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An emended description of *Neofusicoccum brasiliense* and characterization of *Neoscytalidium* and *Pseudofusicoccum* species associated with tropical fruit plants in northeastern Brazil

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Abstract

The Botryosphaeriaceae family is one of the most important groups of plant pathogenic fungi associated with tropical woody species such as *Anacardium occidentale* (cashew) and *Mangifera indica* (mango), which are fruiting species with broad distributions and high economic importance in north-eastern Brazil. This study was designed to characterize species of this family associated with cashew, mango and *Psidium guajava* (guava) that cause dieback and stem and branch cankers. Characterization comprised phylogenetic, morphological, physiological and pathogenic features. The phylogenetic study combined sequences of the internal transcribed spacer (ITS) of the rDNA and partial elongation factor 1-α (EF1-α) regions of DNA. Measurements of fungal conidia and colony growth on different culture media and at different temperatures were conducted. Pathogenicity tests were also performed through inoculation of different host species. By combining the sequences of the referenced genes, it was possible to identify the following Botryosphaeriaceae species: *Neofusicoccum kwambonambiense*, *Neoscytalidium hyalinum* and *Pseudofusicoccum stromaticum* as the first report in cashew, *Neoscytalidium hyalinum* in mango and *Neofusicoccum brasiliense* as the first report in guava. Examination of *N. brasiliense* revealed conidia, providing the first morphological description of the species. *Neofusicoccum kwambonambiense* did not sporulate, but the other isolates produced conidia with dimensions comparable to those described in the literature. All isolates were virulent to mango fruits and young cashew and "caja-umbu" plants, causing necrotic lesions and gum exudation by the hosts. The isolates of *Neofusicoccum* were the most aggressive in the inoculated hosts.

Keywords: *Anacardium occidentale*, Botryosphaeriaceae, fungi, *Mangifera indica*, molecular phylogeny, *Psidium guajava*

Introduction

Family Anarcadiaceae includes tropical fruits of high socio-economic importance in Northeast Brazil (Pickel 2008). Of 80 genera and 600 species present in the family, approximately 13 genera and 68 species occur in Brazil (Barroso 1991), among which the cashew tree (*Anacardium occidentale*) and the mango tree (*Mangifera indica*) stand out among the species of highest economic and social importance (Marques *et al.* 2013b). These plant species are either native, such as the cashew, or, in the case of mango introduced a long time ago by the Portuguese. Recently, novel methods of cultivation have rendered plants vulnerable to abiotic stress and thereby favour the occurrence of severe epidemics caused by opportunistic fungi (Freire 1991, Tavares 2002).

Diseases caused by fungi of the family Botryosphaeriaceae stand out, both in this and other pathosystems in Northeast Brazil, because they are polyphagous pathogens that take advantage of physiologically weakened plants growing under stressful conditions. Therefore, this pathogen-host interaction is the target of several studies aimed at the identification and characterization of the pathogenic species involved (Costa *et al.* 2010, Lima *et al.* 2013, Marques *et al.* 2013a,b, Abdollahzadeh *et al.* 2013, Ismail *et al.* 2012).

Symptoms such as cankers, gummosis and dieback of Anacardiaceae plants have long been associated with infection by *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (Freire *et al.* 2011, Pavilic *et al.* 2004). However, early

pathogenic information and recent phylogenetic studies based on molecular techniques provided clear evidence of the association of a complex of cryptic species of *Lasiodiplodia* and other genera of Botryosphaeriaceae, such as *Neofusicoccum* Crous Slippers & A.J.L. Phillips 2006, *Neoscytalidium* Crous & Slippers 2006, and *Pseudofusicoccum* Mohali, Slippers & Wingf. (Sakalidis *et al.* 2013, Marques *et al.* 2013b, Machado *et al.* 2014, Berraf-Tebbal *et al.* 2014, Gonçalves *et al.* 2016).

The Botryosphaeriaceae is a cosmopolitan and polyphagous group of fungi (Denman *et al.* 2000, Machado *et al.* 2014). Ecologically this family includes non-host-specific pathogens, saprophytes, endophytes, and latent pathogens, and are generally known as weak pathogens (Denman *et al.* 2000, Sakalidis *et al.* 2013, Slippers & Wingfield 2007).

In the asexual phase, *Neofusicoccum*, *Pseudofusicoccum* and *Neoscytalidium* show *Fusicoccum*-type conidia, with narrow, ellipsoid to slightly ovoid and thin-walled forms, which makes them morphologically indistinguishable (Crous *et al.* 2006). The asexual phase is the most common form found in nature; it is easily isolated and exhibits abundant mycelial growth on most culture media but shows poor sporulation (Crous *et al.* 2006, Phillips *et al.* 2013). Morphological characteristics are insufficient for identification at the genus level, so molecular analyses are required to achieve a precise diagnosis (Phillips *et al.* 2013).

The phylogeny of this fungal family undergoes frequent updates due to the description of new species. In addition, reports of new pathogen-host associations and new information regarding their geographic distributions are frequently reported (Phillips *et al.* 2013, Sakadilis *et al.* 2013). Phylogenetic studies indicate that the combined analysis of ITS sequences and partial sequences of the EF1 alfa genes are used to delimit species within these genera (Marques *et al.* 2013a, Phillips *et al.* 2013, Machado *et al.* 2014).

The association of these genera with pathosystems has recently increased (Lopes *et al.* 2014, Gonçalves *et al.* 2016). Twenty-two species of *Neofusicoccum* (Berraf-Tebbal *et al.* 2014), two of *Neoscytalidium* and six of *Pseudofusicoccum* have been reported in Brazil (Phillips *et al.* 2013). In this country, they were isolated in endophytic association with several trees in the "caatinga" ecosystem in Ceará state and have been shown to be potential pathogens when inoculated on mango fruits and "caja-umbu" (*Spondias mombin × S. tuberosa*) trees (Gonçalves *et al.* 2016). In terms of their pathogenic behaviour, they have been associated with dieback and stalk rot in mango fruits (Marques *et al.* 2013b), while *Neoscytalidium hyalinum* has been reported in association with black rot of cassava (*Manihot esculenta*) (Machado *et al.* 2014b) and *Jatropha curcas* (Machado *et al.* 2014a).

In cashew and other commonly grown fruit trees in Northeast Brazil, the occurrence of canker and dieback was associated only with *Lasiodiplodia* species until recently. Two new species of Botryosphaeriaceae involved were phylogenetically characterized and described in association with these symptoms (Coutinho *et al.* 2017). In view of such evidence, the present study aimed to characterize the phylogenetic and pathogenic attributes of isolates morphologically identified as belonging to the group of *Fusicoccum*-like associated with cashew, mango and guava (*Psidium guajava*) plants in Northeast Brazil.

