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ARTICLE

New *Phaeoacremonium* species isolated from sandalwood trees in Western Australia

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Abstract: Thirty-eight *Phaeoacremonium* isolates collected from pruning wounds of tropical sandalwood in Western Australia were studied with morphological and cultural characteristics as well as phylogenetic analyses of combined DNA sequences of the actin and β-tubulin genes. Three known *Phaeoacremonium* species were found, namely *P. alvesii*, *P. parasiticum,* and *P. venezuelense*. *Phaeoacremonium venezuelense* represents a new record for Australia. Two new species are described: *P. luteum* sp. nov. can be identified by the ability to produce yellow pigment on MEA, PDA, and OA, the predominance of subcylindrical to subulate type II phialides, and the mycelium showing prominent exudate droplets observed as warts; and *P. santali* sp. nov. which can be separated from other species producing pink colonies on MEA by the predominance of type I and II phialides, the distinct brownish olive colonies in OA, and slow growth.

Key words:
actin
β-tubulin
DNA phylogeny
Santalum
systematics
Togninia

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INTRODUCTION

Tropical Sandalwood (*Santalum album*) is one of the world's most valuable tropical tree species and demand has led to the overexploitation of natural sandalwood stands, in Timor from which it originates, and in India where it became naturalized over 2000 yr ago (Harbaugh & Baldwin 2007). To combat this destruction, plantations have been established in various countries including Australia, with commercial plantations established in 1999. The Ord River Irrigation Scheme near Kununurra has a Tropical sandalwood plantation estate presently occupying approximately 5 000 ha.

Sandalwood is a slow growing root hemi-parasite. The valuable sandalwood oil has been reported to start developing in the heartwood from 5 yr onwards. Plantation diseases usually only become a risk factor in plantation systems after two to three generations. However, disease risk in tropical plantation systems can become an issue in the first generation (Barry 2002, Barbour *et al*. 2010).

There have been few reports of fungal diseases infecting the Tropical sandalwood. Reports have been made of the presence of *Phytophthora cinnamomi* and in the early 1990s, an isolation of *Ganoderma steyartanum* was undertaken from host species, but not from Tropical sandalwood (Len Nelson pers. comm.). More recently Rural Industries Research and Development Corporation (RIRDC) supported an investigation by Barbour *et al*. (2010) into the identification of the heartwood rot in Tropical sandalwood and the impact on oil levels within the heartwood. Several rot fungi were isolated and sections suggested that the fungi were entering the branches and main stem *via* wounds made during pruning or when branches are damaged. The immediate response to this knowledge was to establish a pruning trial examining the effect of tree age and timing (season) of pruning on infection development. The pruned trees were destructively harvested after 6 and 12 mo, and over 70 endophytes, canker, and rot fungi recovered. Among these fungi were several *Phaeoacremonium* isolates.

The genus *Phaeoacremonium* was established by Crous *et al*. (1996), and 40 species have been described so far (Crous *et al*. 1996, Dupont *et al*. 2000, Groenewald *et al*. 2001, Mostert *et al*. 2005, 2006, Damm *et al*. 2008, Essakhi *et al*. 2008, Graham *et al*. 2009, Gramaje *et al*. 2009, 2012, Úrbez-Torres *et al*. 2014), including three species originally described as *Togninia* species with *Phaeoacremonium* asexual morphs: *T. africana* and *T. griseo-olivacea* (Damm *et al*. 2008), and *T. vibratilis* (Réblová & Mostert 2007). Several species of this genus have been studied intensively because of the involvement of these taxa in two complex fungal diseases of grapevine, namely Petri disease in young vines and esca disease in adult vines (Mostert *et al*. 2006), as well as with human infections, so-called phaeohyphomycoses (Mostert *et al*. 2005). However, numerous species of *Phaeoacremonium* have also been associated with disease

Table 1. The described *Phaeoacremonium* (and *Togninia* sexual morphs) species known from soil and host plants other than *Vitis vinifera*, and their worldwide distribution. * = epithets not yet transferred to *Phaeoacremonium.*

