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Five *Colletotrichum* species are responsible for mango anthracnose in northeastern Brazil

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Abstract *Colletotrichum* species are the most important and widespread form of decay affecting mango fruit worldwide. In this study, *Colletotrichum* species associated with fruit anthracnose isolated from mango in northeastern Brazil were subject to molecular and morphological analyses. The partial sequences of the glyceraldehyde-3-phosphate dehydrogenase gene of 143 *Colletotrichum* isolates was amplified, as an initial measure of genetic diversity. A subset of 47 isolates, selected to represent the range of genetic diversity and geographic origin, were further sequenced using the partial actin, β-tubulin, calmodulin, glutamine synthetase genes and rDNA-ITS region. The multilocus sequence analysis, together with a critical examination of the phenotypic characters, revealed four previously described species (*Colletotrichum asianum*, *Colletotrichum fructicola*, *Colletotrichum tropicale* and *Colletotrichum karstii*) and one new species. The new species is introduced as *Colletotrichum dianesei* and formally described, illustrated and compared with similar taxa. Only *C. asianum* and *C. karstii* have previously been reported from mango, while the other species represent the first report associated with the mango fruits worldwide. All species are reported for the first time associated with the mango fruits in Brazil.

Keywords Multilocus phylogeny · Morphology · New species · Plant disease

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Introduction

The mango (*Mangifera indica* L.) is an important fruit crop in Brazil and other tropical and subtropical countries of the world (Evans and Mendoza 2009). In Brazil, the main areas of cultivation are in the northeastern region and in these areas are mainly produced for export. In 2010, Brazil exported 124,694 tons of mangoes worth approximately US$ 119,929 million (Anuário Brasileiro de Fruticultura 2012). Mango is affected by a number of diseases at all stages of its development, from seedlings in the nursery, to the fruits in storage or transit (Ploetz 2003; Prakash 2004). Anthracnose, caused by species of *Colletotrichum*, is the most important disease of mango in Brazil; the disease limits productivity and reduces fruit quality, thus directly affecting fruit export (Santos Filho and Matos 2003). In India, losses due to anthracnose have been estimated to be 2–39 % (Prakash 2004).

The genus *Colletotrichum* includes a number of plant pathogens of major importance, causing diseases of a wide variety of woody and herbaceous plants (Hyde et al. 2009; Wikee et al. 2011; Cannon et al. 2012). Identification of *Colletotrichum* species was previously based on morphology and to a lesser extend host association (Hyde et al. 2009). A polyphasic approach, using combined sequence analysis of multiple loci, coupled with morphological data, is now recommended for accurate species identification in the genus (Cai et al. 2009, 2011). Ko Ko et al. (2011) noted the need to resurvey plant

pathogens such as *Colletotrichum* with molecular based approaches, as previous data based on morphology was likely to be incorrect. The genus was recently voted the worlds eighth most important group of plant pathogenic fungi, based on perceived scientific and economic importance (Dean et al. 2012).

Anthracnose caused by *C. gloeosporioides* is the most important and widespread form of decay affecting mango fruit worldwide (Jeffries et al. 1990; Prusky and Keen 1993; Freeman et al. 1998; Ploetz 2003). However, following epitypification of *C. gloeosporioides* (Cannon et al. 2008), Phoulivong et al. (2010) were able to show that *C. gloeosporioides* sensu stricto was actually not a common pathogen on fruits in the tropics and it was not the cause of mango anthracnose in Laos and Thailand.

In Brazil, anthracnose of mango is attributed exclusively to *C. gloeosporioides* (Santos Filho and Matos 2003; Ribeiro 2005) and there are no studies on the molecular characterization of *Colletotrichum* species associated with mango. Therefore, the objective of this study was to characterize species of *Colletotrichum* from a large number of isolates associated with mango anthracnose in the northeastern Brazil based on DNA sequence data, morphology, and pathogenicity and virulence tests.

Material and methods

Sampling and fungal isolation

During 2010 and 2011, mango fruits showing anthracnose symptoms were collected in 10 orchards (70 samples per orchard) located in São Francisco Valley, Assú Valley and Zona da Mata Pernambucana, northeastern Brazil. The orchards from Zona da Mata Pernambucana did not receive any fungicide applications, and other two orchards received at least one spray with methyl benzimidazole carbamates (MBC), demethylation inhibitor (DMI) or other fungicides. Samples were recovered from the cultivar Tommy Atkins. Fruit tissues were surface disinfested in 70 % ethanol for 30 s and 1 % NaClO for 1 min. Samples were then rinsed in sterile distilled water for 30 s and dried before small pieces (4–5 mm) of tissue were taken from the margin between necrotic and apparently healthy tissue to be plated onto potato dextrose agar (PDA) (Acumedia, Lansing, USA) amended with 0.5 g l^{-1} streptomycin sulfate (PDAS). Plates were incubated at 25 °C with a 12–h photoperiod provided by fluorescent light for 5 to 7 days. The growing edges of any fungal hyphae developing from the tissues were then transferred aseptically to PDA. When direct examination showed that the fungus was sporulating on the mango fruits, spore masses were picked off with a sterilized wire loop and streaked on the surface of water agar. After incubation overnight (25 °C), single germinated spores were picked up with a sterilized needle and transferred to PDA (Abang 2003; Chomnunti et al. 2011). The fungi were identified following sporulation. One-hundred and forty-three isolates were morphologically identified as *Colletotrichum* spp. (Sutton 1980) and single spore cultures were obtained using the procedure described by Goh (1999). Pure cultures were stored in sterilized water in Eppendorf tubes at 5 °C and stock cultures were stored in PDA slants at 5 °C in the dark.

DNA extraction

Isolates were grown on PDA for 7 days at 25 °C with a 12-h photoperiod. Using a sterile 10 μl pipette tip, a small amount of aerial mycelia was scraped from the colony surface and genomic DNA was extracted using the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., Union City, USA) following the manufacturer's instructions. DNA concentrations were estimated visually in agarose gel by comparing band intensity with a DNA ladder 1 kb (Axygen).

PCR amplification and DNA sequencing

All strains in this study were amplified for the glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene, as an initial measure of genetic diversity. A subset of 47 isolates, selected to represent the range of genetic diversity and geographic origin (Table 1), were further sequenced for partial actin (ACT), β-tubulin (TUB2), calmodulin (CAL), glutamine synthetase (GS) genes and rDNA-ITS (ITS) region were amplified by polymerase chain reaction (PCR) reaction.

The PCR amplification for GPDH, CAL and GS were carried out using the primers-pair GDF1 and GDR1 (Templeton et al. 1992), CL1 and CL2 (O'Donnell et al. 2000), and GSF1 and GSR1 (Stephenson et al. 1997), respectively. The partial ACT, TUB2 and ITS region were amplified with the primers-pair ACT512F and ACT783R (Carbone and Kohn 1999), Bt2a and Bt2b (Glass and Donaldson 1995), and ITS 1 and ITS 4 (White et al. 1990), respectively.

