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Diversity of *Colletotrichum* causing anthracnose on *Psidium guajava* in varied Brazilian physiographic regions

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Abstract

Anthracnose, caused by several *Colletotrichum* species, is a major restricting factor for guava production, but no comprehensive study of the causal agent has been conducted from the plant host centre of diversity. This work characterizes isolates from guava, mainly from the fruit, but also leaves and flowers, representing most Brazilian physiographies according to the partial sequences of the rDNA-ITS, *ApMAT*, *TUB2*, *HIS3* and *GAPDH* gene regions. In addition, the pathogenicity and aggressiveness to fruits of two widely planted guava varieties (SLG and RM) are described. Guava-derived *Colletotrichum* isolates were found in five complexes: gloeosporioides, acutatum, boninense, gigasporum and orchidearum. The gloeosporioides complex was the most prevalent (81%), followed by the acutatum complex (14%). A total of 16 *Colletotrichum* species were naturally associated with guava anthracnose: *C. aeschynomenes*, *C. asianum*, *C. chrysophilum*, *C. fructicola*, *C. gigasporum*, *C. gloeosporioides*, *C. karsti*, *C. melonis*, *C. musae*, *C. nymphaeae*, *C. paranaense*, *C. siamense*, *C. sojae*, *C. syzygicola*, *C. theobromicola* and *C. tropicale*. Apart from *C. nymphaeae* and *C. gloeosporioides*, all the remaining 14 taxa are reported for the first time in *P. guajava*. The most aggressive species belonged to the *C. gloeosporioides* complex. *C. siamense* was the most prevalent, especially in warmer regions, followed by *C. chrysophilum*, mostly in temperate environments. The most aggressive species were *C. siamense*, *C. chrysophilum*, *C. fructicola* and *C. tropicale*. Fruits of the variety SLG were consistently more resistant to anthracnose than the fruits of RM.

KEYWORDS
aggressiveness, disease aetiology, diversity, guava, multilocus phylogeny

1 | INTRODUCTION

Guava (*Psidium guajava*) is a tropical fruit crop within the myrtle (*Myrtaceae*) family with the centre of origin in the Americas, widely cultivated over tropical regions, and with a worldwide production of around 40 million tonnes. India is the world's largest grower, with Mexico and Brazil ranking among the largest producers in the Americas (Angulo-López et al., 2021). Anthracnose is the most important postharvest guava disease in Brazil and worldwide (Fischer et al., 2011). The disease is present in all regions where guava fruit is grown, and an updated compilation of species so far associated to the disease has been presented by Zakaria (2021). Severe symptoms are observed in maturing or ripe fruits, but are also found in green fruit, causing fruit drop and fruit decay of great economic

significance (Lim & Manicom, 2003). Lesions also occur in flowers, leaves, petioles and young branches.

Colletotrichum is an important phytopathogenic fungal genus worldwide (Dean et al., 2012), harbouring plant pathogens across the tropics, subtropics and temperate zones. Anthracnose of tropical fruit crops is economically significant for avocado (*Persea americana*), mango (*Mangifera indica*), citrus (*Citrus* spp.), banana (*Musa* spp.), passionfruit (*Passiflora edulis*) and papaya (*Carica papaya*) (Zakaria, 2021). Hyde, Cai, Cannon, et al. (2009) and Hyde, Cai, McKenzie, et al. (2009) listed 66 provisionally accepted *Colletotrichum* species and possibly 19 additional names. Several successive revisions of the genus have been published in recent decades and the taxonomy is constantly updated (e.g., Cai et al., 2009; Cannon et al., 2012; Damm et al., 2019; Damm, Cannon, Woudenberg, & Crous, 2012; Damm, Cannon, Woudenberg, Johnston, et al., 2012; Hyde, Cai, Cannon, et al., 2009; Hyde, Cai, McKenzie, et al., 2009; Jayawardena et al., 2016; Liu et al., 2014; Weir et al., 2012). More recently, Jayawardena et al. (2021) provided an account of 248 accepted species divided into 14 species complexes and 13 singleton species, whereas Talhinhas and Baroncelli (2021) counted 257 accepted species. However, the most recent update recognizes 340 *Colletotrichum* species (Talhinhas & Baroncelli, 2023).

The guava anthracnose causal agents in Brazil have been traditionally identified as *C. gloeosporioides* sensu lato and *C. acutatum* s. l. (Peres et al., 2002). In the *C. acutatum* complex, Bragança et al. (2016) found *C. abscissum* and *C. nymphaeae* from a limited sampling of isolates from the state of São Paulo, in the southeast of the country. There is one record of *C. simmondsii* as a guava fruit pathogen in Brazil by Cruz et al. (2015), with no mention of geographic origin of the isolate. Two other *Colletotrichum* species, one collected in Italy (*C. psidii*, in the *C. gloeosporioides* complex; Weir et al., 2012) and the other from India (*C. guajavae*, in the *C. acutatum* complex; Damm, Cannon, Woudenberg, & Crous, 2012) have also been associated with *Psidium* species but have not been recorded in the Americas (Zakaria, 2021), nor have they been demonstrated to be pathogenic to guava. Given the wide distribution of the pathogen in Brazil, which is understood to be the centre of diversity of the host plant, a comprehensive survey of guava anthracnose, including the geographic prevalence, and the comparative aggressiveness of the *Colletotrichum* species to guava is warranted. Finally, the response of guava varieties to the disease has not yet been systematically measured.

Here, we report on the diversity of *Colletotrichum* occurring in guava in Brazil, based on a broad collection of isolates mostly collected in small orchards, from all physiogeographic regions, and employing multilocus phylogenetic analysis. The study includes samples collected from spontaneous guava tree specimens that are ubiquitous throughout Brazil, and from other wild *Myrtaceae* specimens. The prevalence of each species by region and their respective aggressiveness to the two most widely planted guava varieties in Brazil are also characterized, with a view to assisting management practices.

2 | MATERIALS AND METHODS

2.1 | Origin, isolation and maintenance of isolates

The survey involved 13 states covering all Brazilian geographic and ecological regions (Table 1). Isolates derived mostly from fruit, but also from leaves and flowers, were collected from 2013 to 2016 from naturally occurring and cultivated specimens of *P. guajava*, together with 10 additional isolates collected from spontaneous wild members of the *Myrtaceae* (*P. firmum*, *Eugenia uniflora* and *Syzygium jambos*), which are also very widespread in the Brazilian territory and could serve as reservoir inoculum for guava infections.

For isolation into pure culture, spores were collected directly from acervuli forming on plant samples maintained in humid chambers (25–29°C). Spores were transferred to water agar (WA) plates and incubated for 24 h at 25°C. Monosporic cultures were obtained from germinated conidia using the hyphal tip procedure, then cultivated for a 7-day period on potato dextrose agar (PDA) plates amended with chloramphenicol (100 mg/L) at 25°C and a 12 h photoperiod. Isolates with cultural characteristics corresponding to *Colletotrichum* were maintained at room temperature in 20 mL glass tubes containing 10 mL sterile distilled water and deposited at the fungal reference collection of the Universidade de Brasília (CCUB). The list of isolates, together with additional supporting information (CCUB ID, NCBI GenBank accessions, host plant, plant organ, year of isolation and geographic location) is provided in Table 2.

2.2 | Species identification and phylogenetic analysis

Fungal DNA for multigenic studies was extracted from 7-day-old PDA colonies (25°C, 12 h photoperiod). Aerial mycelium was transferred to 1.5 mL microtubes and then macerated in liquid nitrogen. Extraction was performed with the NucleoSpin Plant II extraction kit (Macherey-Nagel). Total DNA samples were resuspended in 50 μL of Tris-EDTA buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA) and stored at −20°C. DNA concentration was estimated visually in 1% agarose gels, comparing band intensities with the 100 bp ladder DNA (Axygen).