***Phaeoacremonium* species**	**Host/Substrate**	**Country and Reference**
P. aleophilum (*T. minima*)	*Actinidia chinensis*	Italy (Crous & Gams 2000)
	Malus domestica	Iran (Arzanlou *et al*. 2014); South Africa (Cloete *et al*. 2011)
	Olea europea	Italy (Crous & Gams 2000); USA (Úrbez-Torres *et al*. 2013)
	Phoenix dactylifera	Iran (Mohammadi 2014)
	Prunus armeniaca	Iran (Arzanlou *et al*. 2014); South Africa (Damm *et al*. 2008)
	Prunus persica	South Africa (Damm *et al*. 2008)
	Prunus salicina	South Africa (Damm *et al*. 2008)
	Prunus pennsylvanica	USA (Hausner *et al*. 1992)
	Pyrus communis	South Africa (Cloete *et al*. 2011)
	Salix sp.	USA (Hausner *et al*. 1992)
	Soil	Spain (Agustí-Brisach *et al*. 2013); USA (Rooney *et al*. 2001)
P. alvesii	*Dodonaea viscosa*	Australia (Mostert *et al*. 2005)
	Olea europea	Italy (Nigro *et al*. 2013)
P. amygdalinum	*Prunus dulcis*	Spain (Gramaje *et al*. 2012)
P. argentinense (*T. argentinensis*)	Soil	Argentina (Crous & Gams, 2000)
P. australiense	*Prunus salicina*	South Africa (Damm *et al*. 2008)
P. fuscum	*Prunus salicina*	South Africa (Damm *et al*. 2008)
P. griseorubrum	*Prunus salicina*	South Africa (Damm *et al*. 2008)
P. inflatipes	*Hypoxylon truncatum*	USA (Mostert *et al*. 2005)
	Nectandra sp	Costa Rica (Groenewald *et al*. 2001)
	Quercus virginiana	USA (Groenewald *et al*. 2001)
	Soil	USA (Rooney *et al*. 2001)
P. iranianum	*Actinidia chinensis*	Italy (Mostert *et al*. 2006)
	Malus domestica	Iran (Arzanlou *et al*. 2013)
	Prunus armeniaca	South Africa (Damm *et al*. 2008)
	Prunus dulcis	Spain (Gramaje *et al*. 2012)
	Pyrus communis	South Africa (Cloete *et al*. 2011)
P. mortoniae (*T. fraxinopennsylvanica*)	*Actinidia chinensis*	Italy (Prodi *et al*. 2008)
	Fraxinus excelsior	Sweden (Groenewald *et al*. 2001)
	Fraxinus latifolia	USA (Eskalen *et al*. 2005)
	Fraxinus pennsylvanica	USA (Hausner *et al*. 1992)
	Prunus salicina	South Africa (Damm *et al*. 2008)
	Pyrus communis	South Africa (Cloete *et al*. 2011)
	Quercus agrifolia	USA (Lynch *et al*. 2013)
P. novae-zealandiae (*T. novae-zealandiae*)	*Cupressus macrocarpa*	New Zealand (Hausner *et al*. 1992)
	Pinus radiata	New Zealand (Hausner *et al*. 1992)
P. parasiticum (*T. parasitica*)	*Actinidia chinensis*	Italy (Di Marco *et al*. 2004)
	Aquilaria agallocha	n.d. (Mostert *et al*. 2006)
	Cupressus sp.	n.d. (Mostert *et al*. 2006)
	Nectandra sp.	Costa Rica (Hawksworth *et al*. 1976)
	Olea europea	Italy (Nigro *et al*. 2013)
	Phoenix dactylifera	Iran (Mohammadi 2014); Iraq (Hawksworth *et al*. 1976)
	Prunus armeniaca	South Africa (Damm *et al*. 2008); Tunisia (Hawksworth *et al*. 1976)
	Prunus avium	Greece (Rumbos 1986)
	Quercus virginiana	USA (Halliwell 1966)
	Soil	Tahiti (Dupont *et al*. 2002); Spain (Agustí-Brisach *et al*. 2013)
P. pallidum	*Prunus armeniaca*	South Africa (Damm *et al*. 2008)
P. pruniculum	*Prunus salicina*	South Africa (Damm *et al*. 2008)
P. rubrigenum (*T. rubrigena*)	*Dactylis glomerata*	Spain (Sánchez-Márquez *et al*. 2007)

Table 1. (Continued).

Phaeoacremonium species	Host/Substrate	Country and Reference
	Olea europea	Italy (Nigro *et al*. 2013)
P. scolyti	*Prunus armeniaca*	South Africa (Damm *et al*. 2008)
	Prunus persica	South Africa (Damm *et al*. 2008)
	Prunus persica var. *nucipersica*	South Africa (Damm *et al*. 2008)
	Prunus salicina	South Africa (Damm *et al*. 2008)
P. subulatum	*Prunus armeniaca*	South Africa (Damm *et al*. 2008)
P. theobromatis	*Theobroma gileri*	Equador (Mostert *et al*. 2006)
P. venezuelense	*Prunus armeniaca*	Spain (Olmo *et al*. 2014)
P. viticola (*T. viticola*)	*Actinidia chinensis*	France (Hennion *et al*. 2001)
	Prunus armeniaca	South Africa (Damm *et al*. 2008)
	Prunus salicina	South Africa (Damm *et al*. 2008)
	Pyrus communis	South Africa (Cloete *et al*. 2011)
	Sorbus intermedia	Germany (Mostert *et al*. 2006)
T. africana *	*Prunus armeniaca*	South Africa (Damm *et al*. 2008)
T. griseo-olivacea *	*Prunus armeniaca*	South Africa (Damm *et al*. 208)
T. vibratilis *	*Fagus sylvatica*	France and Hungary (Réblová & Mostert 2007)
	Prunus padus	Sweden (Réblová & Mostert 2007)
	Sorbus sp.	Italy (Réblová & Mostert 2007)

n.d. = no data.

symptoms of a number of woody hosts other than grapevine worldwide (Table 1).

The aim of the present study was to identify isolates of *Phaeoacremonium* collected from pruning wounds of sandalwood trees and to characterise those that appeared to be morphologically and genetically different from known species of the genus.

MATERIAL AND METHODS

Sampling and fungal isolation

Two sandalwood plantations were selected (1-yr-old and 5-yr-old). In each plantation 40 trees were selected for pruning, with the condition that more than two branches required pruning per tree. Twenty were used for the “post wet-season” pruning treatment completed on the 31 May 2011, and the remaining 20 trees were pruned for the ”pre wet-season” treatment on the 4 November 2011. Branches were pruned using secateurs, and when required pruning saws, with cuts made as close to the stem as possible without causing damage to the stem bark. Trees were pruned to a maximum of one half of tree height.

Each plantation was harvested at two time intervals, representing approximately 12 and 18 mo after pruning. Trees were cut at least 5 cm below and above the last recognizable pruning wounds and then labelled, stored in plastic polyweave bags, and transported to Perth by road freight. A bandsaw was used to cut cross sectional discs around the selected pruning wound, with cuts made approximately 1.5 cm from the edges of the wound. Samples were selected from each tree with preference from the largest to the smallest open wound. Where open wounds were not present, preference was in order of the largest to the smallest scar tissue.

