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Characterization of *Phaeoacremonium* isolates associated with Petri disease of table grape in Northeastern Brazil, with description of *Phaeoacremonium nordesticola* sp. nov.

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Abstract This study aims to identify and characterize species of *Phaeoacremonium* associated with Petri disease of table grapes in three regions in the Northeastern Brazil, to investigate the distribution of the species in these regions and to evaluate their pathogenicity and aggressiveness in excised green shoots of table grapes. Fungal identifications were made using a combination of morphology together with a phylogenetic analysis based on portions of the β-tubulin (TUB2) and actin (ACT) genes. Three species of *Phaeoacremonium* (*Pm.*) were identified: *Pm. minimum, Pm. nordesticola* sp. nov. and *Pm. parasiticum*. *Phaeoacremonium minimum* and *Pm. parasiticum* had previously been reported in grapevine. *Phaeoacremonium minimum* was the most prevalent species. All species of *Phaeoacremonium* were pathogenic on detached shoots of table grape, with *Pm. minimum* being the most aggressive.

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Keywords *Phaeoacremonium* · *Vitis Vinifera* · *Vitis Labrusca* · Trunk disease · Phylogeny · Aggressiveness

Introduction

Table grape (*Vitis* spp.) is an important fresh fruit exported by Brazil. In 2013, 43,181 t of table grapes were exported and accounted for US$ 131 million (FAO 2016). The Northeastern region is responsible for 99% of Brazilian exports of table grapes, where 9600 ha are cultivated. The São Francisco Valley, located in the semi-arid region of Bahia and Pernambuco States, is the main grapevine growing area in the region, accounting for 98% of the production (Lazzarotto and Fioravanço 2013). In the Siriji Valley, located in the tropical humid region of Pernambuco State, table grapes have been grown for over 40 years with a production intended only for the local market (Araújo and Ramalho 2009). In 2011, a new production pole of table grapes was started in the Baixo Jaguaribe Valley, located in the semi-arid region of Ceará State, but the plants have not yet reached the production stage. In São Francisco and Baixo Jaguaribe Valleys are planted European cultivars (*Vitis vinifera* L.) grafted onto rootstock, while in Siriji Valley are planted own rooted American cultivars (*Vitis labrusca* L.).

Northeastern Brazil is a tropical region, thus the management systems for table grape production are adapted to the specific environmental conditions of a tropical viticulture. In both the dry and wet tropics, the

growth and cropping cycle of the vine can be manipulated to extend from 5 to 12 months by a combination of pruning, modifying vine water status and the use of chemical regulators. Thus, it is possible to achieve two and a half to three vegetative cycles per year (Camargo et al. 2008; Possingham 2008; Correia et al. 2013).

A wide range of fungal diseases impact on grapevine production. Among them, grapevine trunk diseases (GTDs) are known to occur wherever grapes are grown (Úrbez-Torres et al. 2014). The sudden increase of GTD incidence worldwide has been traditionally associated with reductions in the availability of efficient fungicides, e.g. sodium arsenite, banned in 2001 (Decoin 2001), and other factors such as changes in cultural practices and vineyard management for increasing grape yield (e.g., increasing plant density, irrigation, mechanization, etc.), less protection of pruning wounds due to the increasing cost of labour or the reduced phytosanitary quality of grapevine propagating material. Petri disease is among the most destructive GTD worldwide (Mugnai et al. 1999; Gramaje and Armengol 2011). Incidence of Petri disease have been worsening in all grape-producing regions since the late 1990s, including Europe (Mugnai et al. 1999), the Near East (Arzanlou et al. 2013), North and South America (Correia et al. 2013; Úrbez-Torres et al. 2014), Oceania (Graham et al. 2009), and South Africa (Mostert et al. 2006b), and causing significant economic losses due to yield and quality reductions and vineyard replanting (Scheck et al. 1998).

The first report of Petri disease in table grapes in Northeastern of Brazil was in 2013, in the São Francisco and Siriji Valleys (Correia et al. 2013). The symptoms of this disease are characterized by reduced vigor of vine, short internodes, stunted growth, chlorotic and/or wilting leaves, occasional sudden vine collapse, and black streaking in xylem tissues and black spots in shoots and trunk (Scheck et al. 1998; Mugnai et al. 1999; Gramaje and Armengol 2011; Correia et al. 2013; Úrbez-Torres et al. 2014).

Petri disease is caused by a combination of *Phaeomoniella chlamydospora* (Gams, Crous, Wingf. e Mugnai) Crous & W. Gams and several species of *Phaeoacremonium* (*Pm.*) W. Gams, Crous & M. J. Wingf. (Scheck et al. 1998; Mugnai et al. 1999; Groenewald et al. 2001; Gramaje and Armengol 2011; Correia et al. 2013; Úrbez-Torres et al. 2014). The genus *Phaeoacremonium* was established by Crous et al. (1996), and since then, 46 species have been identified based on morphological along with molecular characters (Gramaje et al. 2015). Species delimitation within *Phaeoacremonium* based solely on cultural and morphological characteristics is challenging, and thus molecular analyses of part of the actin and β-tubulin gene regions have been used routinely for species delineation (Mostert et al. 2006a; Damm et al. 2008; Essakhi et al. 2008; Gramaje et al. 2009, 2012, 2014, 2015; Úrbez-Torres et al. 2014; Raimondo et al. 2014; Ariyawansa et al. 2015).

Phaeoacremonium species have worldwide distribution and wide host range, including woody plants, insect larvae and humans (Mostert et al. 2006a). Twenty-eight species have been isolated from grapevine (Gramaje et al. 2015). Of these, *Pm. minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous appears to be the most widely distributed and the most common in grapevines (Crous et al. 1996; Larignon and Dubos 1997; Mugnai et al. 1999; Groenewald et al. 2001; Mostert et al. 2006b; Essakhi et al. 2008; Martín et al. 2014). Other species that have also been isolated in relatively high frequencies from grapevines include *Pm. parasiticum* (Ajello, Georg & C.J.K Wang) W. Gams, Crous & M.J. Wingf. and *Pm. viticola* J. Dupont (Mostert et al. 2006b). In Brazil, three species were reported in grapevine, *Pm. minimum*, *Pm. parasiticum* (Correia et al. 2013) and *Pm. angustius* W. Gams, Crous & M.J. Wingf. (Gava et al. 2010).

The increasing economic importance of Petri disease caused by *Phaeoacremonium* in grapevines and the recent discovery of several new species of this fungus associated with other woody host plants (Damm et al. 2008; Gramaje et al. 2012, 2014) led us to question what species of *Phaeoacremonium* may be associated with Petri disease of table grape in São Francisco, Siriji and Baixo Jaguaribe Valleys, Northeastern Brazil. The disease etiology is crucial for epidemiological studies and for a better understanding of the distribution and importance of individual species, as well as finding effective management strategies for each pathogen. Therefore, the objectives of this study were: (a) to identify and characterize species of *Phaeoacremonium* associated with Petri disease of table grapes in São Francisco, Siriji and Baixo Jaguaribe Valleys, (b) to investigate the distribution of the species in these regions, and (c) to evaluate their pathogenicity and aggressiveness in excised green shoots of table grapes.

