

Diversity and pathogenicity of *Colletotrichum* species isolated from soursop in Colombia

Elizabeth Álvarez · Lederson Gañán · Alberto Rojas-Triviño · Juan F. Mejía · Germán A. Llano · Alonso González

Accepted: 6 January 2014 / Published online: 25 January 2014
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Abstract Anthracnose, caused by *Colletotrichum* species is a highly limiting disease for the production of the tropical fruit tree crop, soursop (*Annona muricata* L.). In this study, 83 single-spore isolates of *Colletotrichum* were obtained from diseased soursop tissues and subjected to a species complex-specific PCR assay. The isolates were identified as *C. gloeosporioides* sensu lato ($n=60$), *C. boninense* s. lat. ($n=22$), or *C. acutatum* s. lat. ($n=1$). A subset of 21 selected isolates was identified to species level by means of a multi-locus phylogenetic analysis using sequences from the ITS region and partial sequences of the actin, β -tubulin-2, glyceraldehyde-3-phosphate dehydrogenase, and chitin synthase-1 genes. The multi-locus phylogenetic analysis resolved *C. theobromicola*, *C. tropicale*, *C. siamense*, and *C. gloeosporioides* sensu stricto in the *C. gloeosporioides* complex; *C. karstii* and one undetermined species in the *C. boninense* complex; as well as one undetermined species in the *C. acutatum* complex. Significant differences in anthracnose severity were observed between *Colletotrichum* species when tested for pathogenicity on

attached twigs of soursop cv. Elita. *Colletotrichum theobromicola* and *C. tropicale* were associated with high and intermediate virulence, respectively, whereas the remaining species were associated with low virulence.

Keywords *Annona muricata* · Anthracnose · Characterization · Phylogenetic analysis · Virulence

Soursop (*Annona muricata* L.), also known as *guanábana* in Spanish, is an important tropical fruit crop that originated in the Neotropics. It is cultivated in Brazil, Colombia, Mexico, Panama, Peru, Puerto Rico and Venezuela, and is also grown in small family orchards in Southeast Asia, Philippines, India, and Hawaii (USA) or other Pacific islands (Love and Paull 2011). As a commercial crop, it is highly remunerative for both small- and medium-scale farmers (ICUC 2002). The fruit ranks as among the world's best-tasting horticultural species, possessing a sweet, creamy flesh and fragrant flavour (Pareek et al. 2011). It is widely consumed in the tropics of America and Asia (Love and Paul 2011).

Both the vegetative and reproductive parts of soursop trees are attacked by a variety of diseases, including the economically significant anthracnose. Characteristic symptoms include dieback of twigs and branches, necrosis of young leaves and stems, flower drop, fruit drop (especially of young fruits), and rot of mature fruit. Crop losses can be as high as 90 % (Álvarez et al. 2004).

Determination of *Colletotrichum* species and their characterization are traditionally based on microscopic

E. Álvarez (✉) · L. Gañán · A. Rojas-Triviño · J. F. Mejía · G. A. Llano · A. González
Plant Pathology, Tropical Fruit Project, International Center for Tropical Agriculture (CIAT),
A.A. 6713, Cali, Colombia
e-mail: e.alvarez@cgiar.org

L. Gañán
Laboratorio de Fitopatología, Departamento de Producción Agropecuaria, Universidad de Caldas,
A.A. 275, Manizales, Colombia

characters, colony morphology, and host-range (Sutton 1992). However, high variability of morphological and cultural characteristics has rendered these criteria unreliable for identifying the pathogen at species level. Furthermore, several *Colletotrichum* species can be found infecting or colonizing the same host plant (Freeman et al. 1998; Lima et al. 2013) and potential cross-infection among different *Colletotrichum* species has been reported (Alahakoon et al. 1994; Freeman et al. 1998; Phoulivong et al. 2012), making accurate identification of the causal agent by symptoms alone difficult. This presents a quandary, because accurate species identification is critical for designing strategic disease management and understanding the pathogen's population structure and dynamics (Freeman et al. 1998).

Molecular techniques help overcome the inadequacies of traditional methods, and have recently been used to identify and characterize *Colletotrichum* species (Than et al. 2008; Cai et al. 2009; Lima et al. 2013; Schena et al. 2013; Huang et al. 2013; Udayanga et al. 2013; Liu et al. 2013b). Analyses of nucleic acids provide the most reliable structure for classifying *Colletotrichum* species, as DNA traits are not directly influenced by environmental factors (Cannon et al. 2000).

In Colombia, anthracnose in soursop is reported to be caused by *Colletotrichum gloeosporioides* (Álvarez et al. 2004). However, following epitypification of *C. gloeosporioides* (Cannon et al. 2008), phylogenetic analysis of multi-locus sequence data showed that *C. gloeosporioides* is a species complex that contains 22 currently accepted species (Weir et al. 2012). Moreover, Phoulivong et al. (2010) reported that *C. gloeosporioides* sensu stricto is not commonly found on tropical fruits.

In our study, we therefore aim to apply multi-locus phylogenetic analysis to identify *Colletotrichum* species associated with soursop anthracnose in Colombia. We also evaluate the fungal species' pathogenicity and characterize their morphology.

Materials and methods

Source of isolates *Colletotrichum* isolates from diseased tissues (including leaves, branches, fruits, and flowers) of soursop trees showing anthracnose symptoms were collected in key production regions of Colombia. To isolate the pathogens and obtain single-spore cultures,

the procedure described by Than et al. (2008) was carried out.

Molecular characterization. DNA extraction Total genomic DNA was extracted from fungal mycelium grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) following the protocol of Damm et al. (2008).

Identifying *colletotrichum* species complexes by PCR *Colletotrichum* species complexes were identified by using PCR amplification of the ribosomal DNA internal transcribed spacer (ITS) region. The primers used to detect the *Colletotrichum* complexes included the ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), coupled with primers specific for *C. acutatum* s. lat. (*CaInt2*; 5'-GGGGAAGCCTCTCG CGG-3') (Sreenivasaprasad et al. 1996), *C. gloeosporioides* s. lat. (*CgInt*; 5'-GGCCTCCCGC CTCCGGGCGG-3') (Mills et al. 1992), and *C. boninense* s. lat. (*Col1*; 5'-GCCGTCCCTGAAA AG-3') (Afanador-Kafuri et al. 2003; Pileggi et al. 2009).

Genomic DNA from strains GND-1, Pass 063, and Tom 12 was used as positive controls in the PCR amplification for *C. gloeosporioides* s. lat., *C. boninense* s. lat., and *C. acutatum* s. lat., respectively. They were kindly provided by Dr Lucía Afanador-Kafuri, curator of the phytopathogenic fungal collection held at the Plant Health Laboratory, Universidad Nacional (Medellín, Colombia). Negative control was the amplification reaction in the absence of DNA.

