FUSE

Fungal Systematics and Evolution

VOLUME 6
DECEMBER 2020
PAGES 305–314

doi.org/10.3114/fuse.2020.06.16

A new species of *Raffaelea* from beetle-infested *Leucaena leucocephala*

M. Procter[1], W.J. Nel[1], S. Marincowitz[1*], P.W. Crous[1,2], M.J. Wingfield[1]

[1]Department of Biochemistry, Genetics & Microbiology; Forestry and Agricultural Research Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
[2]Westerdijk Fungal Biodiversity Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

*Corresponding author: seonju.marincowitz@fabi.up.ac.za

Key words:
Ambrosia symbiont
new species
Ophiostomatales
taxonomy

Abstract: Species of *Raffaelea* (*Ophiostomatales*: *Ascomycota*) are obligate symbionts of ambrosia beetles, some of which pose a substantial threat to forest trees. *Leucaena leucocephala* is a small mimosoid tree species that is considered as an invasive weed in most of its introduced range globally. During a field expedition on the French island of Réunion, dying *L. leucocephala* trees were observed. Samples were taken from these trees and isolations made from symptomatic wood tissues that included beetle tunnels, but in the absence of the beetles themselves. Multiple isolates of a fungus resembling a *Raffaelea* species were obtained from the discoloured wood associated with the beetle tunnels. To determine their identity, microscopic examination was performed and DNA sequences for three gene regions (ITS, LSU, *TUB*) were obtained. Phylogenetic analyses based on these gene regions revealed that the isolates represent a new species of *Raffaelea*, described here as *R. borbonica sp. nov.* A pathogenicity test was conducted with the fungus, which was shown to cause lesions on the inoculated seedlings, but with a low level of aggressiveness.

Citation: Procter M, Nel WJ, Marincowitz S, Crous PW, Wingfield MJ (2020). A new species of *Raffaelea* from beetle-infested *Leucaena leucocephala*. *Fungal Systematics and Evolution* **6**: 305–314. doi: 10.3114/fuse.2020.06.16
Effectively published online: 4 August 2020
Corresponding editor: A.J.L. Phillips

INTRODUCTION

Leucaena leucocephala is a mimosoid tree species native to southern Mexico and central America (CABI 2019). In various parts of the world including Réunion island where it has been introduced, the tree is considered an invasive weed. This is largely due to its ability to easily colonize disturbed sites; outcompeting indigenous vegetation (MacDonald *et al*. 1991, CABI 2019). The tree also has positive attributes being used for land restoration, natural wind barriers, fodder for animals and as a source of pulp for paper production (Olckers 2011, Anupam *et al*. 2016, Rasat *et al*. 2016, Hakimi *et al*. 2017, CABI 2019). Despite these beneficial properties under some conditions, countries such as South Africa utilise biological control to prevent *L. leucocephala* from spreading as an invasive (Olckers 2011).

Raffaelea, a genus in the family *Ophiostomataceae* (*Ophiostomatales*, *Ascomycota*), accommodates obligate ambrosia beetle symbionts (Harrington *et al*. 2010, Dreaden *et al*. 2014). Most species in the genus are saprotrophic. But some, such as the causal agents of oak wilt in Japan (*R. quercivora*) (Kubono & Ito 2002) and Korea (*R. quercus-mogolicae*) (Kim *et al.* 2009), and laurel wilt in the USA (*R. lauricola*) (Harrington *et al*. 2008) are important tree-killing pathogens. The genus is characterised as having reduced conidiogenous structures and budding secondary conidia; possibly facilitating their dispersal by ambrosia beetles (Gebhardt & Oberwinkler 2005, Harrington *et al*. 2010, De Beer & Wingfield 2013). Until recently *Raffaelea* was known as an asexual genus, but the recent discovery of a sexual morph in one species, and the inclusion of two sexual morphs previously described in *Ophiostoma* have altered this view (Dreaden *et al*. 2014, Musvuugwa *et al*. 2015).

Prior to the wide applications of DNA sequencing technology, species of *Raffaelea* were often confused with those in *Ambrosiella*. This is because species of both genera share convergent morphological traits adapted to their ambrosia beetle-associated lifestyle. Consequently, many *Raffaelea* spp. were initially treated in *Ambrosiella* and *vice versa*. This trend continued until DNA sequence data revealed that these genera reside in two separate orders of the *Sordariomycetes*; the *Ophiostomatales* (*Raffaelea*) and the *Microascales* (*Ambrosiella*) (Harrington *et al*. 2010, De Beer & Wingfield 2013, De Beer *et al*. 2013).

During a survey of fungi on the French island of Réunion in 2015, dying *L. leucocephala* trees were observed (Fig. 1A). Upon closer inspection, the trees were found to have insect tunnels resembling those produced by ambrosia beetles, but insects were no longer present. The wood tissues associated with these tunnels were clearly stained (Fig. 1B, C). The aim of this study was to isolate and identify the fungus responsible for the wood staining and to consider its pathogenicity.