Each 50-μl polymerase chain reaction (PCR) mixture included 21 μl of PCR-grade water, 1 μl of DNA template, 1.5 μM of each primer, and 1 μl of PCR Master Mix (2×) (0.05 u μl^{-1} de *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP; Thermo Scientific, Waltham, USA). PCR reactions were carried out in a thermal cycler (Biocycler MJ 96; Applied Biosystems, Foster City, USA). The cycling parameters for GPDH and GS consisted of a denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min and a final cycle at 72 °C for 10 min. The cycling parameters for CAL were initiated at 94 °C for 2 min followed by 35 cycles at

Table 1 Strains of *Colletotrichum* studied in this paper with details of culture collection, host, location and GenBank accessions of the sequences generated

Species	Culture Accession No.[a]	Host	Location	GenBank Accession No.[b]					
				ACT	CAL	GPDH	ITS	GS	TUB2
Colletotrichum aenigma	ICMP 18608*	*Persea americana*	Israel	JX009443	JX009683	JX010044	JX010244	JX010078	JX010389
C. aeschynomenes	ICMP 17673*, ATCC 201874	*Aeschynomene virginica*	USA	JX009483	JX009721	JX009930	JX010176	JX010081	JX010392
C. alatae	CBS 304.67*, ICMP 17919	*Dioscorea alata*	India	JX009471	JX009738	JX009990	JX010190	JX010065	JX010383
C. alienum	IMI 313842, ICMP 18691	*Persea americana*	Australia	JX009580	JX009664	JX010018	JX010217	JX010074	JX010385
C. annellatum	CBS 129826, CH1*	*Hevea indica*, leaf	Colombia	JQ005570		JQ005309	JQ005222		JQ005656
C. asianum	MFLU 090232	*Coffea arabica*	Thailand	FJ903188	FJ917501	FJ972571	FJ 972605	FJ 972586	FJ 907434
	MFLU 090234*	*Coffea arabica*	Thailand	FJ907421	FJ917503	FJ972573	FJ 972615	FJ 972598	FJ 907436
	CMM4056	*Mangifera indica*	Brazil, São Francisco Valley	KC533720	KC517232	KC517165	KC329789	KC430871	KC517277
	CMM 4057	*Mangifera indica*	Brazil, São Francisco Valley	KC533747	KC517233	KC517168	KC329792	KC430897	KC517278
	CMM 4058	*Mangifera indica*	Brazil, Zona da Mata	KC533748	KC517238	KC517173	KC329797	KC430874	KC517282
	CMM4059	*Mangifera indica*	Brazil, Zona da Mata	KC533750	KC517239	KC517174	KC329798	KC430875	KC517283
	CMM4060	*Mangifera indica*	Brazil, Zona da Mata	KC533749	KC517241	KC517176	KC329799	KC430876	KC517285
	CMM4061	*Mangifera indica*	Brazil, Zona da Mata	KC533736	KC517235	KC517170	KC329794	KC430872	KC517280
	CMM4062	*Mangifera indica*	Brazil, Zona da Mata	KC533721	KC517236	KC517171	KC329795	KC430873	KC517281
	CMM4063	*Mangifera indica*	Brazil, Zona da Mata	KC533722	KC517240	KC517175	KC329771	KC430902	KC517284
	CMM4064	*Mangifera indica*	Brazil, Zona da Mata	KC533724	KC517243	KC517178	KC329801	KC430901	KC517287
	CMM4065	*Mangifera indica*	Brazil, Zona da Mata	KC533723	KC517242	KC517177	KC329800	KC430877	KC517286
	CMM4066	*Mangifera indica*	Brazil, Zona da Mata	KC533751	KC517234	KC517169	KC329793	KC430909	KC517279
	CMM4067	*Mangifera indica*	Brazil, Zona da Mata	KC533725	KC517244	KC517179	KC329803	KC430878	KC517288
	CMM4068	*Mangifera indica*	Brazil, Zona da Mata	KC533752	KC517237	KC517172	KC329796	KC430898	KC517291
C. beeveri	CBS 128527, ICMP 18594*	*Brachyglottis repanda*	New Zealand	JQ005519		JQ005258	JQ005171		JQ005605
C. boninense	CSSN1	*Crinum asiaticum*	China	GQ856774		GQ856743	GQ485597		GQ849437
	MAFF 305972*	*C. asiaticum* var. *sinicum*	Japan	HM582001		HM585386	HM585399		HM585421
C. brasiliense	CBS 128501, ICMP 18607*	*Passiflora edulis*, fruit	Brazil	JQ005583		JQ005322	JQ005235		JQ005669
	CBS 128528, ICMP 18606	*Passiflora edulis*, fruit	Brazil	JQ005582		JQ005321	JQ005234		JQ005668
C. brassicicola	CBS 101059, LYN 16331*	*Brassica oleracea*	New Zealand	JQ005520		JQ005259	JQ005172		JQ005606
C. colombiense	CBS 129817	*Passiflora edulis*, leaf	Colombia	JQ005521		JQ005260	JQ005173		JQ005607
	CBS 129818*	*Passiflora edulis*	Colombia	JQ005522		JQ005261	JQ005174		JQ005608
C. constrictum	CBS 128504, ICMP 12941*	*Citrus limon*	New Zealand	JQ005586		JQ005325	JQ005238		JQ005672
	CBS 128503, ICMP 12936	*Solanum betaceum*	New Zealand	JQ005585		JQ005324	JQ005237		JQ005671
C. cymbidiicola	CBS 123757, MAFF 306100	*Cymbidium* sp.	Japan	JQ005516		JQ005255	JQ005168		JQ005602
	IMI 347923*	*Cymbidium*	Australia	JQ005514		JQ005253	JQ005166		JQ005600
C. dacrycarpi	CBS 130241, ICMP 19107*	*Dacrycarpus dacrydioides*	New Zealand	JQ005584		JQ005323	JQ005236		JQ005670
C. dianesei	CMM4077	*Mangifera indica*	Brazil, São Francisco Valley	KC517295	KC517203	KC517156	KC329773	KC430903	KC517248
	CMM4078	*Mangifera indica*	Brazil, São Francisco Valley	KC533745	KC517205	KC517158	KC329775	KC430886	KC517250
	CMM4079	*Mangifera indica*	Brazil, São Francisco Valley	KC533746	KC517206	KC517159	KC329776	KC430893	KC517251
	CMM4080	*Mangifera indica*	Brazil, São Francisco Valley	KC517297	KC517207	KC517160	KC329777	KC430885	KC517252
	CMM4081, MFLU 1300056	*Mangifera indica*	Brazil, São Francisco Valley	KC517304	KC517218	KC517166	KC329790	KC430880	KC517263

Table 1 (continued)

