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Discovery of the eucalypt pathogen *Quambalaria eucalypti* infecting a non-*Eucalyptus* host in Uruguay

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Abstract. *Quambalaria eucalypti*, a serious pathogen of *Eucalyptus*, is believed to be native to Australia and introduced into various southern hemisphere countries, including Uruguay. In this study we record the discovery of *Q. eucalypti* causing leaf lesions on *Myrceugenia glaucescens*, a tree native to Uruguay. The identity of the pathogen was confirmed using DNA sequence comparisons of the internal transcribed spacer (ITS) region of the rDNA operon, as well as morphological characteristics. This is the first record of the pathogen on a host other than *Eucalyptus*. It clearly indicates a disturbing example of an alien invasive pathogen having undergone a host-shift from non-native *Eucalyptus* to a native tree in Uruguay.

Additional keywords: host jump, Myrtaceae, plant pathogens.

Introduction

The eucalypt pathogen *Quambalaria eucalypti* (Wingf., Crous & Swart) Simpson (Simpson 2000) was first reported from nurseries in South Africa where it caused shoot lesions and leaf spots on commercially propagated *Eucalyptus grandis* Hill: Maid. clones (Wingfield *et al*. 1993). It was considered of minor importance in South Africa until 2005, when it was observed causing serious stem disease in 1-year-old *E. nitens* Maiden plantations (Roux *et al*. 2006). In South America, the pathogen was first reported from Uruguay infecting twigs of *E. globulus* ssp. *globulus* Labill (hereafter referred to as *E. globulus*) in 1999 (Bettucci *et al*. 1999), but severe damage has not been observed in this country. *Quambalaria eucalypti* was first found in Brazil in 2000, causing shoot and leaf lesions on *Eucalyptus* spp. (Alfenas *et al*. 2001). It is currently responsible for significant disease problems during clonal propagation of *Eucalyptus* (Alfenas *et al*. 2004; Andrade *et al*. 2005).

The genus *Quambalaria* is well known in Australia, particularly due to the damage that *Q. pitereka* and *Q. coyrecup* cause on species of *Corymbia* (Simpson 2000; Paap *et al*. 2008; Pegg *et al*. 2008). These fungi are confined to the eucalypts, most of which are native to Australia, and it is intriguing that *Q. eucalypti* has not been found in Australia until very recently. The discovery of the fungus causing leaf spots and stem lesions on *Eucalyptus* spp. in Queensland and New South Wales by Pegg *et al*. (2008) adds credence to the view that *Quambalaria* spp. are pathogens native to Australia (Wingfield *et al*. 1993; de Beer *et al*. 2006; Roux *et al*. 2006; Paap *et al*. 2008; Pegg *et al*. 2008).

In 2007, disease surveys were conducted in native forests in Uruguay and leaf lesions resembling symptoms caused by *Quambalaria* were observed on *Myrceugenia glaucescens* (Camb.) Legrand et Kausel, a Myrtaceae tree native to the country. The discovery of a possible *Quambalaria* sp. on a host other than *Eucalyptus* was of concern. The aim of this study was to identify the causal agent of the disease and to consider the possibility that a host jump from exotic *Eucalyptus* to a native tree, might have occurred.

Methods

Sampling, symptom description and fungal morphology

In June 2007, infected leaves were collected from native *Myrceugenia glaucescens* in the province of Tacuarembo, Uruguay. In the laboratory, lesions were described, excised, surface-disinfested in 70% ethyl alcohol for 30 s, rinsed twice in sterile distilled water, blotted dry on sterile filter paper, and plated onto 2% malt extract agar (MEA) (2% malt extract, 1.5% agar; Oxoid, Basingstoke, England) in Petri plates. Plates were incubated at room temperature (~20°C) for 1 week. Three single hyphal-tip cultures were obtained from emerging colonies. For morphological characterisation, mycelium, conidiophores and conidia were mounted in lactic acid on microscope slides and examined under a Nikon Eclipse E600 light microscopic and photographed with a Nikon Digital Camera DXM1200F (Nikon Inc., Melville, NY). A set of 50 measurements were made of all taxonomically relevant structures.