Materials and methods

Sampling and fungal isolation

The samples used in this study were obtained from plants exhibiting cankers or dieback symptoms on stems and branches in different regions throughout northeastern Brazil, from July to November 2013.

Infected tissues or organs were transported to the Laboratory of Phytopathology of Embrapa Agroindústria Tropical, Fortaleza, Ceará state. Fungal isolation, culturing, and incubation were done according to procedures described earlier (Coutinho *et al.* 2017). Monosporic cultures were obtained from sporulating colonies, transferred to potato dextrose agar (PDA) slants and stored in a refrigerator at 5 °C.

Sporulating cultures in a potato carrot agar (PCA) medium amended with sterilized pine needles (Mohali *et al.* 2005) were examined using light microscopy, and isolates with *Fusicoccum*-like conidium morphology were subjected to DNA extraction, amplification by polymerase chain reaction (PCR) and sequencing of amplified regions.

Molecular characterization

DNA extraction and amplification were conducted at the Molecular Biology Laboratory of Embrapa Agroindústria Tropical. Selected isolates were grown in potato dextrose broth for 7 days in still culture (Coutinho *et al.* 2017). The mycelial mass produced was dried at room temperature and used for DNA extraction following a previously described protocol (Cavalcanti *et al.* 2004). Extracted DNA was quantified using a NanoDrop® 2000c spectrophotometer

(Thermo Fisher Scientific, Wilmington, DE, USA), version 1.0, and portions of 10 ng μL^{-1} were stored in a -20 °C freezer.

The polymerase chain reaction (PCR) mixtures contained genomic DNA (10 ng μL^{-1}), 1x buffer, dNTP (0.2 mM), MgCl$_2$ (1.5 mM), each primer (1.5 mM), Taq Flexi DNA (2.5 U/μL), and sterile deionized water. The ITS primers were ITS1 and ITS4 (White *et al.* 1990) and EF1-688F and EF1-1251R primers were used to amplify part of the EF1-α (Alves *et al.* 2008). The thermal cycle of the PCR consisted of 96 °C for 1 min followed by 35 cycles of 30 s at 94 °C (denaturation), 1 min at 58 °C (EF1-α and ITS regions), 1.5 min at 72 °C (annealing) and 10 min at 72 °C (final extension). The PCR products were separated by electrophoresis in 1.5% agarose gels in 1x Tris-borate EDTA (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer, stained with ethidium bromide (0.5 μg mL^{-1}) for 1 min and visualized under UV light in a transilluminator.

After verification of the amplified bands, 40 μL aliquots of PCR product from each sample were sent to Macrogen (Seoul, South Korea) to be purified and sequenced in forward and reverse directions with the same primers.

Phylogenetic analyses

Sequences were manually corrected using the BioEdit software (Hall 2012) The corrected ITS and EF1-α sequences were separately aligned using the CLUSTALW procedure in the BioEdit software (Table 1). After this, the sequences were concatenated in the BioEdit and aligned together with sequences retrieved from the National Center for Biotechnology Information (NCBI), including a sequence of *Macrophomina phaseolina* used as an outgroup. This alignment was corrected manually, and gaps (-) were used whenever necessary for missing data. Phylogenetic relationships were inferred with the Bayesian method using the Markov chain Monte Carlo procedure for each separated gene, and substitution models of nucleotides were determined by the MrMODELTEST 2.3 (Posada & Buckley 2004) program according to the Bayesian information criterion (BIC). The multiple alignment (ITS + EF1-α) was analysed using MrBayes v.3.1.1 program (Ronquist & Heulsenbeck 2003) with 100 million generations by including each phylogenetic tree of the sample space from each run every 500 generations.

Phylogenetic trees were visualized using FigTree program (Rambaut 2009) and were exported for editing in graphical programs. The obtained sequences (Table 1) were deposited in GenBank at the NCBI site, and the multiple alignments were deposited in the TreeBASE (http://www.treebase.org/) platform under number 19422.

Morphological and physiological characterization

Pycnidia were produced on colonies grown on sterile Petri plates containing PCA medium supplemented with pine needles, incubated for 4 weeks at 25 °C and under a 12 h photoperiod of ultraviolet light.

Fruiting bodies were manually removed and disrupted using scalpels, mounted on slides, stained with a drop of lactophenol and then observed through a light microscope. Thirty measurements were taken from all possible morphological characters displayed (conidia, paraphyses and conidiogenous cells). Images were obtained using a ICc5 131 Axiocam digital camera coupled to a microscope (Carl Zeiss AG Imager.A2, Göttingen, Germany) and a Motic Image Plus 2.0 imaging device (Motic Group Co., Beijing, China).

The growth of all isolates under different temperatures was evaluated by growing them on PDA and storing them under a 12 h photoperiod. Temperatures of 5, 10, 15, 20, 25, 30, 35 and 40 °C were evaluated. Means of mycelial growth were subjected to regression analysis to determine the optimal growth temperature of each species.

The effect of the culture media on growth of some isolates was evaluated using the following media: oats-agar (AvA, 75 g of oats, 17 g of agar, 1 L of water), potato dextrose agar (synthetic PDA, 39 g L^{-1}), PCA (20 g of potato, 20 g of carrot, 17 g of agar, 1 L of water), V8 juice (100 mL of V8, 2 g of CaCO$_3$, 17 g of agar, 900 mL of water), malt agar extract (MEA, 20 g of malt extract, 20 g of dextrose, 1 g of peptone, 20 g of agar, 20 g of dextrose, 1 L of water) and a medium based on green cashew branches in agar and dextrose (200 g of green cashew branches, 17 g of dextrose, 17 g of agar, 1 L of water), because most isolates came from cashew in the survey. The plates were maintained at 25 °C and a photoperiod of 12 h. The diameter of the colonies was measured in two perpendicular directions for two consecutive days. The maximum and daily rate of mycelial growth were estimated.

All *in vitro* trials were conducted in a completely randomized design (DIC), where each plate represented an experimental unit. The mean values were subjected to analysis of variance (ANOVA), followed by the Scott-Knott test at the 5% level of significance. Data from the test of the effects of different culture media were analysed as a 5-by-3 factorial arrangement in order to determine the interactions.

TABLE 1. Isolates of Botryosphaeriaceae species used in this study.