PCRs were carried out with specific primers for the following genomic regions: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-tubulin 2 (*TUB2*), rDNA ITS, histone 3 (*HIS3*) and *ApMAT* regions, with primers listed in Table 3, recommended by Templeton et al. (1992), O'Donnell and Cigelnik (1997), Gardes and Bruns (1993), White et al. (1990), Crous et al. (2004) and Rojas et al. (2010). Thermocycling reactions were performed in a total volume of 25 μL, consisting of 16 μL of autoclaved Milli-Q water, 2.5 μL 10 × Taq DNA polymerase buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 2 μL 10 mM dNTPs, 1 μL of each respective primer (10 μM), 0.75 μL $MgCl_2$ (50 mM), 0.75 μL of recombinant Taq DNA polymerase (Invitrogen, 5 U/μL) and 2 μL of each fungal DNA sample.

TABLE 1 Distribution of *Colletotrichum* isolate collection according to geographic and ecological regions in this study.

Brazilian state	Physiographic region	Primary biome	Number of isolates
Pará (PA)	North	Amazon tropical rain forest	14
Maranhão (MA)	Northeast	Transitional tropical forest	4
Pernambuco (PE)	Northeast	Atlantic tropical rain forest	10
Bahia (BA)	Northeast	Atlantic tropical rain forest	3
Mato Grosso (MT)	Centre West	Transitional Amazon forest and cerrado savannah	3
Mato Grosso do Sul (MS)	Centre West	Cerrado savannah	1
Goiás (GO)	Centre West	Cerrado savannah	28
Distrito Federal (DF)	Centre West	Cerrado savannah	49
Minas Gerais (MG)	Southeast	Atlantic subtropical rain forest	7
São Paulo (SP)	Southeast	Atlantic subtropical rain forest	13
Paraná (PR)	South	Atlantic subtropical rain forest	1
Santa Catarina (SC)	South	Atlantic subtropical rain forest	33
Rio Grande do Sul (RS)	South	Atlantic subtropical rain forest	5
Total			171

Thermocycling for *GAPDH* followed an initial denaturation at 94°C for 2 min; followed by 35 DNA denaturation cycles at 94°C for 45 s, primer annealing at 60°C for 45 s and extension at 72°C for 60 s; and a final extension at 72°C for 7 min. For *TUB2* and ITS regions, initial denaturation was conducted at 94°C for 2 min; followed by 34 DNA denaturation cycles at 94°C for 1 min, primer annealing at 55°C for 30 s and extension at 72°C for 60 s; and a final extension at 72°C for 10 min (Lima et al., 2013). For *HIS3*, initial denaturation was at 96°C for 5 min; followed by 30 cycles at 96°C for 30 s, 52°C for 30 s and 72°C for 60 s; with a final 5 min extension at 72°C (Crous et al., 2004). For *ApMAT* amplification, initial denaturation was at 95°C for 5 min; followed by 10 denaturation cycles at 95°C for 30 s, 62°C for 30 s (decreasing by 1°C each cycle) and 72°C for 1 min; followed by 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 10 min, as described by Doyle et al. (2013).

Amplicons were separated on 1% agarose gels in Tris acetate-EDTA buffer (TAE), stained with ethidium bromide and analysed under UV light. PCR products were sequenced by Macrogen (Seoul, South Korea).

For the phylogenetic analysis, consensus sequences were mounted using the Geneious v. 8.1 software (Biomatters Ltd), employing high-quality forward and reverse sequences for each isolate and target region. Sequence alignments were conducted for each genomic region using the ClustalW software (Thompson et al., 1994).

GAPDH sequences of all isolates were initially compared with the *Colletotrichum* sequences database deposited at GenBank (NCBI, USA; http://www.ncbi.nlm.nih.gov) using the BLAST algorithm. Next, *GAPDH* sequences were analysed by Bayesian inference, to estimate genetic diversity, by geographic origin. Representative isolates were then selected for further study. For the multigenic phylogenetic analysis, a concatenated study using the amplicons of partial gene sequences of *GAPDH*, *ApMAT*, *TUB2*, rDNA-ITS and *HIS3* was performed on this subset of isolates, together with reference isolates for each complex, available in Figure 1. Sequences for the phylogenetic trees were built separately for each *Colletotrichum* complex using Geneious v. 8.1 and MEGA v. 7.0 software (Tamura et al., 2011). Alignments were concatenated and converted to nexus and Phylip format in Sequence Matrix v. 1.8 (Vaidya et al., 2011) and used to build the multilocus phylogenetic tree. Phylogenetic analysis was inferred using the maximum-likelihood (ML) approach. Analysis was performed using RAXML-HCP2 v. 7.0.4 (Stamatakis, 2014) implemented on the CIPRES cluster (https://www.phylo.org/portal2/home.action). ML analyses were carried out with 1000 bootstrap pseudoreplicates under the GTR-GAMMA model (-m GTRGAMMA -p 12345 -k -f a -N 1000 -x 12345). Alignments and trees were deposited at GitHub repository and made available for public access. The individual and concatenated alignments along with estimated phylogeny are available at https://github.com/andersonvieira12/Colletotrichum-spp.-in-guava-in-Brazil.

2.3 | Fruit tissue colonization and aggressiveness bioassays

A total of 22 guava-derived isolates, selected to represent the genetic diversity observed by the *GAPDH* partial gene analysis and geographic regions, were tested for pathogenicity and aggressiveness. Four isolates from other species within the *Myrtaceae* were also included (381ARC from *P. firmum*, 249PT from *Eugenia* sp., 418JR from *Syzygium* sp. and 602JAM from *S. jambos*) in order to acquire information about the ability of these isolates to cause guava anthracnose. Bioassays were carried out on fruits of two *P. guajava* varieties, Cortibel SLG (a.k.a. Semi Lisa Grande or Gigante)

TABLE 2 Identification, host, species, collection organ, year of collection and GenBank accessions of selected *Colletotrichum* isolates.

