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SHORT RESEARCH NOTES

First record of *Passalora calotropidis* in Australia and its generic position

Peter M. Wilkinson[A,D], *Skye Thomas-Hall*[B], *Thomas S. Marney*[C] and *Roger G. Shivas*[C,E]

[A]Tropical Weeds Research Centre, Department of Natural Resources and Mines, Natal Downs Road, PO Box 187, Charters Towers, Qld 4820, Australia.
[B]CRC for Tropical Plant Pathology, Botany Department, The University of Queensland, St Lucia, Qld 4072, Australia.
[C]Plant Pathology Herbarium, Department of Primary Industries and Fisheries, 80 Meiers Road, Indooroopilly, Qld 4068, Australia.
[D]Current address: c/- 105 Albion Street, Warwick, Qld 4370, Australia.
[E]Corresponding author. Email: roger.shivas@dpi.qld.gov.au

Abstract. *Passalora calotropidis* has been found for the first time in Australia on rubber bush (*Calotropis procera*) in northern Queensland where it was associated with a damaging leaf spot disease. Analysis of sequence data of the ITS region indicated that *P. calotropidis* belonged to a group that consisted of species of *Pseudocercospora*. The generic position of *P. calotropidis* and its potential for biological control are discussed.

Additional keywords: ITS, biological control.

Rubber bush or calotrope (*Calotropis procera*, Asclepiadaceae) originates from the African and Indian tropics and has spread to become pantropical (Mabberley 1998). It is a vigorous, invasive weed of much of northern Australia and was thought to have been inadvertently introduced as padding in camel saddles during a gold rush in north-east Queensland. It has been recorded between Cairns and Normanton since 1935 (Hall 1967). First Australian herbarium records are from the early 1940s (Forster 1992). Rubber bush has proven to be a difficult weed to manage and has become especially problematic on alluvial flats and areas degraded through cultivation and overgrazing.

In November 2002 the senior author collected diseased leaves of rubber bush growing on sand dunes, overlooking a tidal estuary extending from the Gulf of Carpentaria, adjacent to the Karumba landing ground, Karumba, north Queensland (17°28′S, 140°50′E, alt. 3 m). This population of rubber bush was mature, producing flowers and seeds, with plants reaching 2–3 m in height. The disease was widespread in this host population. This report provides evidence that the causal organism is *Passalora calotropidis* (Ellis & Everh.) U. Braun, a pathogen not previously known to occur in Australia. Cultures of *P. calotropidis* provided an opportunity to use molecular studies to determine the phylogenetic relationships of this fungus with morphologically similar species.

In the early stages, the disease expresses as a dark lesion on either surface of the leaf with a halo of chlorotic yellow tissue. As the lesions increase in size, the chlorotic zone spreads, bounded by the leaf veins (Fig. 1*A*). On the lower leaf surface, the dark centres grow in size and eventually become covered in mycelium, bearing spores in poorly defined concentric rings. As the disease progresses, the entire leaf becomes yellow, followed by abscission. In the absence of supporting leaves, the branch tips die back, extending to the whole branch as the disease advances.

The leaf spots were covered with fascicles of pale brown conidiophores emerging from substomatal stromata, 10–100 × 4–6 µm, mostly simple, occasionally branched; the conidiogenous scars were mostly inconspicuous and not markedly thickened; conidia were pale brown, 1–5 septate, 20–75 × 5–8 µm, mostly with an unthickened

