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Species of the *Colletotrichum acutatum* complex associated with anthracnose diseases of fruit in Brazil

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ABSTRACT

Although *Colletotrichum acutatum* was recently investigated and shown to be a species complex comprising about 30 species, the name is still used in its broad sense for anthracnose pathogens of fruits in Brazil. In this study, a multilocus molecular analysis was carried out based on a dataset of ITS, HIS3, GAPDH, CHS-1, TUB2 and ACT sequences of *Colletotrichum* strains belonging to the *C. acutatum* species complex from fruits collected in different regions in Brazil combined with sequences of ex-type and other reference strains of species belonging to this complex. The strains were revealed to belong to *Colletotrichum nymphaeae*, *Colletotrichum melonis*, *Colletotrichum abscissum* and one new species, namely *Colletotrichum paranaense*, from apple and peach. Morphological descriptions of the new species and a strain closely related to but diverging from *C. melonis* are provided. From the data presently available, the most common species on apple fruits in Brazil is *C. nymphaeae*. In a pathogenicity test, strains of all four species caused lesions on detached apple, peach and guava fruits, except for strain CBS 134730 that did not infect guava fruits.

Introduction

Colletotrichum species are economically important plant pathogens, especially in tropical, subtropical and temperate regions, where they affect a wide range of plant hosts (Sutton 1992). The most common symptoms associated with *Colletotrichum* infections are sunken necrotic lesions, on which often orange conidial masses are produced, and that are

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referred to as anthracnose (Freeman et al. 1998). *Colletotrichum* species are considered as major pathogens associated with pre- and post-harvest fruit diseases, which cause yield losses mainly in tropical and subtropical areas (Baylei & Jeger 1992; Hyde et al. 2009; Phoulivong et al. 2010). Bitter rot, for example, is considered as one of the most important diseases of apple fruit that can cause up to 50 % yield loss (Sutton 1990).

In the pre-molecular era, the taxonomy of *Colletotrichum* species was predominantly based on morphological and cultural characters such as size and shape of conidia and appressoria, presence or absence of setae, colony colour and growth rate (von Arx 1957; Sutton 1980, 1992). However, these characters are not always reliable for species differentiation due to their variability under changing environmental conditions (Cai et al. 2009).

To date much attention has also been given to conidium morphology (shape and dimensions). For instance, the acute conidial ends resulting in the typical fusiform shape are supposed to be one of the most important morphological features of *Colletotrichum acutatum* (Simmonds 1965). However, the name *C. acutatum* was applied to many species with more or less fusiform conidia, most of them being closely related to *C. acutatum* s. str., and conidial shape can show significant variation within the species and even among the strains of the same species (Damm et al. 2012). Several studies have demonstrated that the fusiform conidial shape is not a consistent feature in the *C. acutatum* species complex. For example, Talhinhas et al. (2002) observed that *Colletotrichum* isolates from *Lupinus* spp. (=*Colletotrichum lupini*) form different proportions of conidia with round ends or one round and one acute end, corresponding with rather cylindrical or clavate shapes. In a recent study, many strains that were previously identified as *Colletotrichum gloeosporioides* based on the more or less cylindrical conidia that are typical for the species (Cannon et al. 2008), were revealed to belong to different species of the *C. acutatum* complex (Damm et al. 2012). Furthermore, there are species with acute-ended conidia that are phylogenetically distinct from the *C. acutatum* complex, for example, *Colletotrichum pseudoacutatum* and *Colletotrichum proteae* (Cannon et al. 2012; Damm et al. 2012; Liu et al. 2013).

Colletotrichum acutatum is known to be genetically highly variable and was divided in infraspecific groups on the basis of molecular data (Guerber et al. 2003; Sreenivasaprasad & Talhinhas 2005). Later it was suggested to be a species complex and separate species were accepted, for example *Colletotrichum clavatum*, *Colletotrichum fioriniae*, and *Colletotrichum phormii* (Farr et al. 2006; Shivas & Tan 2009; Faedda et al. 2011). Only recently, the *C. acutatum* species complex was revised applying a multilocus molecular approach on a large number of strains from numerous hosts worldwide, recognising 31 species (Damm et al. 2012).

Bitter rot of apple is a serious disease of this crop caused by numerous species within the *C. acutatum* species complex. *C. fioriniae* seems to be the species within the *C. acutatum* complex most frequently associated with apples in the USA, while strains from New Zealand belong to *Colletotrichum acerbum*, *C. fioriniae*, *Colletotrichum pyricola* and *Colletotrichum salicis* (Guerber et al. 2003; Damm et al. 2012). Most of the *Colletotrichum* strains from apple causing bitter rot in Croatia were identified as *C. fioriniae* and some as *C. clavatum* (synonym of *Colletotrichum godetiae*) based on ITS sequences (Ivic et al. 2012). A first report of apple bitter rot in the United Kingdom has been also associated with *C. godetiae* (Baroncelli et al. 2014). A further strain had the same ITS sequence as strains BBA 65797 and IMI 345581, the latter previously identified as *C. salicis* by Damm et al. (2012). However, related species may have the same ITS sequence, as this locus is not always informative at the species level in the *C. acutatum* species complex. Other strains from apples in Europe included in the study of Damm et al. (2012) also belong to *C. fioriniae* and *C. godetiae*. In contrast, strains from apple in Japan identified by Sato & Moriwaki (2013) based on a multilocus analysis belong to *C. nymphaeae* and *C. godetiae*. Strains from bitter rot of apple in Korea in the study of Lee et al. (2007) probably belong to *C. fioriniae* and a species of *C. acutatum* clade 2 (*C. nymphaeae* and related species) according to Damm et al. (2012). However, previously isolated strains from apple and other fruits from Brazil belonging to the *C. acutatum* species complex were only identified as *C. acutatum* s. lat. (González et al. 2006; Giaretta et al. 2010). If sequences of these strains were generated at all, only ITS sequences are available on GenBank that do not allow accurate species identification within this complex. Therefore, the name *C. acutatum* is still used in its broad sense in Brazil (Serra et al. 2011; Barquero Quirós et al. 2013; Ciampi-Guillardi et al. 2013; de Souza et al. 2013). To date, only few studied species within the *C. acutatum* species complex occurring in Brazil have been identified, including a disease report of *C. nymphaeae* causing apple bitter rot in southern Brazil (Velho et al. 2014). The aim of the present study, therefore, was to identify *Colletotrichum* strains belonging to the *C. acutatum* species complex associated with different fruit crops from different localities in Brazil by means of multilocus molecular as well as morphological data, and to describe the new species encountered.

Material and methods

Isolates

A total of 17 *Colletotrichum* strains isolated from anthracnose symptoms on apple, peach and guava fruits from different localities in Brazil and tentatively identified as *Colletotrichum acutatum* s. lat. based on conidial morphology was included in this study (Table 1). Type material of new species recognised in this study was deposited in the fungarium of the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands. Subcultures of the ex-type and other isolates used for morphological and sequence analyses are maintained in the culture collections of the CBS and CPC (personal collection of Pedro Crous), Utrecht, The Netherlands and in the *Colletotrichum* collection of the Mycology laboratory, Escola Superior de Agricultura 'Luiz de Queiroz', in Piracicaba, Brazil.

Phylogenetic analyses

Genomic DNA used in this study was extracted according to Murray & Thompson (1980). The PCR reactions were performed in a total volume of 12.5 μL using a 2720 Thermal Cycler (Applied Biosystems, MA, USA). The 5.8S nuclear

Table 1 – Strains of *Colletotrichum* spp. in the *C. acutatum* complex studied with accession numbers, host, country and GenBank numbers.