Initial ID[a]	CCUB ID	Host	*Colletotrichum* species	Plant organ	Geographical origin (county, state)[b]	Year collected	GenBank accession number				
							ITS	*β-tub*	*His*	*ApMAT*	*GAPDH*
49	4993	*Psidium guajava*	*C. siamense*	Fruit	Tijuca, SC	2014	OR429700	OR475874	—	—	OR475953
251PT		*Eugenia uniflora*	*C. siamense*	Fruit	Recife, PE	2014	OR429756	OR475926	—	OR475850	OR476009
15	4988	*P. guajava*	*C. siamense*	Fruit	Lago Sul, DF	2014	OR429686	OR475859	—	OR475798	OR475937
122	2231	*P. guajava*	*C. siamense*	Fruit	Gama, DF	2013	OR429708	OR475882	—	OR475811	OR475961
39	2232	*P. guajava*	*C. siamense*	Fruit	Votorantim, SP	2014	OR429693	OR475867	—	OR475802	OR475946
07	4986	*P. guajava*	*C. siamense*	Fruit	Brazlândia, DF	2014	OR429682	OR475855	—	OR475797	OR475933
43	4991	*P. guajava*	*C. siamense*	Fruit	Lago Sul, DF	2014	OR429696	OR475870	—	—	OR475949
242		*P. guajava*	*C. siamense*	Fruit	Serra Dourada, BA	2016	OR429731	OR475901	—	OR475831	OR475984
206	4998	*P. guajava*	*C. siamense*	Fruit	Turuçu, RS	2014	OR429722	OR475895	—	OR475821	OR475975
203	2222	*P. guajava*	*C. siamense*	Fruit	Turuçu, RS	2014	OR429719	OR475892	—	OR475818	OR475972
24	4990	*P. guajava*	*C. siamense*	Fruit	Candangolândia, DF	2014	—	OR475861	—	OR475801	OR475940
212	5001	*P. guajava*	*C. siamense*	Fruit	Altamira, PA	2014	OR429725	—	—	OR475824	OR475978
248PT	2080	*E. uniflora*	*C. siamense*	Fruit	Brasília, DF	2014	OR429753	OR475923	—	OR475848	OR476006
284		*P. guajava*	*C. siamense*	Fruit	MA	2016	OR429751	OR475921	—	OR475846	OR476004
249 PT	2212	*E.uniflora*	*C. siamense*	Fruit	Brasília, DF	2014	OR429754	OR475924	—	OR475849	OR476007
285		*P. guajava*	*C. siamense*	Fruit	MA	2016	OR429752	OR475922	—	OR475847	OR476005
117		*P. guajava*	*C. siamense*	Fruit	Gama, DF	2013	OR429705	OR475879	—	OR475809	OR475958
110	2235	*P. guajava*	*C. siamense*	Fruit	Brasília de Minas, MG	2013	OR429704	OR475878	—	OR475808	OR475957
129	4995	*P. guajava*	*C. siamense*	Leaf	Gama, DF	2013	OR429709	OR475883	—	OR475812	OR475962
252PT		*E.uniflora*	*C. siamense*	Fruit	Brasília, DF	2014	OR429757	—	OR452457	OR475851	OR476010
134	4996	*P. guajava*	*C. siamense*	Fruit	Cassilândia, MS	2013	OR429710	OR475884	—	OR475813	OR475963
230	2083	*P. guajava*	*C. siamense*	Fruit	Frutal, MG	2016	OR429729	—	—	OR475829	OR475982
253		*P. guajava*	*C. siamense*	Fruit	Novo S. Joaquim, MT	2014	OR429735	OR475905	—	OR475835	OR475988
252		*P. guajava*	*C. siamense*	Fruit	Novo S. Joaquim, MT	2014	OR429734	OR475904	—	OR475834	OR475987
251		*P. guajava*	*C. siamense*	Fruit	Recife, PE	2014	OR429733	OR475903	—	OR475833	OR475986
237	2226	*P. guajava*	*C. siamense*	Fruit	Serra Dourada, BA	2016	OR429730	OR475900	—	OR475830	OR475983
205		*P. guajava*	*C. siamense*	Leaf	Altamira, PA	2014	OR429721	OR475894	—	OR475820	OR475974
254	2220	*P. guajava*	*C. siamense*	Fruit	Novo S. Joaquim, MT	2014	OR429736	OR475906	—	OR475836	OR475989
210	4999	*P. guajava*	*C. siamense*	Fruit	Petrolina, PE	2014	OR429723	OR475896	—	OR475822	OR475976

TABLE 2 (Continued)

Initial ID[a]	CCUB ID	Host	*Colletotrichum* species	Plant organ	Geographical origin (county, state)[b]	Year collected	GenBank accession number				
							ITS	*β-tub*	*His*	*ApMAT*	*GAPDH*
211	5000	*P. guajava*	*C. siamense*	Fruit	Ananindeua, PA	2014	OR429724	OR475897	–	OR475823	OR475977
217	2223	*P. guajava*	*C. siamense*	Fruit	Altamira, PA	2014	OR429727	OR475898	–	OR475826	OR475980
228		*P. guajava*	*C. siamense*	Fruit	Altamira, PA	2014	–	–	–	OR475828	–
245		*P. guajava*	*C. siamense*	Fruit	Serra Dourada, BA	2016	OR429732	OR475902	–	OR475832	OR475985
215	2079	*P. guajava*	*C. siamense*	Fruit	Belém, PA	2014	OR429726		–	OR475825	OR475979
118		*P. guajava*	*C. asianum*	Leaf	Gama, DF	2013	OR429706	OR475880	–	OR475810	OR475959
149	2077	*P. guajava*	*C. tropicale*	Fruit	Recife, PE	2014	OR429711	OR475885	–	OR475814	OR475964
193	2074	*P. guajava*	*C. tropicale*	Leaf	Camocim de São Felix, PE	2014	OR429714	OR475888	–	OR475815	OR475967
197	2227	*P. guajava*	*C. tropicale*	Leaf	Camocim de São Felix, PE	2014	OR429716	OR475890	–	OR475817	OR475969
29	2251	*P. guajava*	*C. aeschynomenes*	Fruit	S. Rita Passa Quatro, SP	2014	–	OR475862	–	–	OR475941
258	2217	*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429740	OR475910	–	OR475839	OR475993
279		*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429747	OR475917	–	OR475843	OR476000
255	2219	*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429737	OR475907	–	OR475837	OR475990
281		*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429749	OR475919	–	–	OR476002
270		*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429745	OR475915	–	OR475842	OR475998
DF18		*P. guajava*	*C. fructicola*	Fruit	Brazlândia, DF	2015	OR429760	OR475929	–	OR475852	OR476013
269		*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429744	OR475914	–	OR475841	OR475997
280	2215	*P. guajava*	*C. fructicola*	Fruit	Jaraguá, GO	2016	OR429748	OR475918	–	OR475844	OR476001
21		*P. guajava*	*C. chrysophilum*	Leaf	S. João Batista, SC	2014	OR429687	OR475860	–	OR475799	OR475938
196	2228	*P. guajava*	*C. chrysophilum*	Leaf	Camocim de São Felix, PE	2014	OR429715	OR475889	–	OR475816	OR475968
47	2086	*P. guajava*	*C. chrysophilum*	Leaf	S. Francisco do Sul, SC	2014	OR429699	OR475873	–	OR475805	OR475952
201		*P. guajava*	*C. chrysophilum*	Leaf	Turuçu, RS	2014	OR429717	–	–	–	OR475970
05		*P. guajava*	*C. chrysophilum*	Fruit	Porto Ferreira, SP	2014	OR429681	OR475854	–	OR475796	OR475932
23	4989	*P. guajava*	*C. chrysophilum*	Fruit	Araquari, SC	2014	OR429688		–	OR475800	OR475939
50	2073	*P. guajava*	*C. chrysophilum*	Flower	Nova Trento, SC	2014	OR429701	OR475875	–	OR475806	OR475954
44	4992	*P. guajava*	*C. chrysophilum*	Fruit	S. Francisco do Sul, SC	2014	OR429697	OR475871	–	–	OR475950
204	2085	*P. guajava*	*C. chrysophilum*	Leaf	Turuçu, RS	2014	OR429720	OR475893	–	OR475819	OR475973
55	2234	*P. guajava*	*C. musae*	Fruit	S. João Batista, SC	2014	OR429702	OR475876	–	OR475807	OR475955

(Continues)

TABLE 2 (Continued)