Materials and methods

Field survey and fungal isolations

During 2012, samples of table grape plants of age ranging from 6 months to 10-years old showing Petri disease symptoms were obtained from 12 vineyards located in the São Francisco, Siriji and Baixo Jaguaribe Valleys (Northeastern Brazil) (Fig. 1). These regions are distant at least 500 km from each other. In each vineyard, 10 grapevines shoots exhibiting reduced vigor of vine, stunted growth, chlorotic and/or wilting leaves, sudden vine collapse, and black streaking in xylem tissues and black spots in shoots were sampled for fungal isolations. Symptomatic wood fragments taken from the margin of internal necroses and brown-black vascular streaking were washed under running tap water, surface-disinfected for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Small pieces (4–5 mm) of tissue were taken from the margin between necrotic and apparently healthy tissue and plated onto malt extract agar (MEA; Acumedia, Lansing, USA) amended with 0.5 g l^{-1} streptomycin sulfate (MEAS). Plates were incubated at 25 °C in the dark for 14 to 21 days. Fungal colonies emerging from plant tissue pieces, characterized by having flat slow-growing cultures on MEA and that were morphologically similar to species of *Phaeoacremonium* (Mostert et al. 2006a) were transferred to potato dextrose agar (PDA; Acumedia) plates and incubated at 25 °C in the dark, with observation after 14 to 21 days. Single-spore cultures were obtained using the procedure described by Goh (1999). Isolates were morphologically identified as *Phaeoacremonium* based on typical characteristics of the genus, namely presence of different types of phialides variable in size and shape observed in the aerial mycelium, and either discrete or integrated in conidiophores, and conidia hyaline, sporulation abundant and aseptate (Mostert et al. 2006a; Gramaje et al. 2015). Pure cultures were stored in sterilized water in Eppendorf tubes at 25 °C in the dark and stock cultures were stored in PDA slants at 5 °C in the dark.

Morphological and cultural characterization

Morphological characters used to distinguish *Phaeoacremonium* species included conidiophore morphology, phialide type and shape size of hyphal warts and conidial size and shape (Mostert et al. 2006a). Colony characters and pigment production on MEA, PDA and oatmeal agar (OA; Difco, Detroit, USA) incubated at 25 °C in the dark were noted after 8 and 16 days. Colony colours were recorded with the colour charts of Rayner (1970). Microscopic observations for all fungi were made from mycelium of colonies cultivated on the respective medium or by using slide culture technique, as explained by Arzanlou et al. (2007) when studying the genus *Mycosphaerella*. Photos were captured with a Nikon DS-Ri2 camera on a Nikon Eclipse Ni-e microscope fitted with Nomarski differential interference contrast optics (Nikon Instruments Europe BV, Netherlands). The length and width of 20 conidiophores, 30 phialides and 50 conidia per isolate were measured with the Nikon measurement module. Mean and standard errors of the measurements, including mean length to width ratio (L/W) of the conidial measurements were calculated.

Isolates were also used to determine the effect of temperature on colony growth of different species. A 5-mm-diameter mycelial plug from the growing margin of a 8-day-old colony was placed in the center of a 90-mm-diameter PDA plate, and three replicates of each isolate were incubated at temperatures ranging from 10 °C to 40 °C in 5 °C intervals in the dark. After 8 and 16-days incubation periods, the colony diameters (mm) were measured in two perpendicular directions. The experiment was done twice. Colony diameters at 16 days were plotted against temperature and a curve was fitted by a cubic polynomial regression ($y = a + bx + cx^2 + dx^3$). Optimal temperature was estimated from the regression equation and numeric summary with TableCurve™ 2D v. 5.01 (SYSTAT Software Inc., Chicago, USA). Optimum temperature was defined as the temperature that produced the maximum mycelial growth. The colony diameter data at 25 °C were used to calculate the mycelial growth rate (mm day^{-1}). One-way analyses of variance (ANOVA) were conducted with data obtained from optimum temperature and mycelial growth rate experiments, 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).

DNA isolation, PCR amplification and sequencing

Using a sterile 10 μl pipette tip, a small amount of aerial mycelium was scraped from the surface of a 7 day old culture on PDA at 25 °C and genomic DNA was

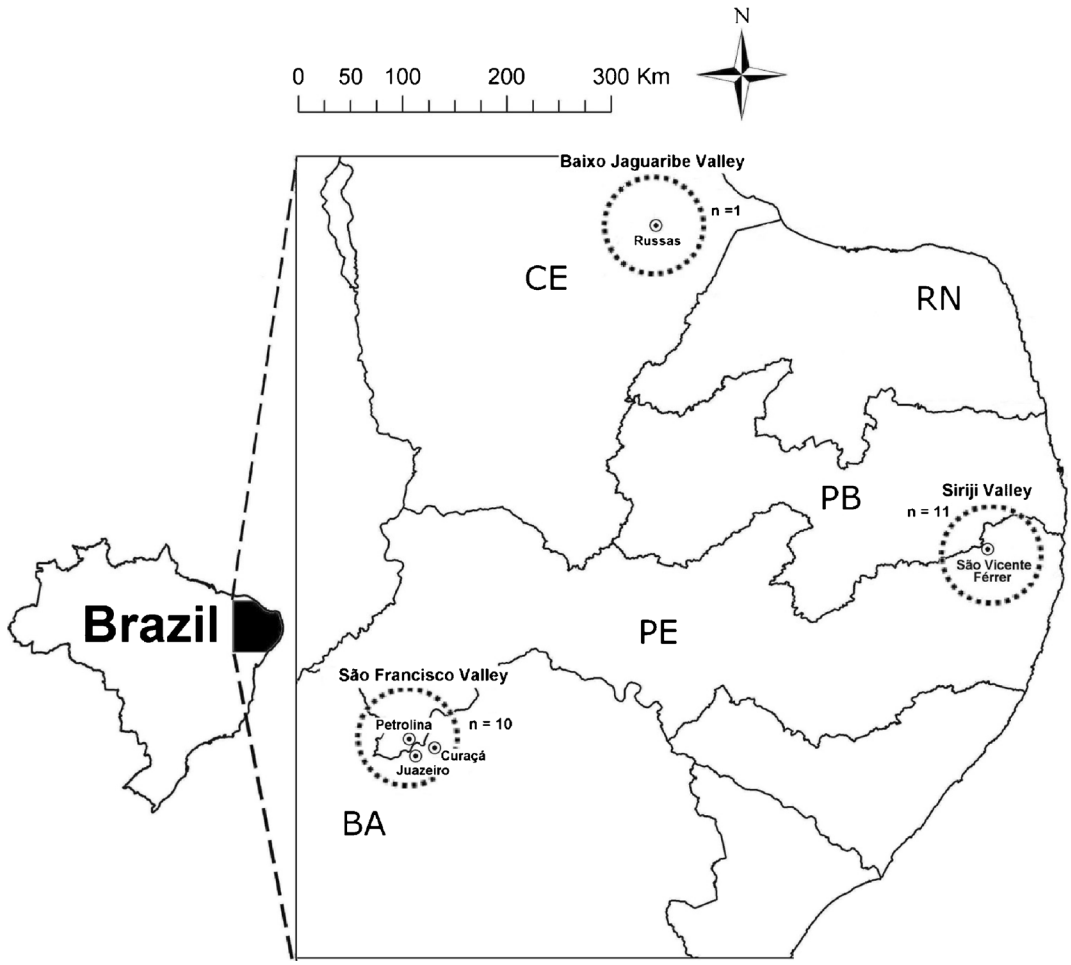

Fig. 1 Collection sites of *Phaeoacremonium* isolates from Northeastern Brazil vineyards, located in the states of Bahia (BA), Pernambuco (PE) and Ceará (CE). The names next to the dots correspond to the cities corresponding to the sampled vineyard. Dotted semicircles represent the regions defined for this study; n = number of isolates in each region