PCR amplifications were performed in a 25- μ l reaction volume, consisting of 10 ng of DNA template, a 1X final concentration of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Inc.), and 0.5 μ M of each primer. The PCR reaction was carried out, using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). The program included an initial step of DNA denaturation at 95 °C for 6 min, followed by 40 cycles consisting of 30 s at 95 °C, 30 s at either 62 °C (for *CgInt* and *CaInt2*) or 60 °C (for *Col1*), and 1.5 min at 72 °C, with a final extension step at 72 °C for 4 min. The PCR products were analyzed by electrophoresis (6 μ l/well), using a 2 % agarose gel stained with SYBR[®] Safe DNA Gel Stain, and then visualized under a Safe Imager[™] 2.0 Blue-Light Transilluminator (Invitrogen, Carlsbad, CA, USA).

Species identification based on multi-locus phylogenetic analyses The complete rDNA ITS region, as well as partial sequences of the actin (ACT), β -tubulin (TUB2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and chitin synthase 1 (CHS-1) genes were amplified and sequenced using the primer pairs ITS-5 + ITS-4 (White et al. 1990), ACT-512F + ACT-783R (Carbone and Kohn 1999), Bt2a + Bt2b (Glass and Donaldson 1995), GDF1 + GDR1 (Templeton et al. 1992), CHS-79F + CHS-354R (Carbone and Kohn 1999), respectively.

The PCR amplifications were performed in a 25- μ l reaction volume, consisting of 10 ng of DNA template, a 1X final concentration of DreamTaq Green PCR Master Mix, and 0.5 μ M of each primer. The reactions were performed with a PTC-100 thermal cycler, using the thermal programs as previously described for the amplification of ITS, ACT, TUB2, and GAPDH (Prihastuti et al. 2009), and for CHS-1 (Weir et al. 2012).

The PCR products were purified by the PEG-NaCl method. The sample was mixed with 1X volume of PEG-NaCl (20 % PEG at MW 6000 and 2.5 M NaCl) and incubated for 15 min at 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 then dissolved in 20 μ l of sterilized distilled water.

The DNA sequences were determined in both directions, using an Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems, Inc., Foster City, CA, USA). Sequencing was performed at the Biotechnology Unit of Iowa State University, USA. The nucleotide sequences were assembled to consensus sequences, edited with ChromasPro v. 1.5 (Technelysium Pty Ltd, Tewantin, QLD, Australia) and aligned, using ClustalW as implemented in MEGA v. 5.1 (Tamura et al. 2011).

For the phylogenetic analyses, molecular data of the targeted genes were compared with sequences of ex-type and other reference strains of accepted *Colletotrichum* species, which were obtained from GenBank (Table 1). Individual gene alignments were concatenated, using FASconCAT v. 1.0 (Kück and Meusemann 2010). Separate partitions were then created for each gene and the model of nucleotide substitution determined by jModelTest 0.1.1 (Posada 2008), according to the corrected Akaike information criterion (AICc).

Bayesian inference was used for phylogenetic reconstruction, using MrBayes v. 3.2.1 (Ronquist et al. 2012).

Metropolis-coupled Markov-Chain Monte Carlo (MCMC) analysis was performed on the dataset with substitution models determined separately for each partition. For the Bayesian analysis, two MCMC chains were run twice for 1×10^7 generations, with trees sampled every 1000 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. Consensus trees were visualized and edited with TreeGraph 2 (Stöver and Müller 2010). Sequences derived in this study were submitted to GenBank (Table 1). Sequence alignments and the consensus trees were edited in Mesquite v. 2.7.5 (Maddison and Maddison 2011) and deposited in TreeBASE (<http://www.treebase.org>) under the accession number S13841.

Pathogenicity trial To evaluate pathogenicity and virulence of *Colletotrichum* species identified, a greenhouse trial was conducted. Scions (twigs) of the soursop cv. Elita were grafted onto sexually reproduced (i.e., seed-based) native rootstocks. When the grafted twigs were 8 months old and about 40 cm high, they were inoculated. Superficial wounds (~7 mm in diameter) were made on stems, using sterilized scalpels. A mycelial plug, with a 6.5-mm diameter, was then taken from the edge of a 15-day-old culture grown on modified Mathur's medium (Freeman et al. 2000). The plug was placed on the wound, with the mycelial surface facing the cambium. Inoculations were made on three parts of the twig, spaced at 10 cm, beginning with the seedling's canopy and finishing at its base, ensuring that their locations were above the grafting point and on young tissues. Once completed, the inoculations were covered with parafilm paper (Parafilm M*; American National Can Company, Norwalk, CT, USA).

All inoculated and control plants (which were mock-inoculated with sterilized Mathur's agar plugs) were incubated for 72 h at 27 to 29 °C and 95 % relative humidity. The plants were then spray-misted for 1 min every hour for 17 days. The isolates were arranged in a randomized complete block design, with three replications per isolate. All trials were replicated twice. The number of isolates inoculated per taxon depended on strain availability, and were treated as subsamples in the statistical analysis. Anthracnose severity was assessed at 23 days after inoculation, using a 0 to 5 point scale, where 0=no visible symptoms, 1=1 to 5 %, 2=5 to 10 %, 3=10 to 25 %, 4=25 to 50 %, and 5=>50 % of twig area showing symptoms. To confirm Koch's

Table 1 GenBank accessions of *Colletotrichum* strains used for the phylogenetic analyses in this study