Species	Accession numbers[a]	Host	Country/location	GenBank numbers[b]					
				ITS	GAPDH	CHS-1	HIS3	ACT	TUB2
C. absissum	CBS 134727, Col 10, CPC 20894, RB239	*Psidium guajava*	Brazil, Cafelândia	KC204988	KC205022	KC205039	KC205003	KC205073	KC205056
	COAD 1877[d]	*Citrus x sinensis*	Brazil, São Paulo	KP843126	KP843129	KP843132	KP843138	KP843141	KP843135
	OCO-ARC-4, RB196	*Citrus x sinensis*	USA, Florida, Arcadia	KT153559	KT153549	KT153544	KT153554	KT153539	KT153564
	IMI 504890, STF-FTP-10, RB197	*Citrus x sinensis*	USA, Florida, Frostproof	KT153558	KT153548	KT153543	KT153553	KT153538	KT153563
C. acerbum	CBS 128530, ICMP 12921, PRJ 1199.3[d]	*Malus domestica*	New Zealand	JQ948459	JQ948790	JQ949120	JQ949450	JQ949780	JQ950110
C. acutatum	CBS 112996, ATCC 56816, STE-U 5292[d]	*Carica papaya*	Australia	JQ005776	JQ948677	JQ005797	JQ005818	JQ005839	JQ005860
	CBS 112980, STE-U 164, RB175	*Pinus radiata*	South Africa	JQ948356	JQ948687	JQ949017	JQ949347	JQ949677	JQ950007
C. australe	CBS 116478, HKUCC 2616[d]	*Trachycarpus fortunei*	South Africa	JQ948455	JQ948786	JQ949116	JQ949446	JQ949776	JQ950106
C. brisbanense	CBS 292.67, DPI 11711[d]	*Capsicum annuum*	Australia	JQ948291	JQ948621	JQ948952	JQ949282	JQ949612	JQ949942
C. chrysanthemi	CBS 126518, PD 84/520[d]	*Carthamus* sp.	Netherlands	JQ948271	JQ948601	JQ948932	JQ949262	JQ949592	JQ949922
C. cosmi	CBS 853.73, PD 73/856[d]	*Cosmos* sp.	Netherlands	JQ948274	JQ948604	JQ948935	JQ949265	JQ949595	JQ949925
C. costaricense	CBS 211.78, IMI 309622	*Coffea* sp.	Costa Rica	JQ948181	JQ948511	JQ948842	JQ949172	JQ949502	JQ949832
	CBS 330.75[d]	*Coffea arabica*, cv. Typica	Costa Rica	JQ948180	JQ948510	JQ948841	JQ949171	JQ949501	JQ949831
C. cuscutae	IMI 304802, CPC 18873[d]	*Cuscuta* sp.	Dominica	JQ948195	JQ948525	JQ948856	JQ949186	JQ949516	JQ949846
C. fioriniae	CBS 125396, GJS 08-140A	*Malus domestica*	USA	JQ948299	JQ948629	JQ948960	JQ949290	JQ949620	JQ949950
	CBS 128517, ARSEF 10222, ERL 1257, EHS 58[d]	*Fiorinia externa*	USA	JQ948292	JQ948622	JQ948953	JQ949283	JQ949613	JQ949943
	IMI 504882, PJ7, RB111[c]	*Fragaria×ananassa*	New Zealand	KT153560	KT153550	KT153545	KT153555	KT153540	KT153565
C. guajavae	IMI 350839, CPC 18893[d]	*Psidium guajava*	India	JQ948270	JQ948600	JQ948931	JQ949261	JQ949591	JQ949921
C. godetiae	CBS 133.44[d]	*Clarkia hybrida*, cv. Kelvon	Denmark	JQ948402	JQ948733	JQ949063	JQ949393	JQ949723	JQ950053
	CBS 193.32, RB019	*Olea europaea*	Italy	JQ948415	JQ948746	JQ949076	JQ949406	JQ949736	JQ950066
C. indonesiense	CBS 127551, CPC 14986[d]	*Eucalyptus* sp.	Indonesia	JQ948288	JQ948618	JQ948949	JQ949279	JQ949609	JQ949939
C. johnstonii	CBS 128532, ICMP 12926, PRJ 1139.3[d]	*Solanum lycopersicum*	New Zealand	JQ948444	JQ948775	JQ949105	JQ949435	JQ949765	JQ950095
C. kinghornii	CBS 198.35[d]	*Phormium* sp.	UK	JQ948454	JQ948785	JQ949115	JQ949445	JQ949775	JQ950105
C. laticiphilum	CBS 112989, IMI 383015, STE-U 5303[d]	*Hevea brasiliensis*	India	JQ948289	JQ948619	JQ948950	JQ949280	JQ949610	JQ949940
C. limetticola	CBS 114.14[d]	*Citrus aurantifolia*	USA, Florida	JQ948193	JQ948523	JQ948854	JQ949184	JQ949514	JQ949844
C. lupini	CBS 109216, BBA 63879	*Lupinus mutabilis*	Bolivia	JQ948156	JQ948486	JQ948817	JQ949147	JQ949477	JQ949807
	CBS 109225, BBA 70884[d]	*Lupinus albus*	Ukraine	JQ948155	JQ948485	JQ948816	JQ949146	JQ949476	JQ949806
	CBS 109226, BBA 71249	*Lupinus albus*	Canada	JQ948158	JQ948488	JQ948819	JQ949149	JQ949479	JQ949809
	CBS 513.97, LARS 401	*Lupinus polyphyllus*	Costa Rica	JQ948157	JQ948487	JQ948818	JQ949148	JQ949478	JQ949808
C. melonis	CBS 159.84[d]	*Cucumis melo*	Brazil	JQ948194	JQ948524	JQ948855	JQ949185	JQ949515	JQ949845
	Col 20	*Malus domestica*	Brazil, Sao Paulo	KC204986	KC205020	KC205037	KC205006	KC205071	KC205054
C. cf. melonis	CBS 134730, CPC 20912, Col 31	*Malus domestica*	Brazil, Rio Grande do Brazil	KC204997	KC205031	KC205048	KC205007	KC205082	KC205065
C. nymphaeae	CBS 112202	*Fragaria* sp.	Spain	JQ948234	JQ948564	JQ948895	JQ949225	JQ949555	JQ949885
	IMI 299103, CPC 18871	*Fragaria vesca*	UK	JQ948231	JQ948561	JQ948892	JQ949222	JQ949552	JQ949882
	CBS 126383, PD 84/121	*Anemone coronaria*	Netherlands	JQ948221	JQ948551	JQ948882	JQ949212	JQ949542	JQ949872
	CBS 127612, DAOM 213709, H-1984	*Fragaria×ananassa*	USA	JQ948230	JQ948560	JQ948891	JQ949221	JQ949551	JQ949881
	CBS 113003, STE-U 4457	*Protea* sp.	South Africa	JQ948209	JQ948539	JQ948870	JQ949200	JQ949530	JQ949860
	IMI 360386, CPC 18925	*Pelargonium graveolens*	India	JQ948206	JQ948536	JQ948867	JQ949197	JQ949527	JQ949857
	IMI 370491, CPC 18932	*Malus pumila*	Brazil	JQ948204	JQ948534	JQ948865	JQ949195	JQ949525	JQ949855
	CPC 20897, Col 13	*Malus domestica*	Brazil, Parana	KC204989	KC205023	KC205040	KC205008	KC205074	KC205057
	CPC 20911, Col 30	*Malus domestica*	Brazil, Rio Grande do Sul	KC204996	KC205023	KC205047	KC205014	KC205081	KC205064
	CPC 20893, Col 9	*Psidium guajava*	Brazil, Cafelândia	KC204987	KC205021	KC205038	KC205005	KC205072	KC205055
	CPC 20898, Col 14	*Malus domestica*	Brazil, Sao Paulo	KC204990	KC205024	KC205041	KC205009	KC205075	KC205058
	CPC 20899, Col 15	*Malus domestica*	Brazil, Sao Paulo	KC204991	KC205025	KC205042	KC205010	KC205076	KC205059

(*continued on next page*)

Table 1 – (*continued*)

Species	Accession numbers[a]	Host	Country/location	GenBank numbers[b]					
				ITS	GAPDH	CHS-1	HIS3	ACT	TUB2
	CPC 20908, Col 27	*Malus domestica*	Brazil, Rio Grande do Sul	KC204994	KC205028	KC205045	KC205012	KC205079	KC205062
	CPC 20915, Col 34	*Malus domestica*	Brazil, Rio Grande do Sul	KC204999	KC205033	KC205050	KC205016	KC205084	KC205067
	CPC 20902, Col 21	*Malus domestica*	Brazil, Sao Paulo	KC204993	KC205027	KC205044	KC205011	KC205078	KC205061
	CPC 20913, Col 32	*Malus domestica*	Brazil, Rio Grande do Sul	KC204998	KC205032	KC205049	KC205015	KC205083	KC205066
	CPC 20910, Col 29	*Malus domestica*	Brazil, Rio Grande do Sul	KC204995	KC205029	KC205046	KC205013	KC205080	KC205063
	CPC 20916, Col 35	*Malus domestica*	Brazil, Rio Grande do Sul	KC205000	KC205034	KC205051	KC205017	KC205085	KC205068
	CPC 20917, Col 36	*Malus domestica*	Brazil, Rio Grande do Sul	KC205001	KC205035	KC205052	KC205018	KC205086	KC205069
	IMI 504889, SA-01, RB190	*Fragaria×ananassa*	Denmark	KT153561	KT153551	KT153546	KT153556	KT153541	KT153566
	CBS 515.78[d]	*Nymphaea alba*	Netherlands	JQ948197	JQ948527	JQ948858	JQ949188	JQ949518	JQ949848
C. orchidophilum	CBS 632.80[d]	*Dendrobium* sp.	USA	JQ948151	JQ948481	JQ948812	JQ949142	JQ949472	JQ949802
C. paranaense	CBS 134729, Col 19, CPC 20901[d]	*Malus domestica*	Brazil, Parana	KC204992	KC205026	KC205043	KC205004	KC205077	KC205060
	IMI 384185, CPC 18937, CPAC 8	*Caryocar brasiliense*	Brazil	JQ948191	JQ948521	JQ948852	JQ949182	JQ949512	JQ949842
	CBS 134728, Col 49, CPC 20928[d]	*Prunus persica*	Brazil, Paranapanema	KC205002	KC205036	KC205053		KC205087	KC205070
C. paxtonii	IMI 165753, CPC 18868[d]	*Musa* sp.	Saint Lucia	JQ948285	JQ948615	JQ948946	JQ949276	JQ949606	JQ949936
C. phormii	CBS 118194, AR 3546[d]	*Phormium* sp.	Germany	JQ948446	JQ948777	JQ949107	JQ949437	JQ949767	JQ950097
	CBS 102054, RB171	*Phormium* sp.	New Zealand	JQ948448	JQ948779	JQ949109	JQ949439	JQ949769	JQ950099
C. pyricola	CBS 128531, ICMP 12924, PRJ 977.1[d]	*Pyrus communis*	New Zealand	JQ948445	JQ948776	JQ949106	JQ949436	JQ949766	JQ950096
C. rhombiforme	CBS 129953, PT250, RB011[d]	*Olea europaea*	Portugal	JQ948457	JQ948788	JQ949118	JQ949448	JQ949778	JQ950108
C. salicis	CBS 607.94[d]	*Salix* sp.	Netherlands	JQ948460	JQ948791	JQ949121	JQ949451	JQ949781	JQ950111
C. scovillei	CBS 126529, PD 94/921-3, BBA 70349[d]	*Capsicum* sp.	Indonesia	JQ948267	JQ948597	JQ948928	JQ949258	JQ949588	JQ949918
	IMI 504891, Coll-25, RB198	*Capsicum annuum*	Taiwan	KT153562	KT153552	KT153547	KT153557	KT153542	KT153567
C. simmondsii	CBS 122122, BRIP 28519[d]	*Carica papaya*	Australia	JQ948276	JQ948606	JQ948937	JQ949267	JQ949597	JQ949927
C. sloanei	IMI 364297, CPC 18929[d]	*Theobroma cacao*	Malaysia	JQ948287	JQ948617	JQ948948	JQ949278	JQ949608	JQ949938
C. tamarilloi	CBS 129811, T.A.3	*Solanum betaceum*	Colombia	JQ948185	JQ948515	JQ948846	JQ949176	JQ949506	JQ949836
	CBS 129812, T.A.4	*Solanum betaceum*	Colombia	JQ948186	JQ948516	JQ948847	JQ949177	JQ949507	JQ949837
	CBS 129813, T.A.5	*Solanum betaceum*	Colombia	JQ948187	JQ948517	JQ948848	JQ949178	JQ949508	JQ949838
	CBS 129814, T.A.6[d]	*Solanum betaceum*	Colombia	JQ948184	JQ948514	JQ948845	JQ949175	JQ949505	JQ949835
Colletotrichum sp.	CBS 129810, T.A.2	*Solanum betaceum*	Colombia	JQ948179	JQ948509	JQ948840	JQ949170	JQ949500	JQ949830
	CBS 101611	Fern	Costa Rica	JQ948196	JQ948526	JQ948857	JQ949187	JQ949517	JQ949847
	CBS 129823, G8	*Passiflora edulis*	Colombia	JQ948192	JQ948522	JQ948853	JQ949183	JQ949513	JQ949843
	CBS 129820, G5	*Passiflora edulis*	Colombia	JQ948183	JQ948513	JQ948844	JQ949174	JQ949504	JQ949834
	CBS 129821, G6	*Passiflora edulis*	Colombia	JQ948182	JQ948512	JQ948843	JQ949173	JQ949503	JQ949833
C. walleri	CBS 125472, BMT(HL)19[d]	*Coffea* sp.	Vietnam	JQ948275	JQ948605	JQ948936	JQ949266	JQ949596	JQ949926