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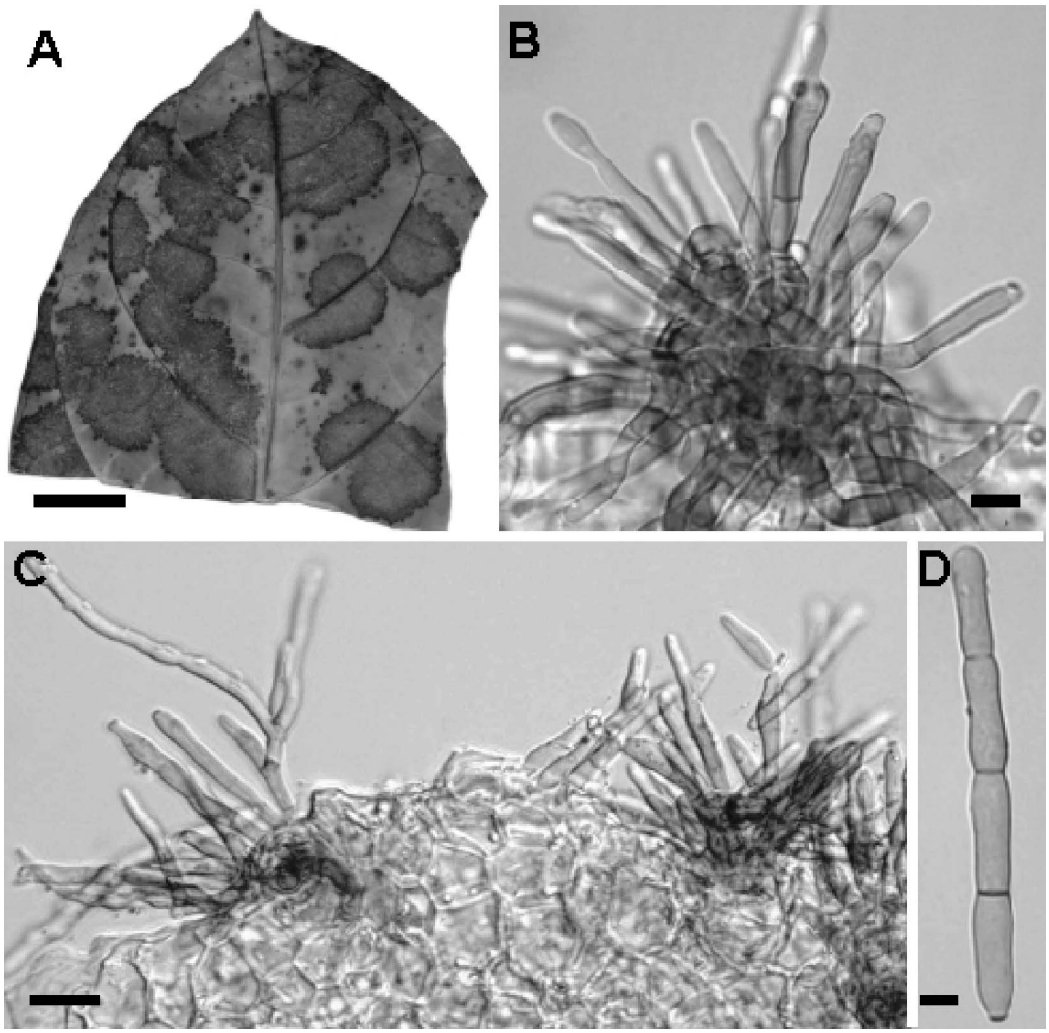

Fig. 1. *Passalora calotropidis* (from BRIP 39358). (*A*) Lesions on leaf of *Calotropis procera*. (*B*) Fasciculate conidiophores. (*C*) Conidiophores arising from substomatal stromata. (*D*) Conidium. Bars A = 2 cm; B = 10 µm; C = 20 µm; D = 5 µm.

hilum (Fig. 1*B–C*). This matched the description of a fungus variously known as *Phaeoramularia calotropidis* (Ellis & Everh.) Kamal, A.S. Moses & R. Chaudhary (1990), *Cercospora calotropidis* Ellis & Everh. given by Chupp (1954) and *Pseudocercospora calotropidis* (Ellis & Everh.) Haldar & Ray (2001).

Cultures of *P. calotropidis* were obtained by transferring conidia from leaf lesions to plates of potato-dextrose agar using a needle. The cultures were incubated at 24°C in the dark for 10 days, followed by 12 h near-UV light/12 h dark cycles for 21 days. After this period the cultures were 3–5 cm in diameter. Nine representative living isolates as well as herbarium material of infected leaves have been lodged in the Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries as BRIP 39185 and BRIP 39358. Hyphae from three isolates were inoculated into potato-dextrose broth and incubated at 25°C in the dark for 14 days. Culture purity was checked microscopically, then hyphae were harvested and washed twice with milli Q water for DNA extraction.