Initial ID[a]	CCUB ID	Host	*Colletotrichum* species	Plant organ	Geographical origin (county, state)[b]	Year collected	GenBank accession number				
							ITS	*β-tub*	*His*	*ApMAT*	*GAPDH*
283	2209	*P. guajava*	*C. gloeosporiodes*	Fruit	MA	2016	OR429750	OR475920	–	OR475845	OR476003
46	2088	*P. guajava*	*C. gloeosporiodes*	Fruit	Porto Belo, SC	2014	OR429698	OR475872	–	OR475804	OR475951
256	2218	*P. guajava*	*C. theobromicola*	Fruit	Jaraguá, GO	2016	OR429738	OR475908	–	OR475838	OR475991
265		*P. guajava*	*C. theobromicola*	Fruit	Jaraguá, GO	2016	OR429743	OR475913	–	OR475840	OR475996
222	2224	*P. guajava*	*C. syzygicola*	Fruit	Ananindeua, PA	2014	OR429728	OR475899	–	OR475827	OR475981
41	2248	*P. guajava*	*C. melonis*	Leaf	S. Francisco do Sul, SC	2014	OR429695	OR475869	–	–	OR475948
8	2250	*P. guajava*	*C. melonis*	Fruit	S. Francisco do Sul, SC	2014	OR429683	OR475856	OR452452	–	OR475934
30	2238	*P. guajava*	*C. melonis*	Fruit	Guaratuba, PR	2014	OR429689	OR475863	OR452453	–	OR475942
32	2076	*P. guajava*	*C. melonis*	Fruit	Tijucas, SC	2014	OR429690	OR475864		–	OR475943
80	2237	*P. guajava*	*C. melonis*	Fruit	S. João Batista, SC	2014	OR429703	OR475877	OR452456	–	OR475956
40	2249	*P. guajava*	*C. melonis*	Leaf	Nova Trento, SC	2014	OR429694	OR475868	OR452455	OR475803	OR475947
36	2233	*P. guajava*	*C. melonis*	Leaf	Juquiá, SP	2014	OR429691	OR475865	OR452454	–	OR475944
DF15	2211	*P. guajava*	*C. melonis*	Fruit	Brazlâindia, DF	2015	OR429759	–	OR452458	–	OR476012
DF13		*P. guajava*	*C. paranaense*	Fruit	Brazlândia, DF	2015	–	OR475928	–	–	–
202	2221	*P. guajava*	*C. nymphaeae*	Fruit	Turuçu, RS	2014	OR429718	OR475891	–	–	OR475971
120	2247	*P. guajava*	*C. nymphaeae*	Fruit	Gama, DF	2013	OR429707	OR475881	–	–	OR475960
9	4987	*P. guajava*	*C. nymphaeae*	Fruit	Canelinha, SC	2014	OR429684	OR475857	–	–	OR475935
250 PT		*P. guajava*	*C. nymphaeae*	Fruit	Brasília, DF	2014	OR429755	OR475925	–	–	OR476008
DF3	2210	*P. guajava*	*C. nymphaeae*	Fruit	Brazlândia, DF	2015	OR429761	OR475930	OR452459	–	OR476014
12	2236	*P. guajava*	*C. nymphaeae*	Fruit	Canelinha, SC	2014	OR429685	OR475858	–	–	OR475936
191	2081	*P. guajava*	*C. karsti*	Fruit	Petrolina, PE	2014	OR429712	OR475886	–	–	OR475965
192	4997	*P. guajava*	*C. karsti*	Fruit	Petrolina, PE	2014	OR429713	OR475887	–	–	OR475966
01	2087	*P. guajava*	*C. karsti*	Fruit	Nova Veneza, GO	2014	OR429680	OR475853	–	–	OR475931
DF01		*P. guajava*	*C. karsti*	Fruit	Brazlândia, DF	2015	OR429758	OR475927	–	–	OR476011
278	2084	*P. guajava*	*C. karsti*	Fruit	Jaraguá, GO	2016	OR429746	OR475916	–	–	OR475999
264		*P. guajava*	*C. sojae*	Fruit	Jaraguá, GO	2016	OR429742	OR475912	–	–	OR475995
263	2075	*P. guajava*	*C. gigasporum*	Fruit	Jaraguá, GO	2016	OR429741	OR475911	–	–	OR475994
38	2078	*P. guajava*	*C. gigasporum*	Fruit	Juquiá, SP	2014	OR429692	OR475866	–	–	OR475945
257		*P. guajava*	*C. gigasporum*	Fruit	Jaraguá, GO	2016	OR429739	OR475909	–	–	OR475992
602JAM	272	*Syzygium jambos*	*C. pseudoacutatum*	Fruit	Brasília, DF	2016	KY344755	KY344754	–	–	KY344753

[a]ID codes as shared with NCBI and as documented by Soares (2017).

[b]Two letter state abbreviation as in Table 1.

TABLE 3 Primers employed for identification of *Colletotrichum* isolates from *Psidium guajava* and other Myrtaceae.

Genomic region	Primer	Sequence (5′–3′)	Reference
GAPDH	GD-F	GCCGTCAACGACCCCTTCATTGA	Templeton et al. (1992)
	GD-R	GGGTGGAGTCGTACTTGAGCATGT	
β-tubulin	T1	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
	T2	TAGTGACCCTTGGCCCAGTTG	
ITS	ITS-1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	ITS 4	TCCTCCGCTTATTGATATGC	White et al. (1990)
HIS3	CYLH3F	AGGTCCACTGGTGGCAAG	Crous et al. (2004)
	CYLH3R	AGCTGGATGTCCTTGGACTG	
ApMAT	CgDL_F6	AGTGGAGGTGCGGGACGTT	Rojas et al. (2010)
	CgMAT1_F2	TGATGTATCCCGACTACCG	

and Cortibel RM (a.k.a. Rugosa Média or Cascão). Asymptomatic, half-ripe fruits (peel colour index # 2; Vieira et al., 2008), selected for uniformity, were washed in running water, surface disinfected with 70% ethanol for 1 min and 1% NaOCl for 5 min, then dried and rinsed with distilled water before inoculation. Inoculation was conducted using the toothpick tip method (wooden toothpicks containing fungal mycelium; Crall, 1952) at 3 mm depth. Control fruits were mock-inoculated with noninfested toothpicks. Fruits were incubated in the dark at 25 ± 2°C. Experimental units were composed of four inoculation points per fungal isolate (per cultivar) in the same fruit, with these replicated five times (five different fruits). Inoculated fruits were initially maintained for 48 h in humid plastic bags (25 ± 2°C), with bags subsequently removed and fruits kept under the same incubation conditions in growth chambers for the observation of symptom development. At 7 and 12 days after inoculation (DAI), lesion diameters were measured with a graduated ruler. At the end of each bioassay, the fungi were reisolated from fruit tissues, cultivated on PDA as described and then compared with the original isolates. Aggressiveness (Andrivon, 1993) was estimated based on the lesion size at 7 and 12 DAI, with analysis of variance and mean comparison (Scott–Knott, $\alpha = 0.05$) conducted with the SASM-AGRI software (https://sasm-agri.software.informer.com/8.1/).

3 | RESULTS

3.1 | Identity of isolates

Colletotrichum spp. were recovered from 161 *P. guajava* plant samples and 10 additional plant species belonging to the *Myrtaceae* (Soares, 2017) covering most of Brazilian biomes and macroclimates (Table 1; Alvares et al., 2014). The 250 bp amplicons of the *GAPDH* gene were used as an initial measure of the isolate genetic diversity, with four main clades identified by Bayesian phylogenetic analysis, corresponding to the following complexes: gloeosporioides ($n = 138$ isolates), acutatum ($n = 24$), boninense ($n = 5$) gigasporum ($n = 3$) and orchidearum ($n = 1$).

The multigenic phylogenetic study with the sequence data for fragments of the five genomic regions *GAPDH*, *ApMAT*, ITS, *TUB2* and *HIS3*, conducted with 82 guava-derived isolates plus four additional *E. uniflora* isolates, identified 16 *Colletotrichum* species (Figure 1).