a CBS: Culture collection of Centraalbureau voor Schimmecultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Working collection of Pedro W. Crous, housed at CBS, Utrecht, The Netherlands; Col: Personal collection of Nelson Massola, housed at ESALQ/USP, Department of Plant Pathology, Piracicaba, Sao Paulo, Brazil; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; BRIP: Plant Pathology Herbarium, Department of Employment, Economic, Development and Innovation, Queensland, Australia; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; CPAC: Collection Cpac-Embrapa at Embrapa-Cerrados, Planaltina, DF, Brasil; HKUCC: The University of Hong Kong Culture Collection, Hong Kong, China; PD: Plantenziektenkundige Dienst Wageningen, Nederland; RB: Personal collection of Riccardo Baroncelli, housed at Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, Pisa, Italy.

b GenBank numbers starting with KC and KT were generated in this study.

c Baroncelli *et al.* (2014).

d Ex-holotype or ex-epitype cultures; Genbank numbers started with JQ and KP were published by Damm *et al.* (2012) and Crous *et al.* (2015), respectively.

ribosomal gene with the two flanking internal transcribed spacers (ITS), an intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and partial sequences of the chitin synthase 1 (CHS-1), actin (ACT), β-tubulin (TUB2) and histone 3 (HIS3) genes were amplified and sequenced using the primers ITS-1F (Gardes & Bruns 1993) and ITS-4 (White *et al.* 1990), GDF1 and GDR1 (Guerber *et al.* 2003), CHS-354R and CHS-79F (Carbone & Kohn 1999), ACT-512F and ACT-783R (Carbone & Kohn 1999), BT2Fd (Woudenberg *et al.* 2009) and Bt-2b (Glass & Donaldson 1995) and CYLH3F and CYLH3R (Crous *et al.* 2004b), respectively. The conditions for PCR of ITS were the same as described by Woudenberg *et al.* (2009), while those for the other genes were carried out with an initial denaturation step at 94 °C for 5 min followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, and a final step at 72 °C for 7 min. The amplicons were visualized in 1 % agarose gels stained with GelRed™ (Biotium, USA). The sequencing was performed using the BigDye terminator sequencing kit v.3.1 (Applied Biosystems, USA) and an ABI PRISM® 3100 DNA sequencer (Applied Biosystems).

The forward and reverse sequences generated were assembled using the software SeqMan v.9.0.4 (DNASTAR®, Madison/USA). Sequences of 59 ex-type and other reference strains of species belonging to the *Colletotrichum acutatum* complex as well as *Colletotrichum orchidophilum* CBS 632.80 (as outgroup), all available on GenBank, were added to the dataset and the sequences aligned with MAFFT v.6.7 (Katoh & Toh 2008). The multiple sequence alignment was manually edited with MEGA v.5.2 (Tamura *et al.* 2011).

Evolution models were estimated in MrModeltest v.3.7 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) using the Akaike information criterion (AIC) for each locus. A Bayesian inference was used to reconstruct the phylogeny based on the multilocus alignment (ITS, HIS3, GAPDH, CHS-1, TUB2, and ACT). The partitioned analysis was performed twice in MrBayers v.3.2 (Ronquist & Huelsenbeck 2003) using the Markov Chain Monte Carlo (MCMC) algorithm to generate phylogenetic trees with Bayesian posterior probabilities (BPP). Four MCMC chains were run simultaneously for random trees for 1×10^7 generations. Samples were taken every 1000 generations. The first 25 % of trees were discarded as burn-in phase of each analysis and posterior probabilities were determined from the remaining trees.

Sequences derived in this study were lodged in GenBank (www.ncbi.nlm.nih.gov/genbank), the alignment in TreeBASE (www.treebase.org/treebase-web/home.html), and taxonomic novelties in MycoBank (www.mycobank.org, Crous *et al.* 2004a).

Morphological analysis

Strains were cultivated on synthetic nutrient-poor agar medium (SNA; Nirenberg 1976) with autoclaved filter paper and *Anthriscus sylvestris* stems placed on the surface and on oatmeal agar medium (OA; Crous *et al.* 2009). The cultures were incubated at 20 °C under near UV light with 12 h photoperiod for 10 d. Measurements of morphological characters were made according to Damm *et al.* (2007). Microscopic preparations were made in clear lactic acid and 30 measurements per structure were made for each strain using a Nikon SMZ1000 dissecting microscope (DM) and a Nikon Eclipse 80i compound microscope using differential interference contrast (DIC) illumination. Appressoria were observed on the reverse side of the plates containing SNA medium. Unless mentioned otherwise, descriptions are based on the ex-type strains and only conidia from conidiomata were included in the morphological examination.

Colony characteristics on SNA and OA medium were observed after the incubation period. To calculate the growth rates, the diameters of colonies were measured after 7 and 10 d. Colony colours were determined according to Rayner (1970).

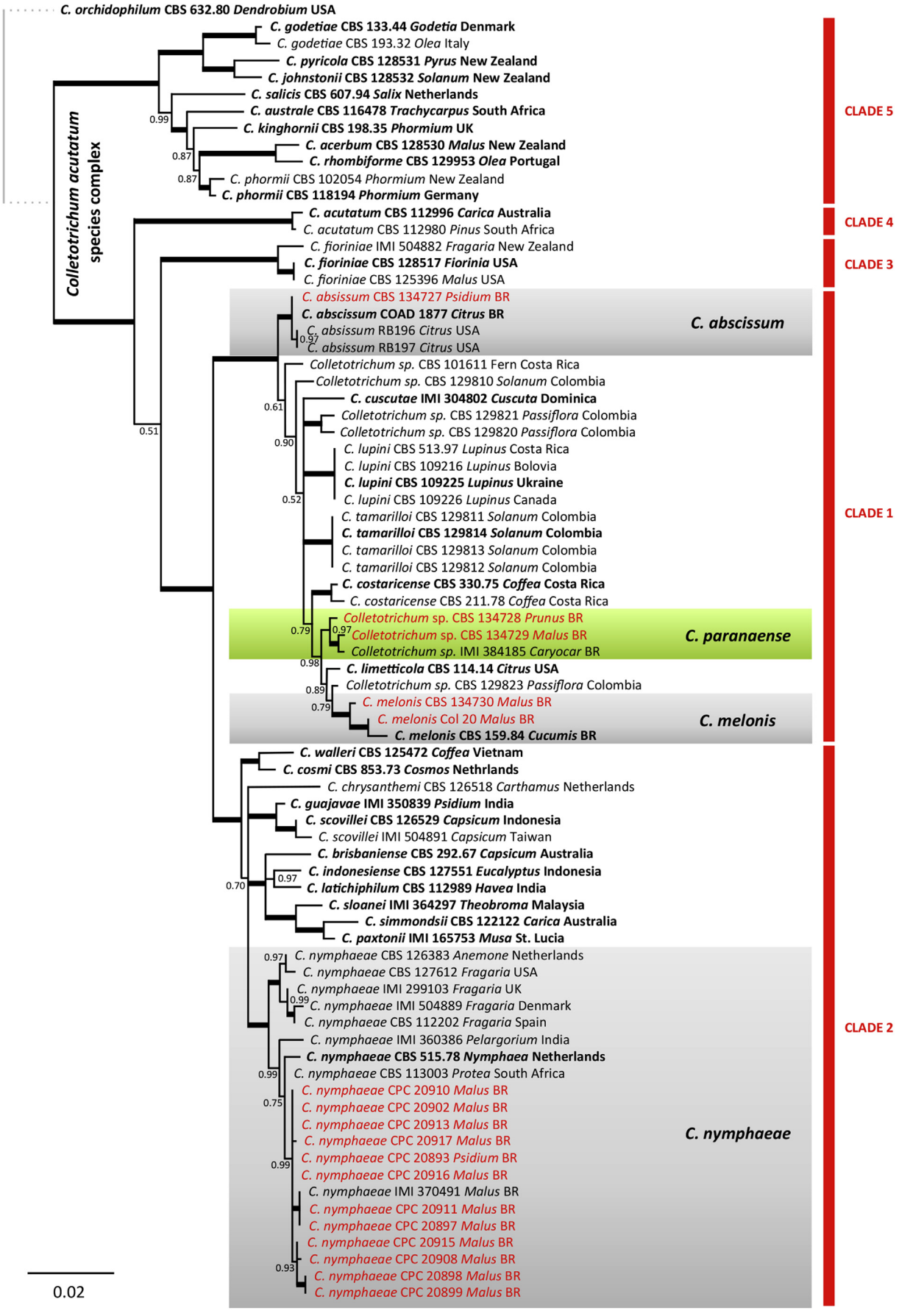
Aggressiveness test

To verify whether the species identified in this work are different in virulence or aggressiveness, a pathogenicity test was conducted by inoculating physiologically mature peach (*Prunus persica* cv. 'Chimarrita'), apple (*Malus domestica* cv. 'Gala') and guava (*Psidium guajava* cv. 'Pedro Sato') fruits. The maturation stage of the fruits was standardised on the basis of peel colour and pulp firmness using a colorimeter (Minolta, model CR-300, Japan) and a penetrometer (Tr Turoni, model 53200, Italy), respectively. Prior to the inoculation, the fruits were immersed in 0.5 % sodium hypochlorite solution for

Table 2 – Pathogenicity test of *Colletotrichum* species on peach, guava and apple fruits.

