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Diversity of *Colletotrichum* species causing onion anthracnose in Brazil

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Abstract Foliar anthracnose is one of the main diseases of onion (*Allium cepa* L.) under tropical and subtropical conditions. Thus far, only *Colletotrichum gloeosporioides* has been reported as the causal agent of this disease in Brazil. However, there are no extensive studies characterizing *Colletotrichum* isolates associated with onion anthracnose in the country. Here, 38 *Colletotrichum* isolates obtained from onion plants displaying foliar anthracnose across major Brazilian onion-producing regions were characterized using morphometric and molecular information. The Bayesian and Maximum Liklihood methods were used for an initial analysis of the β-tubulin gene (*tub2*) sequences of all isolates, resulting in the discrimination of nine haplotypes. Three haplotypes grouped with the reference species of the *C. acutatum* complex and six with the *C. gloeosporioides* complex. Sequences of either the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), actin (*act*), and calmodulin genes or the intergenic spacer (IGS) region between DNA lyase (*apn*2) gene and the mating-type *mat*1–2-1 locus were used to characterize a subset of isolates representing these nine distinct *tub*2 gene haplotypes. These analyses revealed five anthracnose-inducing *Colletotrichum* species, including three members of the *C. acutatum* species complex (*C. nymphaeae*, *C. scovillei*, and *C. tamarilloi*) and two of the *C. gloeosporioides* species complex (*C. fructicola* and *C. theobromicola*). Bioassays confirmed that all these *Colletotrichum* species are pathogenic to onion, inducing typical anthracnose symptoms on bulbs and leaves. Twenty-six out of 38 isolates were identified as *C. theobromicola*, indicating this fungus as the prevalent anthracnose pathogen under Brazilian conditions. This *Colletotrichum* species diversity will affect anthracnose management strategies, including chemical and cultural control as well as the identification and deployment of onion cultivars with species-specific and/or wide-spectrum tolerance/resistance.

Keywords *Allium cepa* · Ascomycota · Tropical fungi · Taxonomy

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Introduction

Foliar anthracnose is one of the major diseases of onions (*Allium cepa* L.) in tropical and subtropical regions (Maranhão et al. 1997; Massola Jr et al. 2016). This disease induces significant yield and quality losses for both bulb and commercial onion seed production. Thus far, only *Colletotrichum gloeosporioides* has been

reported as the causal agent of this disease in Brazil (Bajungu 1979; Wordell Filho et al. 2006; Vila Nova et al. 2011). The expression of anthracnose symptoms is diverse and highly complex, varying according to the onion cultivar, phenological stage and organ of the plant. In some cases, well-defined, depressed lesions occur on the blades and sheaths of the leaves. These lesions can increase in size and become oval with a predominant pinkish color. The symptoms may evolve to punctuated spots of dark coloration. Damping-off of seedlings can be observed when highly infested seeds are used (Wordell Filho et al. 2006). In some special circumstances, leaf twisting symptoms can be observed giving a serpentine-like aspect to the leaves (Hill 1996). Typical bulb deformations associated with onion anthracnose have been named by Brazilian growers as either "cigar" or "hot dog", which expression may involve mixed infections with distinct root pathogens (Wordell Filho et al. 2006; Massola Jr et al. 2016). As the disease progresses, dark green to black stroma may develop on the bulbs.

The classical taxonomic approach of *Colletotrichum* species is based exclusively upon descriptive morphometrical criteria such as conidial shape and size, conidia germination, presence/absence of setae, aspect of the colonies as well as the host range and the type of lesion present in the affected host tissues (Sutton 1980; Cannon et al. 2012). Because morphometric and biological attributes are very often variable and plastic, fungal characterization based solely upon these criteria are not reliable, precluding a correct description of many novel *Colletotrichum* species. In this context, the phylogenetic analyzes involving the sequence information of distinct genomic regions in combination with morphological and pathogenicity traits can provide more precise descriptions of *Colletotrichum* species complexes (Cai et al. 2009). This combination of classical and multilocus molecular approaches is allowing the identification of many previously undescribed species within this genus (Weir et al. 2012; Damm et al. 2012; Cannon et al. 2012; Doyle et al. 2013; Hyde et al. 2014; Vieira et al. 2014). The correct identification of *Colletotrichum* species is essential for a more accurate understanding of the epidemiology and for the development of more efficient control strategies (Phoulivong 2011).

Few studies are available in the literature involving extensive characterization of *Colletotrichum* isolates associated with onion anthracnose employing multilocus phylogeny. Likewise, only two studies were carried out assessing the molecular diversity of the causal agents of onion anthracnose in Brazil, encompassing a reduced number of isolates and covering a relatively narrow geographical area. One study employed morphological and serological information to characterize six *Colletotrichum* isolates (Bajungu 1979). A second study employed Random Amplified Polymorphic DNA (RAPD) marker system in combination with genomic information of the rDNA ITS region to investigate the genetic diversity of 14 onion-infecting isolates from Pernambuco State (Vila Nova et al. 2011). In this context, the main objective of the present study was to conduct morphometrical and multilocus analyses of 38 anthracnose-causing *Colletotrichum* isolates collected in several geographical areas across major onion-producing regions of Brazil. The main objective of this assessment was to acquire a more precise overview about the diversity of the causal agents of this important onion disease under Neotropical conditions.

Material and methods

Colletotrichum isolates associated with onion foliar anthracnose A collection of 38 monosporic isolates of *Colletotrichum* was used in the present study. These isolates were obtained from onion plants displaying one or more typical foliar anthracnose symptoms in several onion-producing regions across eight Brazilian States and the Federal District (Fig. 1), from 1998 to 2014. These isolates were preserved in the collection of phytopathogenic fungi and oomycetes of Embrapa Vegetable Crops (Brasília-DF, Brazil) by the Castellani's method (Castellani 1939) in a cold room at 10 °C in the absence of light. This collection is also cryopreserved at −80 °C. For the present work, isolates were recovered by culturing them in Petri dishes containing PDA (Potato-Dextrose-Agar) medium.

Fungal DNA extraction The isolates were first cultivated in PDA medium for one week at 25 °C with a photoperiod of 12 h. After this period, a portion of aerial mycelium was removed from each Petri dish and the genomic DNA was extracted using 2X CTAB buffer and organic solvents with minor adaptations (Boiteux et al. 1999). DNA concentration was estimated with the microvolume spectrophotometer, Nanodrop (NanoDrop Lite, Thermo Scientific®, USA), while DNA integrity

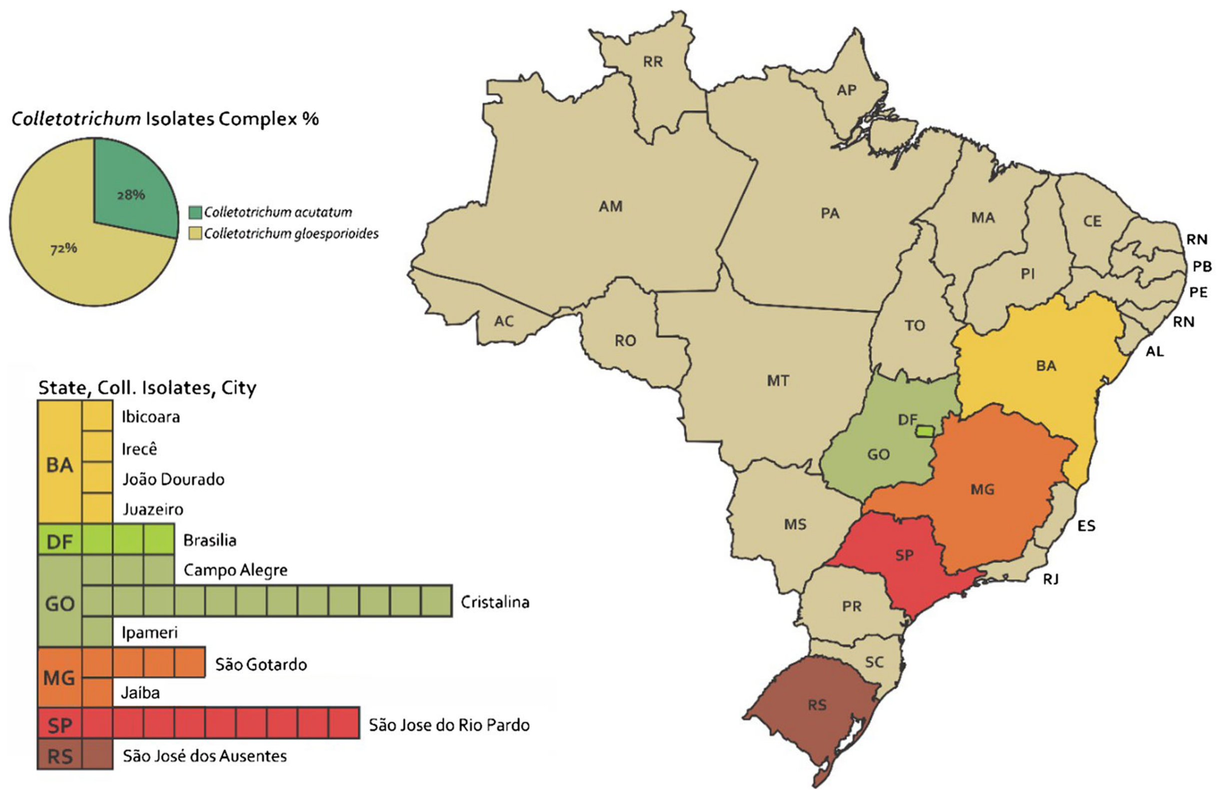

Fig. 1 Geographical distribution and cities where the 38 *Colletotrichum* isolates associated with foliar anthracnose were collected across the onion-producing regions of Brazil. The acronyms of the subset of States where the isolates were collected are: Bahia (BA), the Federal District (DF), Goiás (GO), Minas Gerais (MG), São Paulo (SP) and Rio Grande do Sul (RS)

was evaluated through electrophoresis using 1.5% agarose gel.

