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.

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
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 appresoria, 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 less fusiform 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 pseudacutatum 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 acerum, 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. (Gonçalves 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 Quiroso et al. 2013; Ciampi-Guillard 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
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\(^a\) 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 CAB International 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 Capc-Embrapa at Embrapa-Cerrados, Planaltina, DF, Brazil; 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, Universita 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.
Species of the Colletotrichum acutatum complex in Brazil

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-35R and CHS-79F (Carbone & Kohn 1999), ACT-512F and ACT-783R (Carbone & Kohn 1999), BT2Fd (Woudenberg et al. 2009) and Bt-2b (Glass & Donaldson 1993) 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).

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 MrBayes 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 1 x 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 Turioni, 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          |
| C. melonis      | Col 20          | apple           | 634.62^A        | 121.26^aB       | 176.78^A      |
| C. cf. melonis  | CBS 134730      | apple           | 484.48^A        | 0               | 97.72^aB      |
| C. paranaense   | CBS 134729      | peach           | 510.50^A        | 84.32^A         | 172.88^A      |
| C. abisissum    | CBS 134727      | guava           | 388.94^A        | 329.98^A        | 191.88^A      |
| C. nymphaeae    | CPC 20897       | apple           | 294.76^A        | 217.45^A        | 10.36^A       |
|                 |                 |                 | 315.96^A        | 13.43^aC        | 136.38^aB     |

- a Means followed by the same lower case letter within a column are not significantly different (p ≤ 0.05); means followed by the same capital letter within a row are not significantly different (p ≤ 0.05).
- b Number of fruits with lesions/number of fruits inoculated.
C. ochrophilum CBS 632.80 Dendobium USA
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. australis CBS 116478 Trachycarpus South Africa
C. kinghornii CBS 198.35 Phormium UK
C. acerum CBS 128530 Malus New Zealand
C. rhombiforme CBS 129593 Olea Portugal
C. phormii CBS 102094 Phormium New Zealand
C. phormii CBS 118194 Phormium Germany
C. acutatum CBS 112996 Carica Australia
C. acutatum CBS 112980 Pinus South Africa
C. floriniae IMI 504882 Fragaria New Zealand
C. floriniae CBS 128517 Florinlia USA
C. floriniae CBS 125396 Malus USA
C. abscissum COAD 1877 Citrus BR
C. abscissum KB196 Citrus USA
C. abscissum RB192 Citrus USA
Colletotrichum sp. CBS 101611 Fern Costa Rica
Colletotrichum sp. CBS 129810 Solanum Colombia
C. cuscute 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 Boliviana
C. lupini CBS 109225 Lupinus Ukraine
C. lupini CBS 109226 Lupinus Canada
C. tamarillo CBS 129811 Solanum Colombia
C. tamarillo CBS 129814 Solanum Colombia
C. tamarillo CBS 129813 Solanum Colombia
C. tamarillo CBS 129812 Solanum Colombia
C. costaricense CBS 330.75 Coffee Costa Rica
C. costaricense CBS 211.78 Coffee Costa Rica
Colletotrichum sp. CBS 134728 Prunus BR
Colletotrichum sp. CBS 134729 Malus BR
Colletotrichum sp. IMI 384185 Caryocar BR
C. illiticoala 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. walleri CBS 125472 Coffee Vietnam
C. cosmi CBS 853.73 Cosmos Netherlands
C. chrysantheni CBS 126518 Carthamus Netherlands
C. guaveae IMI 350839 Psidium India
C. scovelli CBS 126529 Capsicum Indonesia
C. scovelli IMI 504891 Capsicum Taiwan
C. brisiandense CBS 292.67 Capsicum Australia
C. indonesiense CBS 127551 Eucalyptus Indonesia
C. laticiphilum 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 126388 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 360385 Pelargonium India
C. nymphaeae CBS 515.78 Nymphaceae 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
Species of the Colletotrichum acutatum complex in Brazil 553

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 × 10⁹ 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 paranaeense. 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 abscessum (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, conidioophores formed directly on hyphae. Septae 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, colarette 1–1.5 µm long, periclinical 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, bulb-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

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

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

**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.

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**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.
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.


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, 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 1–1.5 μm diam, collarette 1–1.5 μm long, periclinal thickening visible. Conidia hyaline, smooth-walled, aseptate, cylindrical, with 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.

**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, reverse pale olivaceous grey floccose-felty aerial mycelium, reverse pale olivaceous grey.
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 fortiniae and a few strains each as Colletotrichum acerbum, Colletotrichum godetiae, Colletotrichum salicus 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 luteum, 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 species 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. fortiniae, C. godetiae and C. salicus) 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. abscessum 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 et al., 2006). On the other hand, the fact that guava can host C. abscessum 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; Phoulvong 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. fortiniae. 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 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. fortiniae 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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