Species	Strain	Original host	Lesion size (mm^2)[a]			Frequency of infection[b]		
			Peach	Guava	Apple	Peach	Guava	Apple
C. melonis	Col 20	apple	634.62^{aA}	121.26^{abB}	176.78^{aB}	10/10	10/10	10/10
C. cf. melonis	CBS 134730	apple	484.48^{abA}	0	97.72^{abB}	10/10	0/10	10/10
C. paranaense	CBS 134729	apple	510.50^{abA}	84.32^{abB}	172.88^{aB}	10/10	2/10	8/10
	CBS 134728	peach	388.94^{bA}	329.98^{aA}	191.88^{aA}	10/10	7/10	10/10
C. abisissum	CBS 134727	guava	294.76^{bA}	217.45^{abAB}	10.36^{bB}	10/10	8/10	1/10
C. nymphaeae	CPC 20897	apple	315.96^{bA}	13.47^{bC}	136.38^{abB}	10/10	4/10	10/10

a Means followed by the same lower case letter within a column are not significantly different ($p \leq 0.05$); means followed by the same capital letter within a row are not significantly different ($p \leq 0.05$).
b Number of fruits with lesions/number of fruits inoculated.


C. orchidophilum CBS 632.80 Dendrobium USA
Colletotrichum acutatum species complex
C. godetiae CBS 133.44 Godetia Denmark
C. godetiae CBS 193.32 Olea Italy
C. pyricola CBS 128531 Pyrus New Zealand
C. johnstonii CBS 128532 Solanum New Zealand
C. salicis CBS 607.94 Salix Netherlands
C. australe CBS 116478 Trachycarpus South Africa
C. kinghornii CBS 198.35 Phormium UK
C. acerbum CBS 128530 Malus New Zealand
C. rhombiforme CBS 129953 Olea Portugal
C. phormii CBS 102054 Phormium New Zealand
C. phormii CBS 118194 Phormium Germany
CLADE 5
C. acutatum CBS 112996 Carica Australia
C. acutatum CBS 112980 Pinus South Africa
CLADE 4
C. fioriniae IMI 504882 Fragaria New Zealand
C. fioriniae CBS 128517 Fiorinia USA
C. fioriniae CBS 125396 Malus USA
CLADE 3
C. absissum CBS 134727 Psidium BR
C. abscissum COAD 1877 Citrus BR
C. absissum RB196 Citrus USA
C. absissum RB197 Citrus USA
C. abscissum
Colletotrichum sp. CBS 101611 Fern Costa Rica
Colletotrichum sp. CBS 129810 Solanum Colombia
C. cuscutae IMI 304802 Cuscuta Dominica
Colletotrichum sp. CBS 129821 Passiflora Colombia
Colletotrichum sp. CBS 129820 Passiflora Colombia
C. lupini CBS 513.97 Lupinus Costa Rica
C. lupini CBS 109216 Lupinus Bolivia
C. lupini CBS 109225 Lupinus Ukraine
C. lupini CBS 109226 Lupinus Canada
C. tamarilloi CBS 129811 Solanum Colombia
C. tamarilloi CBS 129814 Solanum Colombia
C. tamarilloi CBS 129813 Solanum Colombia
C. tamarilloi CBS 129812 Solanum Colombia
C. costaricense CBS 330.75 Coffea Costa Rica
C. costaricense CBS 211.78 Coffea Costa Rica
Colletotrichum sp. CBS 134728 Prunus BR
Colletotrichum sp. CBS 134729 Malus BR
Colletotrichum sp. IMI 384185 Caryocar BR
C. paranaense
C. limetticola CBS 114.14 Citrus USA
Colletotrichum sp. CBS 129823 Passiflora Colombia
C. melonis CBS 134730 Malus BR
C. melonis Col 20 Malus BR
C. melonis CBS 159.84 Cucumis BR
C. melonis
CLADE 1
C. walleri CBS 125472 Coffea Vietnam
C. cosmi CBS 853.73 Cosmos Nethrlands
C. chrysanthemi CBS 126518 Carthamus Netherlands
C. guajavae IMI 350839 Psidium India
C. scovillei CBS 126529 Capsicum Indonesia
C. scovillei IMI 504891 Capsicum Taiwan
C. brisbaniense CBS 292.67 Capsicum Australia
C. indonesiense CBS 127551 Eucalyptus Indonesia
C. latichiphilum CBS 112989 Havea India
C. sloanei IMI 364297 Theobroma Malaysia
C. simmondsii CBS 122122 Carica Australia
C. paxtonii IMI 165753 Musa St. Lucia
C. nymphaeae CBS 126383 Anemone Netherlands
C. nymphaeae CBS 127612 Fragaria USA
C. nymphaeae IMI 299103 Fragaria UK
C. nymphaeae IMI 504889 Fragaria Denmark
C. nymphaeae CBS 112202 Fragaria Spain
C. nymphaeae IMI 360386 Pelargorium India
C. nymphaeae CBS 515.78 Nymphaea Netherlands
C. nymphaeae CBS 113003 Protea South Africa
C. nymphaeae CPC 20910 Malus BR
C. nymphaeae CPC 20902 Malus BR
C. nymphaeae CPC 20913 Malus BR
C. nymphaeae CPC 20917 Malus BR
C. nymphaeae CPC 20893 Psidium BR
C. nymphaeae CPC 20916 Malus BR
C. nymphaeae IMI 370491 Malus BR
C. nymphaeae CPC 20911 Malus BR
C. nymphaeae CPC 20897 Malus BR
C. nymphaeae CPC 20915 Malus BR
C. nymphaeae CPC 20908 Malus BR
C. nymphaeae CPC 20898 Malus BR
C. nymphaeae CPC 20899 Malus BR
C. nymphaeae
CLADE 2
0.99
0.87
0.87
0.57
0.61
0.51
0.90
0.52
0.79
0.97
0.98
0.89
0.79
0.70
0.97
0.97
0.99
0.99
0.75
0.99
0.93
0.02

3 min, then rinsed twice in sterile distilled water and air dried in the laminar flow cabinet.

Strains CBS 134727, CPC 20897, CPC 134729, CPC 134730, CPC 134728 and Col 20 (Table 2) were grown on PDA for 7 d at 26 °C under near UV light (12 h photoperiod), to induce sporulation (Cai *et al.* 2009). After incubation, spores were harvested by adding 10 mL sterile distilled water to each culture followed by scraping the surface with a sterile brush. The resulting spore suspensions were filtered through sterile cheesecloth and the spore concentration was adjusted to 1×10^5 mL^{-1} using a haemocytometer.

The fruits were placed in a plastic box with a lid containing water-soaked cotton wool and inoculated by wounding the fruits with a sterile needle and placing 40 μL spore suspension on the wound. Control fruits were inoculated with sterile distilled water. The plastic boxes were kept in an incubation room at 25 °C and 12 h photoperiod. After 48 h, the lid of the box was removed and the boxes remained in the room for another 5 d. At the 7th d the lesion size was measured and the fungus re-isolated from the margin of the lesion.

The experimental design was randomised with 10 fruits (replicates). Lesion length data were subjected to analysis of variance with the statistical programme 'R' v. 3.0.1 (R Core Team 2013) and the means of each treatment compared using Tukey's test at 95 % of probability.

Results

Phylogenetic analysis

The molecular analysis of 76 isolates of *C. acutatum* s. *lat.* and the outgroup (*Colletotrichum orchidophilum*, strain CBS 632.80) was performed on a sequence alignment with 2222 characters, of which 1702 were conserved, 200 were parsimony-uniformative and 320 were parsimony-informative. The gene boundaries were: ITS: 1–549, HIS3: 550–936, GAPDH: 937–1203, CHS-1: 1204–1482, TUB2: 1483–1974, ACT: 1975–2222. Based on the AIC criteria, the following evolution models were selected for the partitioned Bayesian inference: GTR+G for ACT and GAPDH, HKY+G for TUB2, K80+I+G for CHS-1, GTR+I+G for HIS3 and GTR+I for ITS.

The phylogeny with the Bayesian posterior probability values (Fig 1) exhibits five main clades with >30 clades, most of which representing previously defined species of the *C. acutatum* complex (Damm *et al.* 2012). The majority of the strains from fruits in Brazil grouped in clade 2, with the ex-epitype strain of *Colletotrichum nymphaeae*. The *C. nymphaeae* clade was well supported with a Bayesian posterior probability value (BPP) of 1.0. However, it showed high intraspecific variability. Within *C. nymphaeae*, one strain from guava (CPC 20893) and all strains from apple in Brazil including one from a previous study (IMI 370491, see Table 1), formed an intraspecific subclade (BPP value 0.99).