Sixty-two isolates were found to belong to the *gloeosporioides* complex, of which 59 were from *P. guajava* and three from *E. uniflora*. The guava-derived isolates in the *gloeosporioides* complex were grouped in 10 clades, corresponding to *C. siamense* ($n = 34$), *C. chrysophilum* ($n = 9$), *C. fructicola* ($n = 8$), *C. tropicale* ($n = 3$), *C. gloeosporioides* ($n = 2$), *C. theobromicola* ($n = 2$), *C. asianum* ($n = 1$), *C. aeschynomenes* ($n = 1$), *C. musae* ($n = 1$) and *C. syzygicola* ($n = 1$). The three isolates from *E. uniflora* belonged to the *C. siamense* clade (248PT, 249PT and 252PT).

Fifteen isolates (14 from guava and one from *E. uniflora*) were observed in the *acutatum* complex. The *P. guajava* isolates were grouped into three clades: *C. nymphaeae* ($n = 5$), *C. melonis* ($n = 8$) and *C. paranaense* ($n = 1$). The *E. uniflora* isolate (250PT) was grouped in the *C. nymphaeae* clade.

All five isolates in the *boninense* complex were obtained from *P. guajava* and were found to belong in the *C. karsti* clade. One guava-derived isolate in the *orchidearum* clade was identified as *C. sojae*. The multigenic phylogenetic analysis in the complex *gigasporum* identified three guava isolates as *C. gigasporum*.

3.2 | Prevalence of *Colletotrichum* species according to geographic region

Figure 2 illustrates the species prevalence of 62 samples belonging to the *C. gloeosporioides* complex (Figure 2a) and 15 samples belonging to the *C. acutatum* complex (Figure 2b), according to physiographic region.

Five samples belonging to *C. karsti* (*C. boninense* complex) were distributed in states of the northeastern and central west regions of Brazil (PE, GO, DF). Of the three samples of *C. gigasporum* (sensu stricto), two were from the central west (GO) and one from the southeast (SP). The only representative of the *C. orchidearum* complex (*C. sojae*) was collected in the central west region (GO).

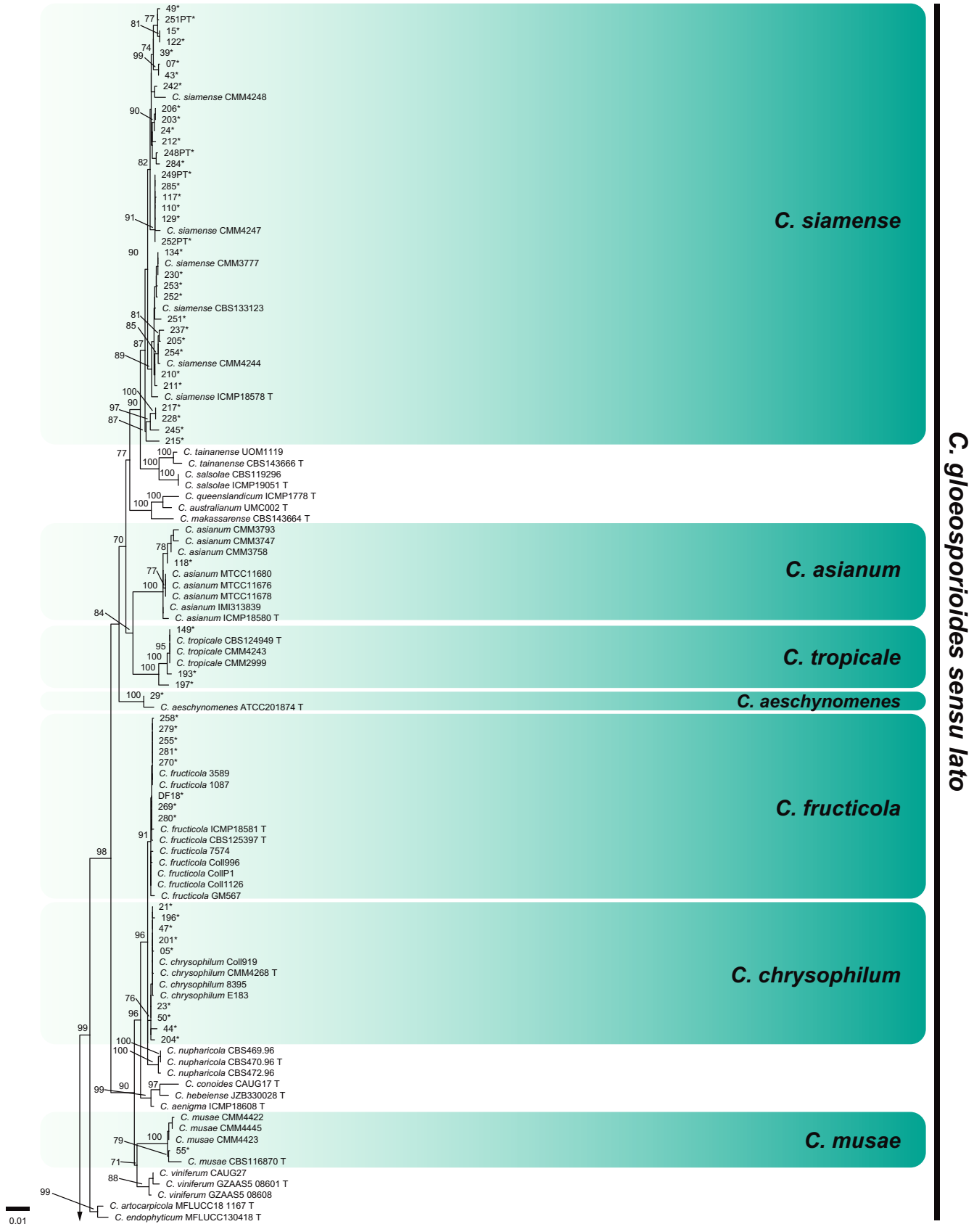

FIGURE 1 Maximum-likelihood tree of *Colletotrichum* inferred from a concatenated alignment of *ACT*, *APN2*/*MAT*-IGS, *GAPDH*, *HIS3* and *TUB2* gene regions. Significant supports (≥70) are shown above the branches. Ex-types are indicated with a 'T' after the culture collection number. Isolates from the present study are indicated with an asterisk. The tree is rooted at the midpoint. The scale bar indicates the average number of substitutions per site.