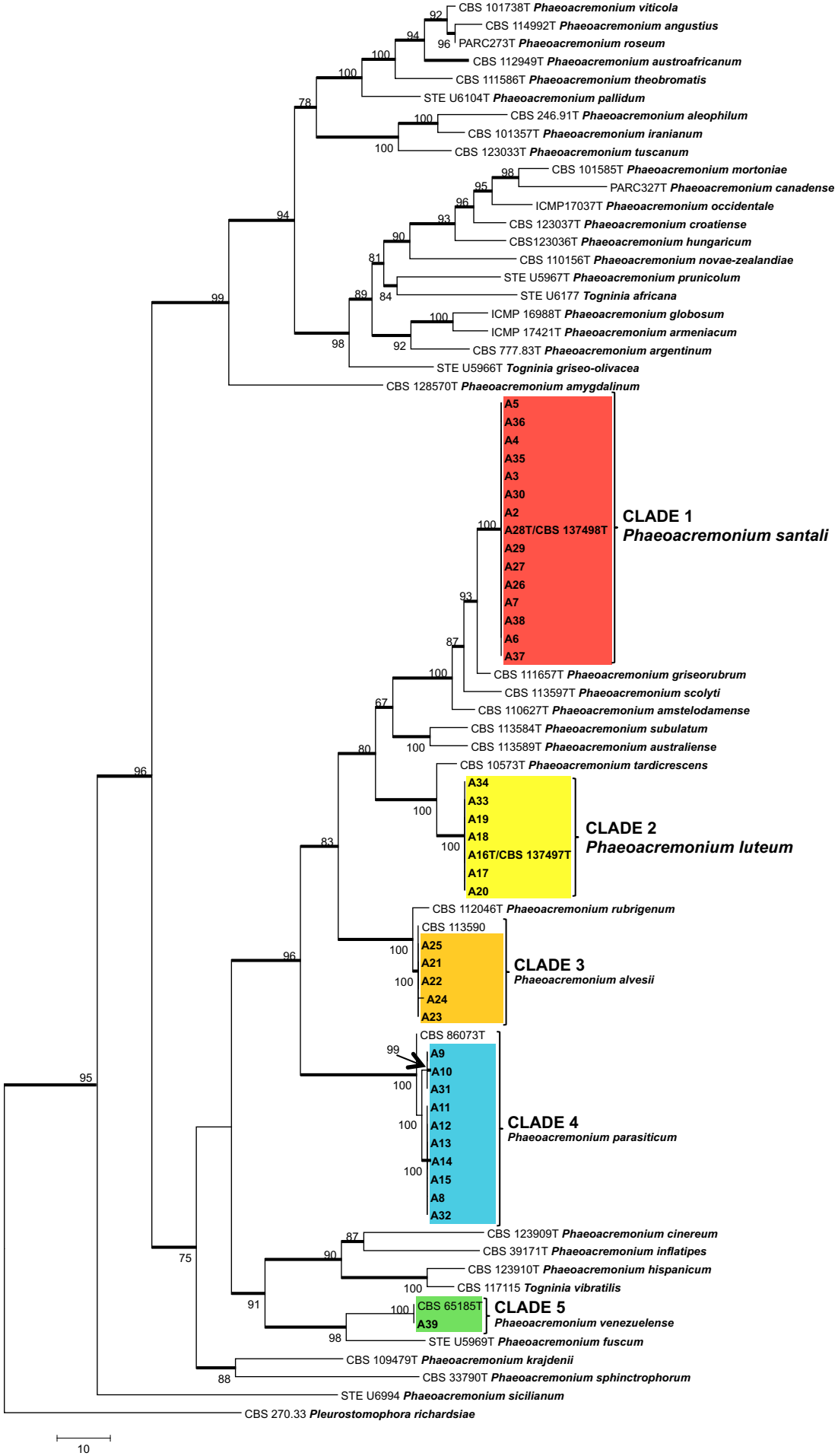
Each wound sample was split longitudinally through the centre of the wound using a chisel cleaned with 70 % ethanol between samples. For each pruning wound, shavings were taken using a sterilised scalpel from the margin between stained and healthy wood. These shavings were then transferred using sterilised forceps onto two media; (1) half strength Potato Dextrose Agar (PDA, Becton Dickinson, Sparks, MD; 19.5 g/L PDA, 7.5 g/L agar) containing 133 µg/mL streptomycin; and (2) Basidiomycete selective medium (5 g/L Bacto peptone (Difco, NSW, Australia), 20 g/L agar, 0.25 g/L $MgSO_4 \cdot 7H_20$, 0.5 g/L K_2HPO_4, 0.016 g/L benomyl, 100 µg/L streptomycin, 2 ml/L 50 % (v/v) lactic acid, and 20 ml/L 95 % ethanol). After 2 wk, representative fungal colonies were transferred onto fresh ½ strength PDA. The cultures were examined regularly and any contaminated cultures were cleaned.

Once clean, all isolates from a single harvest time were subcultured on the same day onto ½ strength PDA. This was to enable comparison of culture morphology. After 2 wk, cultures were grouped based on morphology and representative isolates from each group were selected for molecular study.

Morphological identification and characterisation

Morphological characters used in this study to distinguish *Phaeoacremonium* species include conidiophore morphology, phialide type and shape, size of hyphal warts, and conidial size and shape. Colony characters and pigment production on MEA, PDA and oatmeal agar (OA; 60 g oatmeal; 12.5 g agar; Difco, Madrid, Spain) (Crous *et al*. 2009) incubated at 25 ºC were noted after 8 and 16 d.

Microscopic observations were made from aerial mycelium of colonies cultivated on MEA or by using the slide culture