All other strains studied grouped in clade 1. Among them, two strains from apple in Brazil (CBS 134730, Col 20) grouped with the ex-holotype strain of *Colletotrichum melonis* (CBS 159.84). However, within *C. melonis*, strains Col 20 and CBS 159.84 formed a sister clade (BPP of 1.0) to strain CBS 134730. Strain CBS 134729, also from apple in Brazil, grouped with a strain from Caryocar, also from Brazil, that was treated as *Colletotrichum* sp. in a previous study (Damm *et al.* 2012). A third strain, CBS 134728 from *Prunus,* also grouped (BPP of 0,97) with this clade, which was recognized as a new species named *Colletotrichum paranaense*. Strain CBS 134727 from *Psidium* grouped with two isolates described in previous studies as the causal agents of postbloom fruit drop (PFD) of sweet orange in Florida, USA (Peres *et al.* 2008) and with a strain also from sweet orange but isolated in Brazil and recently described as *Colletotrichum abscissum* (Crous *et al.* 2015).

Taxonomy

Based on the multilocus molecular analysis, the strains studied here belong to four species within the *Colletotrichum acutatum* species complex, including one species that proved to be new to science and is described below. Additionally, descriptions are also provided of strain CBS 134730 treated as *C.* cf. *melonis* and the strain CBS 134727 recognized as the new species described by Pinho & Pereira (2015).

Colletotrichum *cf.* melonis *(Fig 2)*

Sexual morph not observed. Asexual morph on SNA. *Vegetative hyphae* 1.5–4 μm diam, hyaline to buff, smooth-walled, septate, branched. *Chlamydospores* not observed. *Conidiomata* not developed, conidiophores formed directly on hyphae. Setae not observed. *Conidiophores* hyaline, smooth-walled, septate, unbranched, to 9 μm long. *Conidiogenous cells* hyaline, smooth-walled, ampulliform to cylindrical, often constricted at the base, 3.5–4 × 5.5–17.5 μm, opening 1–1.5 μm diam, collarette 1–1.5 μm long, periclinal thickening visible. *Conidia* hyaline, smooth-walled, aseptate, cylindrical to clavate, both ends acute, sometimes one end round, (7–)9–13 (–16) × (3–)3.5–4.5 (–5.5) μm, mean ± SD = 11.1 ± 2.2 × 3.9 ± 0.5 μm, L/W ratio = 2.8. *Appressoria* single, medium to pale brown, bulled-shaped to clavate and sometimes globose to obovoidal, the edge entire or sometimes lobate, (4.5–)6–14.5 (–20.5) × (4–)4.5–6 (–7) μm, mean ± SD = 10.4 ± 4.1 × 5.4 ± 0.7 μm, L/W ratio = 1.9.

Asexual morph on *Anthriscus stem*. *Conidiomata*, acervular, conidiophores formed on a cushion of pale brown angular

Fig 1 – Bayesian inference phylogenetic tree reconstructed from a combined ITS, HIS3, GAPDH, CHS-1, BTU2 and ACT sequence alignment of 76 isolates of the *C. acutatum* species complex including the outgroup. Bayesian posterior probability (BPP) values/bootstrap values (above 0.85 or 85) are shown at the nodes. The thickened nodes represent BPP of 1. Isolates obtained in this study are emphasized in red font. Ex-type cultures are emphasized in bold font. New species are indicated with green boxes. *C. orchidophilum* CBS 632.80 is used as outgroup. Main clades within the *C. acutatum* species complex from Damm *et al.* (2012) are indicated in red by 1–5. The scale bar represents the number of expected changes per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

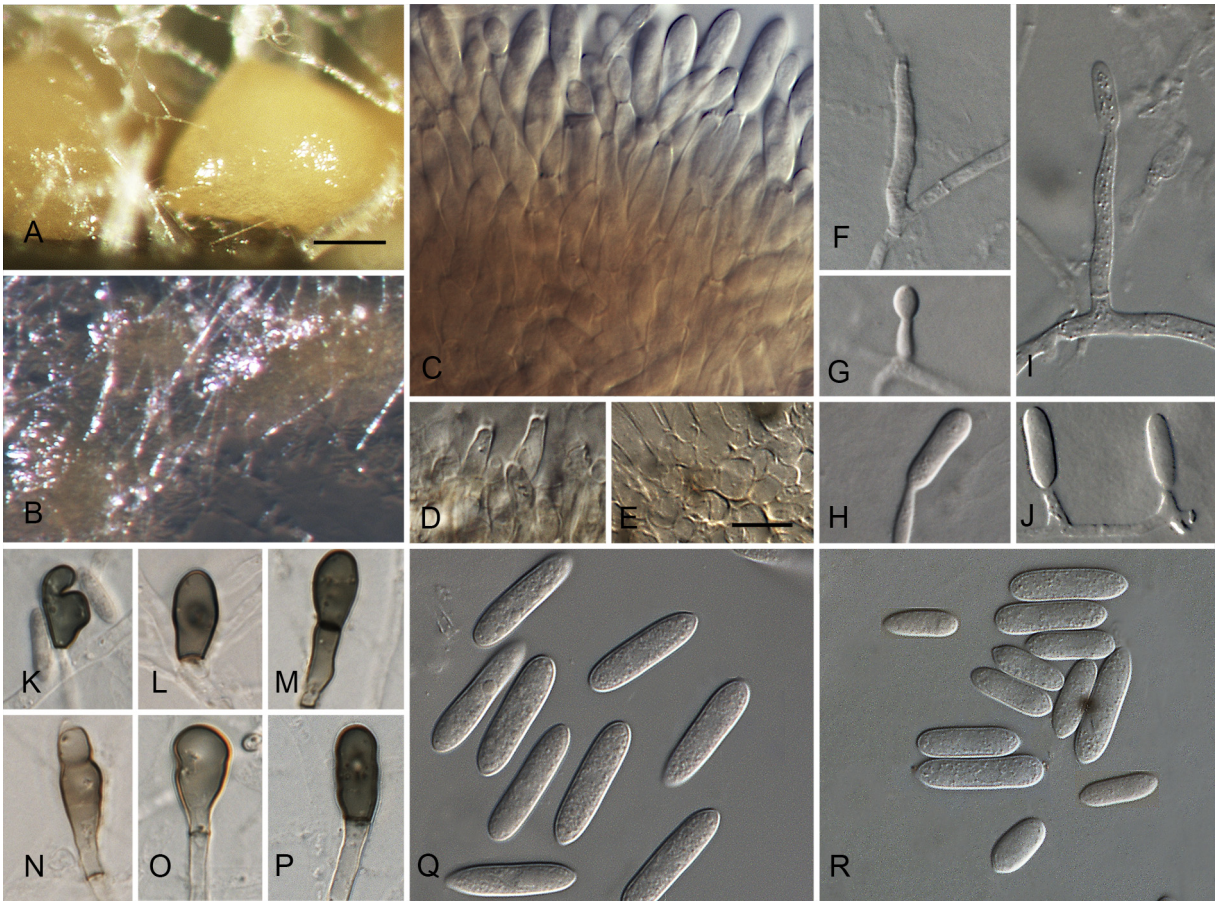


Fig 2 – Colletotrichum cf. *melonis* (from strain CBS 134730). A–B. conidiomata; C–F, H–M. conidiophores; G. angular cells; N–S. appressoria; T–U. conidia; a, c–g, t. from *Anthriscus* stem; b, h–s, u. from SNA. a–b. DM; c–u. DIC. – Scale bars: a = 100 μm; e = 10 μm; scale bar of a applies to a–b; scale bar of e applies to c–u.

cells. *Setae* not observed. *Conidiophores* hyaline, septate, branched, to 35 μm long, *Conidiogenous cells* hyaline, smooth–walled, cylindrical to obclavate, 8.5–15.5 × 3–3.5 μm, opening 1–1.5 μm diam, collarette, 1–1.5 μm long, periclinal thickening visible. *Conidia* hyaline, smooth–walled, aseptate, cylindrical, both ends acute, sometimes one end round, (8–) 11.5–15 (–17.5) × (2.5–)4–5 (–5) μm, mean ± SD = 13.3 ± 1.6 × 4.4 ± 0.4 μm, L/W ratio = 3.0.

Culture characteristics: Colonies on SNA flat with entire edge, hyaline to buff, *Anthriscus* stem partly covered by white floccose aerial mycelium and orange conidia mass, reverse same colours, growth rate 22.5–23 mm in 7 d and 34–34.5 mm in 10 d. Colonies on OA slightly umbonate with entire edge, saffron to olivaceous grey, almost entirely covered by orange conidia mass, partly covered by floccose pale olivaceous grey aerial mycelium, reverse salmon, growth rate 20.5–21 mm in 7 d and 32–32.5 mm in 10 d. Conidia in mass orange.

Specimens examined: BRAZIL, Rio Grande do Sul, from fruit anthracnose of *Malus domestica*, S. Alves (living culture CBS 134730 = CPC 20912 = Col 31).

Notes: Strain CBS 134730 showed differences in morphology with the original strain. Conidiophores from *C.* cf. *melonis* on SNA were unbranched, not degenerate and conidiogenous cells often constrict at the base and smaller, while these characters in *Colletotrichum melonis* were branched, degenerating rapidly, branched and the constriction of the conidigenuos cells was not mentioned. Also, conidia from *C.* cf. *melonis* were frequently acute in both ends, rarely observed in *C. melonis*, showed smaller L/W ratio and bigger appressoria.