Specie	Isolates	Host	Origin	Collection	GenBank accession number ITS	TEF1-α
Fusicoccum atrovirens	CMW 22682	*Pterocarpus angolensis*	Africa	J. Mehl/J. Roux	FJ888476	FJ888457
F. atrovirens	CMW 22674	*P. angolensis*	Africa	J. Mehl/J. Roux	FJ888473	FJ888456
F. fabicercianum	CMW 24703	*Eucalyptus grandis*	China	M.J Wingfield/ X.D.Zhou	HQ332195	HQ332211
F. fabicercianum	CMW 27094	*Eucalyptus sp.*	China	M.J Wingfield	HQ332197	HQ332213
F. ramosum	CBS122069	*E. camaldulensis*	Australia	D. Pavlic	EU144055	EU144070
Macrophomina phaseolina	PD112	*Prunus dulcis*	Australia	D. Pavlic/MJ. Wingfield	GU251105	GU251237
Neofusicoccum algeriense	CBS137504	*Vitis vinifera*	Algeria	A. Berraf-Tebbal	KJ657702	KJ657715
N. algeriense	ALG9	*V. vinifera*	Algeria	A. Berraf-Tebbal	KJ657704	KJ657721
N. andinum	CBS 117453	*Eucalyptus sp.*	Venezuela	S. Mohali	AY693976	AY693977
N. andinum	CBS 117452	*Eucalyptus sp.*	Venezuela	M.J. Wingfield	DQ306263	DQ306264
N. arbuti	CBS 116131	*Arbutus menziesii*	UEA	M. Elliott	GU251152	GU251284
N. arbuti	CBS 117090	*A. menziesii*	UEA	M. Elliott	GU251154	GU251286
N. australe	CMW 6837	*Acacia sp.*	Australia	M.J Wingfield	AY339262	AY339270
N. australe	CMW 6853	*Sequoiadendron*	Australia	M.J Wingfield	AY339263	AY339271
N. batangarum	CMW 28363	*Terminalia catappa*	Africa	D. Begoude/J. Roux	FJ900607	FJ900653
N. batangarum	CMW 283205	*T. catappa*	Africa	D. Begoude/J. Roux	FJ900608	FJ800654
N. brasiliense	CMM 1269	*Mangifera indica*	Brazil	M.W Marques	JX513629	JX513609
N. brasiliense	**IBL447**	***Psidium guajava***	**Cruz/CE/ Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247455**	**KT247457**
N. brasiliense	CMM 1285	*M. indica*	Brazil	M.W Marques	JX513628	JX513608
N. cordaticola	CMW 13992	*Syzigium cordatum*	Africa	D. Pavlic	EU821898	EU821868
N. cordaticola	CMW 14056	*S. cordatum*	Africa	D. Pavlic	EU821903	EU821873
N. eucalyptorum	CBS 115791	*E. grandis*	Africa	H. Smith	AF283686	AY236891
N. eucalyptorum	CMW 10126	*E. grandis*	Africa	H. Smith	AF283687	AY236892
N. eucalypticola	CMW 6217	*E. rossii*	Australia	M.J. Wingfield	AY 615143	AY615135
N. eucalypticola	CMW 6539	*E. rossii*	Australia	M.J. Wingfield	AY 615141	AY615133
N. kwambonambiense	CMW 14023	*S. cordatum*	Africa	D. Pavlic	EU821900	EU821870
N. kwambonambiense	CMW 14140	*S. cordatum*	Africa	D. Pavlic	EU821949	EU821889
***N. kwambonambiense*[a]**	**IBL220**	***Anarcadium occidentale***	**Pio IX/PI/ Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247454**	**KT247456**
N. luteum	CMW 9076	*Malus × domestica*	Nova Zelândia	S.R. Pennycook	AY 236946	AY236893
N. luteum	CBS 110299	*V. vinifera*	Portugal	A.J.L Phillips	AY 259091	AY573217
N. macroclavatum	WAC 12445	*E. globulus*	Australia	T.I Burguess	DQ093197	DQ093218
N. macroclavatum	WAC 12446	*E. globulus*	Australia	T.I Burguess	DQ093198	DQ093219
N. mangiferum	CMW 7024	*M. indica*	Australia	G.I Johnson	AY615185	DQ093221
N. mediterraneum	PD 311	*Olea europea*	Itália	C. Lazzizera	GU251175	GU251307
N. mediterraneum	PD 312	*Eucalyptus sp.*	Grecia	M.J Wingfield/ A.J.L Phillips	GU251176	GU251308

TABLE 1. (Continued)

Specie	Isolates	Host	Origin	Collection	GenBank accession number	
					ITS	TEF1-α
N. occulatum	CBS 128008	*E. grandis*	Australia	T.I Burgess	EU7301030	EU339509
N. occulatum	MUCC 296	*E.s pellita*	Australia	T.I Burgess	EU301034	EU339512
N. parvum	PD 106	*Prunus dulcis*	UEA	T.J Michailides	GU251139	GU251271
N. parvum	CMM 1291	*M. indica*	Brazil	M.W Marques	JX513633	JX513613
N. ribis	CMW7054	*Ribis rubrum*	UEA	B. Slippers	AF241177	AY236879
N. ribis	CMW 7772	*Ribis sp.*	UEA	B. Slippers/G. Hudler	AY236925	AY236877
N. ursorum	CBS 122811	*E. arboretum*	Africa	H.M Maleme	FJ752746	FJ752709
N. ursorum	CMW 23790	*E. arboretum*	Africa	H.M Maleme	FJ752745	FJ75270
N. umdonicola	CMW 14058	*S. cordatum*	Africa	D. Pavlic	EU821934	EU821874
N. umdonicola	CMW 14060	*S. cordatum*	Africa	D. Pavlic	EU821935	EU821875
N. viticlavatum	STE-U 5044	*V. vinifera*	Africa	F. Halleen	AY343381	AY 343342
N. viticlavatum	STE-U 5041	*V. vinifera*	Africa	F. Halleen	AY343380	AY343341
N. vitifusiforme	STE-U 5050	*V. vinifera*	Africa	J.M. Van Niekerk	AY343382	AY343344
N. vitifusiforme	STE-U 5252	*V. vinifera*	Africa	J.M. Van Niekerk	AY343383	AY343343
Neoscytalidium hyalinum	PD 103	*Ficus carica*	UEA	T.J. Michailides	GU251106	GU251238
Ne. hyalinum	WAC 13284	*M. indica*	Australia	J. Ray	GU172382	GU172414
Ne. hyalinum[a]	**IBL89**	**M. indica**	**Varjota/ CE/Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247460**	**KT247458**
Ne. hyalinum[a]	**IBL272**	**A. occidentale**	**Pio IX/ PI/Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247461**	**KT247459**
Ne. novaehollandiae	WAC 13275	*M. indica*	Australia	J. Ray	GU172400	GU172432
Ne. novaehollandiae	WAC 13303	*M. indica*	Australia	J. Ray	GU172398	GU172430
Pseudofusicoccum ardesiacum	WAC 13299	*M. indica*	Australia	J. Ray	GU172404	EU144075
P. ardesiacum	CBS 122062	*Adansonia gibbosa*	Australia	D. Pavilic	EU144060	GU172437
P. adansoniae	CBS 122054	*Eucalyptus sp.*	Australia	D. Pavilic	EF585532	EF585570
P. adansoniae	WAC 13299	*M. indica*	Australia	J. Ray	GU172404	GU172436
P. kimberleyense	CBS 122061	*F. opposita*	Australia	D. Pavilic	EU144059	EU144074
P. kimberleyense	WAC 13293	*M. indica*	Australia	J. Ray	GU172406	GU172438
P. stromaticum	CMW 13435	*E. hybrid*	Venezuela	S. Mohali	DQ436935	DQ436936
P. stromaticum	CMM 3953	*M. indica*	Brazil	M.W Marques	JX464102	JX464109
P. stromaticum	CMM 3961	*M. indica*	Brazil	M.W Marques	JX464103	JX464110
P. stromaticum[a]	**IBL500**	**A. occidentale**	**Pio IX/ PI/Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247464**	**KT247462**
P. stromaticum[a]	**IBL36**	**A. occidentale**	**Quixadá/ CE/Brazil**	**I.B.L Coutinho/ J.S Lima**	**KT247465**	**KT247463**
P. olivaceum	CMW22639	*P. angolensis*	Africa	J. Mehl & J Roux	FJ888463	FJ888439
P. olivaceum	CMW20881	*P. angolensis*	Africa	J. Roux	FJ888459	FJ888437
P. violaceum	CMW20436	*P. angolensis*	Africa	J. Roux	FJ888458	FJ888440

