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

M. Procter¹, W.J. Nel¹, S. Marincowitz¹*, P.W. Crous¹,², M.J. Wingfield³

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

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

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**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.

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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.
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 dH₂O) 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 (Ronquist 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.**

| Species                        | CMW | CBS or others | ITS Accession Numbers | LSU Accession Numbers | TUB Accession Numbers |
|-------------------------------|-----|---------------|-----------------------|-----------------------|-----------------------|
| *R. aguacate*                 |     | Raff. sp. 272 | MT633065              | MT629748              |                       |
|                               | 38067 | 141672       |                       |                       |                       |
| *R. albimanens*               | 25532 | 271.70        | MT633066              | MT629749              | MT644111              |
| *R. amasae*                   | 25542 | 116694        |                       |                       |                       |
| *R. ambrosiae*                | 25533 | 185.64        | MT633067              | MT629751              | MT644094              |
|                               | 25533 | 185.64        | MT633068              | MT629752              | MT644095              |
| *R. arxii*                    | 25534 | 273.70        |                       |                       |                       |
|                               | 25534 | 273.70        | 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              |
|                               | 51772 | V1b M9        | MT633082              | MT629772              | MT644103              |
|                               | 51552 | V1b M10       | MT633079              |                       |                       |
|                               |      | IV4b M11      |                       |                       |                       |
|                               |      | IV4b M12      | MT633058              | MT629741              | MT644104              |
|                               |      | IV4b M13      | MT633059              | MT629742              | MT644106              |
|                               | 51723 | IV4b M14      | MT633060              | MT629743              | MT644105              |
|                               | 51724 | 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 |              |                       |                       |                       |
|                               |      | 139943        |                       |                       |                       |
| *R. cf. campbellii*           |      | Hulcr7355     |                       |                       |                       |
| *R. canadensis*               | 25536 | 168.66        | GQ225699              | MT629755              |                       |
| *R. crotosorata*              | 44793 | 141675        | KX267138              | MT629756              |                       |
| *R. cyclorhipidia*            | 44790 | 141676        | MT633069              | MT629757              |                       |
| *R. ellipticospora*           | 38056 | 121569        | MT633070              | MT629758              |                       |
|                               |      | C2345         |                       |                       |                       |
| *R. fusca*                    | 38798 | 121570        |                       |                       |                       |
| *R. lauricola*                | 36261 | PL159         |                       |                       |                       |
| *R. montetyi*                 | 25537 | 463.94        |                       |                       |                       |
|                               |      | 451.94        |                       |                       |                       |
| *R. quercivora*               | 36263 | 122982        | MT633072              | MT629762              | MT644090              |
|                               |      | 122982        | MT629762              |                       |                       |
|                               |      | Hulcr7167     |                       |                       |                       |
| *R. quercus-mongolicia*       | 37749 | KACC44403     | MT633073              | MT629764              |                       |
|                               | 37751 | KACC44405     | MT633074              | MT629763              | MT644091              |

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Table 1. (Continued).

| Species                  | CMW$^1$ | CBS or others | ITS  | LSU   | TUB   |
|--------------------------|---------|---------------|------|-------|-------|
| *R. rapaneae*            | 40357$^7$ | 140084        | KT192596 | KT182930 | –     |
| *R. santorai*           | 25539$^9$ | 399.67        | MT633075 | MT629765 | EU977476 |
| *R. seticollis*         | 1031$^1$ | 634.66        | MT633076 | MT629766 | –     |
| *R. subalba*             | 38797$^7$ | 121568        | –     | MT629767 | –     |
| *R. cf. subalba*         | –       | –             | –     | –     | KJ909305 |
| *R. subfuscus*           | –       | –             | –     | –     | –     |
| *R. sulcata*             | 25540$^1$ | 806.70        | MH859951 | EU177462 | EU977477 |
| *R. sulphurea*$^2$      | 25529   | 380.68$^6$    | MT633077 | MT629768 | MT644092 |
| *R. xyleborina*          | 45859$^2$ | 111557        | MT633078 | MT629769 | KX267124 |
| *Raffaelea* sp. A (PL1001) | –   | –             | –     | KJ909293 | KJ909295 |
| *Raffaelea* sp.          | 38062   | –             | –     | –     | –     |
| *Ophiostoma piliferum*$^4$ | JGI   | –             | –     | –     | –     |
| *O. quercus*             | 2465    | 117912        | MT633063 | MT629746 | MT644089 |
| *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 Musvuuwga 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).
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 crossoptarsa* 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 mononematous, 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.
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.

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

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