Species	Culture Accession No.[a]	Host	Location	GenBank Accession No.[b]					
				ACT	CAL	GAPDH	ITS	GS	TUB2
	CMM4082, MFLU 1300057	*Mangifera indica*	Brazil, São Francisco Valley	KC517296	KC517204	KC517157	KC329774	KC430895	KC517249
	CMM4083, MFLU 1300058*	*Mangifera indica*	Brazil, São Francisco Valley	KC517298	KC517209	KC517194	KC329779	KC430894	KC517254
	CMM4084	*Mangifera indica*	Brazil, Assú Valley	KC517307	KC517228	KC517201	KC329811	KC430890	KC517273
	CMM4085	*Mangifera indica*	Brazil Assú Valley	KC533740	KC517230	KC517196	KC329813	KC430891	KC517275
	CMM4086	*Mangifera indica*	Brazil, Assú Valley	KC533742	KC517229	KC517195	KC329812	KC430883	KC517274
	CMM4087	*Mangifera indica*	Brazil, Assú Valley	KC533741	KC517231	KC517197	KC329814	KC430887	KC517276
C. dianesei	CMM4088, MFLU 1300059	*Mangifera indica*	Brazil, São Francisco Valley	KC517300	KC517210	KC517162	KC329781	KC430900	KC517255
	CMM4089, MFLU 1300060	*Mangifera indica*	Brazil, São Francisco Valley	KC517302	KC517211	KC517163	KC329783	KC430879	KC517256
	CMM4090	*Mangifera indica*	Brazil, São Francisco Valley	KC533735	KC517208	KC517161	KC329778	KC430899	KC517253
	CMM4091	*Mangifera indica*	Brazil, Assú Valley	KC533737	KC517221	KC517180	KC329804	KC430908	KC517266
	CMM4092	*Mangifera indica*	Brazil, Assú Valley	KC533739	KC517226	KC517192	KC329809	KC430907	KC517271
	CMM4093	*Mangifera indica*	Brazil, Assú Valley	KC533734	KC517225	KC517164	KC329808	KC430892	KC517270
	CMM4094	*Mangifera indica*	Brazil, São Francisco Valley	KC517294	KC517202	KC517155	KC329772	KC430884	KC517247
	CMM4095	*Mangifera indica*	Brazil, São Francisco Valley	KC517305	KC517219	KC517167	KC329791	KC430881	KC517264
	CMM4096, MFLU 1300061	*Mangifera indica*	Brazil, Assú Valley	KC533733	KC517223	KC517190	KC329806	KC430896	KC517268
	CMM4097, MFLU 1300062	*Mangifera indica*	Brazil, Assú Valley	KC533732	KC517222	KC517189	KC329805	KC430888	KC517267
	CMM4098	*Mangifera indica*	Brazil, Assú Valley	KC533738	KC517224	KC517191	KC329807	KC430889	KC517269
	CMM4099	*Mangifera indica*	Brazil, Assú Valley	KC517303	KC517212	KC517200	KC329784	KC430904	KC517257
	CMM4100	*Mangifera indica*	Brazil, Assú Valley	KC517306	KC517227	KC517193	KC329810	KC430882	KC517272
C. fructicola	MFLU 090228*	*Coffea arabica*	Thailand	FJ907426	FJ917508	FJ972578	FJ 972603	FJ 972593	FJ 907441
	MFLU 090226	*Coffea arabica*	Thailand	FJ907427	FJ917509	FJ972579	FJ972602	FJ972592	FJ907442
	CMM4069	*Mangifera indica*	Brazil, São Francisco Valley	KC517299	KC517245	KC517188	KC329780	KC430906	KC517289
	CMM4070	*Mangifera indica*	Brazil, São Francisco Valley	KC517301	KC517246	KC517187	KC329782	KC430910	KC517290
	MFLU 090226	*Coffea arabica*	Thailand	FJ907427	FJ917509	FJ972579	FJ 972602	FJ 972592	FJ 907442
C. gloeosporioides	CBS 953.97	*Coffea arabica*	Thailand	FJ 907430	FJ 917512	FJ 972582	FJ 972609	FJ 972589	FJ 907445
C. hippeastri	CBS 241.78, IMI 304052	*Hippeastrum* sp.	Netherlands	JQ005580		JQ005319	JQ005232		JQ005666
	CBS 125376, CSSG1*	*Hippeastrum vittatum*	China	JQ005579		JQ005318	JQ005231		JQ005665
C. horii	NBRC 7478, ICMP 10492*	*Diospyros kaki*	Japan	JX009438	JX009604	GQ329681	GQ329690	JX010137	JX010450
C. karstii	CORCG6(CGMCC3.14194)	*Vanda* sp.	China	HM581995		HM585391	HM585409		HM585428
	CORCK1	*Calanthe argenteo-striata*	China	HM581991		HM585387	HM585406		HM585424
	CMM4101	*Mangifera indica*	Brazil, São Francisco Valley	KC533743		KC517198	KC295235		KC517292
	CMM4102	*Mangifera indica*	Brazil, São Francisco Valley	KC533744		KC517199	KC295236		KC517293
C. musae	CBS 116870*	*Musa* sp.	USA	JX009486	JX009661	JX009936	JX010189	JX010087	JX010397
C. novae-zelandiae	CBS 128505, ICMP 12944*	*Capsicum annuum*	New Zealand	JQ005576		JQ005315	JQ005228		JQ005662
	CBS 130240, ICMP 12064	*Citrus* sp.	New Zealand	JQ005577		JQ005316	JQ005229		JQ005663
C. nupharicola	CBS 470.96*, ICMP 18187	*Nuphar lutea* subsp. *polysepala*	USA	JX009437	JX009663	JX009972	JX010187	JX010088	JX010398
C. oncidii	CBS 129828*	*Oncidium* sp.	Germany	JQ005517		JQ005256	JQ005169		JQ005603
	CBS 130242	*Oncidium* sp.	Germany	JQ005518		JQ005257	JQ005170		JQ005604
C. parsonsiae	CBS 128525, ICMP 18590*	*Parsonsia capsularis*	New Zealand	JQ005581		JQ005320	JQ005233		JQ005667

Table 1 (continued)

Species	Culture Accession No.[a]	Host	Location	GenBank Accession No.[b]					
				ACT	CAL	GAPDH	ITS	GS	TUB2
C. petchii	CBS 378.94*	*Dracaena marginata*	Italy	JQ005571		JQ005310	JQ005223		JQ005657
	CBS 379.94	*Dracaena marginata*,	Italy	JQ005572		JQ005311	JQ005224		JQ005658
C. phyllanthi	CBS 175.67, MACS 271*	*Phyllanthus acidus*	India	JQ005569		JQ005308	JQ005221		JQ005655
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	JX009447	JX009691	JX009934	JX010276	JX010104	JX010414
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	JX009562	JX009696	JX009916	JX010242	JX010093	JX010403
C. siamense	MFLU 090230*	*Coffea arabica*	Thailand	FJ907423	FJ917505	FJ972575	FJ972613	FJ972596	FJ907438
	MFLU 090231	*Coffea arabica*	Thailand	FJ907422	FJ917504	FJ972574	FJ972614	FJ972597	FJ907437
	MFLU 090229	*Coffea arabica*	Thailand	FJ907420	FJ917502	FJ972572	FJ972604	FJ972599	FJ907435
	ICMP 12567	*Persea americana*	Australia	JX009541	JX009697	JX009940	JX010250	JX010076	JX010387
	DAR 76934, ICMP 18574	*Pistacia vera*	Australia	JX009535	JX009707	JX010002	JX010270	JX010080	JX010391
	ICMP 18121	*Dioscorea rotundata*	Nigeria	JX009460	JX009715	JX009942	JX010245	JX010092	JX010402
C. siamense	ICMP 17795	*Malus domestica*	USA	JX009506	JX009703	JX010051	JX010162	JX010082	JX010393
	CBS 125378, ICMP 18642*	*Hymenocallis americana*	China	GQ856775	JX009709	JX010019	JX010278	JX010100	JX010410
	CBS 130420, ICMP 19118*	*Jasminum sambac*	Vietnam	HM131507	JX009713	HM131497	HM131511	JX010105	JX010415
C. theobromicola	CBS 124945, ICMP 18649*	*Theobroma cacao*	Panama	JX009444	JX009591	JX010006	JX010294	JX010139	JX010447
C. torulosum	CBS 128544, ICMP 18586*	*Solanum melongena* New	Zealand	JQ005512		JQ005251	JQ005164		JQ005598
	CBS 102667	*Passiflora edulis*	New Zealand	JQ005513		JQ005252	JQ005165		JQ005599
C. tropicale	CBS 124949, ICMP 18653*	*Theobroma cacao*	Panama	JX009489	JX009719	JX010007	JX010264	JX010097	JX010407
	MAFF 239933, ICMP 18672	*Litchi chinensis*	Japan	JX009480	JX009722	JX010020	JX010275	JX010086	JX010396
	CMM4071	*Mangifera indica*	Brazil, São Francisco Valley	KC533726	KC517213	KC517181	KC329785	KC430868	KC517258
	CMM4072	*Mangifera indica*	Brazil, São Francisco Valley	KC533727	KC517214	KC517182	KC430866	KC430866	KC517259
	CMM4073	*Mangifera indica*	Brazil, São Francisco Valley	KC533730	KC517217	KC517185	KC329788	KC430870	KC517262
	CMM4074	*Mangifera indica*	Brazil, São Francisco Valley	KC533728	KC517215	KC517183	KC329786	KC430867	KC517260
	CMM4075	*Mangifera indica*	Brazil, Zona da Mata	KC533731	KC517220	KC517186	KC329802	KC430905	KC517265
	CMM4076	*Mangifera indica*	Brazil, São Francisco Valley	KC533729	KC517216	KC517184	KC329787	KC430869	KC517261
C. truncatum	CBS120709	*Capsicum frutescens*	India	GQ856783		GQ856753	GQ485593		GQ849429
	CSSX2	*Crinum asiaticum*	China	GQ856766		GQ856750	GQ485595		GQ849424