Sequence numbers in bold were obtained in the present study. aEx-type cultures obtained in this study. Abbreviations: USA: United States of America; PI: Piauí; EC: Ceará; ITS: internal transcribed spacer from the ribosomal DNA region; TEF1-α: part of the gene coding for the translation elongation factor 1-α; GenBank: NCBI (National Center for Biotechnology Information) database, available at http://www.ncbi.nlm.nih.gov/

Pathogenic characterization

Mango fruits cv. Tommy Atkins and stems of young plants of cashew and "caja-umbu" were inoculated with the six isolates identified in this study. The methodology used for the inoculation of fruits was described by Marques *et al.* (2013b) where fruits were gently wounded in the middle region with the tip of three sterile pins to a 3 mm depth prior to placing a 3 mm agar plug containing mycelium grown on PDA of the isolate to be tested. Non-colonized agar discs were used as control treatments. For young plants, the methodology used for inoculation was described by Lima *et al.* (2013), and consisted of making a 2 mm diameter hole in the stem of the plant at approximately 15 cm from the bottom with an electric drill. Then, a 2 mm diameter agar plug containing mycelium grown on PDA was introduced into the hole, placing it in contact with the vascular system of the plant. The hole was covered with petroleum jelly and sealed with Parafilm. In the control treatment, the plants were drilled and inoculated with PDA medium agar plugs only. Both were conducted under the same experimental conditions proposed by Coutinho *et al.* (2016). In fruits, the diameter of the lesions was measured daily for 7 days using a digital caliper. In plants, the evaluation was made on 15 days after inoculation by measuring the necrotic lesion caused by the infection both longitudinally and perpendicularly. The mean lesion diameters in the fruits on the 4th day and in the stems of the young plants on the 15th day after inoculation were used in the statistical analysis of the data. The experiments were conducted in a completely randomized design consisting of six replicates, where each replicate was composed of one fruit or one young plant of each plant species. Data from the disease progression in mango fruits were evaluated based on nonlinear regression equations to verify the pathogenic ability of each isolate.

Results

Sampling and fungal isolation

This study resulted from a large sampling of tropical fruit plants showing typical symptoms induced by fungi in the Botryosphaeriaceae, such as gummosis and die-back. The sampling encompassed the Ceará (73.6%), Paraíba (1.9%), Pernambuco (5.7%), Piauí (15.1%) and Rio Grande do Norte (3.8%) states in the Northeast region of Brazil. The plants sampled were cashew (41.5%), *Spondias* (26.4%), mango (7.5%), coconut (3.8%), Annonaceae (11.3%) and other tropical fruit plants (9.4%). From this sampling, 108 isolates of Botryosphaeriaceae were obtained, with 102 representing *Lasiodiplodia* species, which were characterized by Coutinho *et al.* (2016). The other six isolates were identified as *Fusicoccum*-like and are discussed in the present study.

Molecular characterization

ITS1 and ITS2, including the 5.8S subunit and partial EF-1α regions were sequenced for six isolates. The alignment dataset comprised sequences from this study and the sequences from ex-type strains from Botryosphaeriaceae species from GenBank (Table 1). The phylogenetic tree generated (Fig. 1) was inferred by Bayesian analysis (IB) using BIC models: TrNef + G for the ITS region and K80 + G for the TEF-1α region.

Alignments were composed of 74 taxa and length of the concatenated sequences was 674 nucleotides. Therefore, out of the 674 characters found in the alignment, 402 were preserved, 258 were variables and 234 were parsimony informative. The phylogenetic tree was composed of 74 taxa, including 22 clades containing *Neofusicoccum* species, six clades containing *Pseudofusicoccum* species, two clades containing *Neoscytalidium* species, three clades containing *Fusicoccum* species and one clade containing *Macrophomina phaseolina*. The isolates identified in this study were grouped with *Neofusicoccum kwambonambiensee* (IBL220) obtained from guava plants located in the state of Ceará, *N. brasiliense* (IBL447) obtained from guava plants located in the state of Ceará, *ditto hyalinum* obtained from cashew trees in the state of Piauí (IBL272) and mango in Ceará state (IBL89), and *P. stromaticum* obtained from a cashew tree in Ceará (IBL36) and Piauí (IBL500) (Table 1). Well-defined clades were formed with low values of Bayesian inference brackets for the clades *N. brasiliense* and *N. kwambonambiense* (Fig. 1).