extracted using the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., Union City, USA) following the manufacturer's instructions. DNA was viewed on 0.8% agarose gels stained with ethidium bromide (0.5 μg ml^{-1}) for 1 min and stored at −20 °C. Portions of the β-tubulin (TUB2) and actin (ACT) genes of *Phaeoacremonium* isolates were amplified as described by Mostert et al. (2006a) using primer sets T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and ACT-512F and ACT-783R (Carbone and Kohn 1999), 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 (2X) (0.05 u μl^{-1} de *Taq* DNA polimerase, reaction buffer, 4 mM MgCl2, 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 ACT gene consisted of a denaturation step at 96 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 80 s and final cycle at 72 °C for 7 min. The cycling parameters for TUB2 gene were initiated at 96 °C for 5 min followed by 36 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 80 s and final cycle at 72 °C for 7 min (Graham et al. 2009). 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 with 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 and sequenced in both directions using a ABI 3730 XL DNA Analyzer (Applied Biosystems) at the Macrogen Inc. (Seoul, Korea).

Phylogenetic analysis

Forward and reverse sequences were assembled using the Staden Package (Staden et al. 1998). Sequences generated in the current study were deposited in GenBank (Table 1).

Sequences of the type and reference strains of *Phaeoacremonium* species of each locus analyzed were downloaded from GenBank and combined with the newly generated sequences. Multiple sequence alignments for each locus independently were performed using the MEGA 7.0.14 (Kumar et al. 2016), and adjustment were manually done in where necessary. Alignment of each locus was loaded in Sequence Matrix v.1.8 (Vaidya et al. 2011) to build the concatenated matrix.

The phylogenies for each locus (ACT and TUB2) and for the concatenated matrix were inferred under the maximum likelihood (ML) criterion. The ML analyses were done in n RAxML - HPC2 (Stamatakis 2014) implemented on CIPRES Science Gateway portal (https://www.phylo.org/portal2/home.action). ML tree searches were performed under the GTRGAMMA model with 1000 pseudoreplicates.

Phylogenetic species recognition

The genealogical concordance phylogenetic species recognition (GCPSR sensu Dettman et al. 2003) criterion was employed to recognize phylogenetic species. A clade was considered an independent lineage if it satisfied the genealogical concordance criteria (the clade was present in the majority of the individual gene trees) or genealogical non-discordance criteria [the clade is strongly supported (bootstrap ≥70) in at least one gene tree, and could not be contradicted in any other individual gene tree at the same level of support].

Novel species were recognized if the clade was recognized as a phylogenetic species by the GCPSR criterion, is strongly support in the multi-locus tree, and did not nest with the type of any previously described species.

Distribution of *Phaeoacremonium* species

Based on the number of isolates of each *Phaeoacremonium* species recorded, the relative frequency of each species in relation to overall number of isolates and to the total number of isolates within each table grape population was calculated.

Pathogenicity tests

Fourteen isolates representing three different *Phaeoacremonium* species were selected to assess their pathogenicity on detached green shoots of cultivar "Isabel" (Table 1). Asymptomatic green shoots of plants not sprayed with fungicides were collected in a commercial vineyard in São Vicente Férrer (Siriji Valley). The shoots were immediately placed into large plastic containers filled with sterile water, with the shoots placed over a plastic grid. The plastic containers were partially sealed with plastic bags and transported to Universidade Federal Rural de Pernambuco. The cut ends were dipped in wax and in the center of each shoot (30 cm long) a superficial wound (~4-mm length, 2-mm deep) was made using a sterilized scalpel. A 4-mm mycelium plug from a 12-day-old PDA culture of each isolate was placed into the wound. Non-colonized PDA agar plugs were used as negative controls. The inoculated area was wrapped with Parafilm (Pechiney Co., Chicago, USA) to prevent rapid dehydration. Inoculated shoots were placed in large plastic containers, as described above, and incubated at 25 °C and 12-h photoperiod in a growth chamber. After 23 days, the Parafilm was removed, the shoots were sliced through lengthwise and the internal lesions visually observed. The isolates were considered pathogenic when the lesioned area advanced beyond the 4-mm diameter inoculated area. The aggressiveness of the isolates was evaluated by measurement of the lesion lengths with a digital calliper (Mitutoyo Co., Kanagawa, Japan). The experiment was arranged in a completely randomized design with ten replicates per treatment (isolate) and one shoot per replicate. The experiment was conducted twice. Differences in aggressiveness of *Phaeoacremonium* species were determined by one-way ANOVA and means were compared by LSD test at the 5% significance level using Statistix v. 9.0.

Table 1 Isolates of *Phaeoacremonium* species used in this study

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no. [b]	
					ACT	BT
Phaeoacremonium africanum	STEU 6177	*Prunus armeniaca*	South Africa	U. Damm	EU128142	EU128100
Pm. alvesii	CBS 110034	*Homo sapiens*	Brazil	L. Mostert	AY579234	AY579301
Pm. amstelodamense	CBS 110627	*H. sapiens*	The Netherlands	J. Bruins	AY579228	AY579295
Pm. amygdalinum	Psp4	*Prunus dulcis*	Spain	D. Gramaje	JN191304	JN191308
Pm. angustius	CBS 114992	*Vitis vinifera*	USA	P. Laringon	DQ173127	DQ173104
Pm. argentinense	CBS 77783	*Soil*	Argentina	A. Martinez	DQ173135	DQ173108
Pm. armeniacum	ICMP 17421	*V. vinifera*	New Zealand	B.S. Weir	EU595463	EU596526
Pm. australiense	CBS 113589	*V. vinifera*	Australia	T. Knaggs	AY579229	AY579296
Pm. austroafricanum	CBS 112949	*V. vinifera*	South Africa	L. Mostert	DQ173122	DQ173099
Pm. cinereum	Pm5	*V. vinifera*	Iran	H. Mohammadi	FJ517153	FJ517161
Pm. croatiense	113Pal	*V. vinifera*	Croatia	B. Cvjetković	EU863514	EU863482
Pm. fraxinopennsylvanicum	CBS 110212	*Fraxinus pensylvanica*	USA	T.E. Hinds	DQ173136	DQ173109
Pm. fuscum	STEU 5969	*Prunus salicina*	South Africa	U. Damm	EU128141	EU128098
Pm. globosum	ICMP 16988	*Vitis* sp.	New Zealnd	U. Damm	EU595466	EU596525
Pm. griseo-olivaceum	STEU 5966	*P. armeniaca*	South Africa	U. Damm	EU128139	EU128097
Pm. griseorubrum	CBS 111657	*H. sapiens*	USA	D. Sutton	AY579227	AY579294
Pm. hispanicum	Pm8	*V. vinifera*	Spain	D. Gramaje	FJ517156	FJ517164
Pm. hungaricum	90Pal	*V. vinifera*	hungary	S. Essakhi	EU863515	EU863483
Pm. inflatipes	CBS 39171	*Quercus virginiana*	USA	R.S. Halliwell	AY579259	AF246805
Pm. iranianum	CBS 101357	*Actinidia chinensis*	Italy	F. Calzarano	DQ173120	DQ173097
Pm. krajdenii	CBS 110118	*V. vinifera*	South Africa	G. van Coller	AY579261	AY579324
Pm. luteum	A33	*Santalum album*	Australia	D. Gramaje	KJ533542	KJ533540
Pm. luteum	A34	*S. album*	Australia	D. Gramaje	KJ533543	KJ533541
Pm. minimum	Y108022z	*V. vinifera*	Spain	L. Martin	JF275893	JF275879
Pm. minimum	STEU 6991	*V. vinifera*	South Africa	L. Mostert	JQ038921	JQ038910
Pm. novae-zealandiae	CBS 114512	*Desmoschoenus spiralis*	New Zealnd	J.R. George	DQ173141	DQ173112
Pm. occidentale	ICMP 17037	*Vitis* sp.	New Zealnd	B.S. Weir	EU595460	EU596524
Pm. pallidum	STEU 6104	*P. armeniaca*	South Africa	U. Damm	EU128144	EU128103
Pm. parasiticum	STEU 6993	*P. armeniaca*	South Africa	Unknown	JQ038927	JQ038916
Pm. parasiticum	CBS 101007	*A. chinensis*	Italy	L. Calzarano	AY579252	AF246804
Pm. pruniculum	STEU 5967	*P. salicina*	South Africa	U. Damm	EU128137	EU128095
Pm. roseum	PARC 281	*V. vinifera*	Canada	J.R. Urbez-Torres	KF764507	KF764659