Genomic DNA was extracted according to the method described by Stewart and Via (1993). PCR was done according to the methods described by White *et al*. (1990), using primers NS7 (GAGGCAATAACAGGT CTGTGATGC) and R635 (GGTCCGTGTTTCAAGACGG) (Johanson and Jeger 1993). PCR products were purified using the UltraClean PCR Clean-up kit (Mo Bio Laboratories USA). Direct sequencing was performed using BigDye V3.1 as described in the manufacturer's directions (Applied Biosystems). Forwards sequence was obtained using 20 ng of purified product with primer ITS5 (GGAAGTAAAAGTCGTAACAAGG) and reverse sequence using primer ITS4 (TCCTCCGCTTATT CATATGC). Sequencing reactions were analysed on an

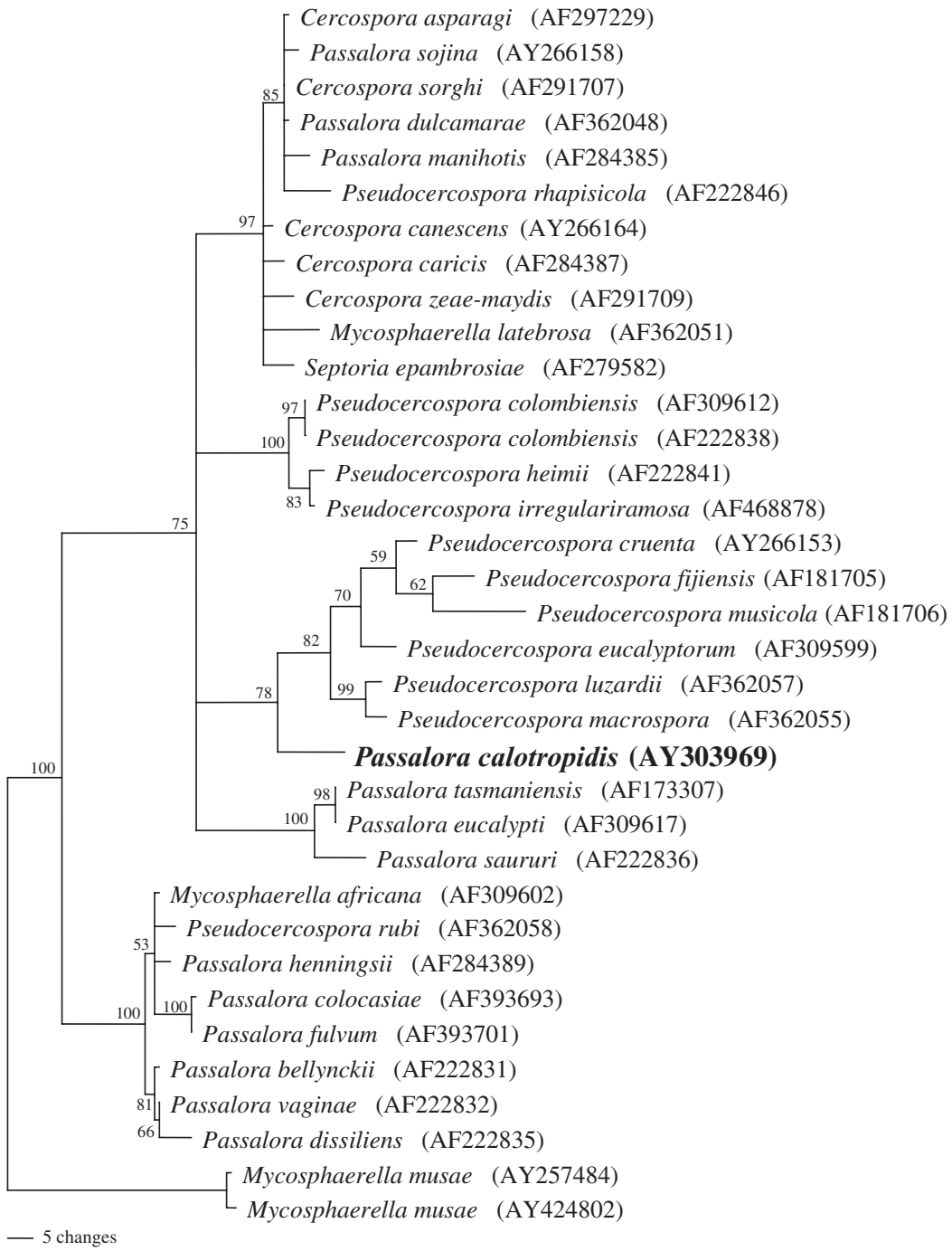

Fig. 2. Phylogenetic relationships of Passalora calotropidis based on conserved nucleic acid sequences in the ribosomal ITS region. GenBank accession numbers are provided in parentheses. Current anamorphic names have been used if available.

