides* isolates: (a) CMA1, (b) CMA2, (c) CMA3, (d) CMA4, (e) CMF1, (f) CME2, (g) CMD2, (h) CMA7. (B) Morphological variability of *Colletotrichum acutatum* isolates: (a) CTI3, (b) CTJ2, (c) CTK11, (d) CTK2. (C) Reference strains: (a) *C. gloeosporioides*,(b) *Colletotrichum fragarie*, (c) *C. acutatum*, (d) *Colletotrichum lupini*.

Table S1 Sampling localities

Table S2 Morphological characteristics determined on potato dextrose agar after 7 days' incubation

Table S3 Sequences retrieved from databases for phylogenetic analyses

Table S4 Average colony growth on potato dextrose agar (PDA), malt agar (MA) and Medium B with tartrate (MB+T), 7 and 15 days after incubation

Table S5 Ammonium tartrate assimilation, sporulation start time and number of conidia produced 7 days after incubation for isolates included in this study. The ability to use ammonium tartrate as a carbon source was determined by observation of pathogen growth and by the production of a purple colour on the media 15 days after incubation at 25°C