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The three cultures showed identical colony and conidial morphology, so a single isolate (UY1718) was selected for phylogenetic analyses. For comparative purposes, an isolate of *Q. eucalypti* from a leaf lesion on *E. globulus* growing in a plantation located in the province of Durazno in Uruguay (UY1036) was included in the study.

DNA extraction, PCR and sequencing

For DNA extractions from isolates UY1036 and UY1718, cultures were grown on 2% MEA at room temperature for 15 days. Mycelium was scraped directly from the surface of the colonies and transferred to Eppendorf tubes (1.5 mL) with 3-mm glass beads and extraction buffer (Qiagen Inc., Valencia, CA). These were vigorously shaken using a vortex mixer and placed in a water bath at 60°C for 1 h. DNA extraction from the mycelial slurry was performed using the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. PCR amplifications were conducted using primers ITS1 and ITS4 (White *et al*. 1990) to amplify the internal transcribed spacer (ITS) region of the rDNA operon. PCR was performed in a 25-µL reaction mixture of 1.0 µL of 0.05% casein, 12.5 µL of Amplitaq Gold PCR Master-Mix (Applied Biosystems, Foster City, CA), 1.0 µL of 10 mM ITS1, 1.0 µL of 10 mM ITS4, 8.5 µL of ddH_2O and 1.0 µL of DNA template of 10 ng/µL. PCR amplifications were performed in a MJ Research PTC 200 DNA Engine Thermal Cycler PCR (MJ Research, Reno, NV) with the following parameters: initial denaturation for 5 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, followed by a final elongation step of 5 min at 72°C, then held at 10°C.

PCR products were stained with SYBR Green nucleic acid dye (MBL International, Woburn, MA) and visualised on a 1.5% agarose gel under UV light. Amplified DNA was purified and prepared for sequencing using ExoSAP-IT PCR clean-up kit (USB Corp., Cleveland, OH) following the manufacturer's instructions. Sequencing reactions were performed using the same primers. Sequences obtained in this study were deposited in GenBank (accessions EU439922 and EU439923 for isolates UY1036 and UY1718, respectively).

Sequences were subjected to BLAST searches in NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified 1 September 2008), and published sequences of related species were downloaded. Sequences were aligned online using the E-INS-i strategy in MAFFT v. 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/, verified 1 September 2008) (Katoh *et al*. 2005), and the aligned dataset deposited in TreeBASE (S2036).

Phylogenetic analyses

Neighbour-joining and maximum parsimony analyses were performed using PAUP v. 4.0b10. (Swofford 2002). The best substitution model for neighbour-joining analysis was determined using Modelltest v. 3.7 (Posada and Crandall 1998). Gaps were treated as missing data and all characters were treated as unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with simple taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. Support for the nodes of the shortest trees was determined by analyses of 1000 bootstrap replicas. Tree length (TL), consistency index (CI), retention index (RI), and homoplasy index (HI) were calculated.

Results

Symptom description

Lesions observed on *M. glaucescens* leaves were characterised by round, brown-reddish spots of variable size between 2 and 4 mm (Fig. 1*a*). The margins of the lesions were generally round and well defined in the adaxial leaf surface and more diffuse in the abaxial surface (Fig. 1*b*, *c*, respectively). Fungal structures were not observed on the lesions and association with wounds was not evident.

Fungal morphology

The morphology of the three pure cultures obtained from the leaf lesions was similar to those described for *Q. eucalypti.* Colonies on MEA were white and finely floccose with aerial hyphae raised up to 1 mm above the colony surface (Fig. 1*d*). Conidiophores arose directly from the mycelium bearing a cluster of conidia. Primary conidia were hyaline, globose to ovoid, of 4–8 × 2–3 µm. Secondary conidia, 2–4 × 1–2 µm, were also observed (Fig. 1*e*).

Phylogenetic analyses

The ITS sequences of the isolates obtained from *M. glaucescens* and *Eucalyptus globulus* (i.e. UY1718 and UY1036, respectively) were identical to each other and grouped consistently with ITS sequences of other *Q. eucalypti* isolates included in the analyses. The two phylogenetic analyses yielded trees with similar topology. From a total of 640 characters, 441 were constant, 11 variable characters were parsimony-uninformative and 188 were parsimony-informative. Heuristic search analysis of the data resulted in one tree (TL = 235 steps; CI = 0.932; RI = 0.968; HI = 0.068). The neighbour-joining tree was chosen for presentation (Fig. 2), displaying bootstrap values for neighbour-joining and maximum parsimony analyses.