CBS 101738T Phaeoacremonium viticola
CBS 114992T Phaeoacremonium angustius
PARC273T Phaeoacremonium roseum
CBS 112949T Phaeoacremonium austroafricanum
CBS 111586T Phaeoacremonium theobromatis
STE U6104T Phaeoacremonium pallidum
CBS 246.91T Phaeoacremonium aleophilum
CBS 101357T Phaeoacremonium iranianum
CBS 123033T Phaeoacremonium tuscanum
CBS 101585T Phaeoacremonium mortoniae
PARC327T Phaeoacremonium canadense
ICMP17037T Phaeoacremonium occidentale
CBS 123037T Phaeoacremonium croatiense
CBS123036T Phaeoacremonium hungaricum
CBS 110156T Phaeoacremonium novae-zealandiae
STE U5967T Phaeoacremonium prunicolum
STE U6177 Togninia africana
ICMP 16988T Phaeoacremonium globosum
ICMP 17421T Phaeoacremonium armeniacum
CBS 777.83T Phaeoacremonium argentinum
STE U5966T Togninia griseo-olivacea
CBS 128570T Phaeoacremonium amygdalinum
A5
A36
A4
A35
A3
A30
A2
A28T/CBS 137498T
A29
A27
A26
A7
A38
A6
A37
CLADE 1
Phaeoacremonium santali
CBS 111657T Phaeoacremonium griseorubrum
CBS 113597T Phaeoacremonium scolyti
CBS 110627T Phaeoacremonium amstelodamense
CBS 113584T Phaeoacremonium subulatum
CBS 113589T Phaeoacremonium australiense
CBS 10573T Phaeoacremonium tardicrescens
A34
A33
A19
A18
A16T/CBS 137497T
A17
A20
CLADE 2
Phaeoacremonium luteum
CBS 112046T Phaeoacremonium rubrigenum
CBS 113590
A25
A21
A22
A24
A23
CLADE 3
Phaeoacremonium alvesii
CBS 86073T
A9
A10
A31
A11
A12
A13
A14
A15
A8
A32
CLADE 4
Phaeoacremonium parasiticum
CBS 123909T Phaeoacremonium cinereum
CBS 39171T Phaeoacremonium inflatipes
CBS 123910T Phaeoacremonium hispanicum
CBS 117115 Togninia vibratilis
CBS 65185T
A39
CLADE 5
Phaeoacremonium venezuelense
STE U5969T Phaeoacremonium fuscum
CBS 109479T Phaeoacremonium krajdenii
CBS 33790T Phaeoacremonium sphinctrophorum
STE U6994 Phaeoacremonium sicilianum
CBS 270.33 Pleurostomophora richardsiae
10

technique, as explained by Arzanlou *et al*. (2007) when studying the genus *Mycosphaerella*. Images were captured with a Nikon camera system (Digital Sight DXM 1200, Nikon, Japan). Structures were mounted in lactic acid, and 30 measurements (1000× magnification) were made. The 5th and 95th percentiles were defined for all measurements with the extremes given in parentheses. Colony colours were determined with the colour charts of Rayner (1970). Cardinal temperatures for growth were obtained by incubating MEA plates in the dark at 5–40 ºC in 5 ºC intervals, also including 37 ºC, human body temperature. Radial growth was measured after 8 d at 25 ºC.

Molecular characterization: DNA isolation and amplification

Fungal mycelium and conidia from pure cultures grown on PDA for 2 wk at 25 ºC in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen with a mortar and pestle. Total DNA was extracted with the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Norcross, GA) following the manufacturer's instructions. DNA was viewed on 0.7 % agarose gels stained with ethidium bromide and stored at -20 ºC.

Approximately 600 bp of the 5' end of the β-tubulin (BT) and approximately 300 bp of the 5' end of the actin (ACT) genes were amplified as described by Mostert *et al*. (2006) using primer sets T1 (O'Donnell & Cigelnik 1997) and Bt2b, and ACT-512F and ACT-783R, (Carbone & Kohn 1999), respectively. PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen (Sequencing Center, Seoul). Sequences were edited using Sequencher software v. 4.7. (Gene Codes, Ann Arbor, MI).

Phylogenetic analyses

The new *Phaeoacremonium* sequences (BT and ACT), together with reference sequences (Mostert *et al*. 2006, Damm *et al*. 2008, Essakhi *et al*. 2008, Graham *et al*. 2009, Gramaje *et al*. 2009, 2012, Úrbez-Torres *et al*. 2014) and the outgroup, *Pleurostomophora richardsiae* (ACT = AY579271, BT = AY579334) obtained from GenBank, were aligned using MAFFT sequence alignment program v. 7 (Katoh & Standley 2013) followed by manual adjustments of the alignments in BioEdit Sequence Alignment Editor v. 7.2.3. A partition homogeneity test of the BT and ACT alignments was conducted with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b (Swofford 2000) to test pairwise congruence between sequence data sets. Phylogenetic analyses of all aligned sequence data were performed with MEGA v. 5.05 software (Tamura *et al*. 2011). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. All characters were unordered and of equal weight.

Maximum parsimony analysis was performed for the combined *Phaeoacremonium* dataset using the heuristic search option with 10 random simple taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5 (Harrison & Langdale 2006). The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC), were calculated.

Sequences derived in this study were lodged at GenBank, the alignments in TreeBASE (www.treebase.org/), and taxonomic novelties in MycoBank (www.MycoBank.org; Crous *et al*. 2004). GenBank accession numbers of the strains collected during this study are listed in Table 2.

RESULTS

Fungal identification

The fungal isolates obtained in this study were characterised by having flat slow-growing cultures on MEA. Different types of phialides that were variable in size and shape were observed in the aerial mycelium, and either discrete or integrated in conidiophores. Sporulation was abundant and conidia hyaline and aseptate. All morphological characters corresponded to the genus *Phaeoacremonium* (Mostert *et al*. 2006). Based on their appearance in culture, the isolates could be assigned to five different clades (Table 2).

Molecular identification and phylogenetic analyses

The partition homogeneity test of the BT and ACT alignments of *Phaeoacremonium* gave a *P*-value of 0.263 indicating that the datasets were congruent and could be combined. The combined sequence dataset consisted of 78 isolates including the outgroup and had 977 characters, of which 535 characters were parsimony-informative, 180 parsimony-uninformative and 262 constant. Sixty equally most parsimonious trees were retained (length = 2539 steps, CI = 0.471, RI = 0.837, RC = 0.399). A tree that closely resembled the strict consensus tree was chosen and is presented in Fig. 1. The isolates of the clade 1 grouped together in a polyphyletic clade with 100 % bootstrap support, with *P. griseorubrum* as closely related species. The isolates of the clade 2 grouped together in a polyphyletic clade with 100 % bootstrap support, with *P. tardicrescens* as closely related species. The isolates of clades 3 and 4, grouped inside the *P. alvesii* and *P. parasiticum* clades respectively, with 99 % bootstrap support.

The BT and ACT sequences of the first clade of *Phaeoacremonium* isolates were 98 % identical to those of *P. griseorubrum* CBS 111657 (GenBank AY579294, AY579227). Differences were found between the first clade of *Phaeoacremonium* isolates and *P. griseorubrum* CBS 111657 sequences with five nucleotides varying in the ACT region and nine nucleotides in the BT region. The BT and ACT sequences of the second clade of *Phaeoacremonium*

Fig. 1. (P. 70). One of 60 most parsimonious trees obtained from heuristic searches of a combined alignment of the TB and ACT gene sequences. Bootstrap support (1 000 replicates) above 60 % are shown at the nodes. *Pleurostomophora richardsiae* was used as outgroup. Ex-type strains for each species are indicated with a 'T' after the strain number. Thickened lines indicate branches present on strict consensus tree.