Fig. 1. A. A dead *Leucaena leucocephala* tree observed on Réunion. **B.** Staining of wood associated with insect tunnelling. **C.** Sap stain in the outer growth rings.

MATERIALS AND METHODS

Fungal Isolation

Pieces of wood including insect tunnels and associated stain were cut from dying *L. leucocephala* trees. Small pieces of stained wood or scrapings from the insect tunnels were transferred to 2 % malt extract agar (MEA: 20 g malt extract, 20 g Difco agar in 1 L dH_2O) amended with the antibiotics streptomycin and cycloheximide (0.4 g streptomycin disulphate and 0.5 g cycloheximide per 1 L, Sigma-Aldrich). The plates were incubated at ambient temperature (approx. 20 °C) until visible fungal growth was observed. Colonies with growth patterns resembling those of *Raffaelea* spp. were sub-cultured on MEA plates, containing the same antibiotics as before, until pure cultures were obtained. The purified cultures were then transferred onto 2 % MEA plates and maintained for further study.

DNA extraction, amplification & sequencing

DNA extraction, amplification, purification and sequencing were performed following the protocols described by Duong *et al*. (2012). These included DNA amplification in 25 µL reaction volumes using FastStart Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany) for the internal transcribed spacer region (ITS), the 28S ribosomal large subunit (LSU), and beta-tubulin (*TUB*) using the primer pairs ITS1F and ITS4 (White *et al*. 1990), LR5 and LROR (Vilgalys & Hester 1990), and T10 and Bt2b (O'Donnel & Cigelnik 1997, Glass & Donaldson 1995). Agarose gel electrophoresis (1 % agarose) was performed on all PCR products to confirm successful amplification. PCR products were cleaned using ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific) following the manufacturer's protocols.

DNA sequencing was performed in both the forward and reverse direction using the same primers as for PCR. Sequencing PCR products were precipitated using the NaOAc/Ethanol precipitation method and analysed on an Applied Biosystems® 3130 (4-capillary) Genetic Analyzer at the sequencing facility of the University of Pretoria (Pretoria, South Africa). Contig sequences were assembled and those produced for the ITS region were used for preliminary identification of isolates by performing BLAST searches against the non-redundant sequence database NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequence data generated in this study were submitted to NCBI GenBank, and those for ex-type isolates will be included in the RefSeq Targeted Loci (RTL) database (Schoch *et al*. 2014) (Table 1). For some species, ITS and LSU sequence data were extracted from whole genomes and these sequences have been deposited in NCBI GenBank.

Phylogenetic analyses

Based on the putative identification of the isolates, datasets were constructed using reference sequences available in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Datasets were aligned using an online version of MAFFT v. 7 (Katoh & Stanley 2013) and the default parameters. Maximum Likelihood (ML) analyses were performed for each individual gene region using raxmlGUI v. 1.3 (Silvestro & Michalak 2012, Stamatakis 2014) with 1 000 bootstrap replicates and the substitution model GTR+I+G. Bayesian Inference analyses were conducted using MrBayes v. 3.2 (Rondquist *et al*. 2012) and run until the standard deviation of frequencies reached a value < 0.01, sampling every 10 000 generations. Run times differed for each gene region: *TUB* (795 000 generations), ITS (6.64 million generations), LSU (6.02 M generations).

Table 1. Isolates used in this study, including multiple isolates of *Raffaelea borbonica sp. nov*.