Table 1 (continued)

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no. [b]	
					ACT	BT
Pm. rubrigenum	CBS 112046	*H. sapiens*	USA	G. Conover	AY579239	AY579305
Pm. santali	CBS 137498	*S. album*	Australia	D. Gramaje	KJ533539	KJ533535
Pm. scolyti	DUCC 407	Barkbeetles	South Korea	M.W. Hyun	KC166686	KC166684
Pm. sicilianum	48Pal	*V. vinifera*	Italy	S. Essakhi	EU863520	EU863488
Pm. sphinctrophorum	CBS 69488	*H. sapiens*	USA	A.A. Padhye	DQ173143	DQ173114
Pm. subulatum	CBS 113584	*V. vinifera*	South Africa	L. Mostert	AY579231	AY579298
Pm. tardicrescens	CBS 110573	*H. sapiens*	USA	Levi	AY579233	AY579300
Pm. tectonae	MFLUCC 14–1129	*Tectona grandis*	Thailand	M. Doilom	KT285560	KT285568
Pm. tectonae	MFLUCC 14–1130	*T. grandis*	Thailand	M. Doilom	KT285561	KT285569
Pm. tectonae	MFLUCC 14–1131	*T. grandis*	Thailand	M. Doilom	KT285562	KT285570
Pm. theobromatis	CBS 111586	*Theobroma gileri*	Ecuador	H.C. Evans	DQ173132	DQ173106
Pm. tuscanum	1Pal	*V. vinifera*	italy	S. Essakhi	EU863490	EU863458
Pm. venezuelense	CBS 65185	*H. sapiens*	Venzuela	M.B. de Albornoz	AY579256	AY579320
Pm. vibratile	CBS 117115	*Fagus sylvatica*	France	J. Fournier	DQ649064	DQ649063
Pm. viticola	Y271031d	*V. vinifera*	Spain	L. Martin	HQ700719	HQ700718
Pm. minimum	CMM 4316[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030788**	**KY030790**
Pm. minimum	CMM 4317	*V. vinifera*	Brazil	M.A. Silva	**KY030789**	**KY030791**
Pm. minimum	CMM 4318[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030792**	**KY012296**
Pm. minimum	CMM 4319	*V. vinifera*	Brazil	M.A. Silva	**KY030793**	**KY012308**
Pm. minimum	CMM 4322[c]	*Vitis labrusca*	Brazil	M.A. Silva	**KY039281**	**KY012297**
Pm. minimum	CMM 4323	*V. labrusca*	Brazil	M.A. Silva	**KY030794**	**KY012298**
Pm. minimum	CMM 4324[c]	*V. labrusca*	Brazil	M.A. Silva	**KY030795**	**KY012299**
Pm. minimum	CMM 4325	*V. labrusca*	Brazil	M.A. Silva	**KY030796**	**KY012300**
Pm. minimum	CMM 4326[c]	*V. labrusca*	Brazil	M.A. Silva	**KY030797**	**KY012301**
Pm. minimum	CMM 4327	*V. labrusca*	Brazil	M.A. Silva	**KY030798**	**KY012302**
Pm. minimum	CMM 4328	*V. labrusca*	Brazil	M.A. Silva	**KY030799**	**KY012303**
Pm. minimum	CMM 4329[c]	*V. labrusca*	Brazil	M.A. Silva	**KY030802**	**KY012304**
Pm. minimum	CMM 4331	*V. labrusca*	Brazil	M.A. Silva	**KY030800**	**KY012305**
Pm. minimum	CMM 4332	*V. labrusca*	Brazil	M.A. Silva	**KY030801**	**KY012306**
Pm. minimum	CMM 4333[c]	*V. labrusca*	Brazil	M.A. Silva	**KY030814**	**KY012307**
Pm. nordesticola	CMM 4312[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030803**	**KY030807**
Pm. nordesticola	CMM 4313[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030806**	**KY030808**

Table 1 (continued)

Taxon	Culture accession no.[a]	Host	Location	Collector	GenBank accession no.[b]	
					ACT	BT
Pm. nordestiicola	CMM 4314[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030804**	**KY030809**
Pm. nordestiicola	CMM 4334[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030805**	**KY030810**
Pm. parasiticum	CMM 4315[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030811**	**KY030785**
Pm. parasiticum	CMM 4320[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030813**	**KY030786**
Pm. parasiticum	CMM 4321[c]	*V. vinifera*	Brazil	M.A. Silva	**KY030812**	**KY030787**

[a] *CBS*Centraalbureauvoor Schimmelcultures, Utrecht, Netherlands; *CMM* Culture Collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil; *DUCC* Dankook University Culture Collection, Cheonan, Korea; *ICMP* International Collection of Microorganisms from Plants, Lincoln, New Zealand; *MFLUCC* Mae Fah Luang University Culture Collection, Chiang Ray, Thailand; *PARC* Culture Collection of the Pacific Agri-Food Research Centre, Summerland, Canada; *STEU* Culture Collection of the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa

[b] Sequence numbers in *bold* were obtained in the present study

[c] Isolates used in pathogenicity tests

Results

Morphological and cultural characterization

Twenty-two isolates of *Phaeoacremonium* were obtained from table grape plants showing Petri disease symptoms in Northeastern Brazil. All species showed morphological features typical of the genus *Phaeoacremonium* (Mostert et al. 2006a), namely presence of different types of phialides observed in the aerial mycelium, and either discrete or integrated in conidiophores, and conidia hyaline and aseptate (Fig. 3). No teleomorph structure was observed during this study.