Table 2. *Phaeoacremonium* species, accession numbers, and collection details of isolates studied.

Species	Isolate[1]	Accesion No.[2]	GenBank Accesions	
			ACT	BT
Phaeoacremonium santali (clade 1)	E2.1B	A2	KF835395	KF823789
	E9.3A	A3	KF835396	KF823790
	E19.2C	A4	KF835397	KF823791
	-	A5	KF835398	KF823792
	E11.2A	A6	KF835399	KF823793
	E15.1A	A7	KF835400	KF823794
	F3.4	A26	KF835401	KF823795
	F6.7	A27	KF835402	KF823796
	F2.3	A28, CBS 137498	KF835403	KF823797
	E7.7	A29	KF835304	KF823798
	F2.4	A30	KF835405	KF823799
	E9.3	A35	KJ533536	KJ533532
	E9.2	A36	KJ533537	KJ533533
	F32.2C	A37	KJ533538	KJ533534
	E38.3B	A38	KJ533539	KJ533535
Phaeoacremonium luteum (clade 2)	F3.7	A16, CBS 137497	KF835406	KF823800
	F2.4	A17	KF835407	KF823801
	F3.3	A18	KF835408	KF823802
	F2.1	A19	KF835409	KF823803
	F2.5	A20	KF835410	KF823804
	E30.SB	A33	KJ533542	KJ533540
	F37.3B	A34	KJ533543	KJ533541
Phaeoacremonium alvesii (clade 3)	F1.2A	A21	KF790540	KF790535
	F11.5B	A22	KF790541	KF790536
	F15.3A	A23	KF790542	KF790537
	F15.5A	A24	KF790543	KF790538
	F5.4A	A25	KF790544	KF790539
Phaeoacremonium parasiticum (clade 4)	E15.3A	A8	KF790555	KF790545
	E8.3B	A9	KF790556	KF790546
	E8.2	A10	KF790557	KF790547
	F11.4E	A11	KF790558	KF790548
	E15.1B	A12	KF790559	KF790549
	E8.3	A13	KF790560	KF790550
	F15.5B	A14	KF790561	KF790551
	F11.2C	A15	KF790562	KF790552
	F11.5C	A31	KF790563	KF790553
	F5.5	A32	KF790564	KF790554
Phaeoacremonium venezuelense (clade 5)	F32.2A	A39	KJ496346	KJ496345

[1] All the isolates were collected in Kununurra (Australia) by TI Burgess.

[2] CBS: Culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

isolates were 97 % identical to those of *P. tardicrescens* CBS 110573 (GenBank AY579300, AY579233). Differences were found between the second clade of *Phaeoacremonium* isolates and *P. tardicrescens* CBS 110573 sequences with six nucleotides varying in the ACT region and 20 nucleotides in the BT region. The BT and ACT sequences of the third clade of isolates had 100 % identity with *P. alvesii* isolates CBS 113590 (GenBank AY579304) and STE-U 6988 (GenBank JQ038925), respectively. A BLASTn search showed that the BT and ACT sequences of the fourth clade of isolates had 100 % identity with isolates previously identified as *P. parasiticum* CBS 860.73 (GenBank AY579253). The BT and ACT sequences of the isolate corresponding to the fifth clade had 100 % identity with *P. venezuelense* isolate CBS 651.85 (GenBank AY579320).