			GenBank Accession Numbers[2]		
Species	**CMW[1]**	**CBS or others**	**ITS**	**LSU**	***TUB***
R. aguacate		Raff. sp. 272 [G]	**MT633065**	**MT629748**	–
	38067[T]	141672	–	–	KJ909297
R. albimanens	25532[T]	271.70 [G]	**MT633066**	**MT629749**	**MT644111**
R. amasae	25542[T]	116694	–	**MT629750**	EU977470
R. ambrosiae	25533[T]	185.64 [G]	**MT633067**	**MT629751**	**MT644094**
	25533[T]	185.64 [G]	**MT633068**	**MT629752**	**MT644095**
R. arxii	25534	273.70	–	**MT629754**	–
	25534	273.70 [G]	MH859604	**MT629753**	–
R. borbonica sp. nov.	51548	IV4a M2	**MT633054**	**MT629736**	**MT644100**
	51549	IV4a M3	**MT633055**	**MT629737**	**MT644109**
	51719	IV4a M4	**MT633056**	**MT629738**	**MT644108**
	51550	IV4a M5	**MT633057**	**MT629739**	**MT644107**
	51720	V1b M7	**MT633080**	**MT629770**	**MT644098**
	51551	V1b M8	**MT633081**	**MT629771**	**MT644097**
	51722	V1b M9	**MT633082**	**MT629772**	**MT644103**
	51552	V1b M10	**MT633079**	–	–
		IV4b M11	–	**MT629740**	**MT644110**
		IV4b M12	**MT633058**	**MT629741**	**MT644104**
	51553[P]	IV4b M13	**MT633059**	**MT629742**	**MT644106**
	51723	IV4b M14	**MT633060**	**MT629743**	**MT644105**
	51724[T]	IV4b M15	**MT633061**	**MT629744**	**MT644099**
	51554	IV2c M22	**MT633052**	**MT629734**	**MT644102**
	51555	IV2c M23	**MT633053**	**MT629735**	**MT644101**
	51556	V3a M28	**MT633083**	**MT629773**	**MT644096**
R. brunnea		378.68	–	EU177457	–
R. campbellii	44800[T]	139943	–	KR018414	KR018444
R. cf. campbellii		Hulcr7355	–	KX267101	KX267112
R. canadensis	25536[T]	168.66	GQ225699	**MT629755**	EU977473
R. crossotarsa	44793[T]	141675	KX267138	**MT629756**	KX267135
R. cyclorhipidia	44790[T]	141676	**MT633069**	**MT629757**	–
R. ellipticospora	38056[T]	121569	**MT633070**	**MT629758**	–
		C2345	–	–	KJ909298
R. fusca	38798[T]	121570	–	EU177449	KJ909301
R. lauricola	36261	PL159	–	**MT629760**	–
		Raff. sp. 570 [G]	**MT633071**	**MT629759**	**MT644093**
R. montetyi	25537[T]	463.94	–	**MT629761**	–
		451.94	–	–	EU977475
R. quercivora	36263	122982 [G]	**MT633072**	**MT629762**	**MT644090**
		122982	–	–	GQ225691
		Hulcr7167	–	–	KX267119
		Hulcr7176	–	–	KX267120
R. quercus-mongolicae	37749[P]	KACC44403	**MT633073**	**MT629764**	–
	37751	KACC44405 [G]	**MT633074**	**MT629763**	**MT644091**

Table 1. (Continued).

Species	CMW[1]	CBS or others	GenBank Accession Numbers[2]		
			ITS	LSU	*TUB*
R. rapaneae	40357[T]	140084	KT192596	KT182930	–
R. santoroi	25539[T]	399.67	**MT633075**	**MT629765**	EU977476
R. seticollis	1031[T]	634.66	**MT633076**	**MT629766**	–
R. subalba	38797[T]	121568	–	**MT629767**	–
		C2401	–	–	KJ909305
R. cf. subalba		Hulcr7375	–	KX267102	KX267113
R. subfusca		Hulcr4717	KX267138	–	KX267122
R. sulcati	25540[T]	806.70	MH859951	EU177462	EU977477
R. sulphurea[1]	25529	380.68[G]	**MT633077**	**MT629768**	**MT644092**
R. xyleborina	45859[T]	Hulcr6099	**MT633078**	**MT629769**	KX267124
Raffaelea sp. A (PL1001)	38062		–	KJ909293	KJ909295
Raffaelea sp.		Hulcr7507	KX267141	–	KX267128
Ophiostoma piliferum[3]	JGI		**MT633063**	**MT629746**	**MT644089**
O. quercus	2465	117912	**MT633064**	**MT629747**	**MT644088**
O. piceae		UAMH11346	**MT633062**	**MT629745**	**MT644087**

[1] T = ex-holotype, P = ex-paratype, G = DNA data extracted from whole genomes.

[2] Sequences generated in this study are in bold.

[3] The genome sequence for *Ophiostoma piliferum* is not yet published, but has been made available on https://mycocosm.jgi.doe.gov/Ophpi1/Ophpi1.home.html.

Morphological characterisation

Growth in culture

A growth study was conducted to determine the optimal growth temperature for the isolated fungus. Three isolates, representing three cultural variants (CMW 51553, CMW 51724, CMW 51555) were used. Isolates were grown on 2 % MEA and the study was performed following the method described by Musvuugwa *et al*. (2015).

Microscopy

Fungal structures were initially mounted in water on microscope slides. The water was later replaced with 85 % clear lactic acid in which visualization and measurements were made. The structures were studied and photographed using differential interference contrast (DIC) microscopy. Where possible, fifty measurements were made for each morphologically characteristic structure. The dimensions were described as minimum-maximum (mean ± standard deviation).

Pathogenicity

A pathogenicity trial was conducted using two isolates of the fungus isolated from infected *L. leucocephala* trees. Seeds were collected from pods on a tree in nature, and after germination these were planted in bags (25 L) containing a soil mixture composed of 1:2:4 mixture of river sand, red top soil and pine bark potting medium. Seedlings were maintained at 25 °C in a phytotron with natural day/night light cycle for approximately 2 yr. Inoculations were made on 2-yr-old saplings.

Eleven saplings were each inoculated with isolates CMW 51553 (M13) and CMW 51724 (M15), and 10 were inoculated with sterile agar plugs to serve as controls. A 3 mm diam. drill bit was used to make a wound of approximately 2 mm deep into the stem of each sapling with the drill bit being sterilized in 80 % ethanol between each sapling. To prepare the inoculum, a 3 mm agar plug was cut from actively growing cultures of the test isolates and placed inside the wound.