<i>Colletotrichum</i> species	Strain code ^a	Host	Location	GenBank accession no. ^b						
				ACT	CHS-1	GAPDH	ITS	TUB2		
<i>C. acerbum</i>	CBS 128530*	<i>Maltus domestica</i>	New Zealand	JQ949780	JQ949120	JQ948790	JQ948459	JQ949780		
<i>C. acutatum</i>	CBS 112996*	<i>Carica papaya</i>	Australia	JQ005839	JQ005797	JQ948677	JQ005776	JQ005839		
<i>C. aenigma</i>	ICMP 18608*	<i>Persea americana</i>	Israel	JX009443	JX009774	JX010044	JX010244	JX010389		
<i>C. aenigma</i>	ICMP 18686	<i>Pyrus pyrifolia</i>	Japan	JX009519	JX009789	JX009913	JX010243	JX010390		
<i>C. aescynomenes</i>	ICMP 17673*	<i>Aeschynomene virgintica</i>	USA	JX009483	JX009799	JX009930	JX010176	JX010392		
<i>C. alatae</i>	ICMP 17919*	<i>Dioscorea alata</i>	India	JX009471	JX009837	JX009990	JX010190	JX010383		
<i>C. alatae</i>	ICMP 18122	<i>Dioscorea alata</i>	Nigeria	JX009470	JX009846	JX010011	JX010191	JX010449		
<i>C. alienum</i>	IMI 313842	<i>Persea americana</i>	Australia	JX009580	JX009754	JX010018	JX010217	JX010385		
<i>C. alienum</i>	ICMP 18621	<i>Persea americana</i>	New Zealand	JX009552	JX009755	JX009959	JX010246	JX010386		
<i>C. annellatum</i>	CBS 129826*	<i>Hevea indica</i>	Colombia	JQ005570	JQ005396	JQ005309	JQ005222	JQ005656		
<i>C. aotearoa</i>	ICMP 18537*	<i>Coprosma</i> sp.	New Zealand	JX009564	JX009853	JX010005	JX010205	JX010420		
<i>C. aotearoa</i>	ICMP 18533	<i>Prunnopythys ferruginea</i>	New Zealand	JX009522	JX009769	JX010026	JX010197	JX010416		
<i>C. asianum</i>	ICMP 18696	<i>Mangifera indica</i>	Australia	JX009576	JX009753	JX009915	JX010192	JX010384		
<i>C. asianum</i>	ICMP 18580*	<i>Coffea arabica</i>	Thailand	JX009584	JX009867	JX010053	FJ972612	JX010406		
<i>C. australe</i>	CBS 116478*	<i>Trachycarpus fortunei</i>	South Africa	JQ949776	JQ949116	JQ948786	JQ948455	JQ949776		
<i>C. beeveri</i>	CBS 128527*	<i>Brachyglottis repanda</i>	New Zealand	JQ005519	JQ005345	JQ005258	JQ005171	JQ005605		
<i>C. boninense</i>	CBS 123755*	<i>Crinum asiaticum</i> var. <i>sinicum</i>	Japan	JQ005501	JQ005327	JQ005240	JQ005153	JQ005588		
<i>C. boninense</i>	CBS 128526	<i>Dacrycarpus dacrydioides</i>	New Zealand	JQ005510	JQ005336	JQ005249	JQ005162	JQ005596		
<i>C. brasiliense</i>	CBS 128501*	<i>Passiflora edulis</i>	Brazil	JQ005583	JQ005409	JQ005322	JQ005235	JQ005669		
<i>C. brasiliense</i>	CBS 128528	<i>Passiflora edulis</i>	Brazil	JQ005582	JQ005408	JQ005321	JQ005234	JQ005668		
<i>C. brassicicola</i>	CBS 101059*	<i>Brassica oleracea</i>	New Zealand	JQ005520	JQ005346	JQ005259	JQ005172	JQ005606		
<i>C. brisbanense</i>	CBS 292.67*	<i>Capsicum annuum</i>	Australia	JQ949612	JQ948952	JQ948621	JQ948291	JQ949612		
<i>C. chrysantemi</i>	IMI 364540	<i>Chrysanthemum coronarium</i>	China	JQ949594	JQ948934	JQ948603	JQ948273	JQ949594		
<i>C. clidemiae</i>	ICMP 18658*	<i>Clidemia hirta</i>	USA, Hawaii	JX009537	JX009877	JX009989	JX010265	JX010438		
<i>C. colombiense</i>	CBS 129818*	<i>Passiflora edulis</i>	Colombia	JQ005522	JQ005348	JQ005261	JQ005174	JQ005608		
<i>C. colombiense</i>	CBS 129817	<i>Passiflora edulis</i>	Colombia	JQ005521	JQ005347	JQ005260	JQ005173	JQ005607		
<i>C. constrictum</i>	CBS 128504*	<i>Citrus limon</i>	New Zealand	JQ005586	JQ005412	JQ005325	JQ005238	JQ005672		
<i>C. cordylinicola</i>	ICMP 18579*	<i>Cordyline fruticosa</i>	Thailand	HM470235	JX009864	JX009975	JX010226	JX010440		
<i>C. cosmi</i>	CBS 853.73*	<i>Cosmos</i> sp.	Netherlands	JQ949595	JQ948935	JQ948604	JQ948274	JQ949595		
<i>C. costaricense</i>	CBS 330.75*	<i>Coffea arabica</i>	Costa Rica	JQ949501	JQ948841	JQ948510	JQ948180	JQ949501		

Table 1 (continued)

Colletotrichum species	Strain code ^a	Host	Location	GenBank accession no. ^b					
				ACT	CHS-1	GAPDH	ITS	TUB2	
<i>C. cuscuteae</i>	IMI 304802*	<i>Cuscuta</i> sp.	Dominica	JQ949516	JQ948856	JQ948525	JQ948195	JQ949516	
<i>C. cymbidicola</i>	IMI 347923*	<i>Cymbidium</i> sp.	Australia	JQ005514	JQ005340	JQ005253	JQ005166	JQ005600	
<i>C. cymbidicola</i>	CBS 123757	<i>Cymbidium</i> sp.	Japan	JQ005516	JQ005342	JQ005255	JQ005168	JQ005602	
<i>C. dacrycarpi</i>	CBS 130241*	<i>Dacrycarpus dacrydioides</i>	New Zealand	JQ005584	JQ005410	JQ005323	JQ005236	JQ005670	
<i>C. fioriniae</i>	CBS 128517*	<i>Fiorinia externa</i>	USA	JQ949613	JQ948953	JQ948622	JQ948292	JQ949613	
<i>C. fructicola</i>	ICMP 18613	<i>Limonium sinuatum</i>	Israel	JX009491	JX009772	JX009998	JX010167	JX010388	
<i>C. fructicola</i>	ICMP 18646	<i>Tetragastris panamensis</i>	Panama	JX009581	JX009874	JX010032	JX010173	JX010409	
<i>C. gloeosporioides</i>	ICMP 17821*	<i>Citrus sinensis</i>	Italy	JX009531	JX009818	JX010056	JX010152	JX010445	
<i>C. gloeosporioides</i>	GM62-L03	<i>Annona muricata</i> , branch	Colombia (Valle del Cauca)	KC512179	KC512158	KC506409	KC512137	KC512200	
<i>C. gloeosporioides</i>	GM78	<i>Annona muricata</i> , leaf	Colombia (Quindio)	KC512180	KC512159	KC506410	KC512138	KC512201	
<i>C. godetiae</i>	CBS 133.44*	<i>Clarkia hybrida</i>	Denmark	JQ949723	JQ949063	JQ948733	JQ948402	JQ949723	
<i>C. guajavae</i>	IMI 350839*	<i>Psidium guajava</i>	India	JQ949591	JQ948931	JQ948600	JQ948270	JQ949591	
<i>C. hippocastri</i>	CBS 125377	<i>Hippeastrum vitatum</i>	China	JQ005578	JQ005404	JQ005317	JQ005230	JQ005664	
<i>C. hippocastri</i>	CBS 241.78	<i>Hippeastrum</i> sp.	Netherlands	JQ005580	JQ005406	JQ005319	JQ005232	JQ005666	
<i>C. horii</i>	ICMP 12942	<i>Diospyros kaki</i>	New Zealand	JX009533	JX009748	GQ329685	GQ329687	JX010375	
<i>C. horii</i>	ICMP 10492*	<i>Diospyros kaki</i>	Japan	JX009438	JX009752	GQ329681	GQ329690	JX010450	
<i>C. indonesiense</i>	CBS 127551*	<i>Eucalyptus</i> sp.	Indonesia	JQ949609	JQ948949	JQ948618	JQ948288	JQ949609	
<i>C. johnstonii</i>	CBS 128532*	<i>Solanum lycopersicum</i>	New Zealand	JQ949765	JQ949105	JQ948775	JQ948444	JQ949765	
<i>C. kahawae</i> subsp. <i>ciggaro</i>	ICMP 18539*	<i>Olea europaea</i>	Australia	JX009523	JX009800	JX009966	JX010230	JX010434	
<i>C. kahawae</i> subsp. <i>kahawae</i>	IMI 319418*	<i>Coffea arabica</i>	Kenya	JX009452	JX009813	JX010012	JX010231	JX010444	
<i>C. karstii</i>	CBS 125468	<i>Coffea</i> sp.	Vietnam	JQ005545	JQ005371	JQ005284	JQ005197	JQ005631	
<i>C. karstii</i>	CBS 128545	<i>Capsicum annuum</i>	New Zealand	JQ005555	JQ005381	JQ005294	JQ005207	JQ005641	
<i>C. karstii</i>	CBS 106.91	<i>Carica papaya</i>	Brazil	JQ005568	JQ005394	JQ005307	JQ005220	JQ005654	
<i>C. karstii</i>	CBS 129824	<i>Musa</i> AAA	Colombia	JQ005563	JQ005389	JQ005302	JQ005215	JQ005649	
<i>C. karstii</i>	CBS 129822	<i>Passiflora edulis</i>	Colombia	JQ005566	JQ005392	JQ005305	JQ005218	JQ005652	
<i>C. karstii</i>	CBS 128550	<i>Annona cherimola</i>	New Zealand	JQ005567	JQ005393	JQ005306	JQ005219	JQ005653	
<i>C. karstii</i>	CBS 128500	<i>Annona cherimola</i>	Mexico	JQ005550	JQ005376	JQ005289	JQ005202	JQ005636	
<i>C. karstii</i>	GM01-L02	<i>Annona muricata</i> , fruit	Colombia, Huila	KC512182	KC512161	KC506412	KC512140	KC512203	
<i>C. karstii</i>	GM40	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512186	KC512165	KC506416	KC512144	KC512207	
<i>C. karstii</i>	GM44-L01	<i>Annona muricata</i> , leaf	Colombia (Quindio)	KC512183	KC512162	KC506413	KC512141	KC512204	