Strain CBS 134730 formed a sister clade with strains Col 20 and CBS 159.84, the ex-holotype strain of *C. melonis* (Fig 1). Although sequences of *C. melonis* strain CBS 159.84 (JQ949845, JQ949515 and JQ949185) were the closest matches in blastn searches on GenBank with the TUB2, ACT and HIS3 sequences of strain CBS 134730 (99 % identity), none of them were identical; they differ in four nucleotides (nt), 1 nt and 3 nt, respectively. The GAPDH and ITS sequences of strain CBS 134730 were identical with those of *Colletotrichum* strain CBS 129823 from *Passiflora edulis* (JQ948512 and JQ948182), while its CHS-1 sequence matched 100 % with JQ948841 from strain CBS 330.75 from *Coffea arabica*, the ex-holotype strain of *Colletotrichum costaricense* (Damm *et al.* 2012). In spite of these sequence differences, we refrain from describing strain CBS 134730 as a new species here, because it is only known from a single strain; it is possible, therefore, that intermediate strains exist

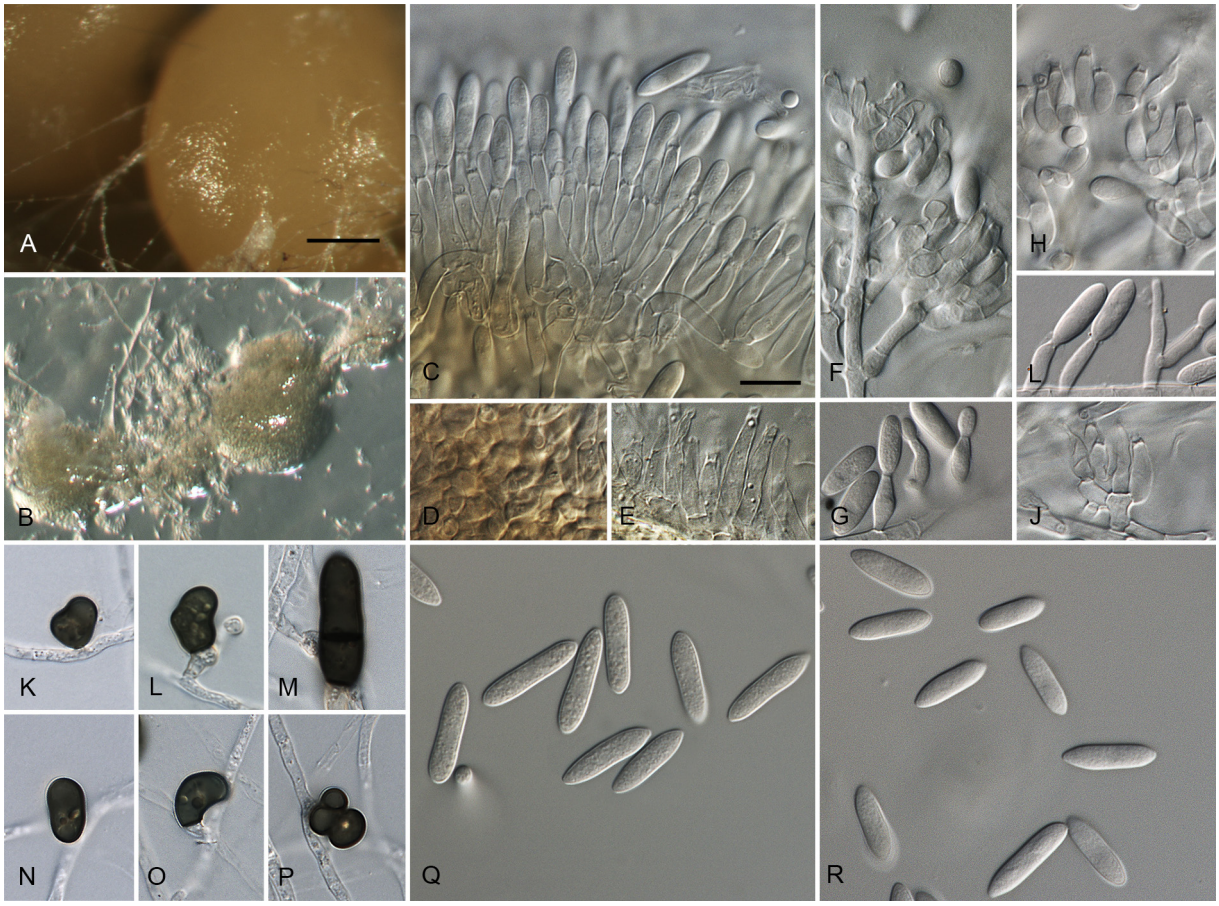


Fig 3 – ***Colletotrichum paranaense*** **(from ex-holotype strain CBS 134729). A–B. conidiomata; C, D, F, J. conidiophores; E. angular cells on the basis of the conidiomata; K–P. appressoria; Q–R. conidia. a, c–e, p. from *Anthriscus* stem; b, f–q, r. from SNA. a–b. DM; c–r. DIC. – Scale bars: a = 100 μm; e = 10 μm; scale bar of a applies to a–b; scale bar of e applies to c–r.**

between Col 20 and CBS 134730, as both strains were isolated from apple in Brazil.

Colletotrichum paranaense *C.A.D. Bragança & Damm, sp. nov* (*Fig 3*)

MycoBank no.: MB814541.

Etymology: Named after the state of Brazil where the species was found, Parana.

Sexual morph not observed. *Asexual morph* on SNA. *Vegetative hyphae* 1–2.5 μm diam, hyaline, smooth-walled, septate, branched. *Chlamydospores* not observed. *Conidiomata* not developed, conidiophores formed directly on hyphae. Setae not observed. *Conidiophores* hyaline, smooth-walled, septate, unbranched, 5–21 μm long. *Conidiogenous cells* hyaline, smooth-walled, elongate-ampulliform to subcylindrical, 4.5–21.5 × 1.5–2 μm, opening 1 μm diam, collarette 1–1.5 μm long, sometimes not visible, periclinal thickening sometimes visible; conidiogenous cells of strain IMI 384185 differed in being broader, measuring 7–14 × 2–3.5 μm and frequently forming polyphialides. *Conidia* hyaline, smooth-walled, aseptate, cylindrical, sometimes slightly constricted in the middle, both ends slightly acute or one end round, (4–)8–15 (–22.5) × (2–)3–4 (–5) μm, mean ± SD = 11.4 ± 3.6 × 3.4 ± 0.6 μm, L/W ratio = 3.4. *Appressoria* single, medium to pale brown, ellipsoidal to obovoidal, the edge entire or sometimes lobate, (4.5–)5.5–10.5 (–15.5) × (3.5–)4.5–7 (–10.5) μm, mean ± SD = 7.9 ± 2.6 × 5.8 ± 1.4 μm, L/W ratio = 1.4.

Asexual morph on *Anthriscus* stem. *Conidiomata*, acervular, conidiophores formed on pale brown, angular basal cells, 3–5.5 μm. *Setae* not observed. *Conidiophores* hyaline, smooth-walled, septate, branched, to 36 μm long. *Conidiogenous cells* hyaline to pale brown, smooth-walled, elongate-ampulliform to cylindrical, 13.5–20 × 3–3.5 μm, opening 1.5–2 μm diam, collarette 1–1.5 μm long, periclinal thickening visible, sometimes distinct. *Conidia* hyaline, smooth-walled, aseptate, straight, cylindrical, both ends slightly acute, sometimes one end round, sometimes slightly constricted in the middle, (8.5–)11–17 (–19.5) × (3–)3.5–4.5 (–4.5) μm, mean ± SD = 14.1 ± 3 × 4.1 ± 0.4 μm, L/W ratio = 3.5; in strain IMI 384185 additionally a low proportion of subglobose, tear-shaped to ellipsoidal conidia observed.

Culture characteristics: Colonies on SNA flat with entire edge, pale honey, filter paper partly covered by pale olivaceous grey, floccose felty aerial mycelium, *Anthriscus* stem partly covered by white to smoke grey aerial mycelium, reverse partly pale