Morphological and physiological characterization

The conidial morphology of isolates was compared with the morphological description of each species, except for *N. kwambonambiensee* (IBL220) (Table 2). All these isolates produced pycnidia in PCA medium plus *Pinus* needles after four weeks of incubation.
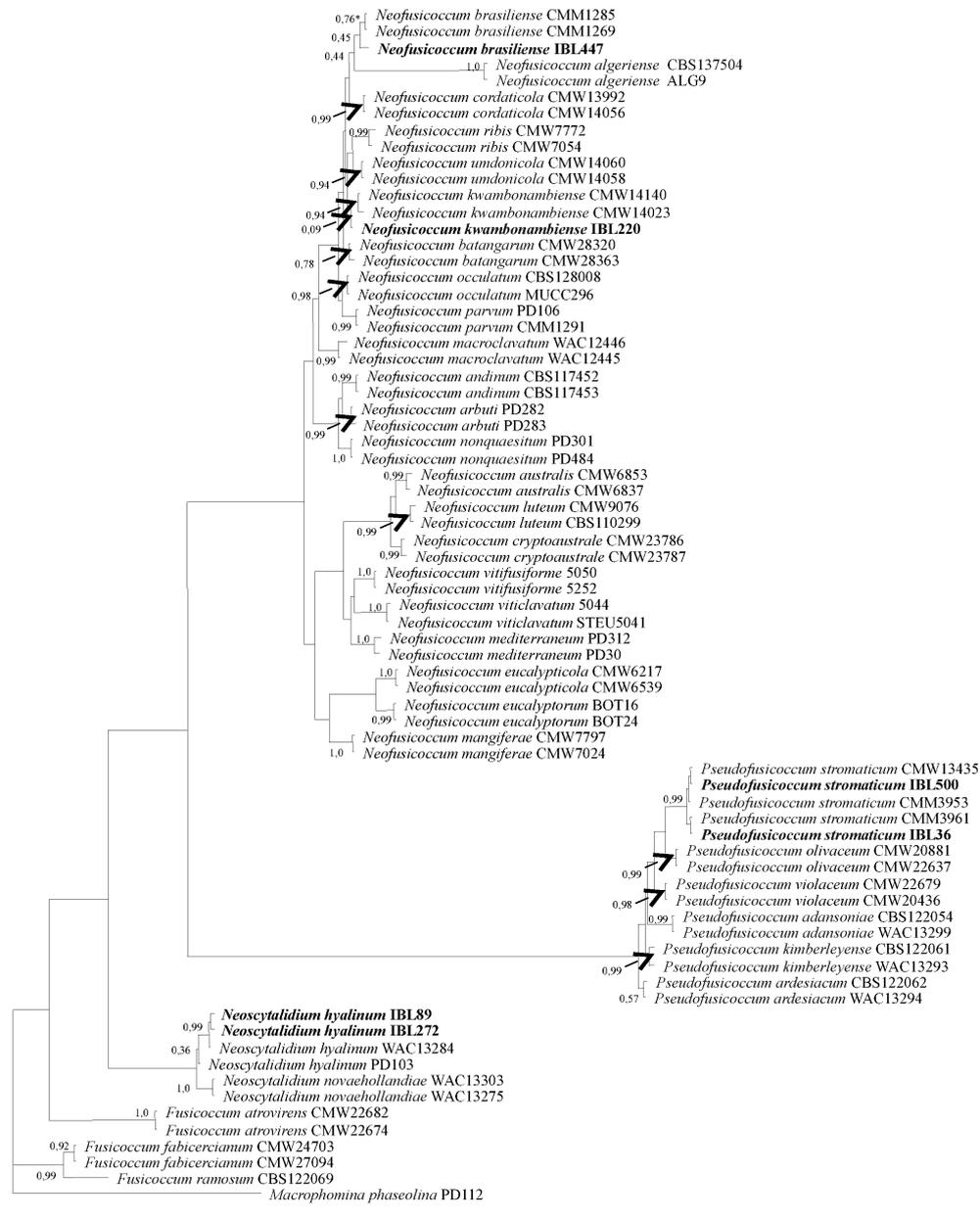
FIGURE 1. Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined sequences of the ITS, TEF-1α. Bayesian posterior probabilities are indicated above the nodes. The tree was rooted to *Macrophomina phaseolina* PD112. The species in this study are highlighted in bold and grey highlight.

TABLE 2 Comparison of morphological characteristics of Botryosphaeriaceae species examined in this study with those in previous studies.

Species	Conidia length (L) × width (W) Size (μm)	L:W	References
Neocytalidium hyalinum	**19,93 (24,83) 27,91 × 8,88 (10,46) 11,93**	**2,37**	**This study**
	10–16(–21)×3.5–6.5	-	Phillips *et al.* 2013
Neofusicoccum brasiliense	**10,72 (21,30) 25,57 × 8,31 (10,18) 11,94**	**2,09**	**This study**
Neofusicoccum kwambonambiense	16 (22,3) 28 × 5 (6,3) 8	3,6	Crous *et al.* 2006
Pseudofusicoccum stromaticum	**20,05 (26,01) 31,46 × 5,18 (6,39) 7,6**	**4,09**	**This study**
	(19–)20–23(–24) × 5,0 (4,0) 6,0	4,0	Mohali *et al.* 2006

The *P. stromaticum* isolates had conidial dimensions close to those of the isolate first described by Mohali *et al.* (2006) and to isolates from the Brazilian territory described by Marques *et al.* (2013b). *N. kwambonambiense* (IBL220) never sporulated. The size of the conidia of the *N. brasiliense* isolate (IBL447) was not compared to that of any other isolates of the same species, since its original description was based only on molecular data (Marques *et al.* 2013).

A quadratic model fitted the growth response to temperature (Table 3). Regression analyses showed that the optimal temperatures for the growth of *N. brasiliense*, *N. kwambonambiense* and one isolate of *P. stromaticum* (IBL36) were below 26 °C. Up to the third day, no isolate reached the diameter of the plate (90 mm). *Ne. hyalinum* and *P. stromaticum* began to grow only above 15 °C, and *Neofusicoccum* spp. began growing at 10 °C. Only the isolates of *Ne. hyalinum* were able to grow at 40 °C.

TABLE 3 Mycelial growth (mm) of Botryosphaeriaceae species at their respective optimal growth temperatures.

Species	Isolates	Optimum temp. $_a$ (°C)	Maximum growth at optimum temp. (mm)$_b$	Equation$_c$	R^2_d
N. brasiliense	IBL447	24,09	86,33	$y = -0,29x^2 + 14,11x - 83,71$	0,85
N. kwambonambiense	IBL220	23,40	70,98	$y = -0,26x^2 + 12,29x - 72,87$	0,73
Ne. hyalinum	IBL89	26,43	85,03	$y = -0,23x^2 + 12,47x - 79,86$	0,78
Ne. hyalinum	IBL272	26,11	82,25	$y = -0,24x^2 + 12,48x - 80,77$	0,75
P. stromaticum	IBL500	26,05	51,24	$y = -0,16x^2 + 8,41x - 58,35$	0,52
P. stromaticum	IBL36	24,24	51,14	$y = -0,1634x^2 + 7,92x - 44,9$	0,82

$_a$ Temperature in which the pathogen reached maximum growth.