[a] *ATCC* American Type Culture Collection, Virginia, USA; *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *CGMCC* China General Microbiological Culture Collection, Beijing, China; *CMM* Culture Collection of Phythopathogenic Fung "Prof. Maria Menezes", Recife, Brazil; *DAR* Queensland Plant Pathology Herbarium (Australia), Queensland, Australia; *ICMP* International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; *IMI* International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; MAFF: *MAFF* Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan; *MFLU* Mae Fah Luang University Herbarium, Thailand; *NBRC* Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan. * ex-type or ex-epitype

[b] *ACT* actin; *TUB-2* partial ß-tubulin (tub2); *CAL* calmoudulin; *GS* glutamine synthetase; *GDPH* glyceraldehydes-3-phosphate dehydrogenase; *ITS* partial rDNA-ITS region

94 °C for 1 min, 54 °C for 30 s, 72 °C for 30 s and a final step at 72 °C for 10 min. The cycling parameters for partial ACT, TUB2 and ITS region consisted of a 2 min denaturing step at 94 °C followed by 34 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final cycle of 10 min at 72 °C.

The PCR amplification products were separated by electrophoresis in 1.5 % agarose gels in 1.0× Tris-acetate acid EDTA (TAE) buffer and were photographed under UV light after staining ethidium bromide (0.5 μg ml^{-1}) for 1 min. PCR products were purified using the AxyPrep™ PCR Cleanup Kit (Axygen) following the manufacturer's instructions. DNA sequencing for GPDH, ACT, CAL, GS, TUB2, CAL genes and ITS region were performed using a ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) at the Sequencing Platform LABCEN/CCB in the Universidade Federal de Pernambuco (Recife, Brazil).

Phylogenetic analysis

The quality of the nucleotide sequences and the contig assembly were carried out using the Staden Package (Staden et al. 1998). Multiple sequence alignments of each gene used Clustal W as implemented in MEGA v.5 (Tamura et al. 2011), and manually adjusted to allow maximum sequence similarity.

Bayesian inference (BI) was used to reconstruct the phylogenetic trees using MrBayes v. 3.2.1 (Ronquist et al. 2012). Bayesian inference has significant advantages over other methods of analysis such as maximum likelihood and maximum parsimony (Archibald et al. 2003) and provides measures of clade support as posterior probabilities rather than random resampling bootstraps (Weir et al. 2012). jModelTest v. 0.1.1 (Posada 2008) was used to carry out the statistical selection of best-fit models of nucleotide substitution using the Bayesian Information Criterion (BIC) (Table 2). The nucleotide multiple sequence alignments of all genes were concatenated and the analysis on the full data set were run twice for 5×10^7 generations. Samples were taken from the posterior every 1,000 generations. Convergence of all parameters was checked using the Tracer program (Rambaut and Drummond 2007). Therefore, the first 25 % of generations were discarded as burn-in.

Sequences of *Colletotrichum* species obtained from GenBank were included in the analyses (Table 1). Seventy–one isolates were used in the initial BI (A) analysis using a concatenated alignment for six: ACT, CAL, GS, GPDH, TUB2 and ITS. *Colletotrichum theobromicola* was used as outgroup in this analysis. A second analysis of BI (B) was carried out to confirm the identity of two isolates of *Colletotrichum karstii*. The second analysis was based on a concatenated alignment of four: ACT, GPDH, ITS and TUB2. *Colletotrichum gloeosporioides* was used as outgroup in this analysis.

Table 2 Nucleotide substitution models used in phylogenetic analyses

Gene	Model - analysis A	Model - Analysis B
ACT	K80+G	K80+G
CAL	TrNef+G	–
GAPDH	HKY+G	K80+G
GS	HKY+G	–
ITS	TIM3ef+I+G	TrNef+G
TUB2	TrNef	K80+G

Sequences derived in this study are deposited in GenBank. Representative isolates of different *Colletotrichum* species obtained in this study were deposited in the Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes" (CMM) at the Universidade Federal Rural de Pernambuco, Brazil and MFLU, Mae Fah Luang University, Thailand.

Morphological studies of *Colletotrichum* species

Based on the fungal species that were identified based on phylogenetic analyses, 47 *Colletotrichum* isolates [*C. asianum* (13), *C. fructicola* (2), *C. tropicale* (6), *C. karstii* (2) and *Colletotrichum* sp. (24)] were further characterized by colony morphology and conidial characteristics. A 4–mm–diameter mycelial plug from the growing margin of a 5-day-old colony was placed in the centre of a 90–mm–diameter PDA plate, and four replicates of each isolate were incubated at 28 °C with a continuous fluorescent light (24–h). The colony colour and diameter from isolates were recorded during 7 days. The colony colour was evaluated using the mycological colour chart (Rayner 1970) and the colony diameter (mm) was measured in two perpendicular directions. The colony diameter data were used to calculate the mycelial growth rate (mm day^{-1}). After 7 days, conidia were mounted in 100 % lactic acid and digital images recorded with a Sansung SDC-415 camera (Sansung Co., Seoul, Korea) on an Olympus BX41 microscope (Olympus Co., Tokyo, Japan). The length and width of 50 conidia per isolate were measured with the Motic Image Plus v. 2.0 image analysis software (Motic Group Co., Beijing, China). For the new *Colletotrichum* species, appressoria were induced using a slide culture technique, in which 10 mm^2 squares of PDA were placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip was placed over the inoculated agar (Johnston and Jones 1997). The length and width of 50 appressoria per isolate were measured with the Motic Image Plus v. 2.0. Mean and standard errors of the conidial and appressoria measurements were calculated.

One–way analyses of variance (ANOVA) were conducted to determine the significance of differences in conidia dimensions and growth rates with *Colletotrichum* species, and

means were compared by Fisher's least significant difference (LSD) test at the 5 % significance level using STATISTIX v. 9.0 (Analytical Software, Tallahassee, USA).

Pathogenicity and virulence in fruits

The isolates used in morphological characterization were selected for pathogenicity and virulence tests on detached fruits of mango (cv. Tommy Atkins) under controlled conditions. The fungal inoculum was produced in Petri dishes containing PDA, which were incubated for 7 days at 28 °C under 12-h photoperiod. Spore suspensions were prepared by adding 20 mL of sterile distilled water to the surface of the cultures, brushing with a soft bristle brush, and filtering through a double layer of cheesecloth. Spore concentration was determined using a hemocytometer and adjusted to 10^6 conidia ml^{-1} with sterile water. Mango fruits at stage three of maturation (Assis 2004), without disease symptoms and which were not treated with fungicides, were washed in running water, surface disinfested in 70 % ethanol for 1 min and 1 % NaClO for 3 min, then rinsed in sterile distilled water. Since non-wounded treatment caused no lesions of *Colletotrichum* (non published data), after drying each fruit was wounded at the medium region by pushing the tip of four sterile pins through the surface of the skin to a depth of 3 mm. Six microliters of conidial suspension were dispensed at wound site on the fruits. Negative controls were fruits treated with sterile distilled water. Inoculated fruits were placed in large plastic containers. Before, the bottom of each container was lined with four paper layers wetted in distilled water to maintain humidity. Each fruit was put on a sterilized Petri dish to avoid direct contact with water. The plastic containers were partially sealed with plastic bags and incubated at 25 °C in the dark. The plastic bags and paper towels were removed after 24–h, and fruits were kept at the same temperature. Fruits were monitored for the onset of symptoms for 10 days. Isolates were considered pathogenic when the lesion area advanced beyond the 4–mm diameter initial injury. The virulence of the isolates was evaluated by measurement of the lesion length at 10 days after inoculation in two perpendicular directions on each fruit. The experiment was arranged in a completely randomized design with six replicates per treatment (isolate) and four fruits per replicate. The experiment was conducted twice. Difference in virulence caused by *Colletotrichum* species was determined by one-way ANOVA and means were compared by LSD test at the 5 % significance level using STATISTIX.