Table 1 (continued)

Colletotrichum species	Strain code ^a	Host	Location	GenBank accession no. ^b					
				ACT	CHS-1	GAPDH	ITS	TUB2	
<i>C. karstii</i>	GM59b	<i>Annona muricata</i> , flower	Colombia (Norte de Santander)	KC512184	KC512163	KC506414	KC512142	KC512205	
<i>C. karstii</i>	GM73	<i>Annona muricata</i> , leaf	Colombia (Tolima)	KC512185	KC512164	KC506415	KC512143	KC512206	
<i>C. kinghornii</i>	CBS 198.35*	<i>Phormium</i> sp.	UK	JQ949775	JQ949115	JQ948785	JQ948454	JQ949775	
<i>C. latticiphilum</i>	CBS 112989*	<i>Hevea brasiliensis</i>	India	JQ949610	JQ948950	JQ948619	JQ948289	JQ949610	
<i>C. limeticola</i>	CBS 114.14*	<i>Citrus aurantifolia</i>	USA	JQ949514	JQ948854	JQ948523	JQ948193	JQ949514	
<i>C. lindemuthianum</i>	CBS 144.31	<i>Phaseolus vulgaris</i>	Germany	JQ005842	JQ005800	JX546712	JQ005779	JQ005863	
<i>C. lupini</i>	CBS 109225*	<i>Lupinus albus</i>	Ukraine	JQ949476	JQ948816	JQ948485	JQ948155	JQ949476	
<i>C. lupini</i>	CBS 129944	<i>Cinnamomum verum</i>	Portugal	JQ949499	JQ948839	JQ948508	JQ948178	JQ949499	
<i>C. lupini</i>	CBS 109217	<i>Lupinus</i> sp.	Germany	JQ949484	JQ948824	JQ948493	JQ948163	JQ949484	
<i>C. lupini</i>	IMI 375715	<i>Lupinus albus</i>	Australia	JQ949482	JQ948822	JQ948491	JQ948161	JQ949482	
<i>C. melonis</i>	CBS 159.84*	<i>Cucumis melo</i>	Brazil	JQ949515	JQ948855	JQ948524	JQ948194	JQ949515	
<i>C. musae</i>	ICMP 17817	<i>Musa sapientum</i>	Kenya	JX009432	JX009815	JX010015	JX010142	JX010395	
<i>C. musae</i>	ICMP 19119*	<i>Musa</i> sp.	USA	JX009433	JX009896	JX010050	JX010146	HQ596280	
<i>C. novae-zelandiae</i>	CBS 128505*	<i>Capsicum annuum</i>	New Zealand	JQ005576	JQ005402	JQ005315	JQ005228	JQ005662	
<i>C. novae-zelandiae</i>	CBS 130240	<i>Citrus</i> sp.	New Zealand	JQ005577	JQ005403	JQ005316	JQ005229	JQ005663	
<i>C. nupharicola</i>	ICMP 17938	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	USA	JX009486	JX009934	JX009936	JX010189	JX010397	
<i>C. nupharicola</i>	ICMP 18187*	<i>Nuphar lutea</i> subsp. <i>polysepala</i>	USA	JX009437	JX009835	JX009972	JX010187	JX010398	
<i>C. nymphaeae</i>	CBS 515.78*	<i>Nymphaea alba</i>	Netherlands	JQ949518	JQ948858	JQ948527	JQ948197	JQ949518	
<i>C. oncidii</i>	CBS 129828*	<i>Oncidium</i> sp.	Germany	JQ005517	JQ005343	JQ005256	JQ005169	JQ005603	
<i>C. oncidii</i>	CBS 130242	<i>Oncidium</i> sp.	Germany	JQ005518	JQ005344	JQ005257	JQ005170	JQ005604	
<i>C. parsonsiae</i>	CBS 128525*	<i>Parsonsia capsularis</i>	New Zealand	JQ005581	JQ005407	JQ005320	JQ005233	JQ005667	
<i>C. paxtonii</i>	IMI 165753*	<i>Musa</i> sp.	Saint Lucia	JQ949606	JQ948946	JQ948615	JQ948285	JQ949606	
<i>C. petchii</i>	CBS 378.94*	<i>Dracaena marginata</i>	Italy	JQ005571	JQ005397	JQ005310	JQ005223	JQ005657	
<i>C. petchii</i>	CBS 125957	<i>Darcaena</i>	Netherlands	JQ005574	JQ005400	JQ005313	JQ005226	JQ005660	
<i>C. phormii</i>	CBS 118194	<i>Phormium</i> sp.	Germany	JQ949767	JQ949107	JQ948777	JQ948446	JQ949767	
<i>C. phyllanthi</i>	CBS 175.67*	<i>Phyllanthus acidus</i>	India	JQ005569	JQ005395	JQ005308	JQ005221	JQ005655	
<i>C. psidii</i>	ICMP 19120*	<i>Psidium</i> sp.	Italy	JX009515	JX009901	JX009967	JX010219	JX010443	
<i>C. pyricola</i>	CBS 128531*	<i>Pyrus communis</i>	New Zealand	JQ949766	JQ949106	JQ948776	JQ948445	JQ949766	
<i>C. queenslandicum</i>	ICMP 1778*	<i>Carica papaya</i>	Australia	JX009447	JX009899	JX009934	JX010276	JX010414	
<i>C. queenslandicum</i>	ICMP 18705	<i>Coffea</i> sp.	Fiji	JX009490	JX009890	JX010036	JX010185	JX010412	