$_b$ Maximum mycelial growth on the third day of incubation at optimum temperature.

$_c$ Regression equation representing the polynomial curve of mycelial growth of each isolates during the temperatures tested: 5, 10, 15, 20, 25,30, 35, 40 ºC.

$_d$ Correlation coefficient

All isolates showed mycelial mass that was whitish on the third day of incubation, and became darker with further incubation. Mycelial growth in the different culture medium according to ANOVA showed interactions among these factors (Fig. 2A).

Potato dextrose agar, V8, MEA and AvA medium favoured *Neoscytalidium hyalinum* and *N. brasiliense* growth without differing from one another, and the same effect was detected for *P. stromaticum* in the V8 medium. The CajuDA and BCA media favoured the mycelial growth of the three studied species, but only *Neofusicoccum* sp. reached 100% of the diameter of petri dishes.

Neoscytalidium hyalinum reached 100% growth on the BDA, V8, MEA and AvA media but reached only 66.66% and 38% growth on CajuDA and BCA, respectively. The growth rate of this species did not differ among the different types of culture media, except for the BCA medium, which provided growth of 10 mm day^{-1}, whereas it provided 35–50 mm day^{-1} for the other species (Fig. 2A,B). The mycelial growth of *Neofusicoccum* sp. was not favoured by the BCA medium (Fig. 2A).

Emended description

Neofusicoccum brasiliense

Phylogenetic description:—M.W. Marques, A.J.L. Phillips & M.P.S. Câmara MycoBank MB80473 (Marques *et al.* 2013).

Morphological description:—I.B.L. Coutinho, C.S. Lima & J.E. Cardoso. **MycoLibrary:**—CMM 4576.

Sexual morph: not observed. **Asexual morph.**: Production of pycnidia after 8 days in pine needles embedded in PCA and sporulation after 17 days under the same conditions. Conidia hyaline, fusiform, base subtruncate to bluntly rounded, non-septate, smooth with granular contents. Dimensions: 10.7–25.6 × 8.3–11.9 μm (mean = 21.3 × 10.2 μm; L:W = 2.1, n = 50). Mycelium abundant, cotonous and dark in BDA, malt extract, oats and V8. Production of red pigment in BDA at 35ºC. Habitat: Necrotic cankers in branches and trunks of *Psidium guajava*.
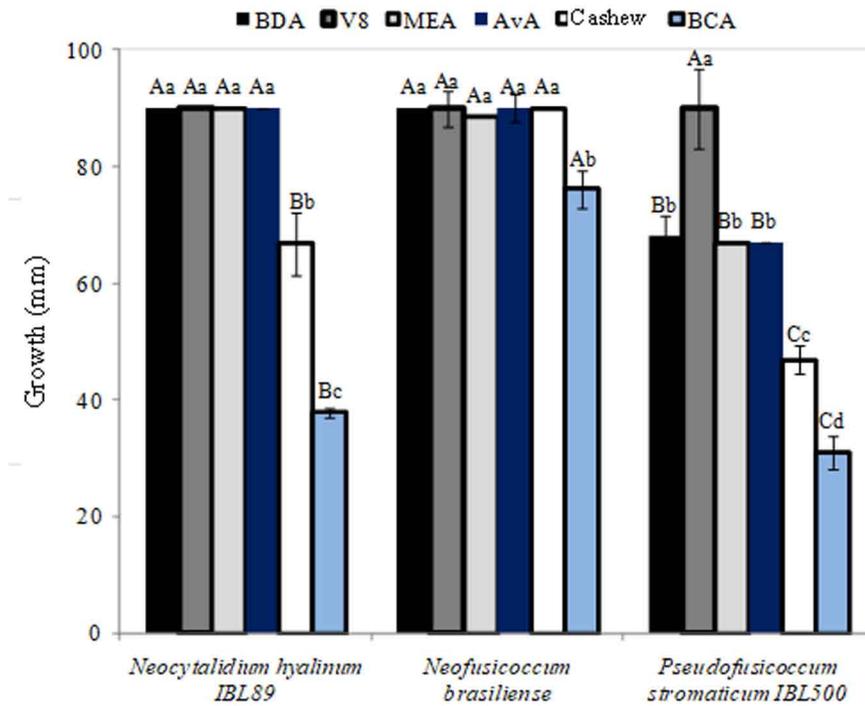
Fig. 2A

BDA ■ V8 □ MEA ■ AvA □ Cashew □ BCA

Fig. 2B
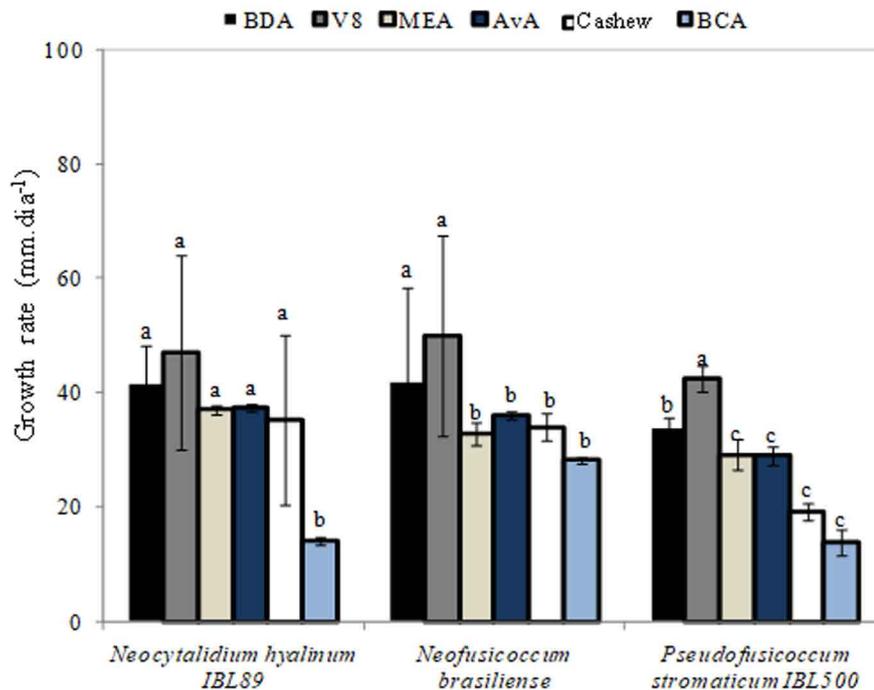
■ BDA ■ V8 □ MEA ■ AvA □ Cashew □ BCA

FIGURE 2. Mycelial growth (A) and *in vitro* growth rate (B) of Botryosphaeriaceae species on the third day of incubation in different culture media. Fig, 2A: There was a positive interaction between the factors of culture media and species. Thus, the same uppercase letters for each culture medium within the species factor indicate no difference among media, and the same lowercase letters within the species factor for each culture medium indicate no difference among species. The means were compared by the Scott-Knott test at P = 0.05. Fig. 2B: There was no positive interaction between the factors of culture media and species. Thus, averages followed by the same lowercase letters within each species do not differ by Scott-Knott's test at P = 0.05.