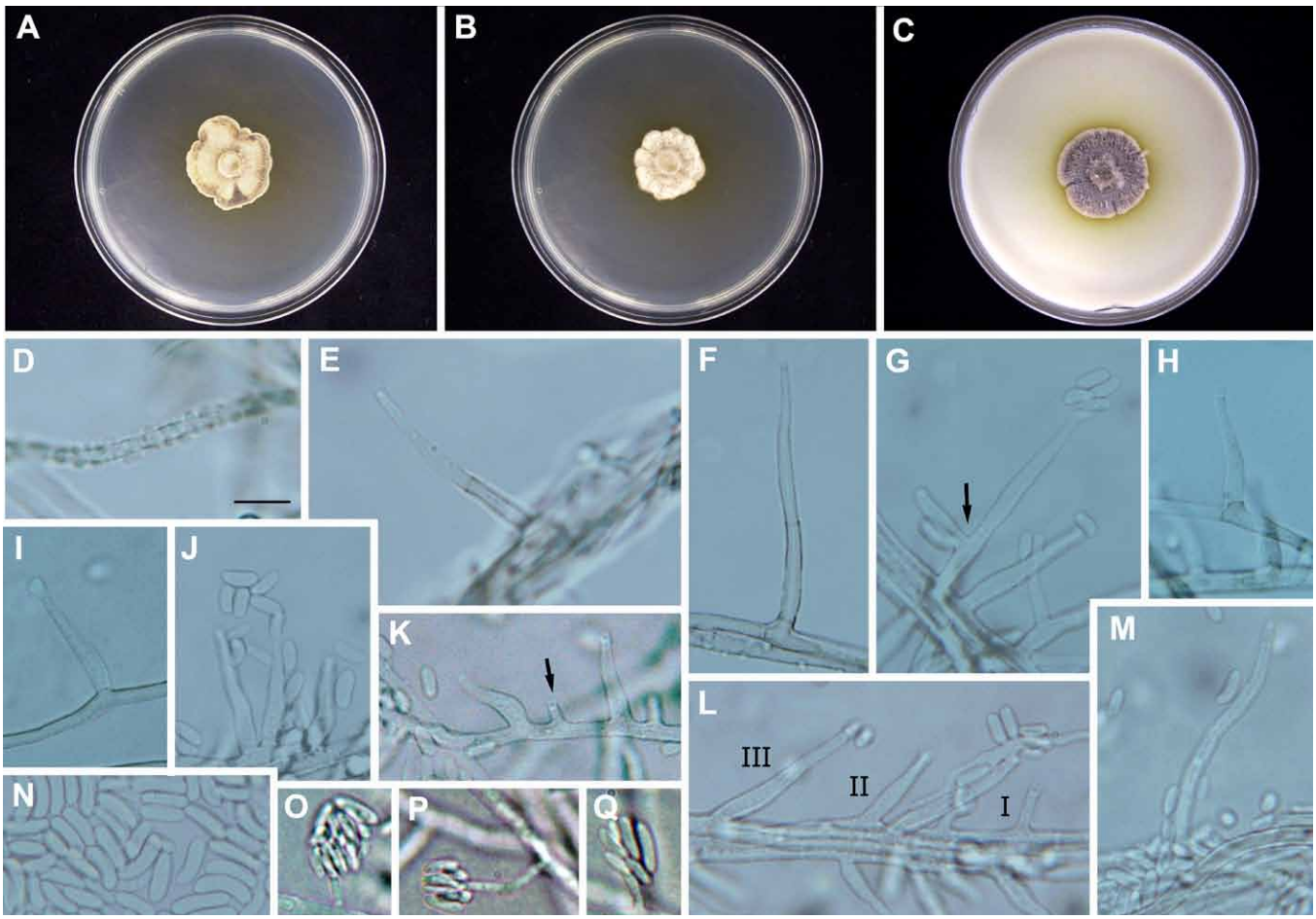


Fig. 2. *Phaeoacremonium luteum* (CBS 137497 – ex-type culture A 16). **A–C.** Sixteen-day-old colonies incubated at 25 ºC on MEA (A), PDA (B) and OA (C). **D–N.** Aerial structures on MEA; **D.** Mycelium showing prominent exudate droplets observed as warts; E–F. Conidiophores; **G.** Conidiophore (indicated by arrow) with terminal and 1 lateral phialide; **H–J.** Type II phialides; **K.** Type I phialide (indicated by arrow); **L.** Type III, type II and type I phialides. **M.** Type III phialide; **N.** Conidia. **O–Q.** Structures on the surface of and in MEA: Phialides with conidia. Bar: D = 10 µm (applies also to E–Q).

TAXONOMY

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

Phaeoacremonium luteum D. Gramaje, T.I. Burgess & J. Armengol, **sp. nov**.
MycoBank MB808419
(Fig. 2)

Etymology: Named after the yellow pigment produced that diffused into the agar ahead of the leading edge of the colony in all culture media.

Diagnosis: *Phaeoacremonium luteum* can be distinguished from the other species producing yellow pigment on MEA, PDA and OA, namely *P. alvesii*, *P. subulatum*, *P. globosum,* and the asexual morph of *Toginia africana*, by the predominance of type II phialides, the mycelium having prominent exudate droplets evident as warts, and slow growth.

Type: **Australia**: *Western Australia*: Kununurra, isolated from *Santalum album* trees, Dec. 2012, *T. I. Burgess* (CBS H-21622 – holotype; CBS 137497 – ex-type culture A16).

Description: Aerial structures *in vitro* on MEA: *Mycelium* consisting of branched, septate hyphae that occurs singly or in bundles of up to 8; hyphae tuberculate with warts to 2 µm diam, verruculose, medium to pale brown and 1.5–2.5 µm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, to 5-septate, sometimes bearing next to the terminal phialide 1–2 lateral ones, medium brown to pale brown, verrucose on the lower part, (15–)15.5–*30*–40(–59) µm long and 1.5–*2.2*–3 µm wide. *Conidiogenous cells* phialides, terminal or lateral, mostly monophialidic, smooth to verruculose, hyaline, collarettes 1.5–2.5 µm long, 1–1.5 µm wide; type I phialides mostly cylindrical, (3–)4–*5*–6(–6.5) × 1.5–2(–2.5) µm; type II phialides predominant, subcylindrical to subulate, (10–)10.5–*13.5*–17 × 2–3.5 µm; type III phialides cylindrical to subcylindrical, 20–*24.5*–30(–32) × 2–*2.5*–3 µm. *Conidia* hyaline, oblong or obovate, some reniform, 4–*5*–6(–7.5) × (1.5–)2–*2.5*–3 (av. = 5 × 2.5) µm, L/W ratio = 2.2. On surface or submerged in the agar: *Phialides* hyaline, mostly

cylindrical, 3–*6*–9(–10) × 1–*1.5*–2 µm. *Conidia* hyaline, mostly allantoid, few reniform, 4.5–*6*(–7) × 1–*1.5*–2, L/W ratio = 4.3.

Culture characteristics: Colonies reaching a radius of 4.5–5 mm after 8 d at 25 ºC. Minimum temperature for growth 15 ºC, optimum 30–35 ºC, maximum 37 ºC. Colonies on *MEA* flat, felty to powdery, with crenate margin; after 8 d and 16 d, brownish olive towards the edge above, buffy brown to buff-yellow in reverse. Colonies on *PDA* flat, felty or woolly textured, with crenate margins; after 8 d and 16 d, buff-yellow and dark greyish brown above, buff-yellow to buffy brown in reverse. Colonies on *OA* flat, with woolly tufts, with entire margin; after 8 d and 16 d olive-grey and brownish vinaceous above, buffy brown to greyish brown in reverse. Yellow pigment produced on MEA, PDA and OA.

Additional cultures examined: **Australia**: *Western Australia*: Kununurra, isolated from *Santalum album* trees, Dec. 2012, *T.I. Burgess A17*, *A18*, *A19*, and *A20*.

Phaeoacremonium santali D. Gramaje, T.I. Burgess, J. Armengol, **sp. nov**.
MycoBank MB808420
(Fig. 3)

Etymology: Named after the host it was isolated from, Tropical sandalwood (*Santalum album*).