Table 1 (continued)

Colletotrichum species	Strain code ^a	Host	Location	GenBank accession no. ^b					
				ACT	CHS-1	GAPDH	ITS	TUB2	
<i>C. rhombiforme</i>	CBS 129953*	<i>Olea europaea</i>	Portugal	JQ949778	JQ949118	JQ948788	JQ948457	JQ949778	
<i>C. salicis</i>	CBS 607.94*	<i>Salix</i> sp.	Netherlands	JQ949781	JQ949121	JQ948791	JQ948460	JQ949781	
<i>C. salsolae</i>	ICMP 19051*	<i>Salsola tragus</i>	Hungary	JX009562	JX009863	JX009916	JX010242	JX010403	
<i>C. scovillei</i>	CBS 126529*	<i>Capsticum</i> sp.	Indonesia	JQ949588	JQ948928	JQ948597	JQ948267	JQ949588	
<i>C. stamense</i>	ICMP 12567	<i>Persea americana</i>	Australia	JX009541	JX009761	JX009940	JX010250	JX010387	
<i>C. stamense</i>	ICMP 18578*	<i>Coffea arabica</i>	Thailand	FJ907423	JX009865	JX009924	JX010171	JX010404	
<i>C. stamense</i>	ICMP 18642	<i>Hymenocallis americana</i>	China	GQ856775	GQ856730	JX010019	JX010278	JX010410	
<i>C. stamense</i>	GM29	<i>Annona muricata</i> , branch	Colombia (Valle del Cauca)	KC512169	KC512148	KC506399	KC512127	KC512190	
<i>C. stamense</i>	GM36-L02	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512171	KC512150	KC506401	KC512129	KC512192	
<i>C. stamense</i>	GM49-L02	<i>Annona muricata</i> , branch	Colombia (Valle del Cauca)	KC512172	KC512151	KC506402	KC512130	KC512193	
<i>C. stamense</i>	GM62-L01	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512178	KC512157	KC506408	KC512136	KC512199	
<i>C. stamense</i>	GM80	<i>Annona muricata</i> , leaf	Colombia (Caldas)	KC512175	KC512154	KC506405	KC512133	KC512196	
<i>C. stamense</i>	GM89-L02	<i>Annona muricata</i> , fruit	Colombia (Sucre)	KC512176	KC512155	KC506406	KC512134	KC512197	
<i>C. sinmondsii</i>	CBS 122122*	<i>Carica papaya</i>	Australia	JQ949597	JQ948937	JQ948606	JQ948276	JQ949597	
<i>C. sinmondsii</i>	CBS 294.67	<i>Carica papaya</i>	Australia	JQ949598	JQ948938	JQ948607	JQ948277	JQ949928	
<i>C. sloanei</i>	IMI 364297*	<i>Theobroma cacao</i>	Malaysia	JQ949608	JQ948948	JQ948617	JQ948287	JQ949608	
<i>C. tamarilloi</i>	CBS 129814*	<i>Solanum betaceum</i>	Colombia	JQ949505	JQ948845	JQ948514	JQ948184	JQ949505	
<i>C. tamarilloi</i>	CBS 129954	<i>Solanum betaceum</i>	Colombia	JQ949509	JQ948849	JQ948518	JQ948188	JQ949509	
<i>C. tamarilloi</i>	CBS 129956	<i>Solanum betaceum</i>	Colombia	JQ949511	JQ948851	JQ948520	JQ948190	JQ949511	
<i>C. tamarilloi</i>	CBS 129811	<i>Solanum betaceum</i>	Colombia	JQ949506	JQ948846	JQ948515	JQ948185	JQ949506	
<i>C. theobromicola</i>	ICMP 18649*	<i>Theobroma cacao</i>	Panama	JX009444	JX009869	JX010006	JX010294	JX010447	
<i>C. theobromicola</i>	ICMP 17895	<i>Annona diversifolia</i>	Mexico	JX009568	JX009828	JX010057	JX010284	JX010382	
<i>C. theobromicola</i>	ICMP 17958	<i>Sylosanthus guianensis</i>	Australia	JX009498	JX009822	JX009948	JX010291	JX010381	
<i>C. theobromicola</i>	ICMP 18566	<i>Olea europaea</i>	Australia	JX009496	JX009801	JX009953	JX010282	JX010376	
<i>C. theobromicola</i>	GM25-L01	<i>Annona muricata</i> , flower	Colombia (Valle del Cauca)	KC512168	KC512147	KC506398	KC512126	KC512189	
<i>C. theobromicola</i>	GM30-L01	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512177	KC512156	KC506407	KC512135	KC512198	
<i>C. theobromicola</i>	GM52-L02	<i>Annona muricata</i> , branch	Colombia (Valle del Cauca)	KC512173	KC512152	KC506403	KC512131	KC512194	
<i>C. theobromicola</i>	GM64-L02	<i>Annona muricata</i> , flower	Colombia (Valle del Cauca)	KC512174	KC512153	KC506404	KC512132	KC512195	
<i>C. ti</i>	ICMP 4832*	<i>Cordyline</i> sp.	New Zealand	JX009520	JX009898	JX009952	JX010269	JX010442	
<i>C. ti</i>	ICMP 5285	<i>Cordyline australis</i>	New Zealand	JX009553	JX009897	JX009910	JX010267	JX010441	

Table 1 (continued)

Colletotrichum species	Strain code ^a	Host	Location	GenBank accession no. ^b					
				ACT	CHS-1	GAPDH	ITS	TUB2	
<i>C. toluosum</i>	CBS 128544*	<i>Solanum melongena</i>	New Zealand	JQ005512	JQ005338	JQ005251	JQ005164	JQ005598	
<i>C. toluosum</i>	CBS 102667	<i>Passiflora edulis</i>	New Zealand	JQ005513	JQ005339	JQ005252	JQ005165	JQ005599	
<i>C. tropicale</i>	ICMP 18653*	<i>Theobroma cacao</i>	Panama	JX009489	JX009870	JX010007	JX010264	JX010407	
<i>C. tropicale</i>	ICMP 18672	<i>Litchi chinensis</i>	Japan	JX009480	JX009826	JX010020	JX010275	JX010396	
<i>C. tropicale</i>	GM04-L01	<i>Annona muricata</i> , flower	Colombia (Huila)	KC512167	KC512146	KC506397	KC512125	KC512188	
<i>C. tropicale</i>	GM33-L01	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512170	KC512149	KC506400	KC512128	KC512191	
<i>C. walleri</i>	CBS 125472*	<i>Coffea</i> sp.	Vietnam	JQ949596	JQ948936	JQ948605	JQ948275	JQ949596	
<i>C. xanthorrhoeae</i>	ICMP 17903*	<i>Xanthorrhoea preissii</i>	Australia	JX009478	JX009823	JX009927	JX010261	JX010448	
<i>Colletotrichum</i> sp.	CBS 129823	<i>Passiflora edulis</i>	Colombia	JQ949513	JQ948853	JQ948522	JQ948192	JQ949513	
<i>Colletotrichum</i> sp.	GM77	<i>Annona muricata</i> , leaf	Colombia (Quindio)	KC512181	KC512160	KC506411	KC512139	KC512202	
<i>Colletotrichum</i> sp.	CBS 123921	<i>Dendrobium kingianum</i>	Japan	JQ005511	JQ005337	JQ005250	JQ005163	JQ005597	
<i>Colletotrichum</i> sp.	GM52-L01	<i>Annona muricata</i> , leaf	Colombia (Valle del Cauca)	KC512187	KC512166	KC506417	KC512145	KC512208	
<i>Glomerella cingulata</i> "f. sp. <i>camelliae</i> "	ICMP 18542	<i>Camellia sasanqua</i>	USA	JX009488	JX009857	JX009994	JX010223	JX010429	