Wounds were sealed using parafilm to prevent drying of the inoculum or contamination. The inoculated saplings were monitored for approximately 6 wk. The bark of the area around the inoculation points was peeled back to expose the lesions. Lesions were measured above and below the inoculation points. To fulfil Koch's postulates, isolations were made from tissue associated with the inoculations, including the controls. Re-isolated cultures from two trees of each treatment and the controls were subjected to DNA sequencing to determine whether the fungus arising from the lesions was the same as that inoculated.

Statistical analyses were performed on lesions using the program R (R Core Team, 2018; https://www.R-project.org/). The data were first analysed using Grubbs test to detect outliers with "outliers" package (Schiffler 1998). Normal distribution and homogeneity of variance were tested by performing a Shapiro-Wilk test and a Bartlett test. The data were then subjected to analysis of variance (ANOVA) and Tukey's honestly significance difference (Tukey's HSD).

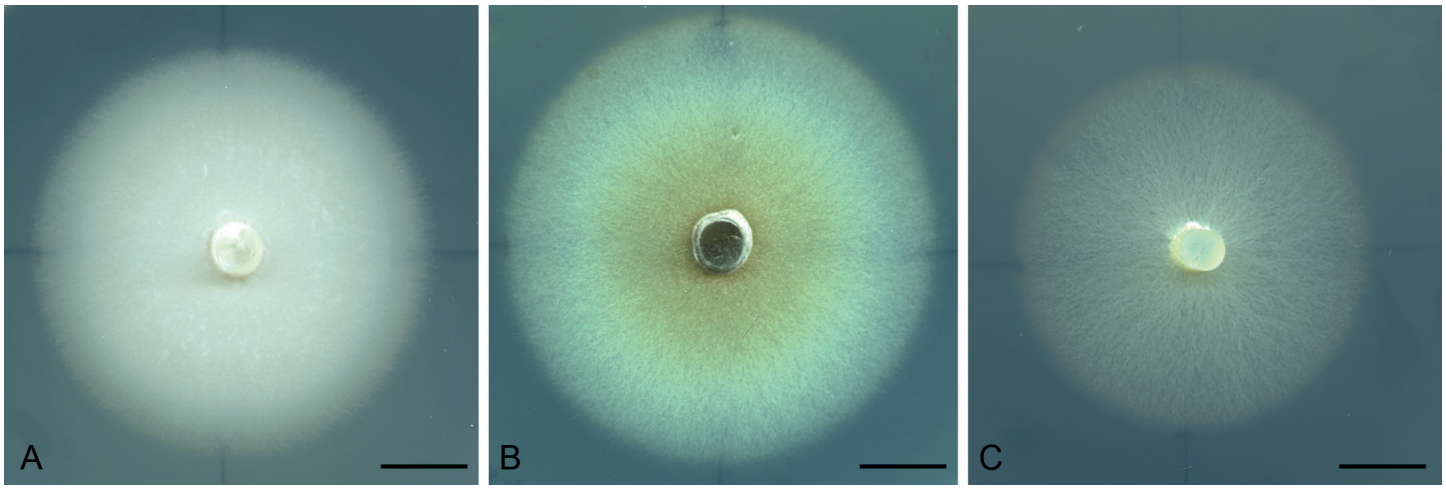

Fig. 2. Three distinct colony morphologies in *Raffaelea borbonica* isolates. Cultures were grown at 25 °C for 12 d in the dark. **A.** CMW 51553. **B.** CMW 51724. **C.** CMW 51555. Scale bars: A–C = 1 cm.

RESULTS

Isolates and phylogenetic analyses

Primary isolations from the wood samples yielded 33 isolates resembling *Raffaelea sensu stricto* as defined by De Beer & Wingfield (2013). These isolates had three different colony morphologies (Fig. 2).

Preliminary identification of the isolates based on the BLAST results of the ITS region confirmed that they were species of *Raffaelea*. Maximum likelihood phylogenetic analyses of the ITS, LSU and *TUB* regions revealed that all isolates were of a single species, regardless of their different colony morphology. They resided in a well-supported lineage sister to *Raffaelea crossotarsa* and were distinct from described species (Fig. 3). Phylogenies produced for the different gene regions using Bayesian inference analyses were highly congruent with those produced by the ML analyses and supported the grouping of the isolates obtained in this study as their own distinct, well supported species.

Pathogenicity

The two isolates used in the pathogenicity trial produced distinct lesions following the 6-wk observation period. Most control seedlings showed small lesions (Fig. 4A), while larger lesion lengths were observed for both isolates (Fig. 4B, C). Lesion lengths above the inoculation point were approximately 10 mm longer on average than those below this point. Exceptionally long lesions were found on two saplings: one inoculated with isolate CMW 51553 and the other with isolate CMW 51724, with one sapling showing fungal spread into the vascular system (Fig. 4D, F).