PCR assays and sequencing of the fungal β-tubulin 2 (tub2) gene PCR assays were carried out initially using a pair of primers targeting the β-tubulin 2 gene (*tub2*), viz. Bt2a (5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′) and βt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3′) (Glass and Donaldson 1995). PCR mix was composed of 3 μL of fungal genomic DNA (10 ng/μL), 2 μL 10X *Taq* DNA Polymerase buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 0.5 μL $MgCl_2$ (50 mM), 2 μL dNTPs (2.5 M each), 0.2 μL of recombinant *Taq* DNA polymerase (Invitrogen®, 5 units/μL), 2.5 μL of each primer (10 μM) and 7.3 μL of ultrapure Milli-Q water, with a final reaction volume adjusted to 20 μL. PCR assays were carried out employing the Verity PCR System (Applied Biosystems). In the PCR assays, the following steps were kept constant across all genomic regions under evaluation: initial denaturation at 94 °C and a final extension at 72 °C for 7 min. The number of haplotypes from the β-tubulin sequences for the *Colletotrichum* species were calculated using DnaSP v5 (Librado and Rozas 2009). A haplotype network to visualize the relationships among haplotypes representing six Brazilian states was reconstructed with PopART (Leigh and Bryant 2015) using the TCS algorithm (Clement et al. 2002) with gaps and missing data excluded.

PCR assays and sequencing of additional genomic regions of isolates representing the nine previously identified tub2 gene haplotypes Altogether nine fungal *tub2* gene haplotypes were separated by DnaSP to represent the total of fully-characterized isolates of our survey. Therefore, only genomic DNAs from these representative isolates obtained for each *tub2* gene haplotype were employed as templates in subsequent PCR assays. Sequence information of following genes/genomic regions were also obtained for this subset of isolates: actin (*act*), the intergenic spacer (IGS) region between the DNA lyase (*apn*2) gene and the mating-type *mat1–2-1* locus

(APN2/MAT-IGS), calmodulin (*cal*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). The primer pair employed to amplify the *act* gene segment was: ACT-512F (5′-ATG TGC AAG GCC GGT TTC GC-3′) and ACT-783R (5’-TAC GAG TCC TTC TGG CCC AT-3′) (Carbone and Kohn 1999). The primers for amplification of the *gapdh* gene were: GDF1 (5’-GCC GTC AAC GAC CCC TTC ATT GA-3′) and GDR1 (5′-GGG TGG AGT CGT ACT TGA GCA TGT-3′) (Templeton et al. 1992). The primers for amplification of the APN2/MAT-IGS region were: CgDL_F6 (5′-AGT GGA GGT GCG GGA CGT T-3′) and CgMAT1_F2 (5′-TGA TGT ATC CCG ACT ACC G-3′) (Rojas et al. 2010). The primers for amplification of the *cal* gene were CL1C (5′-GAA TTC AAG GAG GCC TTC TC-3′) and CL2C (5’-CTT CTG CAT CAT GAG CTG GAC-3′) (O'Donnell et al. 2000). All temperatures of each cycle were used according to the original works, except for the *cal* and *gapdh* genes, where the annealing temperatures 59 °C and 60 °C were employed, respectively. For the APN2/MAT-IGS region the following PCR cycling program was used: initial step of 95 °C for 3 min, 10 cycles (Step Down function) of 95 °C for 45 s, 60 °C for 45 s (reducing 1 °C for each cycle), 72 °C for 1 min, 35 cycles of 95 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min and final extension at 72 °C for 10 min. PCR reactions were performed with a final volume of 20 μL containing 11.1 μL of Milli-Q water, 1 μL of dNTP (Invitrogen®, Germany), 1.5 μL of each primer, 2 μL PCR buffer 10X, 0.6 μL of $MgCl_2$, 0.3 μL of *Taq* DNA polymerase (Invitrogen®, Germany) and 2 μL of genomic DNA. The reactions were carried out in a MyCycler® Thermal Cycler (BioRad®, USA). PCR products were separated on electrophoresis using 1.5% agarose gel in 1X Tris-Acetate acid EDTA (TAE) and photographed in UV light after staining with ethidium bromide (0.5 μg mL^{-1}) for 5 min. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) following the manufacturer's protocol. Sequencing of the genomic regions previously amplified were performed by Macrogen Inc. (Seoul, South Korea).

Phylogenetic analyses Sequence assemblies were performed separately in Geneious R8 software (Kearse et al. 2012) and alignments with MAFFT plugin, also with Geneious R8. The *tub2* gene sequences were initially analyzed for all 38 isolates. Ambiguities and other errors were verified in the corresponding electropherograms and then either removed, resequenced and/or corrected manually. The Bayesian inference method was chosen for the phylogenetic analysis using MrBayes (Huelsenbeck and Ronquist 2001) plugin in Geneious R8 (Kearse et al. 2012). The MEGA X (Kumar et al. 2018) program was used to select substitution model according to the Akaike Information Criterion for each analysis, resulting in HKY + I as the most appropriate for *tub2*. The sequences were compared with reference sequences obtained from GenBank (Table 1). Initial analyses, using the information derived from the *tub2* genomic region, indicated that the isolates could be subdivided into two species complexes: *C. acutatum* and *C. gloeosporioides*. Because of this, a tree was generated with all the isolates used in this study along with a set of reference isolates from species of both the *C. gloeosporioides* complex and the *C. acutatum* complex (Fig. 2). The MEGA X (Kumar et al. 2018) program was again used to select substitution model according to the Akaike Information Criterion, resulting in HKY + G as the most appropriate for *C. acutatum* species complex and GTR + G + I for *C. gloeosporioides* species complex. A subset of isolates (corresponding to each of the nine initially identified *tub2* gene haplotypes) were selected (Table 2) and used to generate two trees: one using sequences corresponding to the genomic regions *tub2* (424 bp in length), *act* (248 bp) and *gapdh* (274 bp) and reference isolates of the *C. acutatum* complex (Fig. 4) and another tree using sequences corresponding to the genomic regions *tub2* (413 bp), *act* (211 bp), *cal* (637), *gapdh* (269 bp) and APN2/MAT-GS (683 bp) and the reference isolates of the *C. gloeosporioides* complex (Fig. 5). In all phylogenetic analyses, the Maximum Likelihood method with RAxML (Stamatakis 2014) plugin in Geneious was used. All maximum likelihood analyses were performed with 1000 bootstrap repetitions.

Morphological characterization of Colletotrichum isolates Based upon the results of the phylogenetic analyses, isolates belonging to each of the nine *Colletotrichum tub2* gene haplotypes were characterized for colony color and morphology as well as morphometric characteristics of the conidia. Disks of 4 mm in diameter were removed from the edges of the colonies (with five days of growth) and placed on plates containing PDA medium. Three replicates were made for each of the nine isolates in a completely randomized experimental *design*. Plates were incubated at 25 °C in

Table 1 Isolates and type-species of the *Colletotrichum acutatum* and *C. gloeosporioides* complexes that were used in the phylogenetic analysis of the *Colletotrichum* isolates associated with onion foliar anthracnose. Asteriskis are indicating the type-species sequences