AB3730XL sequencer by the Australian Genomic Research Facility, Brisbane. Forwards and reverse sequences were aligned and edited using Sequencher 3.0 (Gene Codes Corporation). The ITS sequences were identical for each of the three cultures tested. This sequence was submitted to GenBank (AF303969).

Sequences for 34 of the closest related species were obtained through a BLAST search (Zhang and Madden 1997) with *Mycosphaerella musae* chosen as the outgroup. Sequences were aligned using Sequencher 3.0. Phylogenetic analysis was performed using Paup 4.0b8, (Swofford 1999). Bootstrap analysis (1000 replicates) was

performed using a parsimonious heuristic search with random addition of sequences (1000 replicates), tree bisection-reconnection, branch swapping and MULPAR effective. The most parsimonious distance tree is presented with the bootstrap values.

Comparison of the ITS region of *P. calotropidis* (Fig. 2), showed that *P. calotropidis* was phylogenetically distinct, differing by 45 nucleotide variations (including gap insertions) from the closest phylogenetic relation *Pseudocercospora luzardii* Furlan. & Dianese. The alignment resulted in 135 parsimony informative characters and 39 variable characters that were parsimony uninformative. The consistency index for the dataset was 0.65 and retention index 0.844. The parsimonious heuristic search associated *P. calotropidis* with a clade of anamorphic *Pseudocercospora* species, and this was given 78% support by the bootstrap analysis.

Braun (2000) re-examined the type of *C. calotropidis* and recombined it into *Passalora* noting that it was very variable and intermediate between *Passalora* (that has fasciculate conidiophores and conidia formed singly), *Phaeoramularia* (that has conidia formed in chains) and *Mycovellosiella* (that has secondary superficial hyphae with solitary conidiophores). Furthermore, Braun (2000) cited *C. calotropidis* as an example, which showed that *Passalora*, *Phaeoramularia* and *Mycovellosiella* must be lumped (Crous *et al*. 2001). The synonyms for *P. calotropidis* listed by Crous and Braun (2003) did not include *Pseudocercospora calotropidis* (Ellis & Everh.) Haldar & Ray.

Pseudocercospora accommodates cercosporoid hyphomycetes with pigmented conidiophores and inconspicuous, unthickened, undarkened conidiogenous loci, and differs from *Passalora*, which has conspicuous, somewhat thickened, darkened conidiogenous loci (Crous *et al*. 2001). Braun (2000) described the conidiogenous loci of *Passalora calotropidis* as conspicuous, slightly thickened and somewhat darkened. Haldar and Ray (2001) described the conidial scar at the tip of the conidiophores of *Pseudocercospora calotropidis* as inconspicuous. The conidiophores that we examined had mostly inconspicuous, and not markedly thickened, conidiogenous loci, which indicate *Pseudocercospora*. Furthermore, our molecular analysis indicated that *P. calotropidis* was phylogenetically distinct yet fell within a clade of *Pseudocercospora* species. An examination of morphological characteristics and sequence data from isolates of *P. calotropodis* from around the world might shed further light on its generic position.

Barreto *et al*. (1999) listed the fungal pathogens of rubber bush and discussed their potential as biocontrol agents. One of these pathogens, *Phaeoramularia calotropidis* (Ellis & Everh.) Kamal, Moses & Chaudhary (= *Passalora calotropidis* (Ellis & Everh.) U. Braun), was mentioned as having been spread or introduced with its host, *Calotropis procera*, throughout the tropics of Central and South America but with no confirmed reports in Australasia. Its absence from Australasia may have been because no mycological study of this host has been undertaken in this region (Barreto *et al*. 1999).

The severity of the disease caused by *P. calotropidis* in northern Queensland indicates that it may have potential as a mycoherbicide for calotrope. The most immediate work that needs to be done is the fulfilment of Koch's postulates and the development of a method to inoculate potted plants.

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