Most of the control saplings displayed small lesions but four had somewhat longer lesions. One of the control saplings showed discolouration of the vascular system (Fig. 4E, G). These data were removed as outliers. The result of the Shapiro Wilk test and Bartlett test showed that the data were normally distributed and had equal variance. Results of the ANOVA indicated that there was a significant within the data difference (P-value < 0.01, at the 95 % confidence level). Based on the results of Tukey's HSD, there was a significant difference between the lesions associated with the inoculated versus the control plants but no significant difference in the lesion lengths produced by either of the two fungal isolates (Fig. 5). Mortality was not observed in any of the inoculated saplings for the duration of the trial.

Re-isolations were performed from the lesion margins on both the control and inoculated saplings. Isolates resembling the *Raffaelea* species were obtained from all 11 saplings inoculated with CMW 51724, six of the saplings inoculated with CMW 51553 and one of the control saplings showing larger lesions. DNA sequence analyses confirmed the identity of the isolates as the same as those used in the inoculations.

Taxonomy

Raffaelea borbonica M. Procter, M.J. Wingf & Marinc., ***sp. nov.*** MycoBank MB836438. Fig. 6.

Etymology: Name refers to the island of Réunion, previously known as *Île Bourbon* where this fungus was collected.

Conidiophores mononematous, micro- or macronematous, arising from vegetative hyphae, mostly simple, occasionally branched once, upright, straight, curved or undulate, tapering towards apex, 27–118 (69±23) µm long, 2–5.5 (4.0±0.7) µm wide near base, occasionally reduced to conidiogenous cell. *Conidiogenous cells* integrated, hyaline, cylindrical or peg-like when micronematous, tapering towards apex, blastic, showing percurrent growth, 3–31 × 1.5–2.5 (17±8 × 2±0.3) µm. *Conidia* hyaline, aseptate, majority oblong with the upper part swollen, apex round, tapering toward base, base truncate, 5–8.5 × 2–4 (6.8±0.7 × 2.9±0.4) µm, yeast-like budding from primary conidia observed in fresh culture.

Typus: **Réunion**, on *Leucaena leucocephala*, Jun. 2015, *M.J. Wingfield* & *P.W. Crous* (**holotype** PREM 62884, living culture ex-holotype CMW 51553 = PPRI 27953).

Additional material examined: **Réunion**, on *Leucaena leucocephala*, Jun. 2015, *M.J. Wingfield* & *P.W. Crous,* PREM 62885, living culture CMW 51724 = PPRI 27954.

Culture characteristics: Cultures on 2 % MEA in dark showing optimum growth at 25 °C reaching 80 mm in 12 d, circular growth with smooth margin, mycelia mostly submerged (aerial