^aCBS Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; ICMP International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; IMI International Mycological Institute; * refers to ex-type or ex-epitype

^bACT: actin; CHS-1: chitin synthase-1; GAPDH glyceraldehydes-3-phosphate dehydrogenase; ITS: complete rDNA-ITS region; TUB-2: partial β -tubulin-2 (tub2) Strains and sequences generated in this paper are in bold, other sequences were obtained from Weir et al. (2012), Damm et al. (2012a, 2012b), O'Connell et al. (2012), Liu et al. (2013a)

postulates, fungal isolates were re-isolated from lesion margins onto PDA media and their morphology compared with the originally inoculated isolates.

Data of anthracnose severity were analyzed, using the general linear models procedure (PROC GLM) in SAS v. 6.0 (Statistical Package, Cary, NC, USA). Means of anthracnose severity among species were compared according to the protected Fisher's least significant difference (LSD) test, with a significance value of $P=0.05$.

Morphological characterization of *Colletotrichum* species Mycelial plugs (5 mm in diameter) were taken from the edges of 5-day-old colonies and transferred to the centre of 9-cm-diameter petri dishes containing PDA medium. The dishes were incubated at 21 °C with a 12/12 h light/dark cycle, using a cool, white, fluorescent light. Colony appearance and culture diameter were evaluated after 10 days' growth. For each isolate, the shape, length, and width of 40 conidia were recorded. Morphological studies were conducted, using a completely randomized block design, with three replications. Morphological data were submitted to analysis of variance (ANOVA) to establish differences between species. Means were compared, using the LSD test ($\alpha \leq 0.05$) of the SAS program, v. 6.0.

Results

Identifying *Colletotrichum* isolates and species complexes A total of 83 single-spore isolates were obtained from soursop tissues showing anthracnose symptoms. The isolates were molecularly identified to the *Colletotrichum* species complex by PCR amplifications of the rDNA ITS region using primers specific to the respective species complexes. Agarose gel analysis showed that: (i) a 450-bp DNA fragment was amplified from 60 isolates and the reference strain GND-1 with primers *CgInt* and *ITS4*, specific to *C. gloeosporioides* s. lat.; (ii) a 490-bp DNA fragment was amplified from only one isolate and the reference strain Tom 12 with primers *CaInt2* and *ITS4*, specific to *C. acutatum* s. lat.; and (iii) a 520-bp DNA fragment was amplified from 22 isolates and the reference strain Pass 063 with primers *Col1* and *ITS4*, specific to *C. boninense* s. lat.

Sequencing and phylogenetic analysis DNA sequences were generated for 21 isolates, representing: (i) different

soursop-producing regions in Colombia, (ii) plant parts (branch, leaf, flower, and fruit), and (iii) *Colletotrichum* species complexes as determined by the above PCR assay.

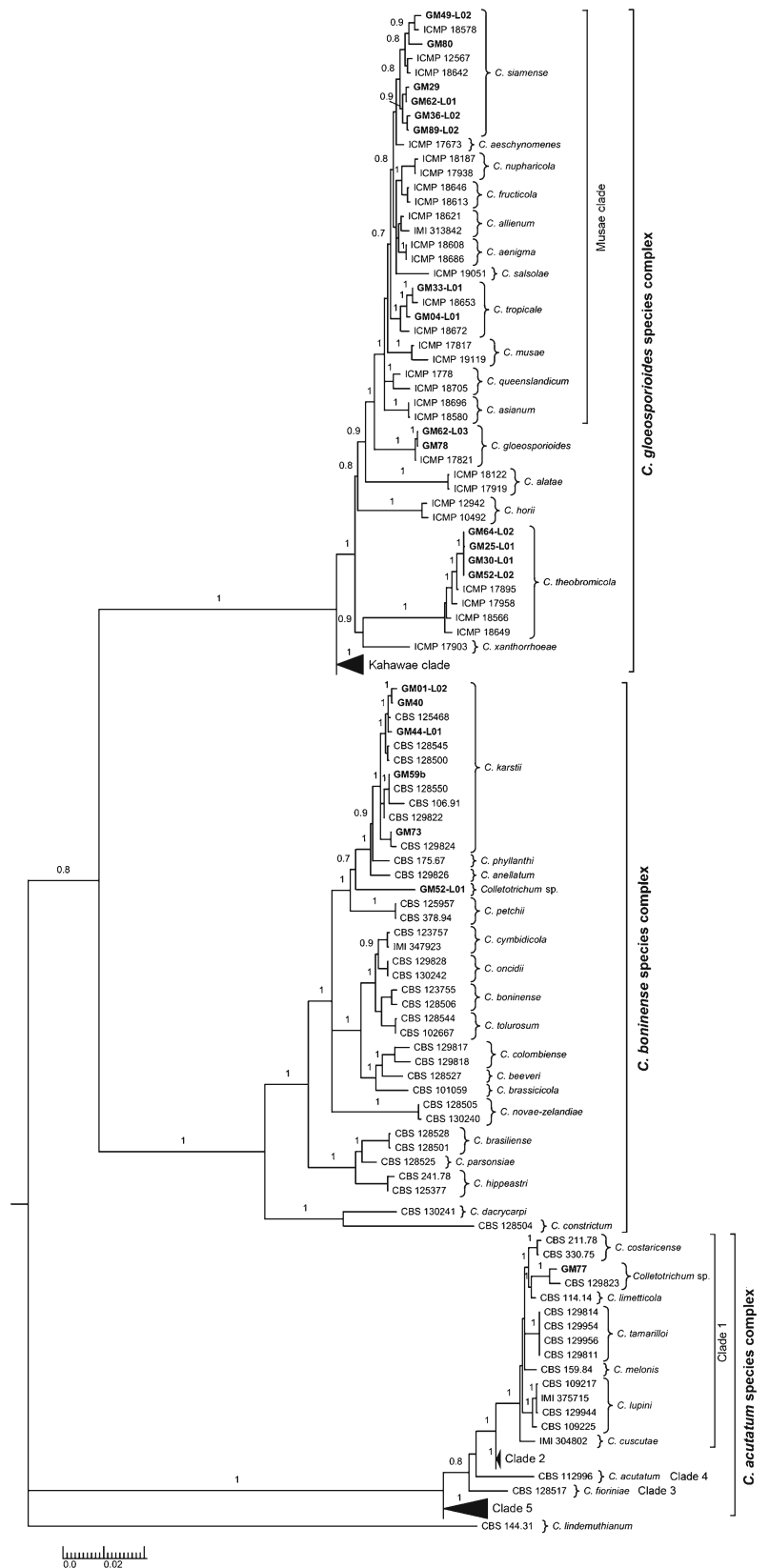
The combined datasets of partial sequences of *ACT*, *CHS-1*, *GAPDH*, and *TUB2*, and the complete sequences of the rDNA–ITS region comprised 1845 characters (including gaps). The gene boundaries and nucleotide substitution models used in the phylogenetic analysis were *ACT*: 1–285 (JC), *CHS-1*: 286–565 (JC), *GAPDH*: 566–867 (K80+I), *ITS*: 868–1243 (SYM+G), *TUB2*: 1424–1845 (K80+G). According to the multi-locus phylogenetic analysis, the isolates were grouped into three well-defined complexes: *C. gloeosporioides* s. lat., *C. boninense* s. lat., and *C. acutatum* s. lat. Thus, results of PCR with specific primers to identify the *Colletotrichum* complexes (*ITS4/CgInt*, *ITS4/CaInt2*, and *ITS4/Col1*) were confirmed.