Geographic distribution:—Ceará State, Brazil.

Specimens examined:—BRAZIL. Ceará State: Cruz city (latitude: -2.91941, longitude: -40.1703 2° 55′ 10″ S, 40° 10′ 13″ E), October 2013, collected by I.B.L Coutinho & J.S. Lima, culture ex-holotype CMM 4576, NCBI GenBank access codes KT247455 (ITS) and KT247457 (EF1-α).

Pathogenicity characterization

All species were virulent when the mango fruits and young plants of cashew and "caja-umbu" were inoculated, but to different degrees of severity depending on the host (Fig. 3). *N. kwambonambiense, N. brasiliense* and *P. stromaticum* (IBL36) were highly aggressive when inoculated into mango fruit, cashew and "caja-umbu" seedlings, causing the greatest lesion length (Fig. 3). In cashew, *Ne. hyalinum* (IBL272) and *P. stromaticum* (IBL500) were the least aggressive, while in mango fruit, *Ne. hyalinum* (IBL272) was moderately aggressive, followed by *Ne. hyalinum* (IBL89) and *P. stromaticum* (IBL500), which were the least aggressive.

In "caja-umbu" seedlings, *Ne. hyalinum* (IBL272) and *Ne. hyalinum* (IBL89) were moderately virulent without significant differences, followed by *P. stromaticum* (IBL500), which was the least virulent isolate among all inoculated hosts (Fig. 3).

The symptoms in mango fruits were seen 3 days after inoculation by any isolate, but *N. brasiliense* was the most virulent species, reaching a maximum growth rate of 18.4 mm day^{-1}. Symptoms in mango fruit were necrotic and watery lesions that grew towards the peduncle and were mummified at the end. The most virulent species stimulated gum exudation by both cashew and "caja-umbu" seedlings, causing superficial cankers in their stem tissues that later progressed to large, necrotic lesions.
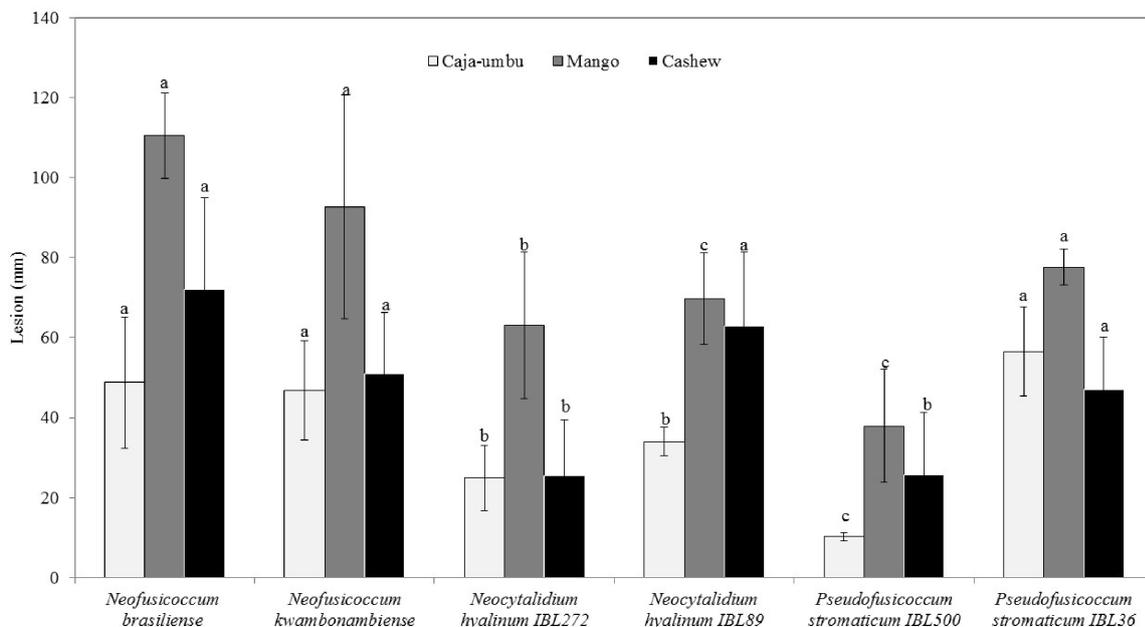

FIGURE 3. Mean lesion length (mm) caused by Botryosphaeriaceae species associated with dieback and stem-end rot in several tropical fruit trees from Northeast Brazil: 45 days after inoculation in stems of young plants of caja-umbu, 15 days after inoculation in stems of young cashew plants and four days after inoculation in injured mango fruits. Bars above columns represent the standard error of the mean. For each host, columns with the same letter do not differ significantly according to Scott–Knott's test at a = 0.05.

Discussion

Four species of Botryosphaeriaceae (*Neoscytalidium hyalinum, Neofusicoccum kwambonambiense, Neofusicoccum brasiliense* and *Pseudofusicoccum stromaticum*) were associated with dieback and canker in the stems and trunks of cashew, mango and guava in Northeast Brazil.

The genera *Neoscytalidium* and *Pseudofusicoccum* were easily identified by their conidial morphology, as they presented characteristics that were comparable to previously described species (Phillips *et al.* 2013). *N. kwambonambiense* failed to sporulate, and morphological features of its conidia could therefore not be assessed.

Until now, the description of *N. brasiliense* was based on molecular data only (Marques *et al.* 2013). The present study provides the first morphological description of *N. brasiliense*. The identification of Botryosphaeriaceae at the species level by means of DNA-based phylogeny (ITS, TEF1-α) and morphological characteristics clearly provide unquestionable support for the taxonomy of this family.

Previously, phylogenetic inferences based on ITS sequences were the most current and valid tools for fungal identification and the establishment of phylogenetic descriptions of the genera *Neofusicoccum*, *Neoscytalidium* and *Pseudofusicoccum*, formerly considered *Botryosphaeria* and *Fusicoccum* anamorphs (Crous *et al.* 2006). Nowadays, Botryosphaeriaceae species are frequently recognized and separated by DNA sequence differences, through multiple phylogenetic inferences, which emphasize protein coding genes such as EF1-α (Abdollahzadeh *et al.* 2010, Phillips *et al.* 2013, Berraf-Tebbal *et al.* 2014). However, for some genera such as *Neofusicoccum*, to distinguish between species, additional genes are required to provide a more robust phylogenetic basis (Abdollahzadeh *et al.* 2010, Phillips *et al.* 2013).