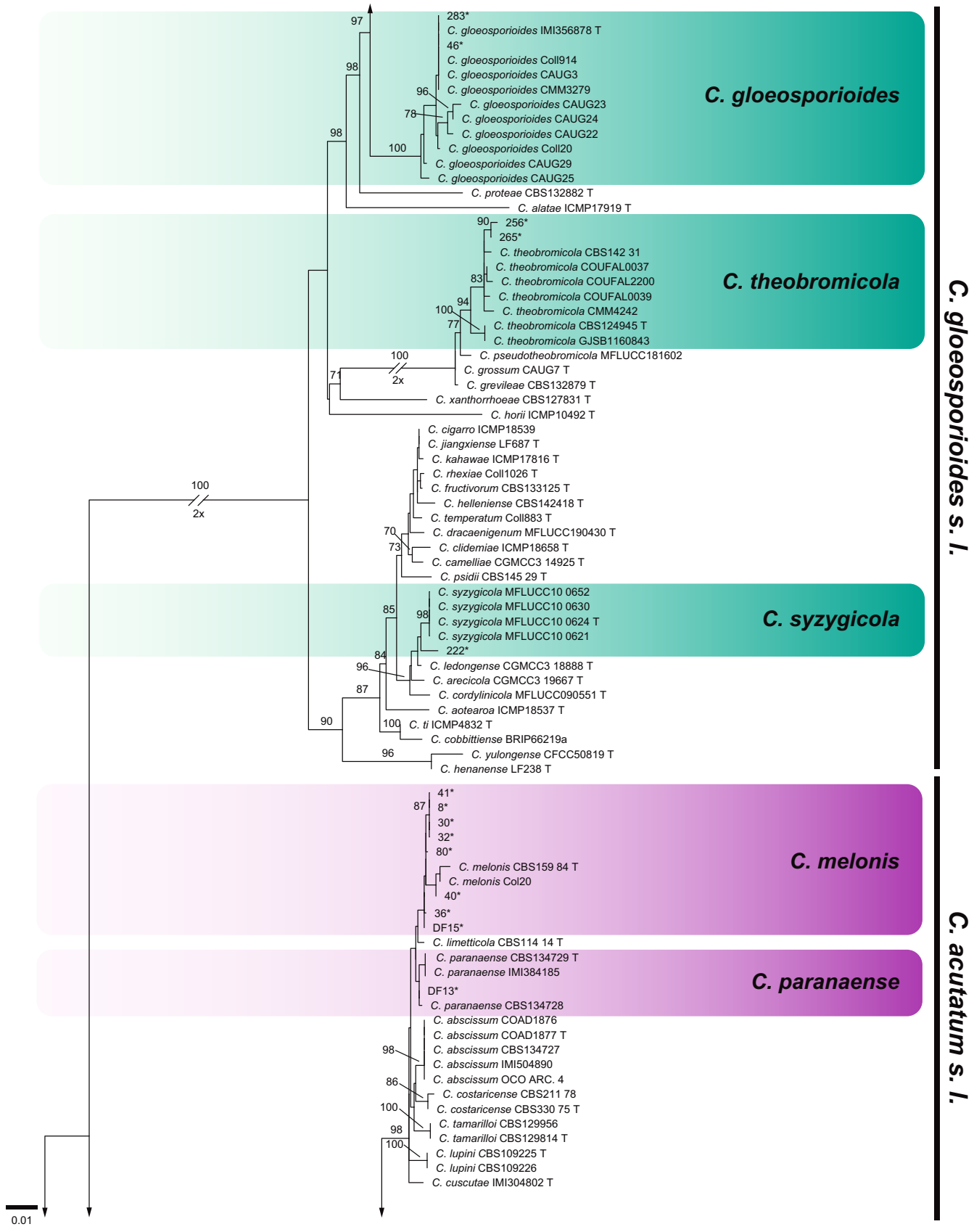

FIGURE 1 (Continued)

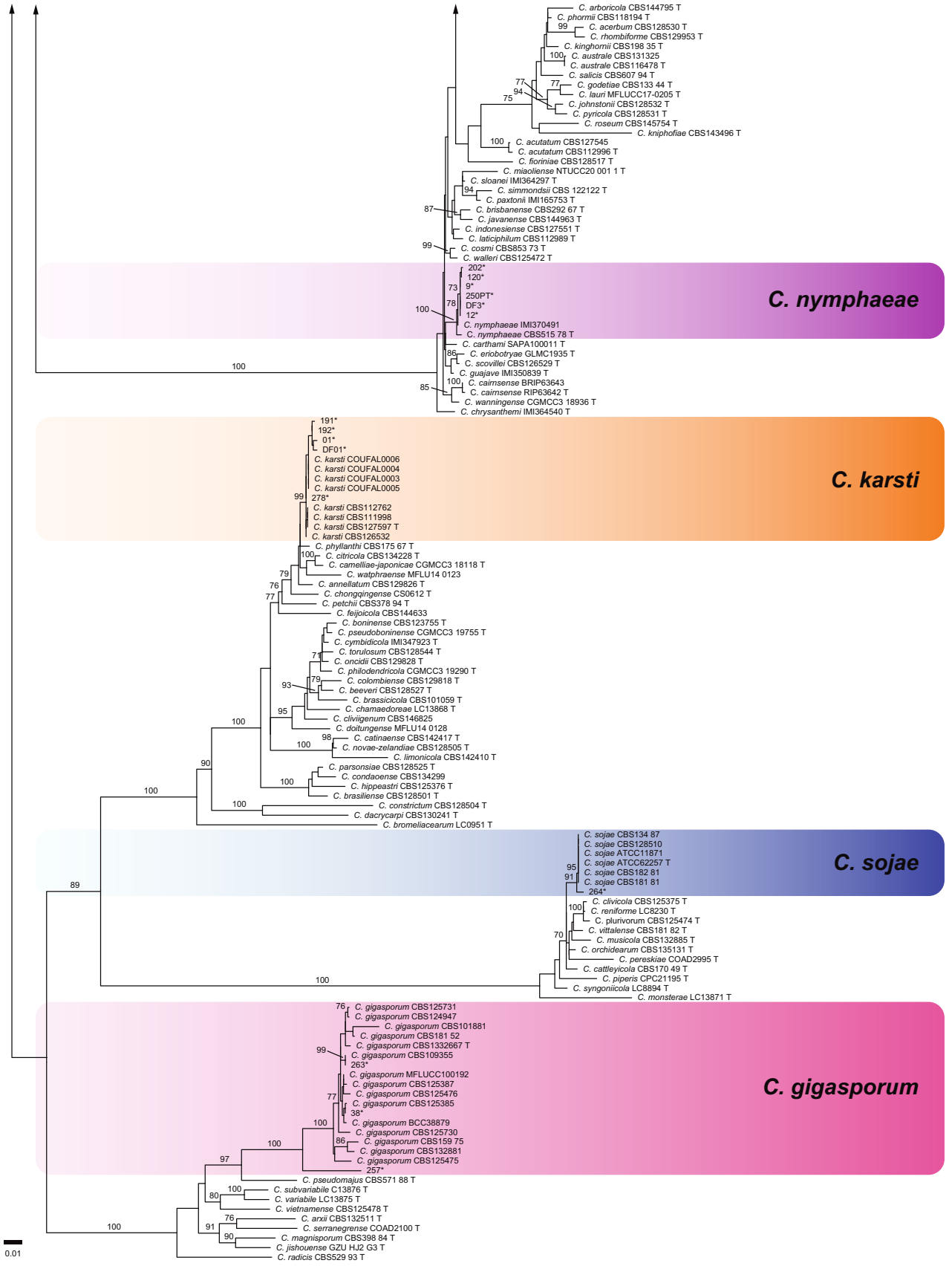

FIGURE 1 (Continued)