Species	Culture	Host	Country	GenBank accession number code				
				APN2/MAT-IGS	gapdh	cal	act	tub2
C. aenigma	ICMP 18608*	*Persea americana*	Israel	–	JX010044	JX009683	JX009443	JX010389
C. aeschynomenes	ICMP 17673*, ATCC 201874	*Aeschynomene virginica*	USA	–	JX009930	JX009721	JX009483	JX010392
C. alatae	CBS 304.67*, ICMP 17919	*Dioscorea alata*	India	KC888932	JX009990	JX009738	JX009471	JX010383
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	KC888927	JX010028	JX009654	JX009572	JX010411
C. aotearoa	ICMP 18537*	*Coprosma* sp.	New Zealand	KC888930	JX010005	JX009611	JX009564	JX010420
C. asianum	ICMP 18580*, CBS 130418	*Coffea arabica*	Thailand	FR718814	JX010053	FJ917506	JX009584	JX010406
C. boninense	MAFF 305972*, ICMP 17904, CBS 123755	*Crinum asiaticum* var. *sinicum*	Japan	–	JX009905	–	JX009583	–
C. clidemiae	ICMP 18658*	*Clidemia hirta*	USA, Hawaii	KC888929	JX009989	JX009645	JX009537	JX010438
C. cordylinicola	MFLUCC 090551*, ICMP 18579	*Cordyline fruticosa*	Thailand	JQ899274	JX009975	HM470238	HM470235	JX010440
C. fructicola	ICMP 18581*, CBS 130416	*Coffea arabica*	Thailand	JQ807838	JX010033	FJ917508	FJ907426	JX010405
C. fructicola (syn. *C. ignotum*)	CBS 125397*, ICMP 18646	*Tetragastris panamensis*	Panama	JQ807839	JX010032	JX009674	JX009581	JX010409
C. fructicola (syn. *Glomerella cingulata* var. *minor*)	CBS 238.49*, ICMP 17921	*Ficus edulis*	Germany	–	JX009923	JX009671	JX009495	JX010400
C. gloeosporioides	IMI 356878*, ICMP 17821, CBS 112999	*Citrus sinensis*	Italy	JQ807843	JX010056	JX009731	JX009531	JX010445
C. gloeosporioides	CBS 273.51*, ICMP 19121	*Citrus limon*	Italy	–	JX009908	JX009630	JX009540	JX010436
C. hippeastri	CBS 241.78, ICMP 17920	*Hippeastrum* sp.	Netherlands	–	JX010054	JX009745	JX009558	–
C. horii	NBRC 7478*, ICMP 10492	*Diospyros kaki*	Japan	–	JX009932	JX009740	JX009485	–
C. kahawae subsp. *ciggaro*	ICMP 18539*	*Olea europaea*	Australia	JQ807840	GQ329681	JX009604	JX009438	JX010450
C. kahawae subsp. *ciggaro* (syn. *Glomerella cingulata* var. *migrans*)	CBS 237.49*, ICMP 17922	*Hypericum perforatum*	Germany	–	JX009966	JX009635	JX009523	JX010434
C. kahawae subsp. ciggaro (syn. *Glomerella rufomaculans* var. *vaccinii*)	CBS 124.22*, ICMP 19122	*Vaccinium* sp.	USA	–	JX010042	JX009636	JX009450	JX010432
C. kahawae subsp. *kahawae*	IMI 319418*, ICMP 17816	*Coffea arabica*	Kenya	–	JX009950	JX009744	JX009536	JX010433

Table 1 (continued)

Species	Culture	Host	Country	GenBank accession number code				
				APN2/MAT-IGS	gapdh	cal	act	tub2
C. musae	CBS 116870*, ICMP 19119	*Musa* sp.	USA	JQ899282	JX010012	JX009642	JX009452	JX010444
C. nupharicola	CBS 470.96*, ICMP 18187	*Nuphar lutea* subsp.*polysepala*	USA	KC888926	JX010050	JX009742	JX009433	HQ596280
C. psidii	CBS 145.29*, ICMP 19120	*Psidium* sp.	Italy	JX145319	JX009972	JX009663	JX009437	JX010398
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	KC888931	JX009967	JX009743	JX009515	JX010443
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	KC888928	JX009934	JX009691	JX009447	JX010414
C. siamense	ICMP 18578*, CBS 130417	*Coffea arabica*	Thailand	KC888925	JX009916	JX009696	JX009562	JX010403
C. siamense (syn. *C. hymenocallidis*)	CBS 125378*, ICMP 18642	*Hymenocallis americana*	China	JQ899289	JX009924	FJ917505	FJ907423	JX010404
C. siamense (syn. *C. jasmini-sambac*)	CBS 130420*, ICMP 19118	*Jasminum sambac*	Vietnam	JQ807842	JX010019	JX009709	GQ856775	JX010410
C. theobromicola	CBS 124945*, ICMP 18649	*Theobroma cacao*	Panama	JQ807841	HM131497	JX009713	HM131507	JX010415
C. theobromicola (syn. *C. fragariae*)	CBS 142.31*, ICMP 17927	*Fragaria* × *ananassa*	USA	KC790726	JX010006	JX009591	JX009444	JX010447
C. theobromicola (syn. *C. gloeosporioides*) f. sp. *stylosantis*	MUCL 42294*, ICMP 17957, CBS 124251	*Stylosanthes viscosa*	Australia	JQ807844	JX010024	JX009592	JX009516	JX010373
C. ti	ICMP 4832*	*Cordyline* sp.	New Zealand	–	JX009962	JX009597	JX009575	JX010380
C. tropicale	CBS 124949*	*Theobroma cacao*	Panama	–	JX009952	JX009649	JX009520	JX010442
C. xanthorrhoeae	BRIP 45094*	*Xanthorrhoea preissii*	Australia	KC790728	JX010007	JX009719	JX009489	JX010407
Glomerella cingulata f. sp. *camelliae*	ICMP 10643	*Camellia* × *williamsii*	UK	KC790689	JX009927	JX009653	JX009478	JX010448
C. acerbum	CBS 128530, ICMP 12921, PRJ 1199.3*	*Malus domestica,*	New Zealand	–	JQ948790	–	JQ949780	JQ950110
C. acutatum	CBS 112996, ATCC 56816, STE-U 5292*	*Carica papaya*	Australia	–	JQ948677	–	JQ005839	JQ005860
C. australe	CBS 116478, HKUCC 2616*	*Trachycarpus fortunei*	South Africa	–	JQ948786	–	JQ949776	JQ950106
C. brisbanense	CBS 292.67, DPI 11711*	*Capsicum annuum*	Australia	–	JQ948621	–	JQ949612	JQ949942
C. chrysanthemi	IMI 364540, CPC 18930	*Chrysanthemum coronarium,*	China	–	JQ948603	–	JQ949594	JQ949924
C. cosmi	CBS 853.73, PD 73/856*	*Cosmos* sp., seed	Netherlands	–	JQ948604	–	JQ949595	JQ949925
C. costaricense	CBS 330.75*	*Coffea arabica,*	Costa Rica	–	JQ948510	–	JQ949501	JQ949831
C. cuscutae	IMI 304802, CPC 18873*	*Cuscuta* sp.	Dominica	–	JQ948525	–	JQ949516	JQ949846
C. fioriniae	CBS 125396, GJS 08-140A	*Malus domestica*	USA	–	JQ948629	–	JQ949620	JQ949950
C. godetiae	CBS 133.44*	*Clarkia hybrida,*	Denmark	–	JQ948733	–	JQ949723	JQ950053

Table 1 (continued)

Species	Culture	Host	Country	GenBank accession number code				
				APN2/MAT-IGS	gapdh	cal	act	tub2
C. guajavae	IMI 350839, CPC 18893*	*Psidium guajava*	India	–	JQ948600	–	JQ949591	JQ949921
C. indonesiense	CBS 127551, CPC 14986*	*Eucalyptus* sp.	Indonesia	–	JQ948618	–	JQ949609	JQ949939
C. johnstonii	CBS 128532, ICMP 12926, PRJ 1139.3*	*Solanum lycopersicum*	New Zealand	–	JQ948775	–	JQ949765	JQ950095
C. kinghornii	CBS 198.35*	*Phormium* sp.	UK	–	JQ948785	–	JQ949775	JQ950105
C. laticiphilum	CBS 112989, IMI 383015, STE-U 5303*	*Hevea brasiliensis*	India	–	JQ948619	–	JQ949611	JQ949940
C. limetticola	CBS 114.14*	*Citrus aurantifolia*	USA, Florida	–	JQ948523	–	JQ949514	JQ949844
C. lupini	CBS 109225, BBA 70884*	*Lupinus albus*	Ukraine	–	JQ948485	–	JQ949476	JQ949806
C. melonis	CBS 159.84*	*Cucumis melo*	Brazil	–	JQ948524	–	JQ949515	JQ949845
C. nymphaeae	CBS 515.78*	*Nymphaea alba*	Netherlands	–	JQ948527	–	JQ949518	JQ949848
C. orchidophilum	CBS 632.80*	*Dendrobium* sp.	USA	–	JQ948481	–	JQ949472	JQ949802
C. paxtonii	IMI 165753, CPC 18868*	*Musa* sp.	Saint Lucia	–	JQ948615	–	JQ949606	JQ949936
C. phormii	CBS 118194, AR 3546*	*Phormium* sp.	Germany	–	JQ948777	–	JQ949767	JQ950097
C. pseudoacutatum	CBS 436.77*	*Pinus radiata*	Chile	–	JQ948811	–	JQ949801	JQ950131
C. pyricola	CBS 128531, ICMP 12924, PRJ 977.1*	*Pyrus communis*	New Zealand	–	JQ948776	–	JQ949766	JQ950096
C. rhombiforme	CBS 129953, PT250, RB011*	*Olea europaea*	Portugal	–	JQ948788	–	JQ949778	JQ950108
C. salicis	CBS 607.94*	*Salix* sp.	Netherlands	–	JQ948791	–	JQ949781	JQ950111
C. scovillei	CBS 126529, PD 94/921–3, BBA 70349*	*Capsicum* sp.	Indonesia	–	JQ948597	–	JQ949588	JQ949918
C. simmondsii	CBS 122122, BRIP 28519*	*Carica papaya*	Australia	–	JQ948606	–	JQ949597	JQ949927
C. sloanei	IMI 364297, CPC 18929*	*Theobroma cacao*	Malaysia	–	JQ948617	–	JQ949608	JQ949938
C. tamarilloi	CBS 129814, T.A.6*	*Solanum betaceum*	Colombia	–	JQ948514	–	JQ949505	JQ949835
C. walleri	CBS 125472, BMT(HL)19*	*Coffea* sp.	Vietnam	–	JQ948605	–	JQ949596	JQ949926

photoperiod of 24 h. This assay was carried out twice. The colony growth (diameter increase) of each isolate was measured for 7 days. At the end of 7 days, the colony color and texture of each isolate were evaluated and according to these criteria they were classified into distinct morphological groups. The diameter of the colony was measured with the aid of a digital caliper (in millimeters) in two perpendicular directions. The mycelial growth of the colonies was expressed in mm day^{-1}. After five days, the microscopic preparation on slides was performed using lactophenol and the images were produced through a Nikon® DS-Ri1 camera coupled to a Nikon® eclipse 80i microscope. The length and width of 50 conidia per isolate were measured with the aid of Motic Image Plus v. 2.0 (Motic Group Co., Beijing, China). In order to determine the differences of the conidial dimensions of the *Colletotrichum* species and the mycelial growth averages, the data were submitted to analysis of variance (ANOVA) and the means were grouped by the Scott-Knott test (Scott and Knott 1974), with significance of 5%, using the Sisvar V. 5.4 software (Ferreira 2014).