In this study, different clades of *Neofusicoccum* sp. that presented low values of Bayesian inference were formed, evidencing the close relationships among them. As for the *Neoscytalidium* and *Pseudofusicoccum* clades, however, the ITS and EF1-α regions showed enough consistency to distinguish species. Based on this information, the phylogenetic analysis of Botryosphaeriaceae should be further explored and standardized in order to guarantee more reproducibility of phylogenetic results. Obviously, the goals of the study would determine the specific genes to be used, considering that for the Botryosphaeriaceae, a combination of ITS and EF1-α genes is commonly used.

This study is the first report of *Ne. hyalinum, Ne. kwambonambiense, N. brasiliense* and *P. stromaticum* associated with cashew plants and *N. brasiliense* associated with guava. The occurrence, distribution and epidemiology of these fungi in fruit plants assume great importance for the development of the tropical fruit industry, especially for cashew and guava.

Botryosphaeriaceae has been reported to be associated with mango trees, and this has revealed the importance of this group of pathogens in relation to yield declines and damage to fruits post harvest (Costa *et al.* 2010, Sakalidis *et al.* 2011, Marques *et al.* 2013, Abdollahzadeh *et al.* 2013). Due to these numerous and frequent occurrences in mango, it is suggested that the centre of origin of these fungi is in the northern hemisphere, even in temperate regions, with further dissemination to tropical regions by the accidental transport of infected plants (Marques *et al.* 2013).

Neofusicoccum parvum and *N. ribis* have been reported to cause post-harvest rot in guava fruits in Brazil (Nogueira Junior *et al.* 2015). The species studied here all had a similar growth response to temperature, with optimum temperature around 25 °C with limiting ranges of ≤10 °C and ≥40 °C. These results are in agreement with studies on the germination of *N. parvum* and *B. dothidea* conidia (Nogueira Junior *et al.* 2015) and the growth of *N. parvum*, *N. brasiliense*, *Ne. hyalinum* and *P. stromaticum* (Marques *et al.* 2013).

Neofusicoccum is commonly associated with numerous woody hosts worldwide (von Arx 1987) and currently has 22 phylogenetically described species (Berraf-Tebbal *et al.* 2014). *N. brasiliense* has been described in Northeast Brazil associated with fruit rot in mango (Marques *et al.* 2013); thus, this is the second report of this species in Brazil and the first in guava branches.

N. kwambonambiense was described for the first time in South Africa in asymptomatic stems, leaves and fruits of dead plants of *Syzygium cordatum* (Pavilic *et al.* 2009, Phillips *et al.* 2013) and was later reported in grapevines in Uruguay (Abreo *et al.* 2013), and to cause fruit rot in post-harvested strawberry in Brazil (Lopes *et al.* 2014). The occurrence of *N. kwambonambiense* associated with symptoms of dieback and canker in stems of cashew plants is unprecedented in Brazilian territory. Such findings become relevant from a pathological point of view, as even at low frequencies and distributions, ecological changes may be occurring to allow the flow of this fungal species from its centre of origin to Brazilian hosts. Both *N. brasiliense* and *N. kwambonambiense* isolates were able to cause necrotic lesions when inoculated on mango fruits and the stems of cashew and "caja-umbu" seedlings.

The Botryosphaeriaceae have been described in association with mango and other woody species, including cashew and "caja-umbu"; however, it is not clear whether these fungi have been fully described in terms of their role as pathogens, especially in woody tropical hosts. This is very important for economic reasons for the development of control measures that ensure good management practices, such as sanitation.

The isolation of Botryosphaeriaceae from asymptomatic tissues evidences the dissemination and infection of tissues through mechanical pruning and the use of infected pruning machinery. The Botryosphaeriaceae are successful as opportunistic endophytic colonizers, which has thus enabled them to occur for long periods of time as latent pathogens causing post-harvest rot (Slippers & Wingfield 2007, Sakalidis *et al.* 2011).

In cashew orchards, in addition to mechanical pruning, propagation via grafting is a routine practice in commercial crop production. Reported studies have shown the dissemination of Botryosphaeriaceae, such as *Lasiodiplodia* spp., in

cashew by infected plant scions used for grafting (Cardoso *et al.* 2009) in addition to through the use of non-disinfested pruning instruments (Cardoso *et al.* 1998). Based on this evidence, a lack of sanitation practices favours the occurrence and the dissemination of Botryosphaeriaceae in orchards of tropical fruit trees.

Diseases caused by *Neoscytalidium* spp., *Ne. hyalinum* (= *Ne. dimidiatum*) and *Ne. novaehollandiae* tend to be common in tropical countries (Phillips *et al.* 2013). *Ne. hyalinum* has been reported in association with freeze-stressed *Citrus* plants in California and with necrotic lesions (Phillips *et al.* 2013). In Italy, it has been detected in branches of *Citrus* spp. causing canker and gummosis (Polizzi *et al.* 2009, 2011). In Brazil, this species has been reported in *Jatropha curcas*, cassava (*Manihot esculenta*) and mango tree (Machado *et al.* 2012, 2014a, b, Marques *et al.* 2013b).

P. stromaticum was originally described in asymptomatic plants as well as in branches and stems of dead plants of *Acacia mangium*, *Eucalyptus* and *Pinus* in Venezuela (Mohali *et al.* 2006, 2007). It is associated with native host plants in Australia, especially in unexplored forests (Pavilic *et al.* 2008). Later, this species was reported in association with declining mango trees in intensively cultivated orchards in Brazil as the second most abundant species in a recent survey, overturning the myth that its distribution was restricted to native hosts of unexplored lands (Marques *et al.* 2012, 2013).

Botryosphaeriaceae is well known to lack host specificity (Slippers & Wingfield 2007), which suggests that the expansion of the distribution, colonization, and speciation of this family has been influenced by environmental factors (Sakadilis *et al.* 2013), such as climate and associated microflora (Pitt *et al.* 2010, Sakadilis *et al.* 2011). Therefore, additional studies are needed to determine the relationship between environmental conditions in different biomes and the presence of such numerous species of Botryosphaeriaceae in diverse hosts in Brazil and around the world. In view of this, it is evident that studies such as this one will stimulate continuing research dealing with the epidemiology of these new reports of Botryosphaeriaceae recently described in the literature.

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