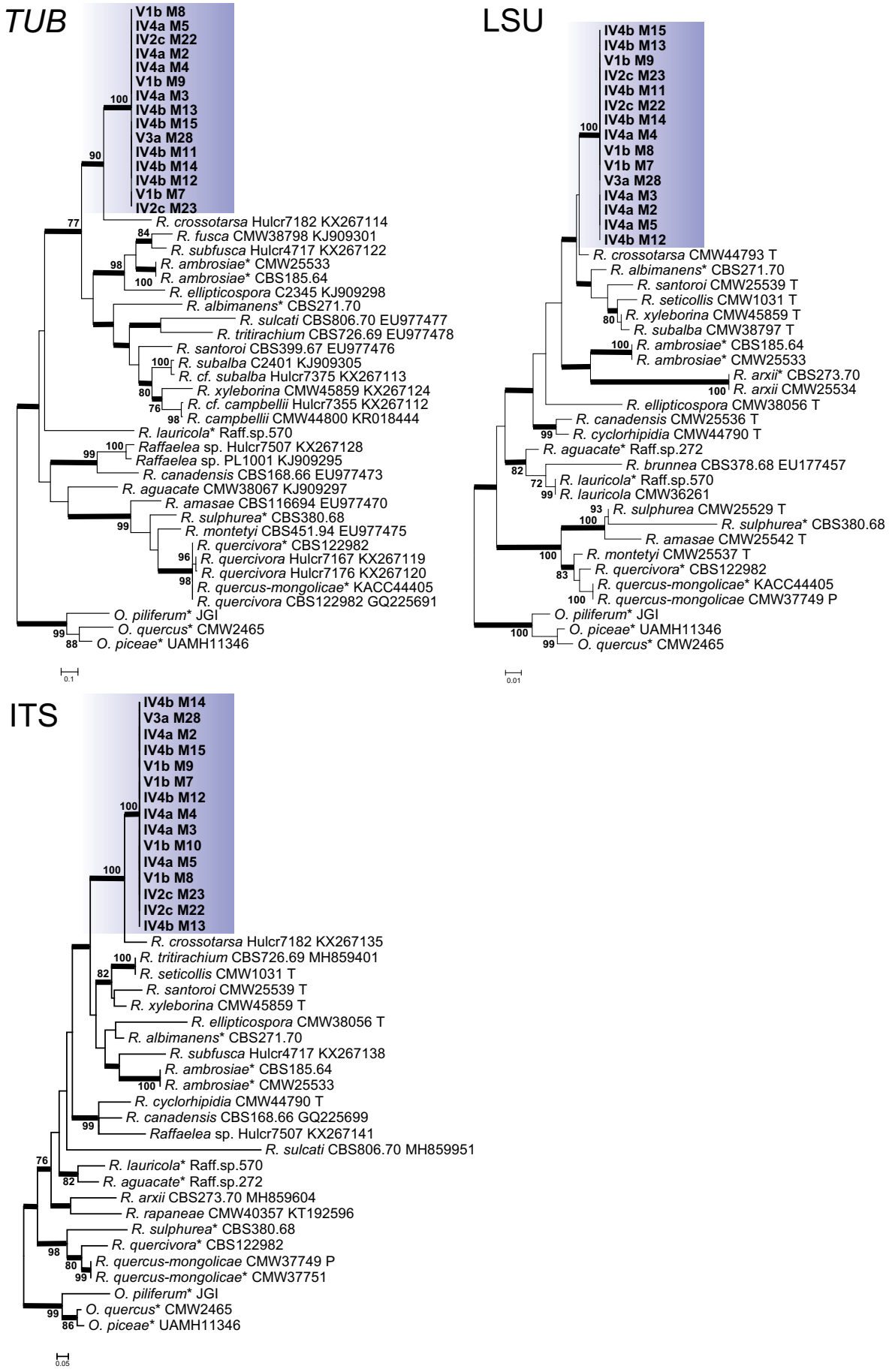

Fig. 3. Phylogenetic trees resulting from maximum likelihood analyses of the *TUB*, LSU and ITS region for species of *Raffaelea.* Bootstrap support values above 70 are indicated on the nodes. Bayesian inference posterior probability values above 0.9 are indicated by bold lines. Sequences generated for the novel species in this study are indicated in **bold**. Asterisks (*) indicate where genome data was used to obtain the sequence used in the analysis. T indicates ex-holotype, P indicates ex-paratype. Species of *Ophiostoma* were used to root the tree.

Fig. 4. Lesions associated with inoculation on *Leucaena leucocephala* and discoloration of the vascular plant tissue. **A.** Control using clean agar. **B.** Isolate CMW 51553. **C.** Isolate CMW 51724 (arrows in A, B and C indicate limits of lesions originating from the agar plug). **D, F.** Inoculated with isolate CMW 51724. **E, G.** A control inoculation.

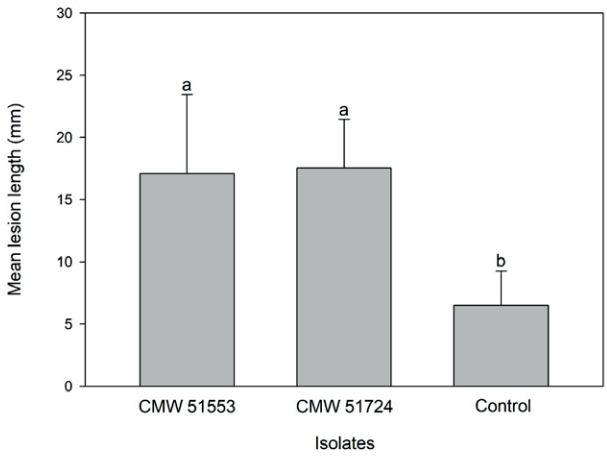

Fig. 5. Bar graph showing mean lesion lengths on inoculated *Leucaena leucocephala* trees. Based on the results of the ANOVA, both inoculated treatments (CMW 51553 and CMW 51724) were significantly different from the control as denoted by **a** and **b**. Inoculated treatments were not significantly different from one another as denoted by **a**. Protruding lines indicate standard deviation.

Fig. 6. Morphological characteristics of *Raffaelea borbonica* (ex-type, CMW 51553 = PPRI 27953). **A.** Culture grown at 25 °C in the dark for 12 d and 120 d. **B.** Mycelial cluster formed in older culture. **C–E.** Conidiophores (arrows). **F.** Conidiogenous cells showing percurrent growth (arrows). **G.** Conidia. **H, I.** Early stage of budding conidium. **J, K.** Later stage of budding conidium. Scale bars: B = 100 µm, C, G = 10 µm, D, E = 5 µm, F, H–K = 2.5 µm.

hyphae sparse), flat, initially whitish to creamy becoming darker with age, sporodochium-like mycelial mass formed in 4-mo-old culture.

Notes: *Raffaelea borbonica* can be distinguished from its closest relative *R. crossotarsa* by conidial shape and in having longer but narrower dimensions. Conidia of *R. borbonica* are predominantly oblong with an enlarged apex, ranging 6.8 × 2.9 µm in average, whereas those of *R. crossotarsa* are globose to ovoid, ranging 6 × 4.9 µm in average.