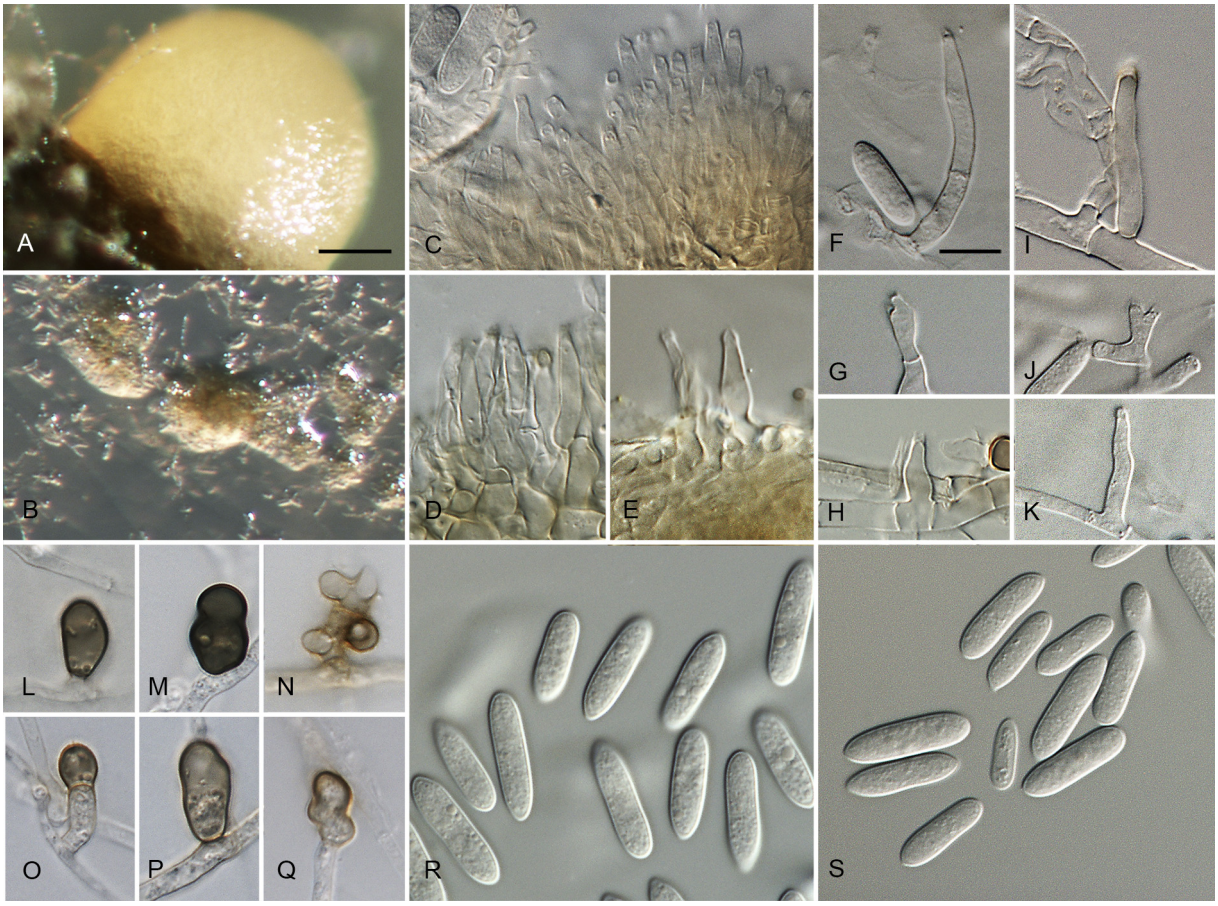


Fig 4 – ***Colletotrichum abscissum* (from strain CBS 134727). A–B. conidiomata; C–K. conidiophores; L–Q. appressoria; R–S. conidia. a, c–e, r. from *Anthriscus* stem; b, f–q, s. from SNA. a–b. DM. c–s; DIC. – Scale bars: a = 100 μm; f = 10 μm; scale bar of a applies to a–b; scale bar of f applies to c–s.**

isabelline to hazel, growth rate 22.5–23 mm in 7 d and 32.5–33 mm in 10 d. Colonies on OA flat with entire edge, covered by pale olivaceous grey to white floccose-felty aerial mycelium and few orange acervuli along the edge, reverse buff to olivaceous grey, honey in the centre, growth rate 21.5–22 mm in 7 d and 30–32 mm in 10 d. Conidia in mass saffron.

Specimens examined: BRAZIL, Paraná, from fruit anthracnose of *Malus domestica*, 2010, L. Mio (CBS H-21122 holotype, culture ex-holotype CBS 134729 = CPC 20901 = Col 19); from *Caryocar brasiliense*, collection date and collector unknown (isolated by J.R.N. Anjos, deposited in CABI 25 July 2000), living culture IMI 384185 = CPC 18937 = CPAC 8; São Paulo, Paranapanema, from fruit anthracnose of *Prunus persica*, 2010, H. Tozze, culture CBS 134728 = CPC 20928 = Col 49.

Notes: The closest described species from *Colletotrichum paranaense* were *Colletotrichum limetticola* *Colletotrichum costaricense*, and *Colletotrichum melonis* (Fig 1). The original strain of *Colletotrichum limetticola* showed conidiophores longer and branched, polyphialides rarely observed and greater L/W ratio. *C. costaricense* showed conidiophores branched, greater L/W ratio, conidia were bigger, appressoria observed in small groups, setae observed and conidiomata not developed. *C. melonis* showed conidiophores branched and degenerating rapidly, periclinal thickening visible, polyphialides not observed, conidia smaller and rarely acute in both ends.

Colletotrichum paranaense can be distinguished from other *Colletotrichum* species by its unique TUB2 and HIS3 sequences. The closest matches in blastn searches on GenBank with the TUB2, GAPDH and HIS3 sequences of strain CBS 134729 were sequences of *Colletotrichum* strain IMI 384185 from *Caryocar brasiliense* (100 %, JQ949842, JQ948521, JQ949182) that is included in this study and considered as *C. paranaense* as well. However, the TUB2 and HIS3 sequences also matched with *Colletotrichum abscissum* (99 % identical, 4 nt differences, KP843135) and *Colletotrichum cuscutae* (1 nt different, JQ949186). The GAPDH sequence of strain CBS 134729 was also identical with that of *Colletotrichum* strain CBS 129821 from *Passiflora edulis* (JQ948512). The CHS-1 sequence was the same as that of *C. costaricense* (CBS 330.75, JQ948841; CBS 211.78, JQ948842), a recently described species from *Coffea* in Costa Rica (Damm *et al.* 2012) and was 99 % identical with *C. paranaense* strain IMI 384185 (JQ948852, all from Damm *et al.* 2012 and included in this study). The ITS region show intraspecific differences as CBS 134728 has identical sequence to *C. limetticola* CBS 114.14 but the other two strains (CBS 134729 and IMI 384185) have unique sequence showing two

different nucleotides compared to the dataset used in this study.

Colletotrichum abscissum *Persoonia* **34**:*237*. 2015 (Fig 4)
Sexual morph not observed. *Asexual morph* on SNA. *Vegetative hyphae* 2–3.5 μm diam, hyaline, smooth-walled, septate, branched. *Chlamydospores* not observed. *Conidiomata* not developed, conidiophores formed directly on hyphae. Setae not observed. *Conidiophores* hyaline, smooth-walled, septate, sometimes branched, 10–29 μm long. *Conidiogenous cells* hyaline, smooth-walled, elongate-ampulliform, 5–17.5 × 2–3 μm, sometimes integrated (not separated from fertile hyphae by a septum), sometimes polyphialides, opening 1–1.5 μm diam, collarette 1–1.5 μm long, periclinal thickening visible. *Conidia* hyaline, smooth-walled, aseptate, cylindrical, both ends round, sometimes with one end acute, (10.5–)12–14.5 (–16) × (3–)3.5–4 (–4.5) μm, mean ± SD = 13.2 ± 1.5 × 3.7 ± 0.4 μm, L/W ratio = 3.6. *Appressoria* single, pale to medium brown, obovoidal, ellipsoidal or clavate, the edge undulate to lobate and sometimes entire, (6–)7–12.5 (–21) × (4.5–)5–6.5 (–7.5) μm, mean ± SD = 9.8 ± 2.9 × 5.9 ± 0.7 μm, L/W ratio = 1.7.

Asexual morph on *Anthriscus* stem. *Conidiomata*, acervular, conidiophores formed on hyaline to pale brown, angular basal cells 5.5–6.5 μm diam. *Setae* not observed. *Conidiophores* hyaline to pale brown, smooth-walled, septate, branched, to 35 μm long. *Conidiogenous cells* hyaline to pale brown, smooth-walled elongate-ampulliform, sometimes attenuated at the base, 12–15 × 2.5–3.5 μm, opening 1 μm diam, collarette pale brown, 1 μm long, periclinal thickening visible. *Conidia* hyaline, smooth–walled, aseptate, cylindrical, with one end acute, sometimes both ends acute, (8.5–)12–16 (–17) × (3–)4–4.5 (–5) μm, mean ± SD = 14 ± 1.9 × 4.3 ± 0.5 μm, L/W ratio = 3.3.

Culture characteristics: Colonies on SNA flat with entire edge, buff, filter paper partly covered by olivaceous felty aerial mycelium, *Anthriscus* stem partly covered by felty aerial mycelium and partly orange due to conidia mass, reverse same colours, growth rate 21–22.5 mm in 7 d and 31–32.5 mm in 10 d. Colonies on OA flat to umbonate with entire edge, olivaceous to pale olivaceous, partly covered by white to olivaceous grey floccose-felty aerial mycelium, reverse pale olivaceous grey to olivaceous grey, growth rate 17–17.5 mm in 7 d and 28–28.5 mm in 10 d. Conidia in mass saffron.

Specimen examined: BRAZIL, Paraná, Cafelândia, from fruit anthracnose of *Psidium guajava*, 2008, H. Tozze (CBS H-21121, culture CBS 134727 = CPC 20894 = Col 10).

Notes: *Colletotrichum abscissum* was described by Pinho *et al.* from *Citrus sinensis* causing postbloom fruit drop in Brazil (Crous *et al.* 2015). Two strains examined by the authors grouped in a well supported clade with ex-type strain COAD 1877. Differences were found between the original strain described by Pinho & Pereira (2015) and the strain described in this study. The original strain not showed polyphialides, conidia were smaller and appressoria from CBS 134727 were bigger and some were clavate. The sequences from the strain examined in this study matched 100 % with *C. abscissum* (KP843139, KP843133, KP843130, KP843127, KP843136). Also, the TUB2 sequence matched with those of *C. costaricense* (99 % identity, 3 nt, CBS 330.75 JQ949831) and *Colletotrichum* sp. strain CBS 129810 (99 % identity, 3 nt, JQ949830). The closest matches with the CHS-1 sequence were *Colletotrichum* strains CBS 129810 from *Solanum betaceum* (100 % identity, JQ948840) and CBS 101611 from a fern (99 % identity, JQ948857) (all from Damm *et al.* 2012 and included in this study). The ITS sequence is identical to strain GM59a (EU734581, *Glomerella acutata* from Colombia, *Annona muricata*, Rojas A *et al.*, unpublished data) and 99 % identical to *Colletotrichum* strain Q003 from *Rubus glaucus* in Colombia (JN715842, Afanador-Kafuri *et al.* 2015).

Damm *et al.* (2012) described a species from guava in India, *Colletotrichum guajavae* that belongs to a different clade within the *Colletotrichum acutatum* species complex than *Colletotrichum abscissum* (Fig 1). In addition, the morphology showed differences as well. Setae were observed in *C. guajavae*, while this character was not observed in *C. abscissum*. Peres *et al.* (2002) isolated a *Colletotrichum acutatum* (s. lat.) strain from guava in Brazil with the same ITS sequence as *C. guajavae* (Damm *et al.* 2012). Another *Colletotrichum* species on guava, *Colletotrichum psidii*, was described in Italy and belongs to the *Colletotrichum gloeosporioides* species complex (Weir *et al.* 2012).