In both analyses, the two sequences obtained in this study grouped with *Q. eucalypti* sequences from Australia and South Africa (Fig. 2), including the sequence from the ex-type isolate of the species. The three other known species of *Quambalaria* also formed distinct, well supported lineages.

Discussion

Results in this study confirmed the presence of *Q. eucalypti* on the native *M. glaucescens* in Uruguay. This is the first report of *Q. eucalypti* infecting a host residing outside of the genus *Eucalyptus*. The pathogen has been known on *Eucalyptus* in Uruguay for some time (Bettucci *et al*. 1999) and the results of this study suggest that it has undergone a host shift to a native tree.

Symptoms observed on *M. glaucescens* were similar to those described by Wingfield *et al*. (1993) on *Eucalyptus*. However, whitish mycelial growth with masses of spores, characteristic of *Q. eucalypti* infections, was not observed on the lesions. Further collecting will determine whether the absence of white pustules is

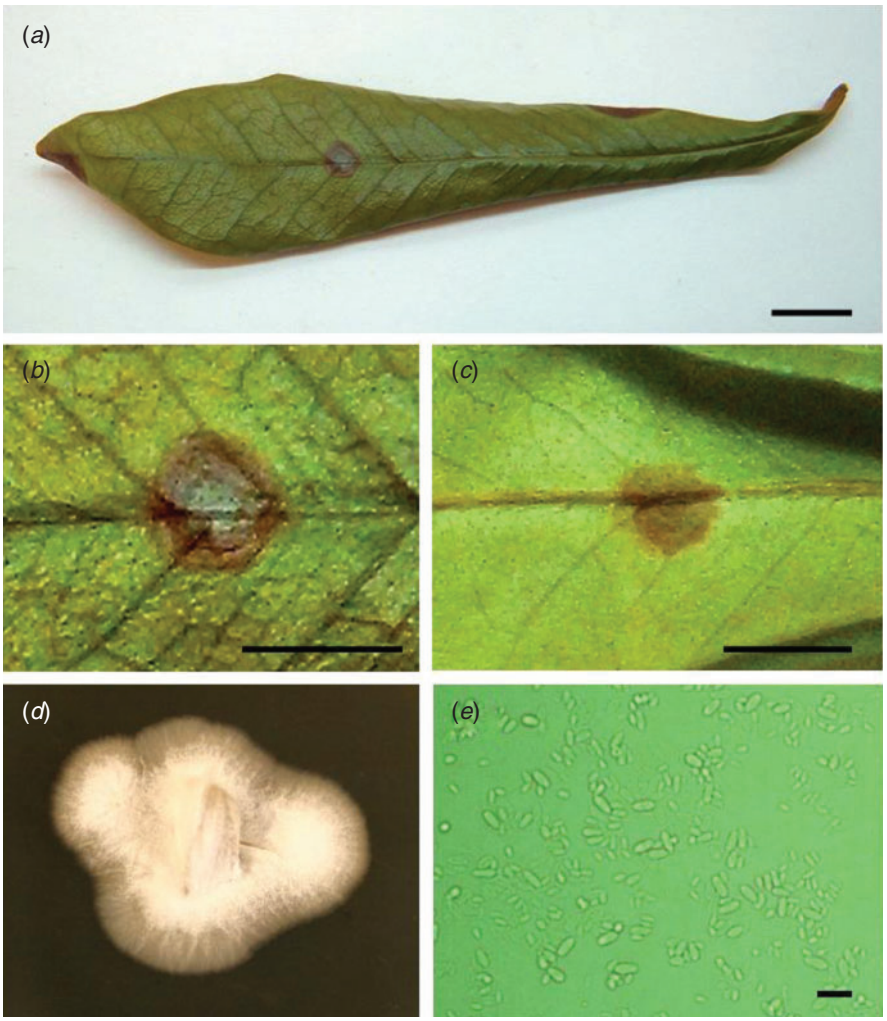

Fig. 1. (*a*) *Quambalaria eucalypti* symptoms on *Myrceugenia glaucescens*, scale bar = 10 mm; (*b*) spot on the adaxial and (*c*) abaxial leaf surface, scale bar = 5 mm; (*d*) white, floccose colony of *Q. eucalypti* isolate UY1718; and (*e*) primary and secondary conidia of isolate UY1718, scale bar = 10 µm.

characteristic for this host or whether this difference was due to environmental conditions occurring before the sampling.