Pathogenicity assay of Colletotrichum isolates from onion via inoculation of the bulbs The bulbs were disinfested according to a previously described protocol (Vila Nova et al. 2011). A subset of isolates representing each of *tub2* gene haplotypes (Table 2) was grown in PDA medium at 25° C in continuous light for 24 h for five days. Afterwards, mycelial discs from each isolate were removed from the edges of the five days old colonies and placed in contact with onion bulbs with the help of autoclaved toothpicks. Each isolate was inoculated into five onion bulbs, each one being tested with two mycelial discs in symmetrical positions on each bulb. The mock-inoculated controls were treated in a similar manner; however, the discs were composed exclusively of PDA. The inoculated bulbs were then kept in moist chambers in trays covered with autoclaved paper towels and moistened with sterile distilled water. Trays remained enclosed by plastic bags for 7 days in the light under a temperature range of 20 ± 5 °C. Fourteen days after inoculation, the diameters of the two lesions of each bulb were measured in two opposite directions. The experimental design was completely randomized with five replications. The data obtained in the 11 treatments for all pathogenicity tests were submitted to analysis of variance (ANOVA) and the means were grouped by the Scott-Knott test (Scott and Knott 1974) with significance of 5%, using Sisvar V. 5.4 software (Ferreira 2014). This test was carried out twice and the results of the two assays were statistically similar ($P > 0.05$). Data were analyzed as a group, representing the average of the two assays.

Fig. 2 Phylogenetic tree of the *tub2* genomic region of 38 onion *Colletotrichum* (Coll) isolates along with GenBank sequences of reference isolates. *Colletotrichum pseudoacutatum* was employed as outgroup. Values on the branches are indicating the posterior probability of Bayesian inference (model HKY + I, 2 million generations and 25% burnin) followed by maximum likelihood bootstraps (model GTR + I, 1000 bootstraps). Values below 60 of either bootstrap or posterior probability were removed. The *Colletotrichum* isolates associated with onion foliar anthracnose employed in the present study are highlighted in blue

Pathogenicity assay of Colletotrichum isolates from onion via leaf inoculation The same subset of selected isolates was transferred to Petri dishes containing oatmeal agar medium and incubated for two weeks at 25° C in continuous light for 24 h for 5 days. Twenty-five mL of 0.01% Tween 20 solution was added to each Petri dish. The mycelium was then dissolved with the aid of a soft bristle brush and the conidial suspension was then filtered in autoclaved double gauze. The suspension was quantified with the aid of a hemocytometer and dilutions were performed to obtain a final concentration of 1×10^{5} conidia/mL. Onion plants cultivar BRS 367, were grown in Styrofoam trays filled with a commercial substrate (Rohrbacher®). Plants with three fully-expanded leaves were transplanted to 1.5-L pots (with four plants each) containing a substrate composed of a soil mixture, burned rice husk and bovine manure in the ratio 3:1:1, enriched with 8 g of a mixture of NPK (formulation 4–30-16) and 7.5 g of dolomitic limestone. The aereal organs of the plants, displaying at least five fully-expanded leaves were inoculated by using a suspension of 1×10^{5} conidia/ml (containing 0.01% Tween) up to the point of drainage. Each plant was sprayed with about 2.5 mL of fungal suspension and then the pots were kept in moist chamber for 48 h. The experiment was carried out twice under greenhouse conditions in a completely randomized design, involving ten treatments (isolates) with three replicates, each one with four plants. Mock-inoculated plants were treated only with a Tween (0.01%) solution.

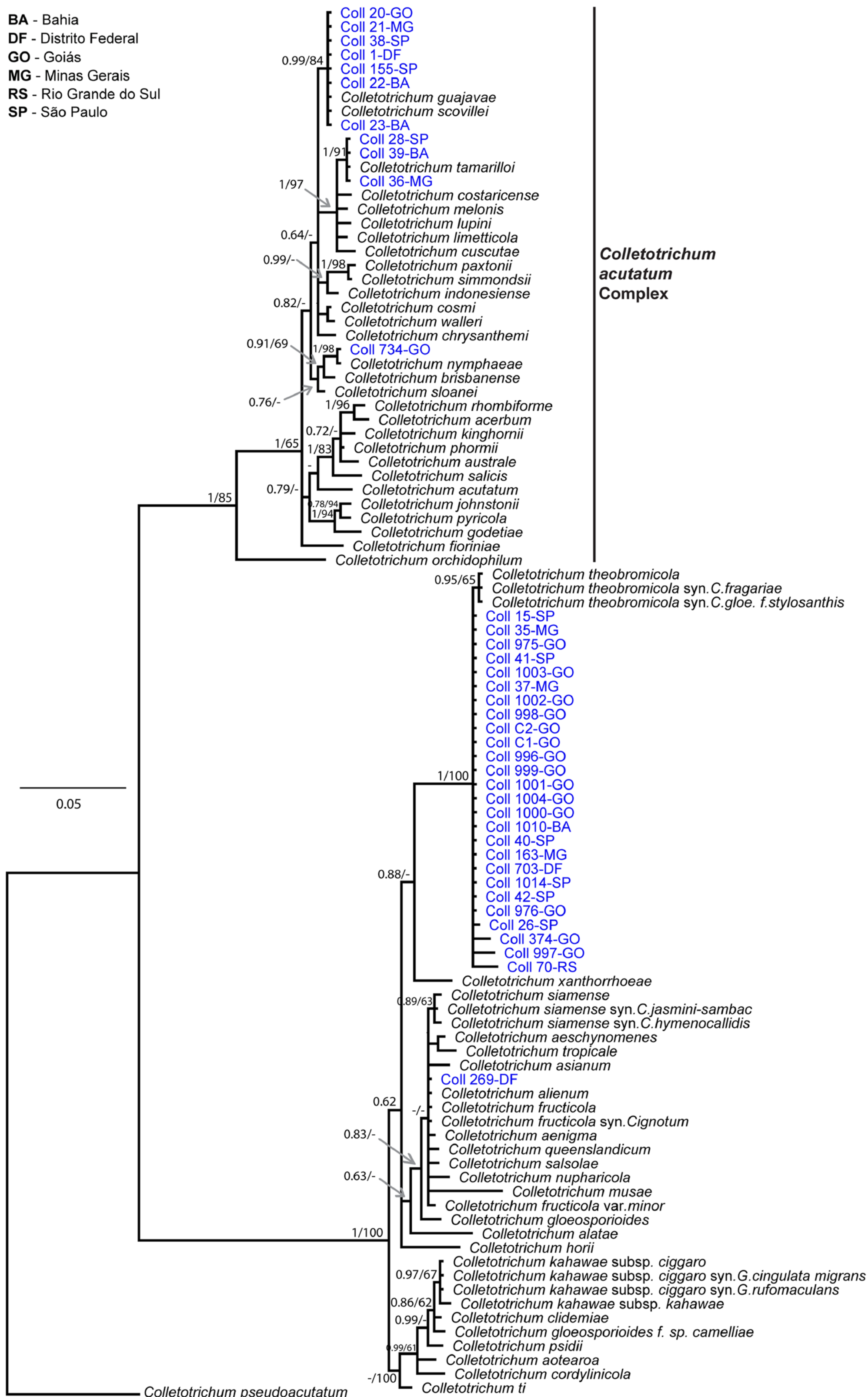
BA - Bahia
DF - Distrito Federal
GO - Goiás
MG - Minas Gerais
RS - Rio Grande do Sul
SP - São Paulo
Coll 20-GO
Coll 21-MG
Coll 38-SP
Coll 1-DF
Coll 155-SP
Coll 22-BA
Colletotrichum guajavae
Colletotrichum scovillei
Coll 23-BA
Coll 28-SP
Coll 39-BA
Colletotrichum tamarilloi
Coll 36-MG
Colletotrichum costaricense
Colletotrichum melonis
Colletotrichum lupini
Colletotrichum limetticola
Colletotrichum cuscutae
Colletotrichum paxtonii
Colletotrichum simmondsii
Colletotrichum indonesiense
Colletotrichum cosmi
Colletotrichum walleri
Colletotrichum chrysanthemi
Coll 734-GO
Colletotrichum nymphaeae
Colletotrichum brisbanense
Colletotrichum sloanei
Colletotrichum rhombiforme
Colletotrichum acerbum
Colletotrichum kinghornii
Colletotrichum phormii
Colletotrichum australe
Colletotrichum salicis
Colletotrichum acutatum
Colletotrichum johnstonii
Colletotrichum pyricola
Colletotrichum godetiae
Colletotrichum fioriniae
Colletotrichum orchidophilum
0.99/84
1/91
1/97
0.64/-
0.99/-
1/98
0.82/-
0.91/69
1/98
0.76/-
1/96
0.72/-
1/65
1/83
1/85
0.79/-
0.78/94
1/94
Colletotrichum acutatum Complex
Colletotrichum theobromicola
Colletotrichum theobromicola syn.C.fragariae
Colletotrichum theobromicola syn.C.gloe. f.stylosanthis
0.95/65
Coll 15-SP
Coll 35-MG
Coll 975-GO
Coll 41-SP
Coll 1003-GO
Coll 37-MG
Coll 1002-GO
Coll 998-GO
Coll C2-GO
Coll C1-GO
Coll 996-GO
Coll 999-GO
Coll 1001-GO
Coll 1004-GO
Coll 1000-GO
Coll 1010-BA
Coll 40-SP
Coll 163-MG
Coll 703-DF
Coll 1014-SP
Coll 42-SP
Coll 976-GO
Coll 26-SP
Coll 374-GO
Coll 997-GO
Coll 70-RS
1/100
0.88/-
0.05
Colletotrichum xanthorrhoeae
Colletotrichum siamense
Colletotrichum siamense syn.C.jasmini-sambac
Colletotrichum siamense syn.C.hymenocallidis
Colletotrichum aeschynomenes
Colletotrichum tropicale
Colletotrichum asianum
Coll 269-DF
Colletotrichum alienum
Colletotrichum fructicola
Colletotrichum fructicola syn.C.ignotum
Colletotrichum aenigma
Colletotrichum queenslandicum
Colletotrichum salsolae
Colletotrichum nupharicola
Colletotrichum musae
Colletotrichum fructicola var.minor
Colletotrichum gloeosporioides
Colletotrichum alatae
Colletotrichum horii
Colletotrichum kahawae subsp. ciggaro
Colletotrichum kahawae subsp. ciggaro syn.G.cingulata migrans
Colletotrichum kahawae subsp. ciggaro syn.G.rufomaculans
Colletotrichum kahawae subsp. kahawae
Colletotrichum clidemiae
Colletotrichum gloeosporioides f. sp. camelliae
Colletotrichum psidii
Colletotrichum aotearoa
Colletotrichum cordylinicola
Colletotrichum ti
Colletotrichum pseudoacutatum
0.89/63
0.62
-/-
0.83/-
0.63/-
1/100
0.97/67
0.86/62
0.99/-
0.99/61
-/100
Colletotrichum gloeosporioides Complex