Results

Phylogenetic analyses

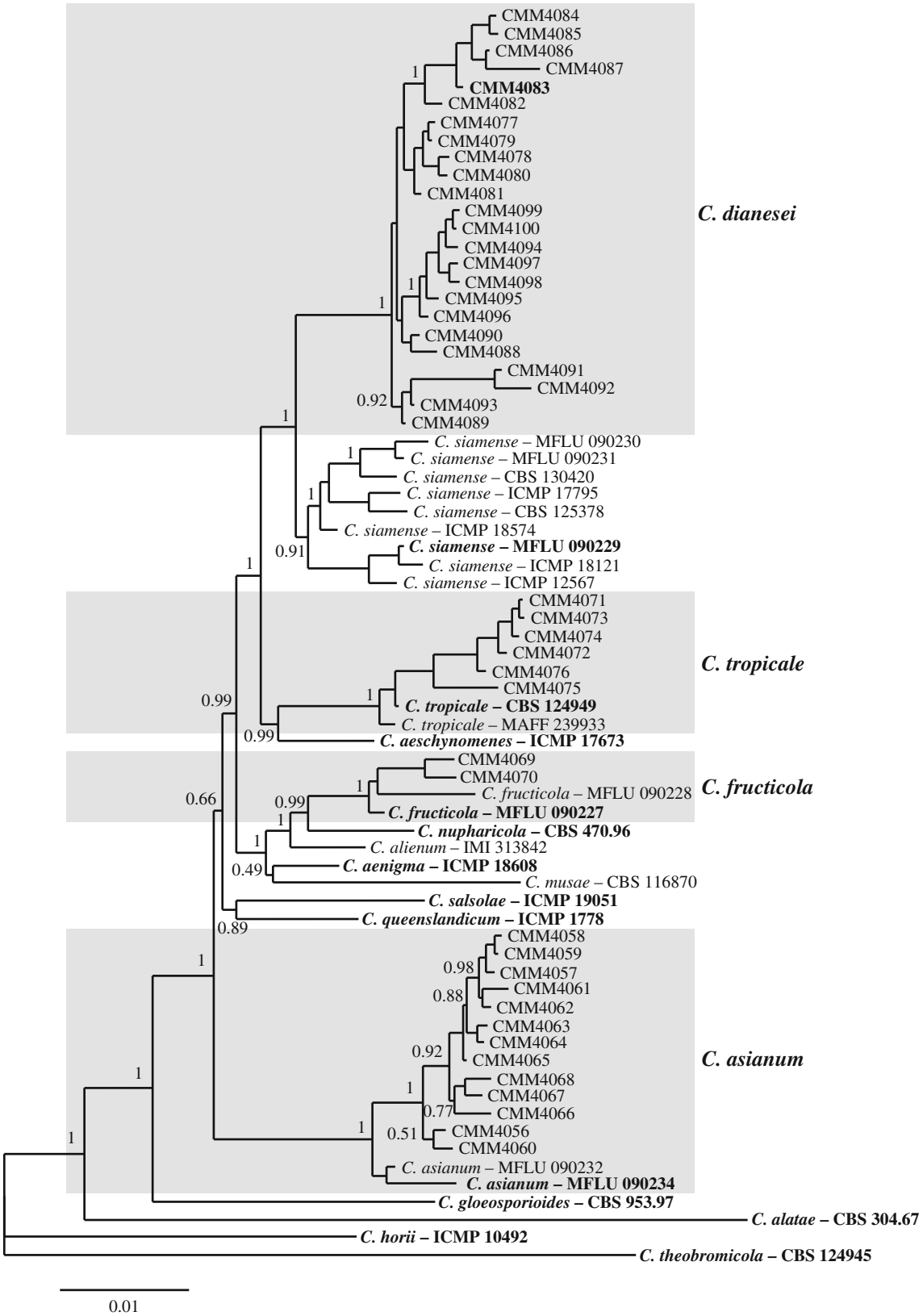
The initial analysis of the partial sequence GPDH gene of the 143 isolates revealed a total of nine haplotypes (H1–H9). Initial analysis of these haplotypes reveal that eight (H1–H8) belong to the *C. gloeosporioides* species complex, all of them within the "Musae clade" sensu Weir et al. (2012). The haplotype 9 (H9) GPDH sequence had a high degree of similarity (99 %) with sequences of type species of *C. karstii* deposited in the GenBank. Forty seven isolates representing all haplotypes were further used for multilocus analysis. Sequences of the ACT gene of the *Colletotrichum* species ranged from 249 to 297 bp. TUB2 gene ranged from 404 to 467 bp. Sequences of the CAL gene ranged from 628 to 707 bp. The sequences of the GPDH gene ranged from 203 to 279 bp. The GS gene ranged from 854 to 1,015 bp. The sequences of the ITS region ranged from 484 to 598 bp.

The Bayesian inference with the haplotypes that belong to the *C. gloeosporioides* complex that was performed with the combined datasets of partial ACT, TUB2, CAL, GS, GPDH and the ITS region comprised 3,220 characters after alignment. The gene boundaries in the alignment were: ACT: 1–293, BT2b: 294–734, CAL: 735–1424, GPDH: 1425–1729, GS: 1730–2647, ITS: 2648–3218. Sequences of *Colletotrichum* type species from the GenBank were included in the analysis (Table 1). The combined dataset resulted in four well supported clades. Three clades corresponded to species previously described: *C. asianum* (13), *C. fructicola* (2) and *C. tropicale* (6). The fourth clade containing the majority isolates (24) did not cluster with any known species, which indicate that these isolates belong to new species (Fig. 1). The BI analysis of the two isolates from haplotype 9 using the combined datasets of ACT, GPDH, ITS and TUB2 cluster these isolates with the type species of *C. karstii* with high posterior probability support (Fig. 2).

The diversity analyses from 143 isolates of *Colletotrichum* spp. show the following haplotype distribution within the species identified in phylogenetic analyses: *Colletotrichum* sp. 74 isolates belong to haplotypes H1 (46), H2 (19) and H4(9); *C. fructicola*: 2 isolates, all in H5; *C. asianum* 58 isolates, H3 (35) and H7 (23); *C. tropicale* 7 isolates, H8 (6) and H6 (1); *Colletotrichum karstii* 2 isolates, all in H9.

Morphological and cultural characterization

The *Colletotrichum* isolates grouped into seven morphotypes, based on colony characteristics (Fig. 3). Twelve percent of all isolates belonged to morphotype 1, which comprised white mycelia lacked conidial masses. Morphotype 2 (29 %) comprised isolates which had pale greenish grey (33""f), in reverse buff (19"f) colonies. Eleven percent of all isolates belonged to morphotype 3 with rosy vinaceous colonies (5"d), in reverse rosy buff (13"f). Isolates belonging to morphotype 4 (6 %) had cinnamon (15"b), in reverse cinnamon (15"i) colonies. Isolates belonging to morphotype 5 (8.3 %) had sky grey colonies (45""d), which in reverse were sky grey (53""d). The morphotype



6 (4.1 %) had pale mouse grey (15”””d) colonies which were pale mouse grey (15”””b) in reverse. Isolates belonging to morphotype 7 (4.1 %) which had orange (13b) colonies which were saffron (13d) in reverse. Isolates

◀ **Fig. 1** A Bayesian inference phylogenetic tree from 72 of *Colletotrichum* isolates using the combined partial sequence data of ACT, CAL, GPDH, GS, ITS and TUB2 genes. The tree shows the phylogenetic relationships of *Colletotrichum* species isolated from *Mangifera indica* and selected *Colletotrichum* species of the "Musae clade" sensu Weir et al. (2012). Bayesian posterior probability values ≥ 0.5 are shown in each node. Ex-type or ex-epitype sequences are emphasized in *bold* font. Culture accession numbers are listed. *Colletotrichum theobromicola* was used as outgroup. The scale bar indicates the number of expected changes per site

identified as *C. asianum* are represented in the morphotypes 1, 2, 3, 4, 5 and 6. The *C. fructicola* isolates were all included in morphotype 1. *C. tropicale* includes the morphotypes 1, 2 and 3. Isolates of *Colletotrichum* sp. comprised morphotypes 1, 2, 3, 4 and 5. The isolates of *C. karstii* belonged to morphotype 7. The species of *Colletotrichum* found in this study show differences in conidial size and growth rates. The previously described species show conidial dimensions and growth rate compatible with what is described in the literature (Table 3).