Within the *C. gloeosporioides* complex, the Bayesian inference analysis positioned the *Colletotrichum* isolates from soursop within four well-resolved and distinct clades (Fig. 1). Most of the isolates belong to two species of the “Musae clade” sensu Weir et al. (2012). In this clade, two soursop isolates (GM04-L01 and GM33-L01) clustered closely together with two reference isolates of *C. tropicale*, while the other six soursop isolates (GM80, GM29, GM36-L02, GM49-L02, GM62-L01, and GM89-L02) grouped with the *C. siamense* reference isolates. The isolates GM25-L01, GM 64-L02, GM30-L01, and GM52-L02 clustered together with five reference isolates of *C. theobromicola*. Finally, the soursop isolates GM62-L03 and GM78 clustered together with the ex-epitype strain of *C. gloeosporioides* s. str. (ICMP 17821).

Within the *C. boninense* complex, *Colletotrichum* isolates GM01-L02, GM40, GM44-L01, GM73, and GM59b from soursop were grouped in a clade that was well resolved, together with five reference isolates of *C. karstii*. Isolate GM52-L01 did not group with any reference species. It was therefore considered as belonging to an undetermined species.

Only one soursop isolate from this study belonged to the *C. acutatum* complex. Isolate GM77 was associated with clade 1 of the *C. acutatum* complex (Damm et al. 2012a) and grouped with *Colletotrichum* sp. strain CBS 129823, which was isolated from *Passiflora edulis* in Colombia and is genetically close to *C. limetticola* (CBS 114.4), with only two base pairs difference in the β -tubulin-2 sequence (Damm et al. 2012a). Thus, the

Fig. 1 Phylogenetic consensus tree based on Bayesian inference, illustrating the relationships of the *Colletotrichum* isolates in the *C. boninense*, *C. gloeosporioides*, and *C. acutatum* complexes. The tree was built, using concatenated sequences of the ACT, CHS-1, GAPDH, ITS, and TUB2 genes, and separate models of DNA evolution. Groups within the *C. gloeosporioides* complex according to Weir et al. (2012; Musae and Kahawae clade) and within the *C. acutatum* complex according to Damm et al. (2012a; Clade 1 to 5) are indicated. *Colletotrichum lindemuthianum* (CBS 144.31) is used as the outgroup



isolate was considered as belonging to an undetermined species.

Pathogenicity trial All 21 *Colletotrichum* isolates produced symptoms on twigs of soursop cv. Elita that were similar to those observed on branches of soursop trees in the field: oval or round black spots, with irregular margins, depressions in the bark; as well as stem rot. Virulence, measured as the percentage of twig area showing symptoms (anthracnose severity), was significantly different ($P < 0.001$) among species. *Colletotrichum theobromicola* and *C. tropicale* respectively showed high and intermediate levels of virulence, whereas the other species showed low virulence (Table 2).

Morphological characterization of *Colletotrichum* species Morphological characteristics are summarized in Table 2. Fungal colonies were grouped into four types, A, B, C and D, according to the texture and colour of aerial mycelia. Statistically significant differences

($P < 0.001$) of conidial and colony size were observed between *Colletotrichum* species collected from soursop in Colombia. Average measurements were similar to those described in the literature (Prihastuti et al. 2009; Rojas et al. 2010; Weir et al. 2012; Damm et al. 2012b; Lima et al. 2013).

Discussion

Colletotrichum is a genus that is currently undergoing taxonomic revision and updating, particularly among its phytopathologically important members (Cannon et al. 2000). This study reports on the occurrence of *Colletotrichum* species associated with anthracnose of soursop in Colombia and their pathogenicity on the susceptible soursop cv. Elita.

The specific primers used in this study were developed as species-specific primers (Mills et al. 1992; Sreenivasaprasad et al. 1996; Afanador-Kafuri et al.

Table 2 Characteristics of *Colletotrichum* species associated with anthracnose of soursop (*Annona muricata*) trees in Colombia

Species	Culture size at 10 days (mm) ^a	Colony type ^b	Conidia			Anthracnose severity (%) ^c
			Shape	Length (µm)	Width (µm)	
<i>C. gloeosporioides</i> s. str. (GM62-L03, GM78)	52.5±2.2ab	A	Cylindrical	13.44±0.08e* (12.5–13.75)	3.28±0.04d (3.12–3.75)	1.1±0.3c
<i>C. karstii</i> (GM01-L02, GM40, GM44-L01, GM59b, GM73)	52.6±4.4b	C	Cylindrical	14.13±0.12 cd (13.0–15.0)	5.12±0.03a (4.96–5.62)	1.9±0.7c
<i>C. siamense</i> (GM29, GM36-L02, GM49-L02, GM62-L01, GM80, GM89-L02)	80.3±5.8d	A	Cylindrical, with obtuse to slightly rounded ends	14.37±0.17c (12.81–16.73)	5.16±0.03a (4.69–6.25)	2.5±0.5c
<i>C. theobromicola</i> (GM25-L01, GM30-L01, GM52-L02, GM64-L02)	43.0±2.5a	B	Cylindrical, sometimes tapering slightly towards the base	16.09±0.33b (14.37–24.4)	3.75±0.05c (2.81–5.62)	75.8±5.0a
<i>C. tropicale</i> (GM04-L01, GM33-L01)	71.0±14.0c	A	Cylindrical	12.19±0.09f (11.25–12.51)	4.22±0.04b (3.75–4.38)	9.6±4.1b
<i>Colletotrichum</i> sp. indet. (GM52-L01)	53.3±3.0ab	D	Cylindrical	17.5±0.0a	3.12±0.10d (2.5–3.75)	2.0±0.5c
<i>Colletotrichum</i> sp. indet. (GM77)	60.7±1.2b	A	Cylindrical, with both ends acute	13.75±0.2de (12.5–15.0)	2.52±0.03e (2.5–3.75)	3.7±1.3c