Table 2 Summary of information of the β-tubulin (*tub2*) gene haplotypes of 38 *Colletotrichum* isolates associated with onion foliar anthracnose in Brazil and GenBank accessions for tub2 as well as four additional genomic regions (Act, GAPDH, APN2/MAT-IGS, and Cal)

Haplotype	Isolate	*Colletotrichum* species Complex	City	State	Tub2	Act	GAPDH	APN2/ MAT-IGS	Cal
1	Coll 28	*C. acutatum*	São José do Rio Pardo	SP	MT012459	–	–	–	–
1	Coll 36	*C. acutatum*	São Gotardo	MG	MT012460	–	–	–	–
1	Coll 39	*C. acutatum*	Juazeiro	BA	MT012461	MN090177	MN090186	–	–
2	Coll 1	*C. acutatum*	Brasília	DF	MT012452	–	–	–	–
2	Coll 20	*C. acutatum*	Cristalina	GO	MT012453	MN090174	MN090184	–	–
2	Coll 21	*C. acutatum*	Jaíba	MG	MT012454	–	–	–	–
2	Coll 22	*C. acutatum*	Ibicoara	BA	MT012455	–	–	–	–
2	Coll 23	*C. acutatum*	João Dourado	BA	MT012456	MN090175	MN090185	–	–
2	Coll 38	*C. acutatum*	São José do Rio Pardo	SP	MT012457	–	–	–	–
2	Coll 155	*C. acutatum*	São José do Rio Pardo	SP	MT012458	–	–	–	–
3	Coll C2	*C. gloeosporioides*	Cristalina	GO	MT012429	–	–	–	–
3	Coll C1	*C. gloeosporioides*	Campo Alegre	GO	MT012428	–	–	–	–
3	Coll 15	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012449	–	–	–	–
3	Coll 35	*C. gloeosporioides*	São Gotardo	MG	MT012448	MN090173	MN090178	MN090160	MN090166
3	Coll 37	*C. gloeosporioides*	São Gotardo	MG	MT012447	–	–	–	–
3	Coll 40	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012446	–	–	–	–
3	Coll 41	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012445	–	–	–	–
3	Coll 42	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012444	–	–	–	–
3	Coll 163	*C. gloeosporioides*	São Gotardo	MG	MT012443	–	–	–	–
3	Coll 703	*C. gloeosporioides*	Brasília	DF	MT012442	–	–	–	–
3	Coll 975	*C. gloeosporioides*	Campo Alegre	GO	MT012441	–	–	–	–
3	Coll 976	*C. gloeosporioides*	Cristalina	GO	MT012440	–	–	–	–
3	Coll 996	*C. gloeosporioides*	Cristalina	GO	MT012439	–	–	–	–
3	Coll 998	*C. gloeosporioides*	Cristalina	GO	MT012438	–	–	–	–
3	Coll 999	*C. gloeosporioides*	Cristalina	GO	MT012437	–	–	–	–
3	Coll 1000	*C. gloeosporioides*	Cristalina	GO	MT012436	–	–	–	–
3	Coll 1001	*C. gloeosporioides*	Cristalina	GO	MT012435	–	–	–	–
3	Coll 1002	*C. gloeosporioides*	Cristalina	GO	MT012434	–	–	–	–
3	Coll 1003	*C. gloeosporioides*	Cristalina	GO	MT012433	–			–
3	Coll 1004	*C. gloeosporioides*	Cristalina	GO	MT012432	–	–	–	–
3	Coll 1010	*C. gloeosporioides*	Irecê	BA	MT012430	–	–	–	–
3	Coll 1014	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012431	–	–	–	–
4	Coll 26	*C. gloeosporioides*	São José do Rio Pardo	SP	MT012427	MN090172	MN090179	MN090161	MN090167
5	Coll 374	*C. gloeosporioides*	Campo Alegre	GO	MT012426	MN090169	MN090181	MN090157	MN090163
6	Coll 734	*C. acutatum*	Cristalina	GO	MT012451	MN090176	MN090183	–	–
7	Coll 997	*C. gloeosporioides*	Cristalina	GO	MT012450	MN090168	MN090182	MN090156	MN090162
8	Coll 269	*C. gloeosporioides*	Brasília	DF	MT012424	MN090170	MN090178	MN090158	MN090164
9	Coll 70	*C. gloeosporioides*	São José do Norte	RS	MT012425	MN090171	MN090180	MN090159	MN090165

Results

Phylogenetic analyses The 38 isolates were initially separated into nine *tub2* gene haplotypes (Table 2). With this initial analysis using *tub2* gene information, fungal species belonging to two *Colletotrichum* complexes (*C. acutatum* and *C. gloeosporioides*) were observed in both Bayesian and Maximum Likelihood

inferences (Fig. 2). These two methods rendered phylogenetic trees with similar topologies. Eleven (28.9%) out of the 38 isolates grouped in the clade of the *C. acutatum* species complex and 27 isolates (71.1%) grouped in the *C. gloeosporioides* species complex. The tub 2 haplotype network (Fig. 3) revealed two distinct clades corresponding the clades already observed in the Fig. 2. The first clade is represented by isolates of the *C. gloeosporioides* species complex, originated from multiple geographic regions, including all sampled states. The second clade is represented by isolates of the *C. acutatum* species complex, also originated from multiple geographic regions, including all sampled states except Rio Grande do Sul. Six out of nine *tub2* haplotypes were represented by only one isolate (Table 2 and Fig. 3).

In this preliminary phylogenetic analysis employing the *tub2* information, three haplotypes (Hap 1, Hap 2, and Hap 6) grouped with the reference species of the *C. acutatum* complex and six haplotypes (Hap 3, Hap 4, Hap 5, Hap 7, Hap 8, and Hap 9) grouped with references of the *C. gloeosporioides* complex. Twenty-six out of the 27 isolates belonging to the *C. gloeosporioides* species complex grouped in a subclade together with the species *C. theobromicola*. The other isolate belonging to the *C. gloeosporioides* complex (Coll 269), grouped in a subclade with the species *C. fructicola* and related species of this complex. Among the eleven isolates that grouped with species of the *C. acutatum* species complex, seven grouped in a subclade with *C. scovillei* and *C. guajavae*, two species with close phylogenetic relationship. Another three isolates grouped in a subclade with *C. tamarilloi*, while the other isolate grouped in a clade with *C. nymphaeae*. Primers used for the APN2/MAT-IGS region failed to amplify in five representative isolates from the haplotypes that grouped with species of the *C. acutatum* complex species. However, amplicons corresponding to such region were observed in all the remaining isolates. The genomic regions *act*, *cal*, and *gapdh* were successfully amplified across all representative haplotype