Taxonomy

Colletotrichum dianesei N. B. Lima, M. P. S. Câmara & S. J. Michereff **sp. nov.** (Fig. 4a–f)

MycoBank: MB803223.

Etymology: named in honor of professor José Carmine Dianese, University of Brasília (Brazil), for his contribution to Brazilian mycology.

Description: colonies on PDA at first white and becoming pale brownish to pinkish, reverse pale yellowish to pinkish, max. of 47.8 mm diam. in 5 days at 28 °C, growth rate 7.60–9.55 mm day^{-1} ($\bar{x} = 8.60 \pm 0.95$, n=6) (Fig. 4a, b). Aerial mycelium greyish white, dense, cottony, absent conidial masses. *Sclerotia* absent. *Acervuli* brown to dark brown present in culture. Setae present but rare (Fig. 4c, d). *Conidia* common in mycelium, one-celled, smooth-walled, hyaline, cylindrical with obtuse to slightly rounded ends, sometimes oblong, 10.5–14.5×4–5.5 μm ($\bar{x} = 12.0 \pm 0.95 \times 4.5 \pm 0.40$, n=50)

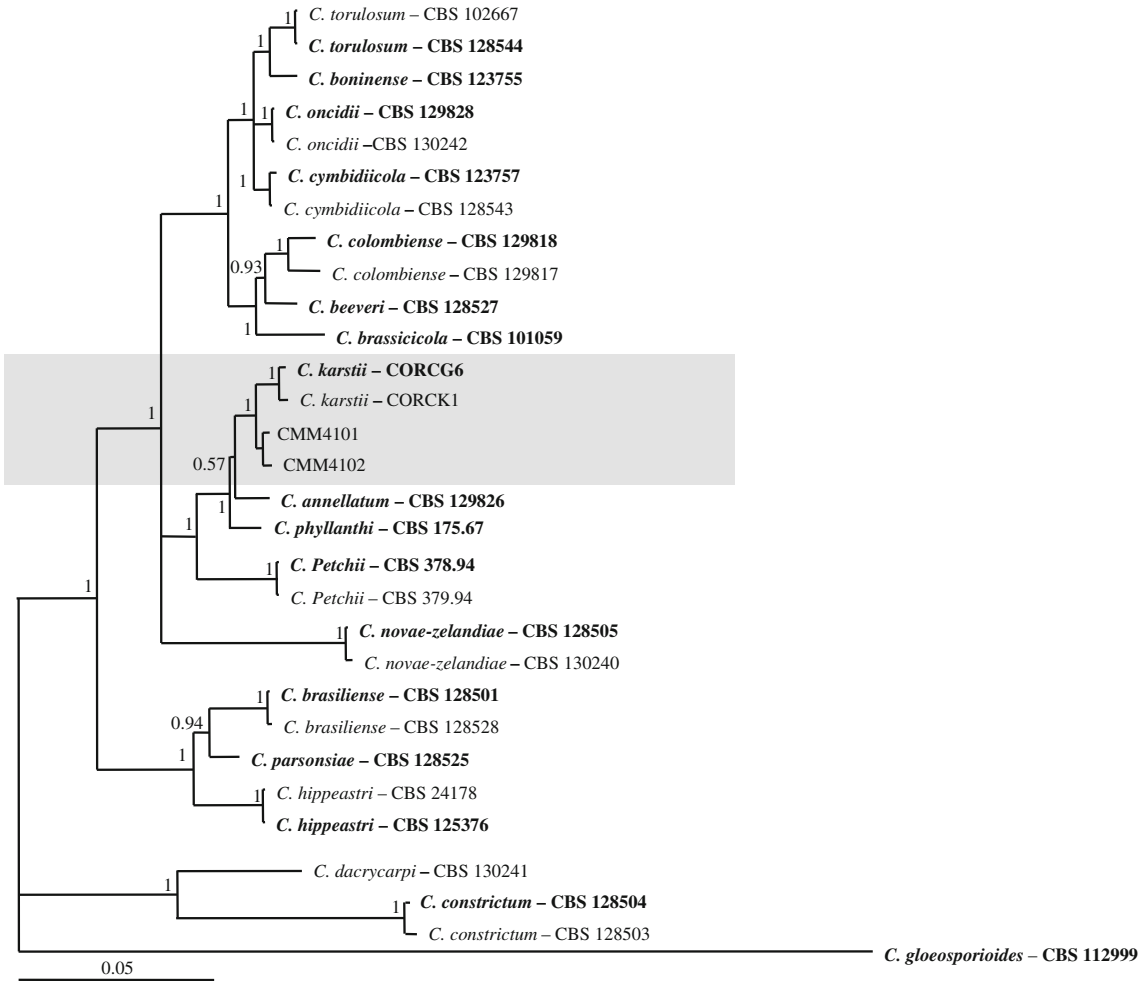


Fig. 2 A Bayesian inference phylogenetic tree of 30 isolates to confirm the identity of two isolates of *Colletotrichum karstii.* The tree was built using concatenated sequences of the partial ACT, GPDH, ITS and TUB2 genes each with a separate model of DNA evolution. Bayesian posterior probability values ≥ 0.5 are shown in each node. Ex-type or ex-epitype sequences are emphasized in *bold* font. Culture accession numbers are listed. *Colletotrichum gloeosporioides* was used as outgroup. The scale bar indicates the number of expected changes per site