DISCUSSION

A new species of *Raffaelea,* described here as *R. borbonica*, was isolated from dying *L. leucocephala* trees in Réunion. Isolation of this fungus from discoloured wood tissue was associated with ambrosia beetle damage. The known association between *Raffaelea* species and these beetles implies that *R. borbonica* was probably transmitted to the trees by the insects (De Beer & Wingfield 2013, Hulcr & Stelinski 2017, Vanderpool *et al*. 2017). However, the absence of the insects in the collected samples precludes the opportunity to link the beetle to *R. borbonica*. To do this, it will be necessary to recollect and determine the species of the beetle responsible for the damage.

Raffaelea borbonica bears close morphological resemblance to other species of *Raffaelea s. str*. (De Beer & Wingfield 2013). It can, however, be distinguished from previously described species based on its oblong to sub-globose conidia. Conidia of *Raffaelea* spp. are more typically ovoid to globose and occasionally T- or Y-shaped (Musvuugwa *et al*. 2015). *Raffaelea* now includes 30 described species (De Beer & Wingfield 2013, Musvuugwa *et al*. 2015, Simmons *et al*. 2016) together with many taxa treated only as "*Raffaelea* sp." and yet to be described (Simmons *et al*. 2016, Li *et al*. 2018).

In inoculation tests, *R. borbonica* was shown to have a low level of aggressiveness when compared to similar studies done with other *Raffaelea* species. For example, trials with *R. lauricola* were conducted in a similar manner as in this study and resulted in mortality within one month (Huges *et al*. 2011, Loyd *et al*. 2020). The disparate responses in some but not all of the inoculated saplings in the present study were most likely due to the fact that the saplings were grown from seeds and were thus genetically distinct from each other. The fact that the *L. leucocephala* trees from which *R. borbonica* was isolated in Réunion were wilting could have been due to the physical damage from the beetle infestation, or a combination of this damage and the low aggressiveness of the fungus. Variable levels of susceptibility of individual trees as seen in the pathogenicity tests could also have been a factor. There were no other obvious factors that might have contributed to the death of trees.

A confounding factor in the inoculation trials was that four control saplings developed lesions. *Raffaelea borbonica* was

not isolated from three of these plants and we assume that the lesions were due to a host-response to the physical damage of wounding. Re-isolation of the fungus from one of the control plants most likely arose from a contamination event. In this regard, Ophiostomatoid fungi, including *Raffaelea* species, are well-known to be transmitted by arthropods such as mites (Roets *et al*. 2008) that could have moved *R. borbonica* to one of the control plants.

Leucaena leucocephala is an aggressive invasive weed in Réunion. A disease of these plants is of interest given that it might provide a means to reduce the invasiveness of the plant. However, results of this study provided no evidence that *R. borbonica* poses a health threat to *L. leucocephala* or that it might offer any opportunity for biological control.

ACKNOWLEDGEMENTS

We thank Mr. Seamus Morgan for assistance in maintaining greenhouse plants, Dr Tuan Duong and Prof. Wilhelm de Beer for suggestions regarding the phylogenetic analyses, and Dr Donghyeon Lee and Dr Hiroyuki Suzuki for assistance with the statistical analyses. Dr Dominik Strassberg provided the opportunity to conduct a survey of fungi in Réunion and Prof. David Richardson assisted in providing logistics for this visit. We appreciate the financial support of the University of Pretoria and the DST/NRF Centre of Excellence in Plant Health Biotechnology (CPHB), South Africa.

Conflict of interest: The authors declare that there is no conflict of interest.

REFERENCES

Anupam K, Sharma AK, Lal PS, *et al.* (2016). Preparation, characterization and optimization for upgrading *Leucaena leucocephala* bark to biochar fuel with high energy yielding. *Energy* **106**: 743–756.

CABI (2019). *Leucaena leucocephala*. In: *Invasive Species Compendium*. Wallingford, UK: CAB International. https://www.cabi.org/isc/datasheet/31634.

De Beer ZW, Wingfield MJ (2013). Emerging lineages in the *Ophiostomatales*. In *The Ophiostomatoid Fungi: Expanding Frontiers* (Seifert KA, De Beer, ZW, Wingfield MJ, eds) CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands: 21–46.

De Beer ZW, Seifert KA, Wingfield MJ (2013) The ophiostomatoid fungi: their dual position in the *Sordariomycetes*. In *The Ophiostomatoid Fungi: Expanding Frontiers* (Seifert KA, De Beer, ZW, Wingfield MJ, eds) CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands: 1–19.

Dreaden TJ, Davis JM, De Beer ZW, *et al.* (2014). Phylogeny of ambrosia beetle symbionts in the genus *Raffaelea*. *Fungal Biology* **118**: 970–978.

Duong TA, De Beer ZW, Wingfield BD, *et al.* (2012). Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. *Mycologia* **104**: 715–732.

Gebhardt H, Weiss M, Oberwinkler F (2005). *Dryadomyces amasae*: a nutritional fungus associated with ambrosia beetles of the genus *Amasa* (*Coleoptera*: *Curculionidae*, *Scolytinae*). *Mycological Research* **109**: 687–696.

Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied Environmental Microbiology* **61**: 1323–1330.

Hakimi MI, Khalil H, Goembira F, *et al.* (2017). Engine-compatible biodiesel from *Leucaena leucocephala* seed oil. *Journal of the Society of Automotive Engineers Malaysia* **1**: 86–93.

Haridas S, Wang Y, Lim L, *et al.* (2013). The genome and transcriptome of the pine saprophyte *Ophiostoma piceae*, and a comparison with the bark beetle-associated pine pathogen *Grosmannia clavigera*. *BMC Genomics* **14**: 373.

Harrington TC, Fraedrich SW, Aghayeva DN (2008). *Raffaelea lauricola,* a new ambrosia beetle symbiont and pathogen on the *Lauraceae*. *Mycotaxon* **104**: 399–404.

Harrington TC, Aghayeva DN, Fraedrich SW (2010). New combinations in *Raffaelea, Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* **111**: 337–361.

Hughes M, Smith JA, Mayfield AE, *et al*. (2011). First report of Laurel Wilt Disease caused by *Raffaelea lauricola* on Pondspice in Florida. *Plant Disease* **95**: 1588–1588.

Hulcr J, Stelinski LL (2017). The ambrosia symbiosis: from evolutionary ecology to practical management. *Annual Review of Entomology* **62**: 285–303.

Jeon J, Kim KT, Song H*, et al.* (2017). Draft genome sequence of the fungus associated with Oak Wilt mortality in South Korea, *Raffaelea quercus-mongolicae* KACC44405. *Genome announcements* **5**: e00797-00717.

Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution* **30**: 772–780.

Kim KH, Choi YJ, Seo ST, *et al.* (2009). *Raffaelea quercus-mongolicae sp. nov.* associated with *Platypus koryoensis* on oak in Korea. *Mycotaxon* **110**: 189–197.

Kubono T, Ito S (2002). *Raffaelea quercivora sp. nov.* associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* **43**: 255–260.

Li Y, Huang Y-T, Kasson MT, *et al*. (2018) Specific and promiscuous ophiostomatalean fungi associated with *Platypodinae* ambrosia beetles in the southeastern United States. *Fungal Ecology* **35**: 42–50.

Loyd AL, Chase KD, Nielson A, *et al*. (2020). First report of Laurel Wilt caused by *Raffaelea lauricola* on *Sassafras albidum* in Tennessee and Kentucky. *Plant Disease* **104**: 567–567.

Macdonald IAW, Thebaud C, Strahm WA, *et al.* (1991). Effects of alien plant invasions on native vegetation remnants on La Reunion (Mascarene Islands, Indian Ocean). *Environmental Conservation* **18**: 51–61.

Masuya H, Manabe R, Ohkuma M, *et al*. (2016). Draft genome sequence of *Raffaelea quercivora* JCM 11526, a Japanese oak wilt pathogen associated with the platypodid beetle, *Platypus quercivorus*. *Genome Announcements* **4**: e00755-16.

Musvuugwa T, De Beer ZW, Duong TA, *et al.* (2015). New species of *Ophiostomatales* from *Scolytinae* and *Platypodinae* beetles in the Cape Floristic Region, including the discovery of the sexual state of *Raffaelea*. *Antonie van Leeuwenhoek* **108**: 933–950.

O'Donnell K, Cigelnik E (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics & Evolution* **7**: 103–116.

Olckers T (2011). Biological control of *Leucaena leucocephala* (Lam.) de Wit (*Fabaceae*) in South Africa: A tale of opportunism, seed feeders and unanswered questions. *African Entomology* **19**: 356–365.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rasat MSM, Wahab R, Mohamed M, *et al.* (2016). Preliminary study on properties of small diameter wild *Leucaena leucocephala* species as

potential biomass energy sources. *ARPN Journal of Engineering and Applied Sciences* **11**: 6128–6137.

Roets F, De Beer ZW, Wingfield MJ, *et al.* (2008). *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. *Mycologia* **100**: 496–510.

Ronquist F, Teslenko M, van der Mark P, *et al.* (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.

Schiffler RE (1998). Maximum Z scores and outliers. *The American Statistician* **42**: 79–80.

Schoch CL, Robbertse B, Robert V, *et al.* (2014). Finding needles in haystacks: linking scientific names, reference specimens and molecular data for *Fungi*. *Database: The Journal of Biological Databases and Curation* **2014**: bau061.

Silvestro D, Michalak I (2012). raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity & Evolution* **12**: 335–337.

Simmons DR, De Beer ZW, Huang Y-T, *et al.* (2016). New *Raffaelea* species (*Ophiostomatales*) from the USA and Taiwan associated with ambrosia beetles and plant hosts. *IMA Fungus* **7**: 265–273.

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.

Vanderpool D, Bracewell RR, McCutcheon JP (2017). Know your farmer: ancient origins and multiple independent domestications of ambrosia beetle fungal cultivars. *Molecular Ecology* **27**: 2077–2094.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, San Diego, California, USA: **8**: 315–322.