Aggressiveness test

All tested strains were able to infect their original host and all other hosts included, except for *C.* cf. *melonis* strain CBS 134730 that did not infect guava fruits (Table 2), while no lesions were formed on control fruits. However, there were differences in the size of the lesions and the frequency of fruits infected. None of the strains formed larger lesions on its original host compared to the other hosts.

All strains tested formed large lesions on all inoculated peach fruits; while their infection frequency and lesion size on guava and apple were variable. *Colletotrichum melonis* (Col 20), caused larger lesions on peach than *Colletotrichum paranaense* (CBS 134728), *Colletotrichum abscissum* (CBS 134727) and *Colletotrichum nymphaeae* (CPC 20897). *C. abscissum* showed no difference in lesion size between peach and guava. That strain infected only one apple; the lesions size of which was smaller then those caused by most other strains tested. *C. melonis* strain Col 20 infected all inoculated guava fruits, while *C.* cf. *melonis* strain CBS 134730, infected only peach and apple. Additionally, *C. nymphaeae* strain CPC 20897 formed smaller lesions on guava fruits than *C. paranaense* strain CBS 134728.

Discussion

A large number of *Colletotrichum* species belonging to the *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* species complexes were recently differentiated, some of them being associated with fruit diseases of apple, including bitter rot (Damm *et al.* 2012; Weir *et al.* 2012). In most of the previous studies on apples in Brazil, the causal agents of bitter rot were reported as *C. gloeosporioides* and *C. acutatum* (or its sexual morph *Glomerella acutata*), referring to these species in their wide sense as species complexes (Serra *et al.* 2011; de Souza *et al.* 2013; Barquero Quirós *et al.* 2013; Ciampi-Guillardi *et al.* 2013). Some of these studies have shown a high genetic variability among strains causing apple bitter

rot in Brazil (González et al. 2006; Giaretta et al. 2010). Based on ITS sequences, Giaretta et al. (2010) differentiated strains from bitter rot of apple in five main clades, indicating the disease to be caused by several species within the *C. acutatum* and *C. gloesporioides* complexes. Our study on *C. acutatum* s. lat. strains from fruits in Brazil confirms at least three species of this complex to be associated with apple fruit diseases.

In the study of Damm et al. (2012), most of the *C. acutatum* s. lat. strains from apple (mainly originating from the USA) were identified as *Colletotrichum fioriniae* and a few strains each as *Colletotrichum acerbum*, *Colletotrichum godetiae*, *Colletotrichum salicis* and *Colletotrichum nymphaeae*, while the latter seems to be more important on other hosts, especially strawberry. The only *C. nymphaeae* strain from apple included in Damm et al. (2012) was also the only strain from apple in South America in their study and originated from Brazil. Velho et al. (2014) tested strains of this species collected in southern Brazil to cause apple bitter rot. Our study suggests *C. nymphaeae* to be the most important species of the *C. acutatum* complex associated with anthracnose diseases of apple in Brazil. Based on ITS sequences, more than half of the *C. acutatum* strains from apple in Brazil in the study of Giaretta et al. (2010) and strains from a disease report of bitter rot of apple in Uruguay by Alaniz et al. (2012) belong to the same main clade within this species complex (indicated as main clade 2 in Damm et al. 2012) and might represent *C. nymphaeae* as well.

The *C. nymphaeae* strains from Brazil included in this study formed a subclade within *C. nymphaeae* that slightly separates them from *C. nymphaeae* strains from other host plants (Fig 1). Furthermore, sequences of strains in this study formed additional subclades, showing the genetic diversity within the species. *C. nymphaeae* has a wide distribution and host range and has been shown to be genetically variable before, with strains from specific hosts forming intraspecific subclades (Damm et al. 2012). However, based on the low support of these subclades and few base pair differences, we refrain from describing further species within *C. nymphaeae*.

While the majority of the strains was identified as *C. nymphaeae*, all other strains, including the newly described species *C. paranaense* and *C. melonis* are part of a main clade within the *C. acutatum* species complex (indicated as main clade 1 in Damm et al. 2012) that comprises closely related species predominantly occurring on various hosts in Central and South America which are well distinguished with GAPDH and TUB2 sequences. *Colletotrichum lupine*, e.g., was originally described (as *Gloeosporium lupini*) on *Lupinus albus* in Brazil (Bondar 1912), but is a commonly occurring species worldwide (Nirenberg et al. 2002; Damm et al. 2012). Most likely, the speciation of *C. lupini* developed in South America as well and spread with the host plant to other parts of the world. Some of the strains from apple in this study were identified as *C. melonis* and a new species, *C. paranaense*. ITS sequences place the remaining strains occurring on apple in Brazil from the study of Giaretta et al. (2010) in the same main clade, although it is not possible to identify them to species level on this basis. No species in this main clade of the *C. acutatum* species complex was previously associated with diseases of apple, peach or guava fruits, and none of the other four species of the *C. acutatum* complex from apple reported by Damm et al. (2012) from other regions in the world was so far found to be associated with apples in Brazil. These species (*C. acerbum*, *C. fioriniae*, *C. godetiae* and *C. salicis*) were not reported from South America at all, except for a few *C. godetiae* strains from other hosts in Chile, Colombia and Mexico that formed an interspecific clade within *C. godetiae*. Thus, the species composition of the *C. acutatum* complex associated with apple bitter rot in Brazil (possibly in South America) is different from other regions in the world. This might be important for plant quarantine.

Strain CBS 134727 of *C. abscissum* grouped together with strains of citrus postbloom fruit drop. This strain was collected on guava fruit with antracnose in a commercial orchard located at Cafelândia city. A previous study revealed that this strain is pathogenic to citrus, causing citrus postbloom fruit drop in 70 % of inoculated flowers (Ramiro et al., unpublished data). Guava volatiles are repellent to *Diaphorina citri*, the vector of citrus huanglongbing (HLB) (Zaka et al. 2010), and intercropping citrus with guava is considered a strategy to control HLB (Beattie el al., 2006). On the other hand, the fact that guava can host *C. abscissum* has obvious epidemiological consequences on citrus postbloom fruit drop outbreaks.

All strains tested for their pathogenicity on fruits in this study were able to infect different fruit hosts. However they differed in their aggressiveness towards them. Several studies have demonstrated the lack of host specificity of *Colletotrichum* species infecting fruits (Peres et al. 2002; MacKenzie et al. 2009; Lakshmi et al. 2011; Phoulivong et al. 2012; Peng et al. 2013; de Souza et al. 2013; Baroncelli et al. 2015). Many *Colletotrichum* species can be associated with different hosts and one host can be affected by different species (Damm et al. 2012, 2014; Weir et al. 2012). In a study of MacKenzie et al. (2009), genetically distinct strains of *C. acutatum* (s. lat.) isolated from strawberry, blueberry, citrus and fern were pathogenic and shown to have differences in aggressiveness with the highest incidence and the biggest lesions being observed on their original host. Based on their TUB2 sequences, Damm et al. (2012) could link the strains from strawberry to *C. nymphaeae* and the strains from blueberry to *C. fioriniae*. Some *Colletotrichum* species are more frequently associated with a specific fruit crop or seem to have a narrow host range, while other species occur on a wide range of hosts.

Regarding the species isolated from apple, none of them seemed to be specific to apple fruits. Based on our pathogenicity data, they can all potentially infect peach and guava (with the exception of *C.* cf. *melonis* strain CBS 134730 that did not infect guava at all), and there is no difference in virulence of the three species on apple. *C. nymphaeae* was recently also identified among strains in the NIAS Genbank from multiple hosts, including several strains from apple and peach in Japan (Sato & Moriwaki 2013). This species caused lesions on fruits of all three hosts tested and apparently also occurs on all of them in nature. But although *C. nymphaeae* and *C. fioriniae* both have a large host range, and some of the hosts are overlapping, there could be still differences in pathogenicity and aggressiveness as the results of MacKenzie et al. (2009) suggest.

The species newly reported in this paper have so far only been isolated from apple, peach and pequi (*Caryocar brasiliense*). However, it was found to be potentially pathogenic also on guava in this study. Future studies will show if *C. paranaense* occurs on this host in nature. The knowledge of cross infection ability of a species is important to investigate its potential host range and, consequently, to support quarantine measures

(Phoulivong *et al.* 2012). The possible plurivorous nature of the species identified in this study might be one of the reasons for the cross-pathogenicity of the strains tested. Another reason could be the inoculation technique and incubation conditions of the pathogenicity test that might establish conditions more favourable for infection than usually occurring in nature. Although wound infection is common practice in pathogenicity tests on fruits (Cai *et al.* 2009; Peng *et al.* 2013), it could have influenced the virulence of the strains. For example, in inoculation experiments by von Arx & van der Velden (1961), the number of fruit hosts infected by *Colletotrichum orbiculare* and "*Glomerella cingulata*" (probably *C. gloeosporioides* s. lat.) was much higher after wound inoculation than without wounding.

The Bayesian tree obtained in this study suggests high genetic variability among the species occurring in Brazil. This can be important knowledge for developing control strategies. For example, the population of a plant pathogen with high genetic variability can evolve rapidly, and this information can be used for predicting how long a control measure is likely to be effective (McDermott & McDonald 1993). Additionally, the correct identification of the pathogen is important for its effective control strategy based on fungicides, because some species are more sensitive to specific groups of chemical compounds than other species (Freeman *et al.* 1998; Sanders *et al.* 2000; Wong & Midland 2007). For example, *C. gloeosporioides* (s. lat.) is considered highly sensitive to benomyl, whereas *C. acutatum* (s. lat.) is comparatively resistant (Freeman *et al.* 1998). However, the individual species within these species complexes need to be tested for their sensitivity for specific fungicides. Furthermore, the accurate identification of the pathogens can improve our understanding of their epidemiology and infection strategy, and provide important knowledge for breeding of resistant cultivars.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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