The discovery of *Q. eucalypti* on a native Uruguayan tree is disturbing, since the fungus is a virulent primary pathogen that has the capacity to cause severe disease (Wingfield *et al.* 1993; Alfenas *et al*. 2004; Andrade *et al*. 2005; Roux *et al*. 2006). Host shifts, such as the one that appears to have occurred with *Q. eucalypti*, are relatively well known amongst tree pathogens (Slippers *et al*. 2005; Woolhouse *et al*. 2005) and many have lead to serious disease epidemics. In the case of the Myrtaceae, the *Eucalyptus* rust caused by *Puccinia psidii* provides a remarkable example of host jump. This pathogen is native to South America and has undergone a host jump from the native *Psidium pomiferum* (syn. *Psidium guajava*) to introduced *Eucalyptus* species in Brazil (Coutinho *et al*. 1998; Glen *et al*. 2007).

Host jumps, such as the one that has apparently occurred with *Q. eucalypti*, are often mediated by close genetic relationships among hosts (Slippers *et al*. 2005). In this case, *E. globulus* and *M. glaucescens* reside respectively in the closely related tribes Eucalypteae and Myrteae, in the Myrtaceae (Wilson *et al*. 2005). However, cases of host jumps occurring more widely in the Myrtales, for example between the Melastomataceae and the Myrtaceae, are emerging (Wingfield 2003) and they raise serious concern for the biosecurity of the Myrtaceae worldwide.

Geographical proximity and opportunities of cross-species transmission are mostly responsible for the appearance of new host–parasite combinations (Roy 2001). Thus, species growing adjacent to infected plants are exposed to increased inoculum pressure that increases the probabilities of eventual infection. In Uruguay, *Eucalyptus* plantations are geographically located close to native Myrtaceae trees, so biotic exchange can easily occur (Pérez 2008). Thus, pathogens introduced with germplasm of *Eucalyptu*s, to promote a growing paper and pulp industry, could threaten the native flora of Uruguay and probably other parts of South America in the future.

During the past 3 years, we have carried out extensive country-wide surveys in Uruguay and have consequently sampled a large number of native Myrtaceae trees. Only a single tree was found