The morphological characteristics observed in *Pm. minimum* were: *colony* colour on MEA honey-brown or beige; *yellow pigment* not produced on MEA and PDA; *mycelium* texture mostly verruculose; *conidiophore* structure mostly short and usually unbranched, 18.9–36.5 × 1.9–2.9 μm ($\bar{x}$ = 23.9 ± 1.7 × 2.4 ± 0.4 μm; *n* = 32); *conidiogenous cells* phialides predominant type II and III; type I phialides cylindrical, 2.5–4.9 × 0.6–1.5 μm ($\bar{x}$ = 4.1 ± 0.7 × 1 ± 0.3 μm; *n* = 38); type II phialides mostly elongate-ampulliform and attenuated at the base, 6.6–11 × 1.3–2.7 μm ($\bar{x}$ = 9.6 ± 1.7 × 1.8 ± 0.5 μm; *n* = 66); type III phialides subcylindrical or elongate-ampulliform and attenuated at the base, 11–19.5 × 1.7–2.6 μm ($\bar{x}$ = 14.9 ± 2.1 × 2.1 ± 0.4 μm; *n* = 52); *conidia* hyaline, mostly oblong-ellipsoid or cylindrical, occasionally reniform, 3.1–7× 1.3–3.4 μm ($\bar{x}$ = 4.8 ± 0.9 × 2.1 ± 0.3 μm; *n* = 165), L/W ratio = 2.3.

The morphological characteristics observed in *Pm. parasiticum* were: *colony* colour on MEA brown with medium brown center; *yellow pigment* produced on MEA and PDA; *mycelium* texture verrucose; *conidiophore* structure mostly long and branched, 27.2–56.1× 2–3.4 μm ($\bar{x}$ = 39.1 ± 8.2 × 2.6 ± 0.5 μm; *n* = 34); *conidiogenous cells* phialides predominant type III; type I phialides cylindrical, occasionally widened at the base, 5.7–6.4 × 1.1–2.2 μm ($\bar{x}$ = 6.0 ± 0.3× 1.6 ± 0.5 μm; *n* = 32); type II phialides subcylindrical, tapering toward the apex, 12–24.3 × 1.4–2.6 μm ($\bar{x}$ = 17.4 ± 3.0 × 2.1 ± 0.4 μm; *n* = 42); type III phialides mostly cylindrical to subulate, 22–40.7× 2.4–3.6 μm ($\bar{x}$ = 29.9 ± 6.3× 2.9 ± 0.4 μm; *n* = 62); *conidia* hyaline, mostly oblong-ellipsoid, sometimes allantoid to broadly oblong, 2.7–6.3× 1.3–2.8 μm ($\bar{x}$ = 4 ± 0.7× 1.8 ± 0.3 μm; *n* = 134), L/W ratio = 2.2.

All species of *Phaeoacremonium* used in this study grew at temperatures ranging from 10 °C to 35 °C. Only one isolate of *Pm. minimum* (CMM 4322) and two isolates of *P. parasiticum* (CMM 4315 and CMM 4321) grew at 40 °C. There were no significant differences ($P > 0.05$) among the *Phaeoacremonium* species in relation to mycelial growth rate (0.22–0.25 mm day^{-1}) and optimum temperature for mycelial growth (29.6–30.1 °C).

Phylogenetic analyses

Three distinct haplotypes were found among the twenty-two isolates based on the DNA sequences. The topologies of each single locus tree were not discordant among them. Alignments of ACT and TUB2 provided topologies with great resolution, and clades of all *Phaeoacremonium* species were recovered with significant support. The individual gene trees resolved the same clades recovered in the multi locus tree (Fig. 2).

Phylogenetic species recognition

The 22 *Phaeoacremonium* isolates from grapevine included in the multi locus analyses were resolved in three independent lineages according to the GCPSR criterion, and could be recognized as phylogenetic species. Fifteen isolates were assigned to the species *P. minimum*. Three isolates were placed within the *P. parasiticum* clade and were assigned to this species. Four isolates nested together in a clade closely related with *P. luteum*, and is described in the present study as a novel species named *Phaeoacremonium nordesticola*. The species description is presented in the taxonomic section.

Taxonomy

Based on the DNA sequence analyses and morphological characters, one species of *Phaeoacremonium* proved distinct from known species, and is described below.

Phaeoacremonium nordesticola M.A. Silva, M.P.S. Câmara & S.J. Michereff **sp. nov.** MycoBank register = 820710; Figs. 3a–p.

Etymology: The name refers to Northeastern Brazilian region, where this species was first found.

Description colonies on PDA at first hazel (17″‘i) to olivaceous buff (21″‘d), becoming greenish glaucous (23″‘i) and olivaceous (21”k) at the surface with the reverse side of the colonies olivaceous buff (21″‘d) after 8 days in the dark at 25 °C. Colonies reaching a radius of 16.7–1.7 mm after 8 days in the dark at 25 °C on MEA. No pigment produced on MEA and PDA. Minimum temperature for growth 10 °C, optimum 29.7 °C, maximum 37 °C. The mycelial growth rate at 25 °C was 0.22 ± 0.02 mm day^{-1}. *Aerial mycelium* consisting of branched, septate hyphae that occurs singly or in bundles of up to 8; hyphae without warts. *Conidiophores* mostly short, usually unbranched, 18.9–36.5 × 1.90–2.9 μm ($\bar{x}$ = 23.9 ± 1.7 × 2.4 ± 0.4 μm; n = 46). *Conidiogenous cells* phialides predominant type II, terminal or lateral, mostly monophialidic; type I phialides cylindrical, 2.5–4.9 × 0.6–1.5 μm ($\bar{x}$ = 4.0 ± 0.4 × 1.1 ± 0.2 μm; n = 36); type II phialides mostly elongate-ampulliform and attenuated at the base, 6.6–11 × 1.3–2.7 μm ($\bar{x}$ = 9.4 ± 1.0 × 1.9 ± 0.4 μm, n = 58); type III phialides cylindrical to subcylindrical, 11–19.5 × 1.6–2.6 μm ($\bar{x}$ = 14.9 ± 21 × 1.9 ± 0.4 μm; n = 42); *Conidia* hyaline, mostly allantoid, few reniform, 2.9–7.5 × 1.1–2.4 μm ($\bar{x}$ = 4.2 ± 0.9 × 1.7 ± 0.3 μm; n = 178), L/W ratio = 2.6.

Sexual morph: not produced in culture.
Substrate: *Vitis vinifera.*
Known Distribution: Brazil (Ceará, Pernambuco).
Type: Brazil, Pernambuco, Petrolina, on *Vitis vinifera* tree, 2012, coll. M. A. Silva (holotype URM register = 89963 dry culture produced on pine needles, ex-type culture URM register = 7391 = CMM 4312).

Additional cultures examined Brazil, Pernambuco, Petrolina, isolated from *Vitis vinifera* trees, 2012, coll. M.A. Silva, CMM 4313 and CMM 4314; Brazil, Ceará, Russas, isolated from *Vitis vinifera* tree, 2012, coll. M.A. Silva, CMM 4334.