Diagnosis: *Phaeoacremonium santali* can be distinguished from the other species producing pink colonies on MEA, namely *P. alvesii*, *P. armeniacum*, *P. griseorubrum*, *P. rubrigenum*, *P. scolyti,* and *P. viticola*, by the predominance of type I and II phialides, the brownish olive colour in OA, and slow growth. Colonies reached a radius of only 6.6–7.5 mm in 8 d at 25 °C on MEA. *Phaeoacremonium griseorubrum* overlaps with *P. santali* in growth rate, but has a temperature maximum for growth of 40 °C, compared with 37 °C in the latter species.

Type: **Australia**: *Western Australia*: Kununurra, isolated from *Santalum album* trees, Dec. 2012, *T.I. Burgess* (CBS H-21621 – holotype; CBS 137498 – ex-type culture A 28).

Description: Aerial structures *in vitro* on MEA: *Mycelium* consisting of branched, septate hyphae that occurs singly or in bundles of up to six; smooth or rarely with warts, verruculose, yellow-brown to hyaline, 2–3.5 µm wide. *Conidiophores* mostly short, usually unbranched, arising from aerial or submerged hyphae, erect to flexuous, to 3-septate, often bearing besides the terminal phialide 1–2 lateral ones, pale brown, smooth to verruculose, (10–)10.5–*15*–27(–31) µm long and 1.5–*2*(–2.5) µm wide. *Conidigoenous cells* phialides, terminal or lateral, mostly polyphialidic, smooth to verruculose, hyaline, collarettes 1.5–2.5 µm long, 1–1.5 µm wide; type I and II phialides predominant, type I phialides cylindrical, occasionally widened at the base, tapering towards the apex, (2–)2.5–*6*–7.5(–8) × 1–*1.5*–2 µm; type II phialides elongate-ampulliform and attenuated at the base, or navicular, tapering towards the apex, (5.5–)6–*7*–9 × 2–*3*–3 µm; type III phialides subcylindrical to navicular, 12–*14*–19(–20) × 1.5–*2*–2.5 µm. *Conidia* hyaline, oblong ellipsoidal, some obovoid or reniform, (3–)4–*4.5*–5(–6) × 1.5–*2*–3 µm, L/W ratio = 2.1. On surface or submerged in the agar: *Phialides* hyaline, mostly cylindrical, 5–*7.5*–10(–12) × 1–*1.5*–2 µm. *Conidia* hyaline, mostly allantoid, 5–*6*–7(–11) × 1–*1.5*–2, L/W ratio = 4.1.

Culture characteristics: Colonies reaching a radius of 6.6–7.5 mm after 8 d at 25 ºC. Minimum temperature for growth 15 ºC, optimum 30 ºC, maximum 37 ºC. *Colonies on MEA* flat, erose or dentate; after 8 d pale rose to pinkish vinaceous towards the edge above, pale rose towards the edge in reverse, after 16 d rosy vinaceous to pinkish buff towards the edge above, vinaceous pink near the centre and pinkish buff towards the edge in reverse. Colonies on *PDA* flat, felt-like with few woolly tufts near the centre, with entire margin; after 8 d pale pinkish buff towards the edge above and in reverse, after 16 d isabelline to oliveacous towards the edge above, violet-brown towards the centre and pale brown to orange-grey towards the edge in reverse. Colonies on *OA* flat, felty to powdery, with entire margin; after 8 d and 16 d dull green to olive green above, brownish olive to dark vinaceous-brown towards the edge in reverse.

Additional cultures examined: **Australia**: *Western Australia*: Kununurra, isolated from *Santalum album* trees, Dec. 2012, *T.I. Burgess A2, A3, A4, A5, A6, A7, A26, A27, A29,* and *A30*.

DISCUSSION

In this study, the integration of morphology, cultural characters, and DNA sequence data revealed the presence of five *Phaeoacremonium* species within pruning wounds of *Santalum album* in tropical Western Australia. *Phaeoacremonium* species were commonly isolated from both 1- and 5-year-old trees and in both harvests, approximately 12 and 18 mo after pruning. Two novel species of *Phaeoacremonium, P. luteum* and *P. santali,* were obtained from sandalwood, bringing the total number of known species of *the genus* to 42.

Micromorphological traits, such as conidiophore morphology, phialide type and shape, size of hyphal warts, and cultural characters are useful in distinguishing *Phaeoacremonium* species (Mostert *et al*. 2005). In addition, molecular analyses of part of the β-tubulin and actin gene regions have been shown to give high phylogenetic resolution within *Phaeoacremonium* in previous studies (Mostert *et al*. 2005, 2006, Réblová & Mostert 2007, Essakhi *et al*. 2008, Graham *et al*. 2009, Gramaje *et al*. 2012). Distinct features of *P. luteum* include its ability to produce yellow pigment on MEA, PDA and OA, the predominance of subcylindrical to subulate type II phialides and the mycelium showing prominent exudate droplets observed as warts. Yellow pigment production on PDA and OA is a common culture characteristic of some *Phaeoacremonium* species, and is considered useful in distinguishing species in the genus, especially on OA, which is an excellent medium to test pigment production (Mostert *et al*. 2006). It is also important to note the slow growth of this species on malt extract agar, with colonies reaching a radius of only 4.5–5 mm after 8 d. *Phaeoacremonium santali*