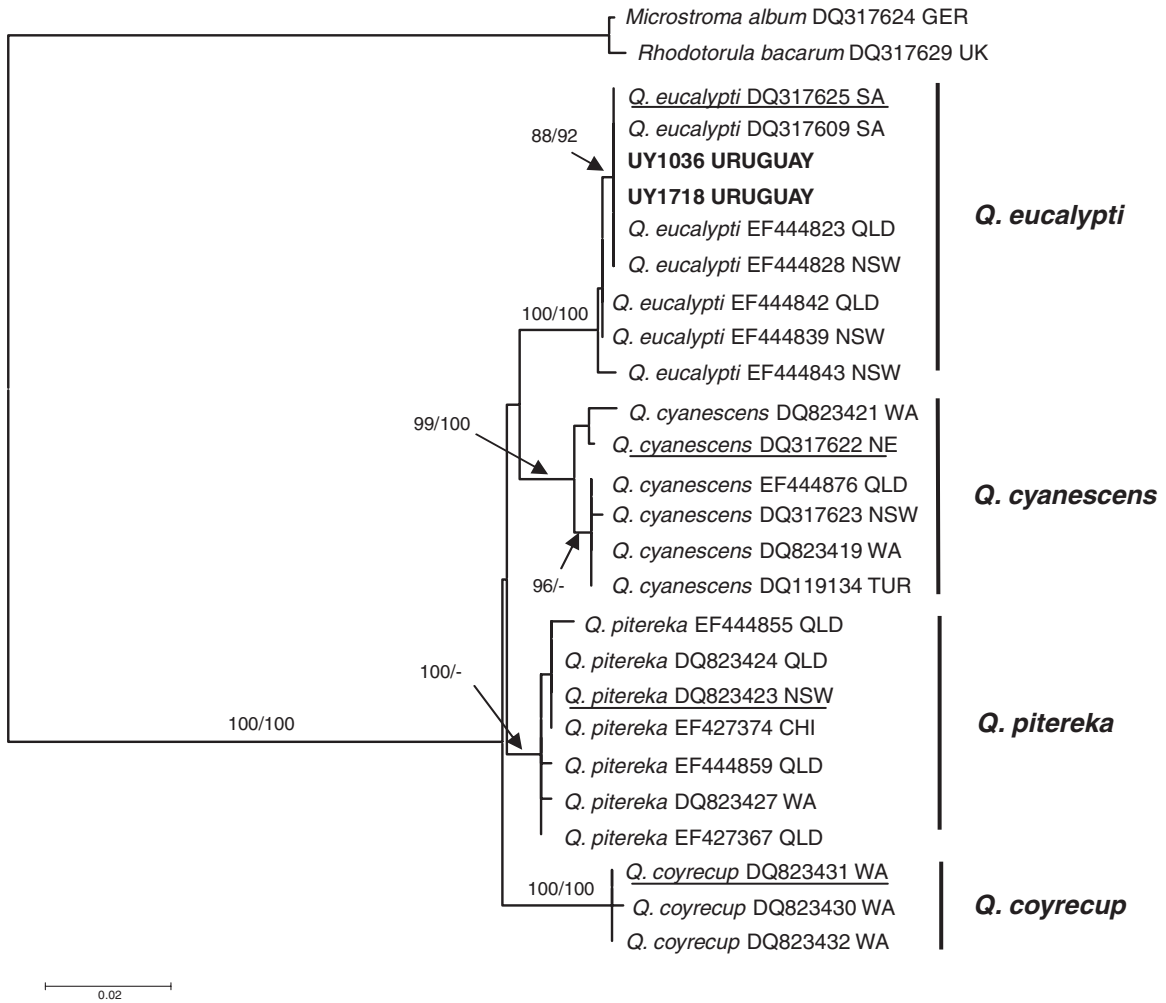

Fig. 2. Neighbour-joining tree from the ITS sequence data obtained using TVM + G model. GenBank accession number and country of origin is shown for each sequence: CHI, China; GER, Germany; NET, Netherlands; NSW, New South Wales, Australia; QLD, Queensland, Australia; SA, South Africa; TUR, Turkey; UK, United Kingdom; WA, Western Australia. Bootstrap values of 1000 replicas of neighbour-joining and maximum parsimony analyses are shown at the branches, respectively. Only bootstrap values higher than 75% are shown. *Microstroma album* and *Rhodotorula bacarum* were used as outgroup taxa. The two isolates obtained in this study are in bold and the ex-type cultures are shown underlined. Branch lengths are scaled and scale bar is 0.02 nucleotide substitutions per site.

with infections of *Q. eucalypti*. At the present time, there is no evidence that an epidemic is emerging, but continued monitoring is needed in Uruguay to assess the importance of *Q. eucalypti* in Uruguay.

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References

Alfenas AC, Zauza EA, Rosa OP, Assis T (2001) *Sporothrix eucalypti*, um novo patogeno do eucalipto no Brasil. *Fitopatologia Brasileira* **26**, 221. [In portugese] doi: 10.1590/S0100-41582001000200021

Alfenas AC, Zauza EA, Mafia RG, Assis TF (2004) 'Clonagem e Doenças do Eucalipto.' (Universidade Federal do Viçosa: Vinosa, Brazil) [In Portugese]

Andrade GC, Alfenas AC, Mafia RG, Maffia LA, Gonçalves RC (2005) Escala diagramatica para avaliação da severidade da mancha foliar do eucalipto causada por *Quambalaria eucalypti. Fitopatologia Brasileira* **30**, 504–509. [In Portugese] doi: 10.1590/S0100-41582005000500007

de Beer ZW, Begerow D, Bauer R, Pegg GS, Crous PW, Wingfield MJ (2006) Phylogeny of Quambalariaceae fam. nov., including important *Eucalyptus* pathogens in South Africa and Australia. *Studies in Mycology* **55**, 289–298.