Notes – Phylogenetically *Pm. nordesticola* is closely related to *Pm. luteum* D. Gramaje, T.I. Burgess & J. Armengol, but cultures of *Pm. luteum* produced yellow pigment on MEA, PDA and OA, which did not occur with *Pm. nordesticola*. Phialides type I (4–6 × 1.5–2 μm), type II (10.5–17 × 2–3.5 μm) and type III (20–30 × 2–3 μm), and conidia (4–6 × 2–3 μm; $\bar{x}$ = 5 × 2.5 μm) of *Pm. luteum* are longer and wider than

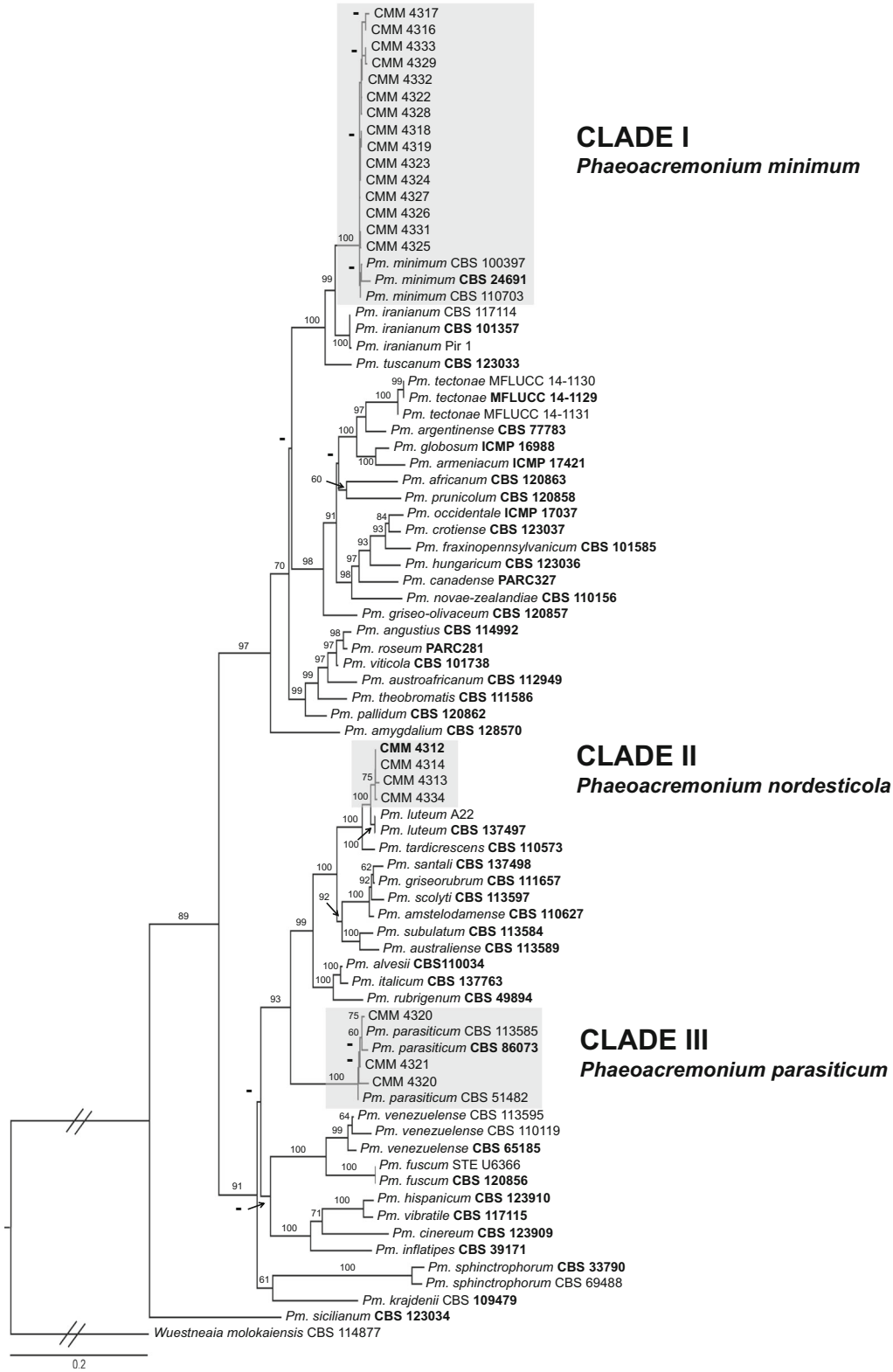
CMM 4317
CMM 4316
CMM 4333
CMM 4329
CMM 4332
CMM 4322
CMM 4328
CMM 4318
CMM 4319
CMM 4323
CMM 4324
CMM 4327
CMM 4326
CMM 4331
CMM 4325
Pm. minimum CBS 100397
Pm. minimum CBS 24691
Pm. minimum CBS 110703
CLADE I
Phaeoacremonium minimum
Pm. iranianum CBS 117114
Pm. iranianum CBS 101357
Pm. iranianum Pir 1
Pm. tuscanum CBS 123033
Pm. tectonae MFLUCC 14-1130
Pm. tectonae MFLUCC 14-1129
Pm. tectonae MFLUCC 14-1131
Pm. argentinense CBS 77783
Pm. globosum ICMP 16988
Pm. armeniacum ICMP 17421
Pm. africanum CBS 120863
Pm. prunicolum CBS 120858
Pm. occidentale ICMP 17037
Pm. crotiense CBS 123037
Pm. fraxinopennsylvanicum CBS 101585
Pm. hungaricum CBS 123036
Pm. canadense PARC327
Pm. novae-zealandiae CBS 110156
Pm. griseo-olivaceum CBS 120857
Pm. angustius CBS 114992
Pm. roseum PARC281
Pm. viticola CBS 101738
Pm. austroafricanum CBS 112949
Pm. theobromatis CBS 111586
Pm. pallidum CBS 120862
Pm. amygdalium CBS 128570
CMM 4312
CMM 4314
CMM 4313
CMM 4334
CLADE II
Phaeoacremonium nordesticola
Pm. luteum A22
Pm. luteum CBS 137497
Pm. tardicrescens CBS 110573
Pm. santali CBS 137498
Pm. griseorubrum CBS 111657
Pm. scolyti CBS 113597
Pm. amstelodamense CBS 110627
Pm. subulatum CBS 113584
Pm. australiense CBS 113589
Pm. alvesii CBS110034
Pm. italicum CBS 137763
Pm. rubrigenum CBS 49894
CMM 4320
Pm. parasiticum CBS 113585
Pm. parasiticum CBS 86073
CMM 4321
CMM 4320
Pm. parasiticum CBS 51482
CLADE III
Phaeoacremonium parasiticum
Pm. venezuelense CBS 113595
Pm. venezuelense CBS 110119
Pm. venezuelense CBS 65185
Pm. fuscum STE U6366
Pm. fuscum CBS 120856
Pm. hispanicum CBS 123910
Pm. vibratile CBS 117115
Pm. cinereum CBS 123909
Pm. inflatipes CBS 39171
Pm. sphinctrophorum CBS 33790
Pm. sphinctrophorum CBS 69488
Pm. krajdenii CBS 109479
Pm. sicilianum CBS 123034
Wuestneaia molokaiensis CBS 114877
0.2

Fig. 2 Maximum likelihood tree of *Phaeoacremonium* species inferred from a concatenated alignment of ACT and TUB2. Significant supports for the Maximum Likelihood analysis (≥70) are shown above the nodes. "-" indicates no-significant support. Branches crossed by diagonal lines are shortened by 50%. Types are emphasized in bold font. Isolates from grapevine are highlighted in red. *Wuestneaia molokaiensis* CBS114877 was used as outgroup. The scale bar indicates the average number of substitutions per site

those of *Pm. nordesticola* (type I phialides: 2.5–4.9× 0.6–1.5 μm; type II phialides: 6.6–11 × 1.3–2.7 μm; type III phialides: 11–19.5 × 1.6–2.6 μm; conidia: 2.9–7.5 × 1.1–2.4 μm, $\bar{x}$ = 4.23 × 1.65 μm). The conidia L/W ratio of *Pm. luteum* (2.2) is smaller that of *Pm. nordesticola* (2.6). *Phaeoacremonium nordesticola* differs from its closest phylogenetic neighbor, *Pm. luteum*, by unique fixed alleles in one loci based on alignments of the separate loci deposited in TreeBase as study S16135: alignments and β-tubulin positions 118 (T), 242 (G), 361 (C), 508 (A) and Actin positions 2 (A), 13 (C), 59 (C), 102 (A), 745 (T) and 259 (A).