^a Diameter of cultures grown on PDA agar at 20 °C for 10 days

^b Colony type: A = Cottony texture, dense grayish-white to white aerial mycelium; B = Compact texture, dense grayish-black aerial mycelium; C = Colony white to pale salmon, with aerial mycelium in dispersed tufts or flat with entire margin; D = Colony hyaline to honey, partly covered with white aerial mycelium

^c Mean of anthracnose severity on 8-to-12-month-old twigs on trees of the susceptible soursop cv. Elita at 23 days after inoculation with mycelial agar plugs. No symptoms were observed in the control plants (mock-inoculated with sterilized agar plugs); these data were excluded from the statistical analysis

* Values with same letters do not differ significantly according to Fisher's LSD test ($P \leq 0.05$)

2003; Pileggi et al. 2009) before these species were shown to be part of species complexes. For this study, the primers reliably differentiated between the isolates at the species complex level, indicating that 73 % belonged to the *C. gloeosporioides* complex, 26 % to the *C. boninense* complex, and only 1 % to the *C. acutatum* complex. The results furthermore indicated that anthracnose of soursop trees in Colombia was mostly associated with species of the *C. gloeosporioides* complex, followed by species of the *C. boninense* complex.

According to Weir et al. (2012), taxa other than *C. gloeosporioides* s. str., *C. fructicola*, and *C. siamense* (which are all recognized as belonging to the *C. gloeosporioides* complex) have one or more bases that do not match those of the CgInt primer. However, such mismatched positions and stringency of the PCR reaction might still result in positive fragment amplification (Weir et al. 2012). We confirmed this in our study: PCR reactions of isolates identified as *C. theobromicola* and *C. tropicale* with primers CgInt and ITS4 resulted in a 450-bp DNA fragment.

Multi-locus molecular analyses as employed in our study have been used in various earlier studies to identify and delimit *Colletotrichum* species attacking different hosts. The analyses are now routinely used as the basis on which to describe new *Colletotrichum* species (Damm et al. 2012a; 2012b; Weir et al. 2012; Lima et al. 2013; Udayanga et al. 2013; Huang et al. 2013; Liu et al. 2013b). Molecular phylogeny, based on multiple gene sequences, is preferred to using either morphology or rDNA–ITS sequences alone (Crouch et al. 2009) because neither adequately resolves taxonomic issues, especially for the species and clades within the *C. acutatum* and *C. gloeosporioides* complexes.

The markers used in the present study were recently accepted for genetic delimitation of species in the genus *Colletotrichum* (Weir et al. 2012; Damm et al. 2012a; 2012b; Cannon et al. 2012) and are also used for molecular identification in the *Colletotrichum* database of the CBS-KNAW Fungal Biodiversity Centre (<http://www.cbs.knaw.nl/colletotrichum>). For our study, this molecular approach reliably differentiated *Colletotrichum* species collected from soursop. According to our analysis, the genetic diversity was high and the *Colletotrichum* isolates associated with soursop anthracnose were distributed across seven taxa, including *C. karstii*, *C. theobromicola*, *C. tropicale*, *C. siamense*, *C. gloeosporioides* s. str.,

Colletotrichum sp. indet. from the *C. boninense* complex (GM52-L01), and *Colletotrichum* sp. indet. from the *C. acutatum* complex (GM77).

As far as we can ascertain, except for *C. karstii* (Damm et al. 2012b), all the known taxa identified (*C. siamense*, *C. gloeosporioides* s. str., *C. theobromicola*, and *C. tropicale*) represent the first reports of these species in Colombia. Moreover, none of the five taxa present a host-specific relationship with soursop trees, as each has been collected from other hosts as well. The five taxa are reported as having worldwide geographic distribution, and some strains have been associated with plant diseases in other agriculturally important crops such as *Theobroma cacao*, *Coffea arabica*, *Persea americana*, *Capsicum annum*, *Mangifera indica*, *Citrus* spp., *Olea europaea*, and *Carica papaya* (Damm et al. 2012b; Weir et al. 2012; Lima et al. 2013; Schena et al. 2013; Udayanga et al. 2013). *Colletotrichum siamense* is considered to be a dominant group of species, associated with pre- and postharvest diseases of a wide range of tropical fruits (Udayanga et al. 2013).

In managing *Colletotrichum* diseases, crop rotation is a highly effective way of promoting healthy crop production (Phoulivong 2011). However, our results indicate that the soursop tree can be a source of inoculum of various *Colletotrichum* species, thus potentially infecting other plant hosts, and conversely. Therefore, decisions on crop rotation and polyculture should be implemented with caution, because of the presence of a diverse community of *Colletotrichum* species. To manage anthracnose in soursop, best cultural practices, combined with chemical control, need to be adopted to ensure plant health (Phoulivong 2011). Although the use of resistant cultivars is probably the most desirable technology for disease control, soursop has not yet received much attention in this research line.

Of the species identified, only *C. tropicale* has been associated with fruit rot of soursop before (Rojas et al. 2010). In addition, isolates of *C. karstii*, *C. theobromicola*, and *C. siamense* have been reported as affecting other *Annona* species (Damm et al. 2012b; Weir et al. 2012; Udayanga et al. 2013).

The pathogenicity test, whereby trees of soursop cv. Elita were artificially inoculated with isolates of the different *Colletotrichum* species, showed that all species were pathogenic, albeit at different levels of virulence. *Colletotrichum theobromicola* and *C. tropicale* possessed higher and intermediate levels of virulence,

respectively, while the remaining *Colletotrichum* species had low levels of virulence.

These findings suggest that, because symptoms of stems (i.e., dieback of twigs and branches, and stem necrosis) cause the highest crop losses, *C. theobromicola* and *C. tropicale* have, economically, the most significant impact on soursop anthracnose in Colombia. Thus, management strategies need to focus on these two species. Similarly, *C. theobromicola* is considered as a primary pathogen in olive (*Olea europaea*). In contrast, isolates of *C. karstii* and *C. siamense* are weakly pathogenic to olive (Schena et al. 2013).

The *Colletotrichum* species identified in our study showed cultural and morphological characteristics similar to those described in previous studies. The cultural appearance of *Colletotrichum* species is highly variable, being determined by diverse factors such as the culture media used, subculture conditions, environmental factors (e.g., temperature, light intensity, and photoperiod), and storage conditions (Weir et al. 2012). Cultural and morphological characteristics therefore cannot be confidently used alone to determine species within a complex (Phoulivong et al. 2010).

The results of this study are relevant because, by demonstrating the diversity and virulence of *Colletotrichum* species infecting soursop, they will facilitate the development and implementation of disease management practices and of more effective quarantine measures to minimize the risk of introducing new species across borders or continents.

Acknowledgments We are grateful to Corporación BIOTEC, Ministerio de Agricultura y Desarrollo Rural de Colombia, Agencia Colombiana de Cooperación Internacional (ACCI), and COLCIENCIAS for their financial support. We thank Dr Jairo Castaño-Zapata for his contributions to this research; Juan B. Cuasquer (Systems Engineer, CIAT); and Elizabeth L. McAdam and Matthew Blair for reviewing the manuscript.

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