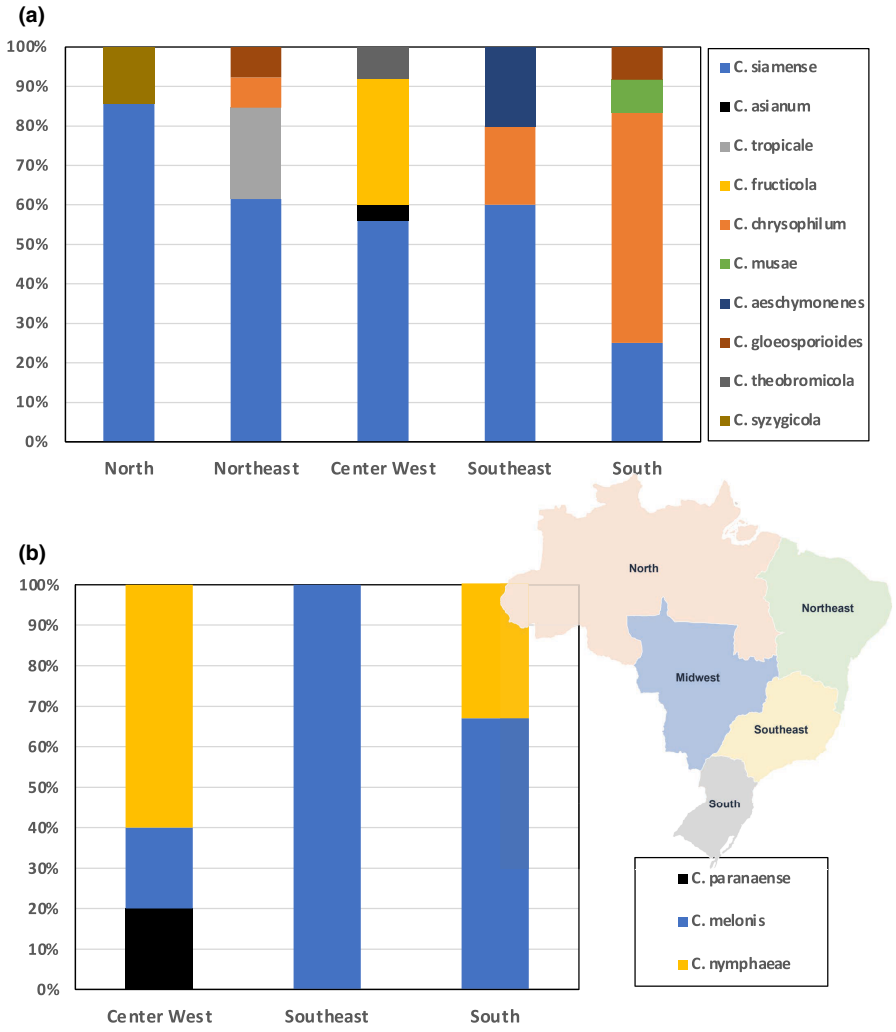

FIGURE 2 Prevalence of *Colletotrichum* species of the (a) *C. gloeosporioides* complex ($n = 62$) and (b) *C. acutatum* complex ($n = 15$) that were associated with guava anthracnose in Brazil, by physiographic region. Members of *C. acutatum* were not found in the northern and northeastern regions.

3.3 | Fruit tissue colonization and aggressiveness

All 26 representatives of the complexes *gloeosporioides*, *acutatum*, *gigasporum*, *boninense*, *orchidearum* and *C. pseudoacutatum* were able to colonize unripe fruits of both guava varieties. Typical anthracnose symptoms developed 7 DAI and progressed further up to 12 DAI, when the assays were terminated (Table 4). Disease symptoms are illustrated in Figure 3 for members of the *C. gloeosporioides* complex. All *Colletotrichum* isolates were recovered by direct isolation from conidial masses of all treatments. Mock-inoculated fruit developed no symptoms.

Although all isolates were able to colonize guava fruit tissue, an extensive variation in aggressiveness (sensu Andrivon, 1993) was observed, as estimated by the speed of tissue colonization. Furthermore, the *Colletotrichum* isolates from the other *Myrtaceae* species (381ARC, 418JR, 249PT and 602JAM; Table 4) were also able to colonize guava fruit, and often very aggressively. No differential interaction in aggressiveness was evident between the pathogen and the host genotypes studied, that is, the most (or least) aggressive isolates to Cortibel RM were also the most (or least) aggressive isolates to Cortibel SLG (Table 4).

Despite the fact that both Cortibel RM and Cortibel SLG were susceptible to the range of isolates tested, the latter (SLG) was consistently less diseased than RM. Overall, mean lesion size in Cortibel SLG was 23% and 16% smaller than in Cortibel RM at 7 and 12 DAI, respectively. While this work did not focus on host resistance, it is worth mentioning that the relative response of the two varieties stood across a wide range of treatments (isolates), indicating that quantitative resistance to *Colletotrichum* is present in the *P. guajava* germplasm.

Four species of the *gloeosporioides* complex, namely *C. siamense*, *C. fructicola*, *C. syzygicola* and *C. tropicale*, were found to be the most aggressive, causing mean lesions 50% (on RM) and 41% (on SLG) larger than the overall mean lesion size calculated for the bulk of isolates 7 DAI. Corresponding mean figures for these four species on RM and SLG at 12 DAI were 48% and 48%, respectively.

4 | DISCUSSION

This is the first broad study to focus on the identity and distribution of the guava anthracnose pathogen in Brazil, based on a collection gathered over a wide area between latitudes 01°27′ S and 31°25′ S and covering a range of diverse physiographic regions. We examined samples obtained mostly from small backyard orchards, but also included representatives of commercial fields and spontaneous trees.

TABLE 4 Aggressiveness of *Colletotrichum* spp. to fruits of guava varieties Cortibel RM and Cortibel SLG, measured by lesion diameter (mm).

			Lesion diameter			
			7 DAI		12 DAI	
Isolate code[a]	*Colletotrichum* complex	Species	Cortibel RM	Cortibel SLG	Cortibel RM	Cortibel SLG
284	*C. gloeosporioides*	*C. siamense*	19.0 a	14.0 a	26.3 b	23.2 a
381ARC		*C. siamense*	16.3 a	14.3 a	30.3 a	23.8 a
418JR		*C. siamense*	17.2 a	12.3 b	28.1 a	23.0 a
254		*C. siamense*	15.9 a	13.4 a	24.2 b	21.6 b
215		*C. siamense*	15.7 a	10.6 c	21.3 c	17.9 c
249PT		*C. siamense*	15.7 a	13.2 a	19.5 c	14.7 d
39		*C. siamense*	11.7 b	10.4 c	21.6 b	18.3 c
203		*C. siamense*	8.6 c	5.3 f	20.4 c	16.1 d
193		*C. tropicale*	16.6 a	8.3 d	31.3 a	15.8 d
222		*C. syzygicola*	16.3 a	10.2 c	25.0 b	20.1 b
204		*C. chrysophilum*	14.3 b	12.2 b	25.0 b	18.9 c
255		*C. fructicola*	15.0 a	9.6 c	25.3 b	17.7 c
265		*C. theobromicola*	9.7 c	6.7 e	15.8 d	13.2 e
46		*C. gloeosporioides*	6.0 d	6.3 e	16.4 d	19.4 c
283		*C. gloeosporioides*	6.0 d	3.8 g	15.0 d	10.0 f
46		*C. asianum*	5.6 d	5.2 f	5.6 f	12.1 e
283		*C. musae*	4.8 d	4.4 f	5.1 f	6.8 g
DF3	*C. acutatum*	*C. nymphaeae*	8.5 c	6.7 e	17.4 d	14.8 d
202		*C. nymphaeae*	7.7 c	3.8 g	11.7 e	7.0 g
40		*C. melonis*	6.5 d	4.9 f	14.8 d	10.9 f
41		*C. melonis*	5.6 d	6.4 e	10.1 e	9.1 f
263	*C. gigasporum*	*C. gigasporum*	4.8 d	4.8 f	5.5 f	5.7 g
257		*C. gigasporum*	3.9 d	4.4 f	4.5 f	7.6 g
264	*C. orchidearum*	*C. sojae*	4.0 d	4.3 f	4.2 f	4.7 h
DF1	*C. boninense*	*C. karsti*	4.5 d	4.7 f	4.5 f	5.7 g
602JAM	n.d.	*C. pseudoacutatum*	4.7 d	4.8 f	7.0 f	6.3 g
Overall average			10.2	7.9	16.8	14.0
Mock-inoculated			2.5 d	2.5 g	2.5 f	2.5 h

Note: Means followed by same letter in columns do not differ (Scott–Knott, $\alpha = 0.05$). DAI, days after inoculation with the toothpick method. Fruits were at the half-ripe stage at inoculation, and bioassays were conducted at 25°C. nd, complex not defined.