Distribution of *Phaeoacremonium* species

Phaeoacremonium minimum was the predominant species isolated from table grape trees (15 isolates) followed by *Pm. nordesticola* (four isolates) and *Pm. parasiticum* (three isolates). The distribution of *Phaeoacremonium* species differed between the three regions of the Northeastern Brazil. The three *Phaeoacremonium* species were found only in São Francisco Valley. *Pm. minimum* and *Pm. parasiticum* were found in Siriji Valley (Fig. 4). A single isolate of *Pm. nordesticola* was obtained in Baixo Jaguaribe Valley.

Pathogenicity tests

All isolates of *Phaeoacremonium* were pathogenic on detached green shoots of grapevine, resulting in visible lesions 23 days after inoculation. The symptoms observed both on the surface and internally were brown dark necrotic lesions which extended upward and downward from the point of inoculation. There were significant ($P \leq 0.05$) differences in aggressiveness among the species. *Phaeoacremonium minimum* was the most aggressive, causing the largest lesion (10.9 ± 1.1 mm), while *Pm. nordesticola* was the less aggressive, causing the smaller lesion (8.6 ± 0.6 mm). *Phaeoacremonium parasiticum* showed intermediate aggressiveness, causing lesion of 9.5 ± 0.9 mm.

Discussion

Table GTDs and the associated pathogens have been little studied worldwide. This study constitutes the first attempt to assess the diversity of *Phaeoacremonium* species on table grapes showing Petri disease symptoms in Brazil. Species identification was based on morphological characters and analysis of partial sequences of actin and β-tubulin genes. Three species were identified, namely *Pm. minimum*, *Pm. parasiticum*, and the new species *Pm. nordesticola*. The colony characteristics and conidiophores, phialides and conidia dimensions of the first two species obtained in this study were similar to those previously described in the literature (Mostert et al. 2006a).

Since 1996, when the genus *Phaeoacremonium* was first established (Crous et al. 1996), 28 species of *Phaeoacremonium* had been isolated from grapevines and identified based on their cultural, morphological, and molecular characters (Crous et al. 1996; Dupont et al. 2000; Groenewald et al. 2001; Mostert et al. 2005; Mostert et al. 2006b; Essakhi et al. 2008; Graham et al. 2009; Gramaje et al. 2009; Úrbez-Torres et al. 2014; Raimondo et al. 2014). Interestingly, the majority of these species (22 out of 28) have been identified within the last 8 years, which may be a response to a significant upsurge in the number of field surveys conducted since the early 2000s spurred by increases in Petri disease incidences in grape growing regions worldwide. Discovery of a novel species in the present study increases to 29 and 47, respectively, the total number of *Phaeoacremonium* species occurring in grapevine and in this genus.

Phaeoacremonium nordesticola is recognized as a new species in the genus *Phaeoacremonium*, closely related to *Pm. luteum*. Considering the phylogenetic data, the isolates of *Pm. nordesticola* formed a clade strongly supported in the maximum-parsimony analysis (100%). *Phaeoacremonium nordesticola* can also be distinguished from *Pm. luteum* based on colony characteristics, mycelial growth and phialides dimensions described for this species (Gramaje et al. 2014). *Phaeoacremonium nordesticola* did not produce yellow pigment on MEA, PDA or OA, which occurs with from

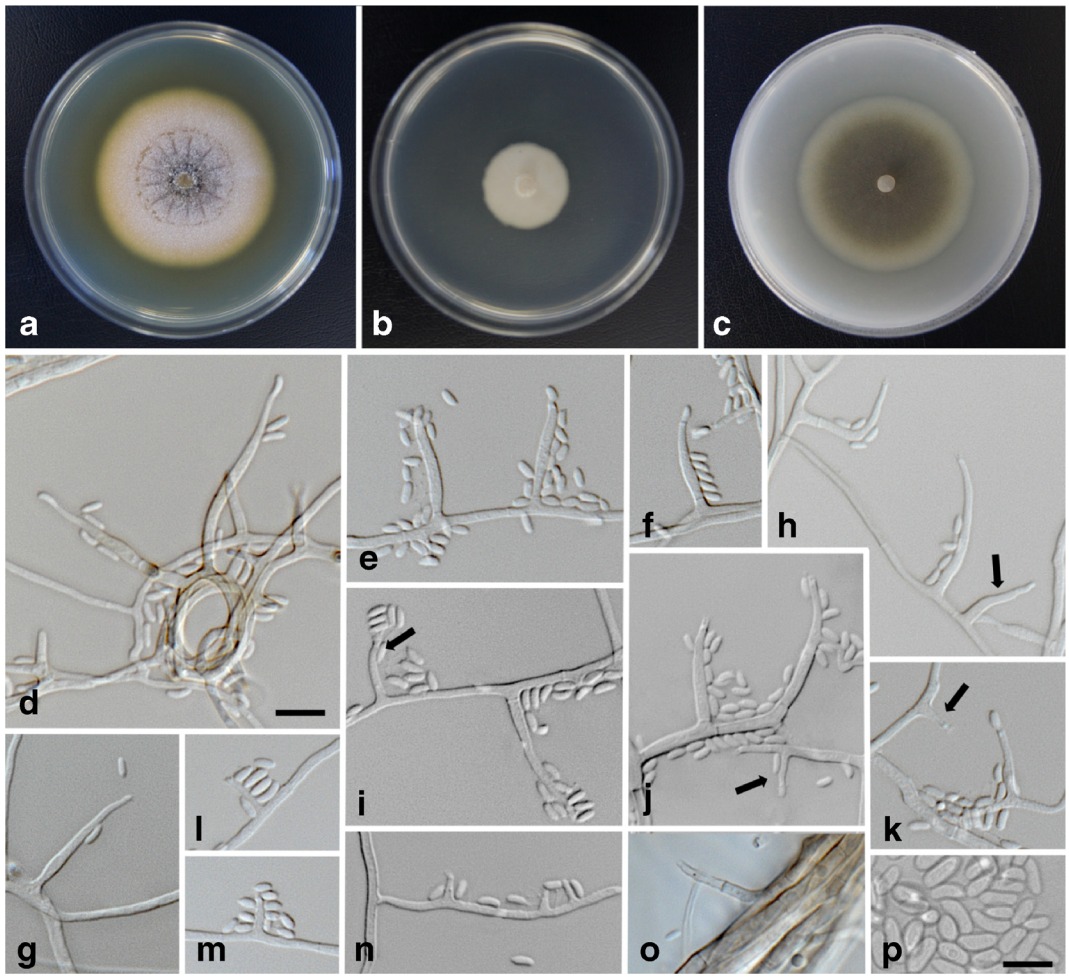

Fig. 3 *Phaeoacremonium nordesticola* holotype (culture CMM 4312). Sixteen-day-old colony grown on MEA (**a**), PDA (**b**) and OA (**c**); (**d-o**) Aerial structures on MEA (D-G) Type III phialides; (**h-i**) Type III and Type II phialides (indicated by arrow); (**j**) Type III and Type I phialides (indicated by arrow); (**k**) Type II and Type I phialides (indicated by arrow); (**l-n**) Type I phialides; (**o**) Single conidiophore; (**p**) Structures on the surface of and in MEA: conidia. Scale bars: **d** – 10 μm; **p** – 5 μm. Scale bar for **d** applies to **d-o**