Bettucci L, Alonso R, Tiscornia S (1999) Endophytic mycobiota of healthy twigs and the assemblage of species associated with twig lesions of *Eucalyptus globulus* and *E. grandis* in Uruguay. *Mycological Research* **103**, 468–472. doi: 10.1017/S0953756298007205

Coutinho TA, Wingfield MJ, Alfenas AC, Crous PW (1998) *Eucalyptus* rust: a disease with the potential for serious international implications. *Plant Disease* **82**, 819–825. doi: 10.1094/PDIS.1998.82.7.819

Glen M, Alfenas AC, Zauza EAV, Wingfield MJ, Mohammed C (2007) *Puccinia psidii*: a threat to the Australian environment and economy – a review. *Australasian Plant Pathology* **36**, 1–16. doi: 10.1071/AP06088

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**, 511–518. doi: 10.1093/nar/gki198

Paap T, Burgess TI, McComb JA, Shearer BL, Hardy GEStJ (2008) *Quambalaria* species, including *Q. coyrecup* sp. nov., implicated in canker and shoot blight diseases causing decline of *Corymbia* species in the southwest of Western Australia. *Mycological Research* **112**, 57–69.

Pegg GS, O'Dwyer C, Carnegie AJ, Burgess TI, Wingfield MJ, Drenth A (2008) *Quambalaria* species associated with plantation and native eucalypts in Australia. *Plant Pathology.* doi: 10.1111/j.1365-3059.2008.01840.x

Pérez CA (2008) Relationship between pathogens of *Eucalyptus* and native Myrtaceas in Uruguay. PhD Thesis, University of Minnesota, St Paul, MN.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics (Oxford, England)* **14**, 817–818. doi: 10.1093/bioinformatics/14.9.817

Roux J, Mthalane ZL, de Beer ZW, Eisenberg B, Wingfield MJ (2006) *Quambalaria* leaf and shoot blight on *Eucalyptus nitens* in South Africa. *Australasian Plant Pathology* **35**, 427–433. doi: 10.1071/AP06044

Roy BA (2001) Patterns of association between crucifers and their flower-mimic pathogens: host jumps are more common than coevolution or cospeciation. *Evolution* **55**, 41–53.

Simpson JA (2000) *Quambalaria,* a new genus of eucalypt pathogens. *Australasian Mycologist* **19**, 57–62.

Slippers B, Stenlid J, Wingfield MJ (2005) Emerging pathogens: fungal host jumps following anthropogenic introduction. *Trends in Ecology & Evolution* **20**, 420–421. doi: 10.1016/j.tree.2005.05.002

Swofford DL (2002) 'PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10a.' (Sinauer Associates: Sunderland, MA)

White TJ, Bruns S, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal genes for phylogenetics. In 'PCR protocols: a guide to methods and applications.' (Eds MA Innis, DH Gelfand, JJ Sninsky, TJ White) pp. 315–322. (Academic Press: San Diego, CA)

Wilson PG, O'Brien MM, Heslewood MM, Quinn CJ (2005) Relationships within Myrtaceae sensu lato based on a matK phylogeny. *Plant Systematics and Evolution* **251**, 3–19. doi: 10.1007/s00606-004-0162-y

Wingfield MJ (2003) Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from *Cryphonectria* canker. *Australasian Plant Pathology* **32**, 133–139. doi: 10.1071/AP03024

Wingfield MJ, Crous PW, Swart WJ (1993) *Sporothrix eucalypti* (sp. nov.), a shoot and leaf pathogen of *Eucalyptus* in South Africa. *Mycopathologia* **123**, 159–164. doi: 10.1007/BF01111267

Woolhouse ME, Haydon DT, Antia R (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in Ecology & Evolution* **20**, 238–244. doi: 10.1016/j.tree.2005.02.009

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