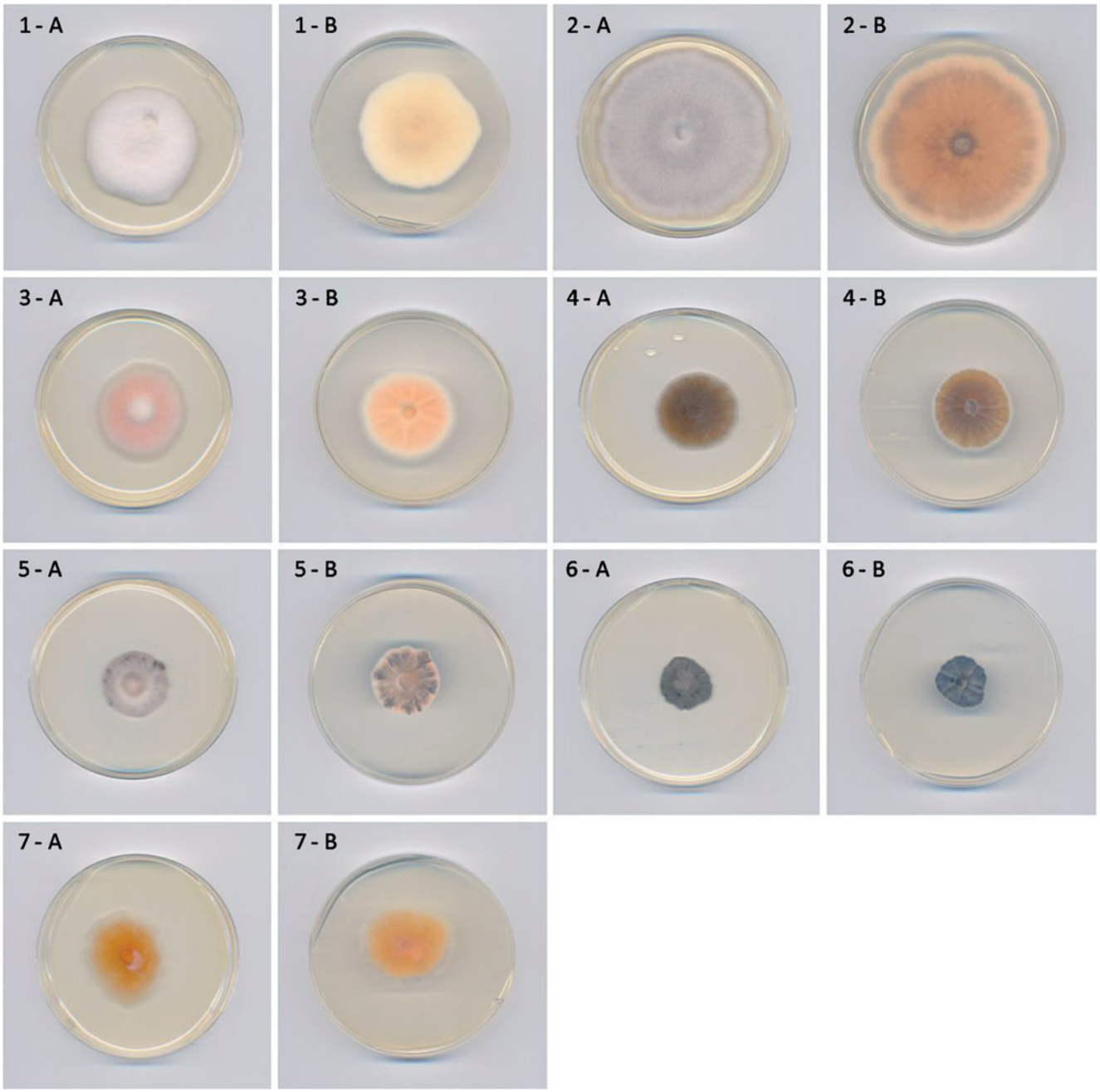


Fig. 3 Morphotypes (1 to 7) of isolates of *Colletotrichum* from mango fruits. Colony characteristics: Plates in A aerial view, reverse view in B

(Fig. 4f). *Appressoria* in slide cultures, brown, ovoid, sometimes clavate, 6.0–10.0×4.5–7.0 μm ($\bar{x} = 7.5 \pm 0.9 \times 5.5 \pm 0.5$, n=50) (Fig. 4e).

Teleomorph: not produced in culture.

Holotype: BRAZIL–Pernambuco, Petrolina, Farm Mapel (40° 34′ 23″, 9° 18′ 40″), on *Mangifera indica* fruits, 11 Apr 2010, Coll. N. Lima (holotype living culture CMM 4083; isotype in MFLU 1300058);

Known distribution: Pernambuco, Brazil.

Additional specimens examined: Additional specimens examined: Brazil, Pernambuco, Petrolina, Farm Mapel (40°34′ 23″, 9°18′40″), on *Mangifera indica* fruit, 11 May 2010, coll. N. B. Lima (paratype living cuture CMM4081, ex-paratype living culture MFLU 1300056). Brazil, Pernambuco, Petrolina, Farm Mapel (40°34′ 23″, 9°18′40″), on *Mangifera indica* fruit,11 May 2010, coll. N. B. Lima (paratype living cuture CMM4082, ex-paratype living culture MFLU 1300057). Brazil, Rio Grande do Norte, Ipanguaçu, Farm São João (36°53′03″, 5°31′29″), on *Mangifera indica* fruit, 9 Jun 2010, coll. N. B. Lima (paratype living cuture CMM4097, ex-paratype living culture MFLU 1300062). Brazil, Rio Grande do Norte, Ipanguaçu, Farm São João (36°53′03″, 5°31′29″), on

Table 3 Summary of morphological data of *Colletotrichum* isolates

Species	Conidia			Growth rate (mm day^{-1})
	Length (μm)	Width (μm)	Shape	
C. asianum	13.40±2.36b (5.60–21.00)	4.31±0.61b (2.70–5.60)	Cylindrical	5.16±1.62c (2.60–10.10)
Colletotrichum dianesei	12.06±1.54c (10.6–14.30)	4.30±0.69b (3.90–5.20)	Cylindrical	8.01±1.71a (4.30–13.38)
C. fructicola[a]	–	–	–	6.56±0.34b (6.10–7.02)
C. karstii	14.52±2,87a (10.40–20.20)	4.90±0.15a (3.80–6.50)	Cylindrical	5.20±0.86bc (3.65–6.44)
C. tropicale	14.12±1.17a (9.4–16.6)	4.82±0.49a (3.6–6.0)	Cylindrical	6.48±1.04b (4.41–8.24)

[a] These isolates did not produced conidia

Mangifera indica fruit, 9 Jun 2010, coll. N. B. Lima (paratype living cuture CMM4096, ex-paratype living culture MFLU 1300061). Brazil, Pernambuco, Petrolina, Farm copa fruit (40°34′00″, 9°23′08″), on *Mangifera indica* fruit, 23 May