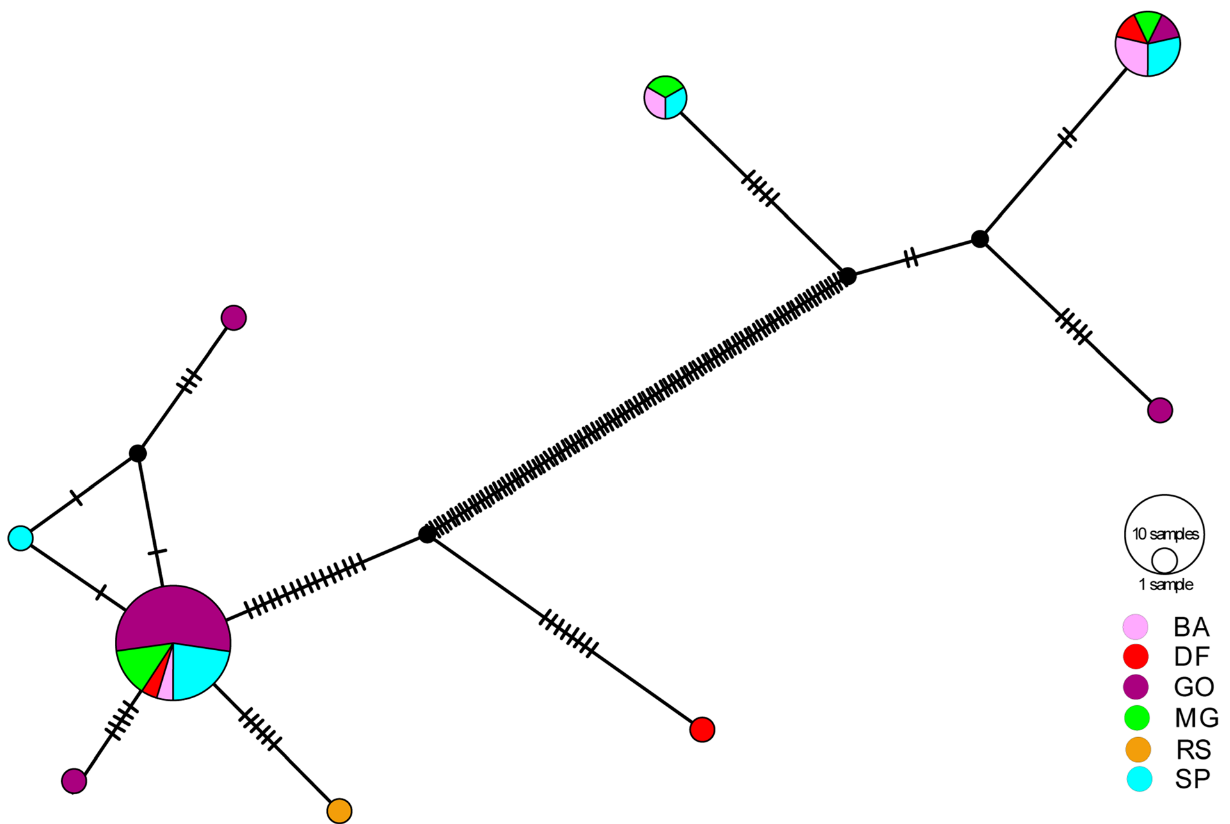

Fig. 3 TCS haplotype network generated for β-tubulin sequences representing six Brazilian states using PopArt. The sizes of the circles are proportional to the frequency of each haplotype. Black circles represent hypothetical haplotypes. Hatch marks along the network branches indicate the number of mutations. Each color represents a Brazilian state

isolates. The concatenated trees corroborated the results of the *tub2* phylogeny. The same set of species belonging to the *C. acutatum* and *C. gloeosporioides* complexes identified in the *tub2* phylogeny were confirmed in this analysis. Three species were found in the *C. acutatum* complex and two species were detected in the *C. gloeosporioides* complex. The *tub2, act*, and *gapdh* genes tree of the four onion isolates of the *C. acutatum* complex displayed well-supported clades with high posterior probability values, indicating that all the isolates used in this tree are bona fide members of the *C. acutatum* complex (Fig. 4). With the maximum value of posterior probability, the representative isolate Coll 39 clustered with *C. tamarilloi*, while isolate Coll 734 grouped with *C. nymphaeae*. Both representative isolates Coll 20 and Coll 23 clustered with *C. scovillei* with posterior probability of 0.96 and separated from *C. guajavae*. The *tub2*, *act*, *cal*, *gapdh* and APN2/MAT-IGS genes tree displays the representative onion isolates and the references of the *C. gloeosporioides* complex (Fig. 5). The isolates Coll 26, Coll 35, Coll 70, Coll 374 and Coll 997 formed a clade associated with the reference isolate of *C. theobromicola* (syn. *C. fragariae*), with posterior probability of 1 (Fig. 5). The Coll 269 isolate grouped to *C. fructicola* with posterior probability of 0.98.

Morphological characterization Based on colony morphology, the representative isolates of *Colletotrichum* were separated into seven different morphological groups. The morphological groups #1, #2, #3, #5, and #7 included the isolates Coll 35, Coll 23, Coll 39, Coll 269 and Coll 20, respectively. The morphological group #4 was represented by the isolate Coll 997, while the morphological group #6 was composed by the isolates Coll 26, Coll 70 and Coll 374. The representative isolate Coll 734 had its morphological analysis compromised due to successive contaminations. The morphological group #1 had a light gray colony with a dark green back and absence of conidia. The morphological group #2 displayed a slightly cottonous colony with pale orange color, with a few orange conidia, and a pale orange reverse. The morphological group #3 displayed colonies with olive color with absence of conidia, and reverse of coloration varying from white to olive. The morphological group #4 presented a colony with a color ranging from gray to white, the reverse of coloration ranging from white to pale orange and with no production of conidia. The morphological group #5 showed a colony with a gray and reverse coloration ranging from gray to white with absence of conidiomata. The morphological group #6 presented a colony with irregular borders and very cottonous of light gray color and the reverse ranging from gray to dark green and absence of conidiomata. The morphological group #7 presented a slightly cottonous colony of olive color with numerous conidiomata of orange color and the reverse of coloration varying from orange to olive.

The mycelial growth rates displayed significant statistical differences among the isolates. Four distinct groups were observed (Table 3). The isolates Coll 269 and Coll 997 displayed the highest average growth (mean of 10.4 mm.day-1), while the Coll 26 isolate had the lowest growth rate, 6.03 mm day^{-1}. The isolates Coll 20, Coll 35, Coll 70 and Coll 23 were in the group with the second highest mycelial growth rate (mean of 9.6 mm day^{-1}). The isolates Coll 374 and Coll 39 showed a mean of 8.55 mm day^{-1}. Regarding the diameter of the colony measured at the end of seven days, all the isolates presented a distribution of means identical to the distributions of the average of the daily mycelial growth rate, grouping equally in four groups.

In relation to the morphological attributes, the isolates Coll 269, Coll 997, Coll 26, Coll 35 and Coll 374 displayed oblong conidia with both obtuse ends, ranging in size from 9.50 μm to 13.00 μm in length and from 3.50 μm to 4.50 μm in width. The isolates Coll 23, Coll 20 and Coll 39 displayed fusiform (sometimes oblong) conidia with one or in some cases, both acute ends, varying in dimensions of 12.50 μm to 15.00 μm in length and 3.50 μm to 4.50 μm in width. The Coll 70 isolate, in PDA medium, displayed an abundant production of a sterile cottonous mycelium, making it impossible to measure the conidia.

Pathogenicity assays in onion bulbs The representative subset of *Colletotrichum* isolates, except Coll 734, were pathogenic, causing typical anthracnose lesions on the onion bulbs seven days after inoculation. Two types of lesions were observed: one firm and other with an aqueous aspect, both with signs of the pathogen. Significant statistical difference was detected among the lesion size means (= aggressiveness) induced by the selected isolates. Isolates Coll 23 and Coll 20 were weakly aggressive and the isolates Coll 39, Coll 997, Coll 70, Coll 269, Coll 35, Coll 374, and Coll 26 were the most aggressive (Table 4). All isolates were re-isolated from the symptomatic bulbs.