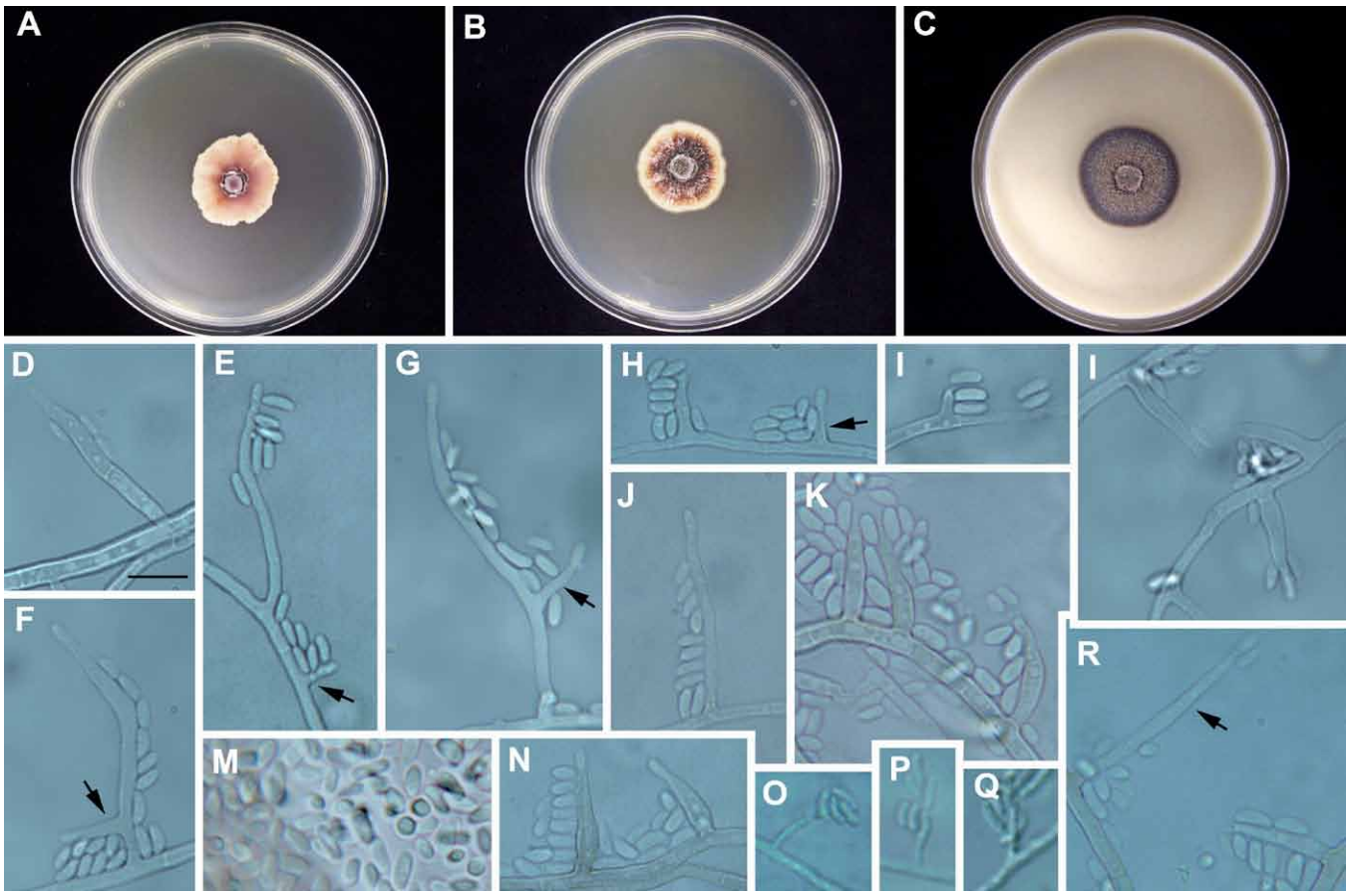


Fig. 3. *Phaeoacremonium santali* (CBS 137498 – ex-type culture A 28). **A–C.** Sixteen-day-old colonies incubated at 25 ºC on MEA (A), PDA (B) and OA (C). **D–N.** Aerial structures on MEA; **D–E.** Conidiophores and type I phialide (indicated by arrow); **F–G.** Conidiophores (indicated by arrows) with terminal and 1 lateral phialide; **H.** Type II and Type I phialides (indicated by arrow); **I.** Type I phialide; **J.** Type III phialide. **K–L.** Type II phialides; **M.** Conidia; **N.** Type II phialides; **O–Q.** Structures on the surface of and in MEA: phialides with conidia; **R.** Type III phialide (indicated by arrow). Bar: D = 10 µm (applies also to E–R).

could be distinguished from the other species producing pink colonies on MEA by the predominance of type I and II phialides, its distinct brownish olive colonies in oatmeal agar, and slow growth.

Growth temperature studies showed that all the isolates of *P. luteum* and *P. santali* had a maximum growth temperature of 37 ºC, suggesting that they have the potential to survive at human body temperature. Several thermotolerant *Phaeoacremonium* species, such as *P. alvesii*, *P. griseorubrum*, *P. krajdenii*, *P. parasiticum,* and *P. venezuelense*, are associated with phaeohyphomycosis in humans and also have been isolated from woody hosts (Mostert *et al*. 2005, Essakhi *et al*. 2008).

In addition to the two new taxa, three previously known species were also found on sandalwood. *Phaeoacremonium alvesii* and *P. parasiticum* had been previously reported from *Dodonaea viscosa* (Mostert *et al*. 2005) and *Vitis vinifera* (Pascoe & Cottral 2000) in Australia, respectively. *Phaeoacremonium venezuelense* represents a new record for Australia and has previously been reported from humans in Brazil (Guarro *et al*. 2006), Canada (Mostert *et al*. 2005), France (Mostofi *et al*. 2012), and Venezuela (de Albornoz 1974), from grapevine in Algeria (Berraf-Tebbal *et al*. 2011) and South Africa (Mostert *et al*. 2005), and more recently from wood decay of apricot trees in Spain (Olmo *et al*. 2014).

Species of *Phaeoacreomonium* obtained in this study were all collected from pruning wounds. Some *Phaeoacremonium* spp., such as *P. aleophilum* and *P. mortoniae*, produce perithecia (i.e. a *Togninia* sexual morph) in old, rotted, vascular tissue of pruning wounds and in deep cracks in cordons, trunks, and spurs of grapevine (Eskalen *et al*. 2005, Rooney-Latham *et al*. 2005, Baloyi *et al*. 2013). Ascospores are released from these overwintering structures by rain and can infect the grapevine through fresh pruning wounds, which are recognized as the main point of entry for *Phaeoacremonium* species into grapevines (Eskalen *et al*. 2005). Of the species found in this study, only *P. parasiticum* has a known sexual morph and could possibly be present as perithecia on sandalwood trees. Aerial inoculum could be released by these ascomata on infected tress, thus becoming a major source of fungal infection. Insect transmission of sexual spores or conidia may also occur (Moyo *et al*. 2014).

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