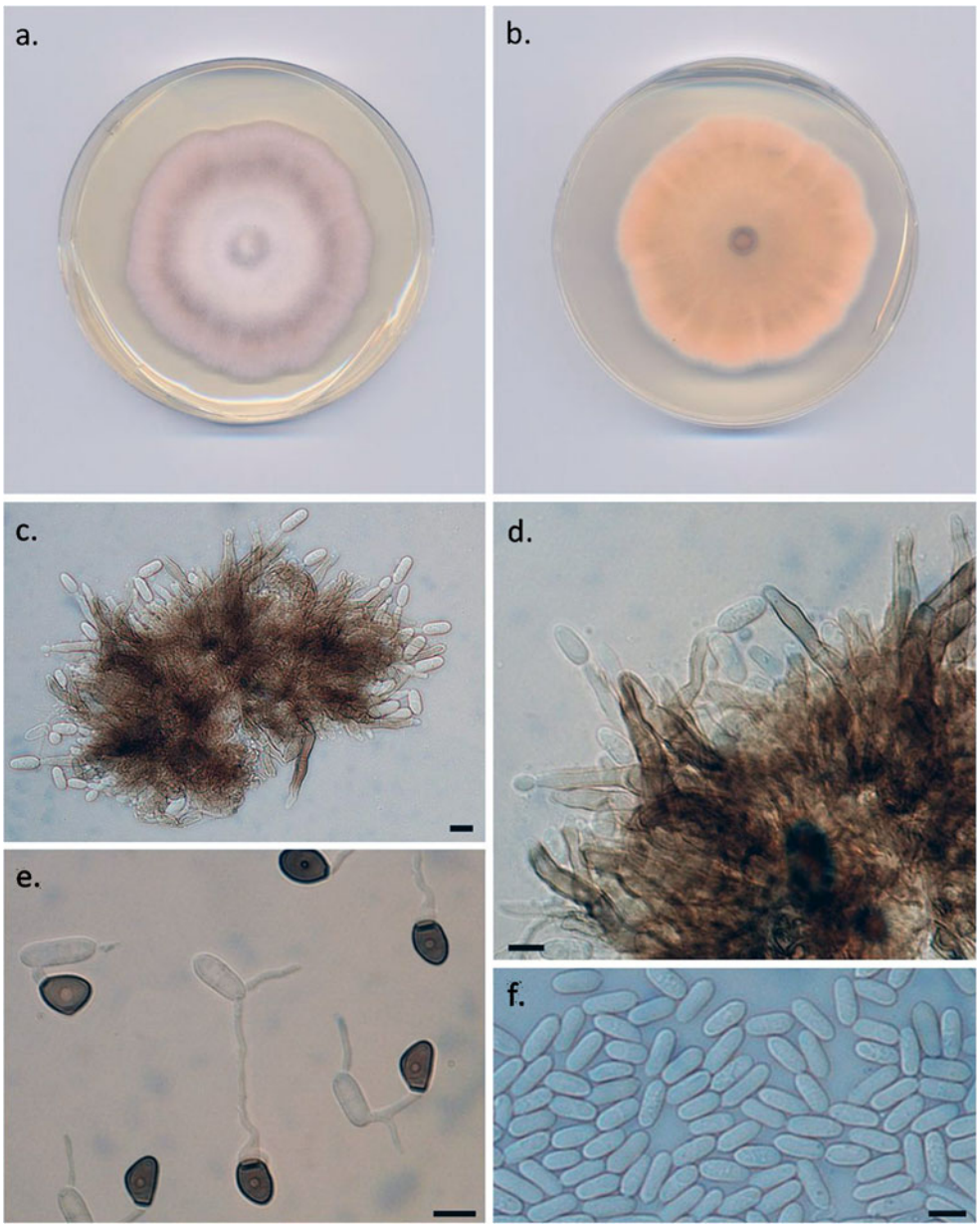


Fig. 4 *Colletotrichum dianesei* (from holotype.). Upper (**a**) and reverse (**b**) sides of cultures on PDA 7 days after inoculation; **c** brown to dark brown conidiomata and dark brown setae; **d** conidiogenous cells; **e** appressoria; **f** conidia. Bars: 10 μm

2010, coll. N. B. Lima (paratype living cuture CMM4089, ex-paratype living culture MFLU 1300060). Brazil, Pernambuco, Petrolina, Farm copa fruit (40°34′00″, 9°23′08″), on *Mangifera indica* fruit, 23 May 2010, coll. N. B. Lima (paratype living cuture CMM4088, ex-paratype living culture MFLU 1300059).

Pathogenicity and virulence in fruits

All isolates of *Colletotrichum* were pathogenic to mango fruits. Ripe fruits affected by anthracnose develop sunken, prominent, dark brown to black decay. *C. asianum* were most virulent. There were no significant (P≤0.05) differences in virulence among the other species (Fig. 5).

Discussion

This study represents the first attempt to characterize *Colletotrichum* species associated with anthracnose of mango fruits in Brazil using a phylogenetic approach. Although *C. gloeosporioides* has previously been shown to be the causal agent of tropical fruit rots, the most striking discovery of this study is that none of the 47 strains isolated from mango fruits with anthracnose symptoms belong to the species *C. gloeosporioides*. Phoulivong et al. (2010) showed that *C. gloeosporioides* was actually not a common pathogen in the tropics and it was not the cause of anthracnose in mango in Laos and Thailand.

Phylogenetic analysis showed that most of the strains included in this study belong to the 'gloeosporioides'complex. Five species of *Colletotrichum* were found associated with anthracnose of mango fruits, *C asianum*, *C. fructicola*, *C. tropicale*, *C. dianesei and C. karstii*, while *C. asianum* and *C. karstii* have been previously recorded from mango (Damm et al. 2012; Weir et al. 2012).

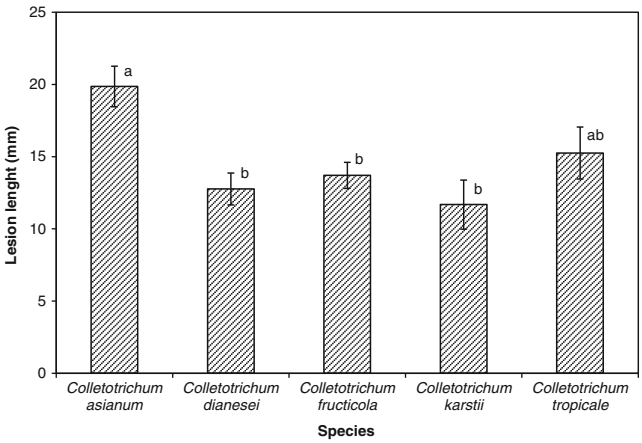


Fig. 5 Mean lesion lengths (mm) caused by *Colletotrichum* species associated with mango anthracnose in northeastern Brazil, 10 days after inoculation with conidia suspension (10^6 conidia/ml) onto wounded fruits of Tommy Atkins cultivar. *Bars* above columns are the standard error of the mean. Columns with same letter do not differ significantly according to Fisher's LSD test ($P \leq 0.05$)

Colletotrichum dianesei was the most frequently isolated species with 51.1 % all the isolates, indicating it is the most frequent *Colletotrichum* species associated to mango in northeastern of the Brazil. Phylogenetic analysis reveals high suppot for the C. dianesei clade, which is closely related to *C. siamense* (Fig. 1). These two species are similar in conidial shape but differ in conidial size, *C. dianesei* having longer conidia with a mean length 12.06 μm, when compared with *C. siamense* (10.18 μm). *Colletotrichum dianesei* also differs from *C. siamense* in growth rate, with 8.60 mm day^{-1} and 9.30 mm day^{-1}, respectively.

Colletotrichum asianum was the second most prevalent species with 27.7 % of all isolates. This species was originally described by Prihastuti et al. (2009) in *Coffea arabica* from Thailand. This is the first report this species associated with the mango fruits in Brazil. However, it is already known from *Mangifera indica* in Australia, Colombia, Japan, Panama and the Philippines (Weir et al. 2012).

Colletotrichum tropicale was described by Rojas et al. (2010) from *Theobroma cacao* in Panamá. This study represents the first report this species associated with the mango fruits worldwide. Rojas et al. (2010) noted that *C. tropicale* has been isolated from a wide range of hosts in forests in tropical America, from rotting fruit as well as leaf endophytes.

Colletotrichum fructicola was originally reported causing coffee berries in Thailand (Prihastuti et al. 2009). This species was also found as a leaf endophyte in several plants in Central America (originally described as *C. ignotum* (Rojas et al. 2010)). *Colletotrichum fructicola* biologically and geographically diverse. The species is presently known from *C. arabica* (Thailand), *Pyrus pyrifolia* (Japan), *Limonium* (Israel), *Malus domestica* and *Fragaria×ananassa* (USA), *Persea americana* (Australia), *Ficus* (Germany), *Malus domestica* (Brazil), *Dioscorea* (Nigeria), and *Theobroma* and *Tetragastris* (Panama) (Weir et al. 2012), and *Vitis* (China) (Peng et al. 2013). This is the first report of this species causing anthracnose in mango worldwide.

Colletotrichum karstii was recently reported from mango from Australia (Damm et al. 2012). It occurs on many host plants and is the most common and geographically diverse species in the *C. boninense* complex. This species was reported in China from *Vanda* sp. leaf and on several other orchids (Yang et al. 2011) and from Citrus leaves (Peng et al. 2012), as well as in infections from *Phalaenopsis* orchid petals in United States (Jadrane et al. 2012). Some isolates from *Passiflora edulis* in Brazil that were initially identified as *C. boninense* (Tozze et al. 2010), but later revealed to be *C. karstii* by GPDH sequences (Damm et al. 2012).

Pathogenicity testing using isolates from the five species of *Colletotrichum* showed that all species were pathogenic

to mango fruits. Symptoms development may vary considerably with factors such as variety and condition of the fruit, humidity and temperature, and the concentration of inoculum (Simmonds 1965; Freeman et al. 1998). This result may not accurately reflect the true virulence potential of these species. Additional research should be conducted to determine the virulence potential of *Colletotrichum* species according to natural infections rather than artificial inoculations.

The *Colletotrichum* isolates from mango analyzed in this study showed high variability based on GPDH gene and the morphological characteristics. Five species were identified, with the majority of the species had more than one haplotype and a high number of morphotypes. The greater the genetic diversity of a population, greater evolutionary potential and hence the more likely it to adapt to changing environmental conditions (McDonald and Linde 2002). That is, the greater the diversity, the greater the chance there is an individual that is adapted to certain restrictive condition covering the population. Accordingly, such information is relevant because it can assist in the implementation of disease control measures more effectively.

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