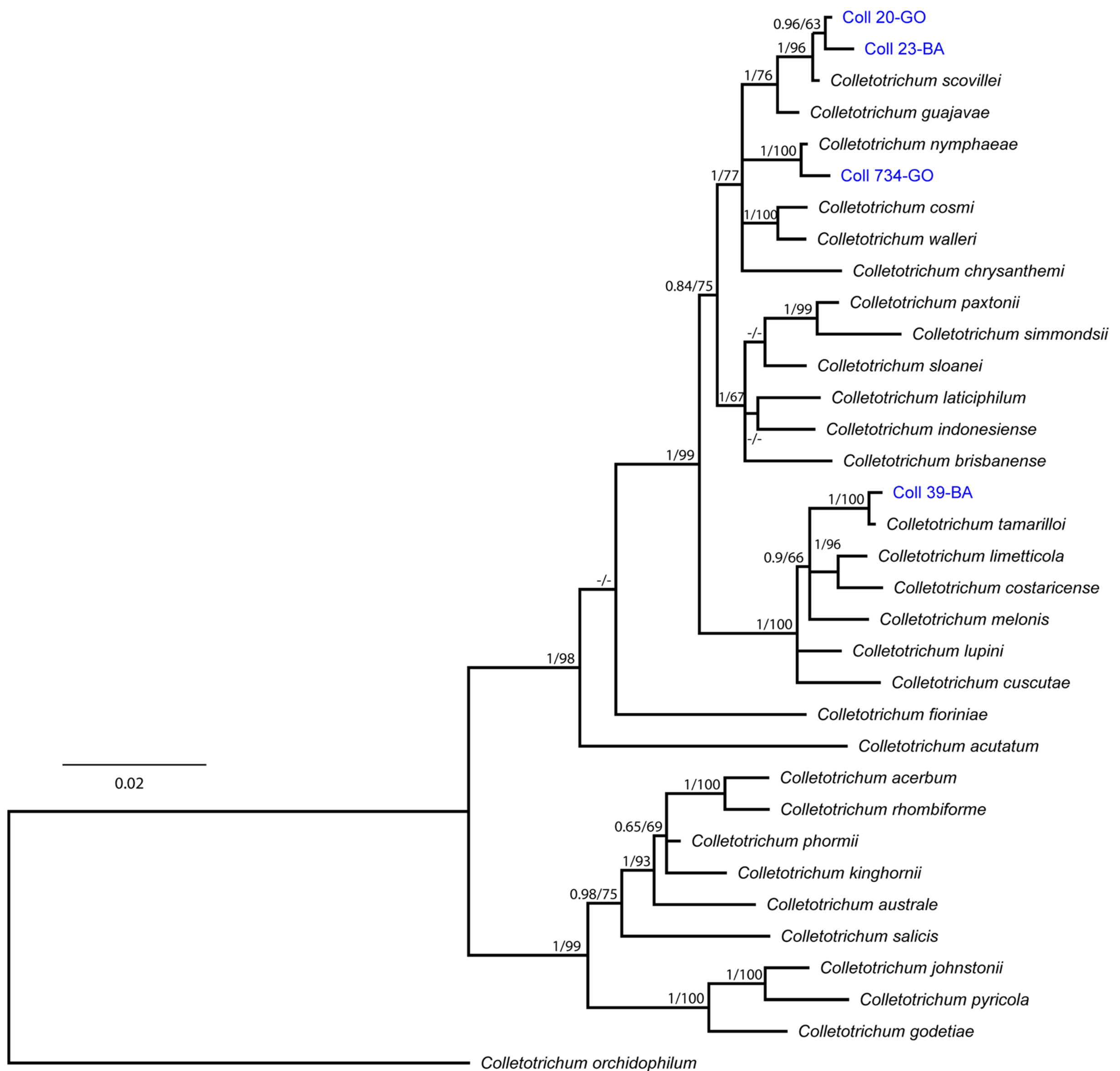

Fig. 4 Phylogenetic tree of concatenated sequences of *tub2, act*, and *gapdh* genes containing four representative isolates plus reference accessions of members from the *Colletotrichum acutatum* complex. *Colletotrichum orchidophilum* was employed as outgroup. Values on branches correspond to posterior probability of Bayesian inference (model HKY + G, 6 million generations and 25% burn-in) followed by maximum likelihood bootstraps (model GTR + G, 1000 bootstraps). Values below 60 of bootstrap or posterior probability were removed. The *Colletotrichum* isolates associated with onion foliar anthracnose employed in the present study are highlighted in blue

Pathogenicity assays in onion leaves All selected *Colletotrichum* isolates were able to induce foliar symptoms in onion plants. However, the means of severity did not display significant statistical difference (data not shown). The severity of the disease was classified as an overall "grade 1", indicating a low rate of aggressiveness of isolates in onion leaves during the evaluation period of the experiment. The isolates were re-isolated in PDA media and they were morphologically similar to the original cultures.

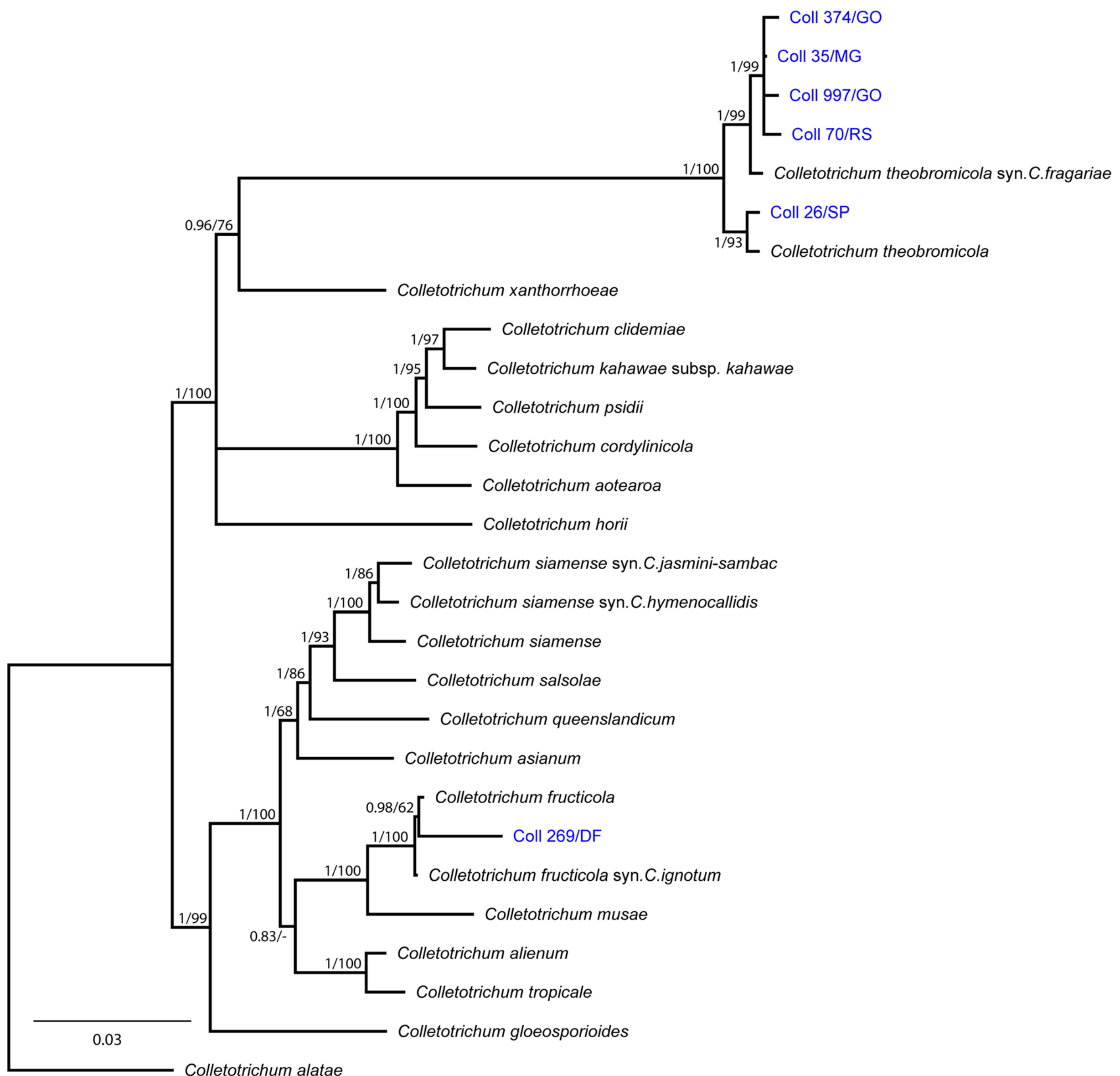

Fig. 5 Phylogenetic tree of concatenated sequences of *tub2, act*, *cal* and APN2/MAT-IGS genomic regions, containing six representative isolates plus reference accessions from members of the *Colletotrichum gloeosporioides* complex. *Colletotrichum alatae* was employed as outgroup. Values on branches correspond to posterior probability of Bayesian inference (model HKY + I, 2 million generations and 25% burn-in) followed by maximum likelihood bootstraps (model GTR + I, 1000 bootstraps). Values below 60 of bootstrap or posterior probability were removed. The *Colletotrichum* isolates associated with onion foliar anthracnose employed in the present study are highlighted in blue

Discussion

Extensive characterization studies of the putative complex of *Colletotrichum* species associated to foliar anthracnose of onion via a combination of morphological and molecular tools are scarce in the literature. These analyses were carried out with a representative collection of 38 isolates obtained from diseased onion plants across major onion-producing areas in four macro geographic regions of Brazil. The results indicated that there

Table 3 Morphological characteristics of representative β-tubulin (*tub2*) gene haplotypes identified in a collection of *Colletotrichum* isolates associated with onion foliar anthracnose in Brazil

Isolate	Color of the colony		Colony texture	Length of conidia (μm)	Conidia width (μm)	Growth rate (mm/d)	Colony diameter (mm)
	Front	Reverse					
Coll 26	light gray	gray to dark green	very cottony	12,30 b	3,77 a	6,03 a	42,16 a
Coll 374	light gray	gray to dark green	very cottony	11,83 b	4,53 d	8,53 b	59,66 b
Coll 39	olive	white to olive	cottony	12,79 c	4,45 d	8,56 b	60,00 b
Coll 20	olive	orange to olive	litte contoured	15,02 d	4,11 c	9,26 c	64,83 c
Coll 35	light gray	dark green	cottony	11,9 b	3,90 b	9,66 c	67,66 c
Coll 70	light gray	gray to dark green	very cottony	–	–	9,76 c	68,50 c
Coll 23	pale orange	pale orange	cottony	13,35 c	3,99 b	9,76 c	68,50 c
Coll 997	light gray	white to pale orange	cottony	9,57 a	3,62 a	10,36 d	72,66 d
Coll 269	gray	gray to white	very cottony	11,81 b	3,67 a	10,50 d	73,33 d

Means followed by the same lowercase letter do not differ significantly from one another by the Scott-Knott's cluster analysis, $p \leq 0.05$

is at least five *Colletotrichum* species as members of this complex. Distinct *Colletotrichum* species have been reported causing anthracnose in onions in the several countries such as *C. coccodes* in USA (Rodriguez-Salamanca et al. 2012; Baysal-Gurel et al. 2014; Hay et al. 2016) and *C. siamense* and *C. truncatum* in India (Chowdappa et al. 2015). However, in our study, despite the diversity of sampled fields in the four main onion producing regions of Brazil, these species were not found associated with the foliar anthracnose of onion. In fact, novel information about the fungal diversity associated with this important tropical and subtropical diseases was obtained. Species belonging to the *C. gloeosporioides* complex are often reported in association with onion anthracnose, while species of the *C. acutatum* complex have not yet been reported in the literature. Therefore, our work is the first formal report of the association of three novel species of the *C. acutatum* complex (*C. scovillei*, *C. nymphaeae* and *C. tamarilloi*) with the foliar anthracnose of onions.