Pm. luteum. Phialides type I, II and III of *Pm. luteum*are longer and wider than those of *Pm. nordesticola*. The conidia L/W ratio of *Pm. luteum* is smaller that of *Pm. nordesticola*.

In this work, *Pm. minimum* was the most frequently isolated species associated with Petri disease of table grape, and also the most widespread species in vineyards of Northeastern Brazil. This species had been reported in table grapes in Northeastern Brazil (Correia et al. 2013), being recognized as the most common species on grapevines worldwide (Mostert et al. 2006b; Essakhi et al. 2008; Martín et al. 2014; Úrbez-Torres et al. 2014). This species has been isolated from other host plants than grapevine, including *Actinidia chinensis* (Crous and Gams 2000), *Cydonia oblonga* (Sami et al. 2014), *Diospyros kaki* (Moyo et al. 2016), *Malus domestica* (Cloete et al. 2011), *Olea europea* (Crous and Gams 2000), *Phoenix dactylifera* (Mohammadi 2014), *Prunus armeniaca* (Damm et al. 2008), *Prunus persica* (Damm et al. 2008), *Prunus salicina* (Damm et al. 2008), *Prunus pennsylvanica* (Hausner et al. 1992), *Pyrus communis* (Cloete et al. 2011) and *Salix* sp. (Hausner et al. 1992).

The other two species isolated in this work, *Pm. parasiticum* and *Pm. nordesticola*, were isolated with similar frequency (18.2%) from table grapes with Petri disease symptoms. *Phaeoacremonium parasiticum* is commonly isolated from grapevines in relatively high

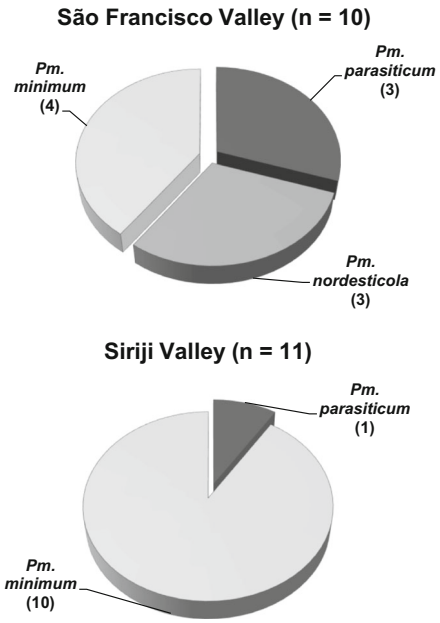

Fig. 4 Frequency of *Phaeoacremonium* species associated with Petri disease of table grape in São Francisco and Siriji Valleys, Northeastern Brazil

frequencies (Dupont et al. 2002; Mostert et al. 2006b) and had been reported in table grapes in Northeastern Brazil (Correia et al. 2013). Besides Brazil, *Pm. parasiticum* was also isolated in Algeria (Berraf-Tebbal et al. 2011), Argentina (Dupont et al. 2002), Australia (Mostert et al. 2005), Chile (Auger et al. 2005), Iran (Mostert et al. 2006a), Italy (Essakhi et al. 2008), Peru (Romero-Rivas et al. 2009), South Africa (Crous et al. 1996), Spain (Aroca et al. 2006), Turkey (Dupont et al. 2000) and USA (Mostert et al. 2006a). This species was also found on other woody hosts as an endophyte or as agent of plant disease, including *A. chinensis* (Di Marco et al. 2004), *Aquilaria agallocha* (Mostert et al. 2006b), *Cupressus* sp. (Mostert et al. 2006b), *Cydonia oblonga* (Sami et al. 2014), *Diospyros kaki* (Moyo et al. 2016), *Nectandra* sp. (Hawksworth et al. 1976), *O. europea* (Nigro et al. 2013), *P. dactylifera* (Hawksworth et al. 1976), *P. armeniaca* (Hawksworth et al. 1976), *Prunus avium* (Rumbos 1986), *P. communis* (Sami et al. 2014), *Quercus virginiana* (Halliwell 1966) and *Santalum album* (Gramaje et al. 2014). *Phaeoacremonium parasiticum* have also been associated with human infections, often causing phaeohyphomycosis (lumps of fungal growth under the skin) (Crous et al. 1996; Mostert et al. 2005; Mostert et al. 2006a). Species of *Phaeoacremonium* are opportunistic pathogens needing a subcutaneous traumatic inoculation or predisposed host to be able to infect and cause disease (Mostert et al. 2006b).

Regarding cultural characteristics, the optimum temperature for mycelial growth for *Phaeoacremonium* species from table grape varied between 29.6 and 30.1 °C, and some isolates of *Pm. minimum* and *Pm. parasiticum* grew at 40 °C, corroborating with information from the literature (Mostert et al. 2006a). The growth of *Pm. parasiticum* at 10 °C observed in this study contrast to information that the minimum temperature for this species is 15 °C (Mostert et al. 2006a). As can be observed, cultural characteristics may vary among isolates of the same species and therefore are of limited value in the determination of species.

Although many new *Phaeoacremonium* species have been identified from Petri disease infected plants, there is a lack of evidence for pathogenicity in most studies (Dupont et al. 2000; Groenewald et al. 2001; Mostert et al. 2006b; Mostert et al. 2006b; Essakhi et al. 2008; Gramaje et al. 2009), making it difficult to evaluate whether the majority of *Phaeoacremonium* species are involved in disease development. All isolates of *Phaeoacremonium* species in this study were able to infect, colonize, and produce lesions in detached grapevine shoots, confirming their status as Petri disease pathogens, including the new species *Pm. nordesticola*. Differences in aggressiveness among *Phaeoacremonium* species inoculated in grapevine, as observed in this study, have been reported previously (Mostert et al. 2006b; Halleen et al. 2007; Aroca and Raposo 2009; Úrbez-Torres et al. 2014).

This article reports three species of the genus *Phaeoacremonium* associated with Petri disease of table grape in Northeastern Brazil. All the species found in Northeastern Brazil have potential to cause disease in table grapes, but *Pm. minimum* was the most aggressive species. Studies are needed on the epidemiology and impact on table grape production together with information referring to ecology, distribution, host range and fungicide sensitivity of all species of *Phaeoacremonium* found in this study.

The results of this study will certainly be crucial to a better formulation of Petri disease control strategies and genetic improvement programs in tropical viticulture.

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