[a]381ARC originally from *Psidium firmum*; 249PT originally from *Eugenia* sp.; 418JR originally from *Syzygium* sp. and 602JAM originally from *Syzygium jambos*. All other isolates from *Psidium guajava*.

Isolates corresponding to *C. gloeosporioides* s. l. and *C. acutatum* s. l., traditionally referred to as the causal agents of guava anthracnose in Brazil, were also the most frequently identified in this study. However, in total, 16 *Colletotrichum* species (sensu stricto) were found associated with *P. guajava*: *C. aeschynomenes*, *C. asianum*, *C. chrysophilum*, *C. fructicola*, *C. gigasporum*, *C. gloeosporioides*, *C. karsti*, *C. melonis*, *C. musae*, *C. nymphaeae*, *C. paranaense*, *C. siamense*, *C. sojae*, *C. syzygicola*, *C. theobromicola* and *C. tropicale*. Of these, only *C. nymphaeae* and *C. gloeosporioides* have been reported previously in guava (Bragança et al., 2016) and the remaining 14 are here reported for the first time.

Nevertheless, it is worth noticing that *C. acutatum* and *C. abscissum*, reported in Brazil by Bragança et al. (2016), and *C. simmondsii*, by Cruz

FIGURE 3 Aggressiveness of isolates of the *Colletotrichum gloeosporioides* complex to *Psidium guajava* 'Cortibel SLG' (1, 3) and 'Cortibel RM' (2, 4) at 7 and 12 days after inoculation (DAI), respectively. (A) Mock-inoculated control. (B–I) *C. siamense*, isolates 39, 284, 215, 254, 203, 418JR, 381ARC and 249PT, respectively. (J) *C. asianum* (118). (K and L) *C. fructicola* (204 and 255). (M) *C. gloeosporioides* s. l. (222). (N) *C. theobromicola* (265). (O) *C. tropicale* (193). (P and Q) *C. gloeosporioides* (46, 283). (R) *C. musae* (55).

et al. (2015), were not found in this survey. Similarly, *C. psidii* or *C. guajavae* mentioned by Zakaria (2021) were not found. These two latter species have not been recorded in the Americas nor have they been demonstrated to be pathogenic to guava.

It is significant that guava and the other *Myrtaceae* included in this study (*P. firmum*, *E. uniflora*, *S. jambos* and *Syzygium* sp.) are endemic botanical species throughout the sampled regions, and that the ubiquity of anthracnose over a wide area indicates that the *Colletotrichum*–*P. guajava* pathosystem is possibly native to Brazil, and probably very ancient. This may partially explain the great species diversity found among the *Colletotrichum* associated with *P. guajava*. Indeed, the fact that isolates from other *Myrtaceae* (such as 381ARC from *P. firmum*, 418JR from *Syzygium* sp., 249PT from *E. uniflora* and 602JAM from *S. jambos*) were found to cause anthracnose on guava support the hypothesis of the endemic association of some of the *Colletotrichum* species with guava and pose practical management concerns. While several of the fungus species discussed in this study occur throughout the world and have wide host ranges (e.g., *C. nymphaeae*, *C. karsti*, *C. fructicola*, *C. gloeosporioides* and *C. siamense* have over 50 and up to 173 hosts), some are much more restricted, both in territory and host range. For instance, *C. melonis* has previously only been reported in Brazil and neighbouring Uruguay and on only three plant species, according to the U.S. National Fungus Collections Databases (https://fungi.ars.usda.gov/). Similarly, *C. paranaense* is reported only from three hosts, again mostly from Brazil. It is likely that these two species are endemic to the Brazilian territory. Furthermore, *C. syzygicola* is the first record in the Americas; this species was previously reported only in Thailand on *Syzygium samarangense*, another species of the *Myrtaceae* family. The detection of *C. asianum*, mostly known as a mango pathogen, and *C. sojae*, a pathogen of leguminous field crops, is also noteworthy. Mango trees are widespread across all Brazilian regions (FAOSTAT, 2023), which makes it one putative source of *C. asianum* inoculum to guava. Likewise, *C. sojae* is a soybean (*Glycine max*) pathogen, a legume planted annually almost uninterruptedly from the southern Amazon to southern Brazil (Dias et al., 2019), with several associated *Colletotrichum* species recorded with it, including *C. sojae* (Boufleur et al., 2021).

The diversity of species found in this study reflects, and even surpasses, the diversity found in earlier studies conducted with other tropical fruit species in a single country, such as mango (Lima et al., 2013) and papaya (Vieira et al., 2022). Even accounting for the worldwide variability of *Colletotrichum* species found on mango ($n = 26$) or papaya ($n = 17$), the list of 16 species detected in this study in one (admittedly very large) country is impressive. Such variability of *Colletotrichum* species helps to explain the ubiquitous presence of guava anthracnose over widely separated regions in diverse physical and ecological environments and over variable host genotypes. Nevertheless, some prevalence patterns among regions are apparent at the species level. For example, *C. siamense* is the most frequent species found in the warm climates of the north, northeast, centre west and southeast regions, while it is in a minority in the more temperate south. Conversely, *C. chrysophilum*, which is also widespread, is dominant in the south, but becomes less common in the warmer regions of the north, northeast and centre west. That may reflect better adaptation of these two species to each physiographic region.

Some of the *Colletotrichum* species found in this study have already been reported infecting other plant hosts in Brazil. Some of these, such as *C. siamense*, *C. fructicola*, *C. chrysophilum*, *C. theobromicola*, *C. tropicale*, *C. gloeosporioides* and *C. karsti*, are ubiquitous plant pathogens, infecting several other tropical fruit crops in Brazil (e.g., Bragança et al., 2014; Lima et al., 2013; Silva et al., 2021; Soares et al., 2020; Veloso et al., 2018; Vieira et al., 2022). Therefore, a flow of *Colletotrichum* isolates between guava (and other *Myrtaceae*) and other botanical families cannot be ruled out.

The bioassays unveiled widespread variation in aggressiveness among the *Colletotrichum* of *P. guajava*. Yet, even if many *Colletotrichum* species were found naturally associated to guava anthracnose, it was clear that those belonging to the *C. gloeosporioides* complex (especially *C. siamense*. *C. chrysophilum* and *C. fructicola*) are the most aggressive and prevalent. *C. siamense* is clearly the most frequently found species in *P. guajava* in Brazil, distributed through all physiographic regions, and also present in other *Myrtaceae*. This species has also been recorded on guava in India, the world's largest producer, as well as in other top producers, such as Indonesia and Mexico (Zakaria, 2021). Therefore, *C. siamense* should be seen as the main target for the development of host genetic resistance. Fortunately, partial resistance to anthracnose appears to be available in the *P. guajava* germplasm, as Cortibel SLG (also referred to as Gigante) consistently developed smaller lesions, which agrees with unpublished reports from growers and extension personnel. Therefore, further investigation is warranted into screening of guava germplasm for resistance to anthracnose.

In conclusion, approximately 81% of the isolates in this study were found in the *gloeosporioides* complex, including the four most aggressive (*C. siamense*, *C. chrysophilum*, *C. fructicola* and *C. tropicale*). *C. siamense*, followed by *C. chrysophilum* and *C. fructicola*, were also the most abundant. However, it is noteworthy that other members of the *gloeosporioides* complex, such as *C. musae*, *C. asianum* and *C. theobromicola*, were much less aggressive. Therefore, the mere identification of the causal agent at the level of the *Colletotrichum* complex is not sufficient for a precise estimate of the risk of production loss at any specific situation; accurate determination of the causal agent at the species level is foremost for successful guava anthracnose management.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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