Table 4 Aggressiveness in onion bulbs of representative β-tubulin (*tub2*) gene haplotypes identified in a collection of *Colletotrichum* isolates associated with onion foliar anthracnose in Brazil

Isolate	Aggressiveness on bulbs	
	Lesion diameter (mm)	Analysis of variance
Control	0,0	D
Coll 734	0,0	D
Coll 23	3,4	C
Coll 20	3,8	C
Coll 39	6,8	B
Coll 997	8,5	B
Coll 70	9,8	B
Coll 35	9,8	B
Coll 269	11,1	B
Coll 374	12	A
Coll 26	15,3	A
F		20,94**
CV(%)		36,16

Means followed by the same letter do not differ significantly from one another by the Scott-Knott's cluster analysis, $p \leq 0.05$

Thus far, only *C. gloeosporioides* (sensu *lato*) has been reported as the causal agent of this disease in Brazil. However, we found here that two distinct species of the *C. gloeosporioides* complex (*C. theobromicola* and *C. fructicola*) are associated with this disease in the country. Twenty-six out of 38 isolates were identified as *C. theobromicola*, indicating this fungus as the prevalent anthracnose pathogen under Brazilian conditions. This fungal species was recently reported as causal agent of foliar anthracnose on welsh onion (*Allium fistulosum* L.) in Brazil (Matos et al. 2017). Other species of *Colletotrichum*, as *C. spaethianum* (Santana et al. 2016) and *C. truncatum* (Matos et al. 2017) were also reported as causal agent of foliar anthracnose on welsh onion in Brazil. However, none of these fungal

species was detected in association with onion in our work, which may indicate the occurrence of host species-specific *Colletotrichum* diversity. Additional surveys will confirm this hypothesis.

The species *C. fructicola*, *C. nymphaeae*, *C. scovillei*, *C. tamarilloi* and *C. theobromicola* have already been reported in Brazil causing anthracnose in distinct crops (Alexandre et al. 2014; Silva et al. 2017; Veloso et al. 2018; Moreira et al. 2019). The species *C. tamarilloi* was reported as the causal agent of anthracnose in scarlet eggplant fruits (Alexandre et al. 2014). The species *C. theobromicola* (syn. *C. fragariae*) and *C. fructicola* were reported as causal agents of cashew anthracnose (Veloso et al. 2018). *Colletotrichum nymphaeae* and *C. scovillei* were reported causing anthracnose on *Capscium* species (Silva et al. 2017) and apple fruits (Moreira et al. 2019), respectively.

Unlike the incongruities that may occur between morphology and phylogeny, two isolates showed similarity across these two analyzes. For example, the isolates Coll 70 and Coll 374 were grouped in the same clade and in the same morphological group (#7). These isolates are phylogenetically close to each other, and similar to *C. theobromicola*, although they were obtained from two geographically very distant regions, São José do Norte-RS and Cristalina-GO. The fact that *Colletotrichum* spp. can be disseminated by contaminated onion seeds (Massola Jr et al. 2016) can explain the presence of the same species causing anthracnose in onions in these two far apart geographic regions of Brazil.

The information derived from only three genes (*tub2*, *act*, and *gapdh*) has been sufficient to obtain well-supported clades for studies involving the genetic diversity of the species from the *C. acutatum* complex (Dean et al. 2012; Vieira et al. 2020). For the *C. gloeosporioides* complex, it is well-known that the APN2/MAT-IGS region in association with other genomic regions such as *act*, *cal*, *chs* (chitin synthetase), *gs* (glutamine synthetase) and *his* (histone) provides crucial information to obtain a precise characterization of isolates (Sharma et al. 2013; Vieira et al. 2020). Therefore, the genomic regions used here were suitable to identify and characterize our sample of isolates.

In the morphological analysis, it was observed that the color and texture of the morphological groups #1, #5, and #6 are congruent with reference isolates of the species employed by Weir et al. (2012) to define the *C. gloeosporioides* species complex. The development of mycelium with white, gray, and olive colors have also been reported in other studies as being a major characteristic associated with the members of the *C. gloeosporioides* species complex (Bernstein et al. 1995; Gunnell and Gubler 1992). The occurrence, however, of colonies with rosy to orange colorations and with the reverse color ranging from white to pink are characteristic of *C. acutatum* species complex (Bernstein et al. 1995; Damm et al. 2012). The morphological groups #2, #3, #4, and #7 were composed of species belonging to this complex and displayed these peculiar morphological characteristics. In addition, the differences in the morphology of the conidia produced in these colonies are in agreement with colony morphology data for most isolates. The isolates included in each morphological group, with presence of colony characteristic of species included in the *C. gloeosporioides* complex, also displayed typical conidia of the members of this complex, without shape variations. However, for the isolates included in the morphological groups #2, #3, #4, and #7 there was no consistence in the conidial shape. Damm et al. (2012) also observed a conspicuous variation of the conidial shape within species of the *C. acutatum* complex, and this fact can be due several reasons. One is that fungi preserved over a long period and subcultured multiple times, may be prone to morphophysiological variations when compared to wild/fresh isolates (Budiie et al. 1999). A subgroup of our *Colletotrichum* isolates from onion has been maintained for over a decade (data not shown). Besides influencing the shape of the conidia, the successive subculture and the preservation during long periods of *Colletotrichum* isolates promote changes in the colony texture. This type of fungal manipulation can result in denser and cottony colonies, which may display considerable loss in the capacity of conidial production (Damm et al. 2012). This condition may explain the fact that the isolates such as Coll 70 and Coll 734 displayed only sterile mycelium. It is important to highlight that these changes in colony morphology are not necessary associated with loss of pathogenicity, as observed here for the isolate Coll 70, which was among the most aggressive in the bioassay using onion bulbs (Table 4). The abnormalities in conidial size and mycelial growth of all isolates found in this study were similar to the overall means described for *Colletotrichum* species. Weir et al. (2012) obtained conidial dimension values quite similar to those obtained in this study after analyzing colonies with 10 days of growth. This

observation indicates that the cultivation period ranging from seven to 10 days may not have strong influence in the overall *Colletotrichum* conidial dimensions.

The differences in symptom expression observed in our bioassay with onion bulbs employing representative isolates of the *C. acutatum* and *C. gloeosporioides* complexes were also observed by Vila Nova et al. (2011) with a distinct collection of *C. gloeosporioides* isolates from Pernambuco State, Brazil. The aqueous type of lesions, observed in both works, indicates activity of extracellular pectinolytic enzymes produced by members of this group of pathogens. According to Bailey et al. (1992), tissue-degrading enzymes can act in the establishment of *Colletotrichum* species infections and are, therefore, important components associated with the divergences observed in aggressiveness across isolates. Vila Nova et al. (2011) also found differences in the aggressiveness of *Colletotrichum* isolates in onion leaves, contrasting with results of our study. The variation in the levels of aggressiveness is a useful information from the onion breeding standpoint, guiding the identification of most suitable isolates for anthracnose resistance selection programs (Reis and Oliveira 2009).

For a long time, the taxonomy of the genus *Colletotrichum* has been problematic due to the absence of uniform criteria to characterize species (Hibbett and Taylor 2013). In addition, morphological characterizations are not always accurate because they deal with variable and plastic characteristics. Changes in the composition of the medium, storage time and the number of times that a given isolate has been subcultured are major factors that alter the results of morphological analyses, leading to imprecise characterization (Cai et al. 2009; Weir et al. 2012).

The results of our work demonstrated the association of at least five *Colletotrichum* species causing onion anthracnose in Brazil. This diversity of *Colletotrichum* species in onions was not identified in previous diversity studies. Thus far, only *Colletotrichum gloeosporioides* has been reported as the causal agent of this disease in Brazil (Bajungu 1979; Wordell Filho et al. 2006; Vila Nova et al. 2011). However, our work is clearly indicating that anthracnose disease is, in fact, caused by a complex of *Colletotrichum* species, with the prevalence of *C. theobromicola* isolates. In this scenario, the identification of a fungal complex in this pathossystem is a relevant contribution since the correct identification of the causal agents is essential for the establishment of more effective anthracnose management strategies, including epidemiological aspects, chemical and cultural control as well as the identification and deployment of onion cultivars with species-specific and/or wide-spectrum tolerance/resistance.

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Compliance with ethical standards

Disclosure of potential conflict of interest The authors do not have any conflict of interest.

Research involving human participants and/or animals Not applicable.

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