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Novel species of *Cercospora* and *Pseudocercospora* (Capnodiales, Mycosphaerellaceae) from Australia

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ABSTRACT

Novel species of *Cercospora* and *Pseudocercospora* are described from Australian native plant species. These taxa are *Cercospora ischaemi* sp. nov. on *Ischaemum australe* (Poaceae); *Pseudocercospora airliensis* sp. nov. on *Polyalthia nitidissima* (Annonaceae); *Pseudocercospora proiphydis* sp. nov. on *Proiphys amboinensis* (Amaryllidaceae); and *Pseudocercospora jagerae* sp. nov. on *Jagera pseudorhus* var. *pseudorhus* (Sapindaceae). These species were characterised by morphology and an analysis of partial nucleotide sequence data for the three gene loci, ITS, LSU and EF-1 α . Recent divergence of closely related Australian species of *Pseudocercospora* on native plants is proposed.

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Introduction

Cercospora Fresen. and *Pseudocercospora* Speg. are large genera of mostly plant pathogenic fungi, containing over 3500 and 1600 species names respectively (www.mycobank.org). The delimitation of species within *Cercospora* and *Pseudocercospora*, as well as the circumscription of the genera, has changed since their establishment by Fresenius (1863) and Spegazzini (1910), respectively. These changes have resulted from critical evaluation of the taxonomic importance of morphology (Chupp 1954; Crous & Braun 2003), host specificity (Deighton 1976, 1979) and molecular phylogeny (Crous et al. 2000). Together these characteristics provide the basis for a stable

taxonomy that resolves both generic and species limits for *Cercospora* (Groenewald et al. 2013) and *Pseudocercospora* (Crous et al. 2013).

The Australian cercosporoid fungi, including *Cercospora* and *Pseudocercospora*, have never been comprehensively revised. Since the application of phylogenetic analysis of DNA sequence data to fungal systematics in the last decade, several new Australian species of *Cercospora* (Crous et al. 2013) and *Pseudocercospora* (Shivas et al. 2009a, 2009b; 2010; Crous et al. 2010a, 2010b 2011; McTaggart et al. 2010) have been discovered and named. An examination of recently collected specimens of leaf spots on some native plants from eastern Queensland, revealed further novel species of *Cercospora* and *Pseudocercospora*.

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Materials and methods

Isolates

Conidia and conidiophores were removed with the tip of a scalpel from leaf spots on freshly pressed herbarium specimens of four native Australian plants, viz., *Ischaemum australe*, *Jagera pseudorhus* var. *pseudorhus*, *Polyalthia nitidissima*, and *Proiphys amboinensis*. The conidia were suspended in a droplet of water and spread on plates of potato dextrose agar (PDA) amended with 100 µg ml⁻¹ streptomycin sulphate, and incubated at room temperature (23–25 °C) under ambient light. From these plates single conidial colonies were established on PDA after 1–3 d.

DNA extraction, amplification and phylogeny

Mycelia were scraped off PDA cultures and macerated with 0.5 mm glass beads (Daintree Scientific) in a Tissue Lyser (QIAGEN). Genomic DNA was then extracted with the Genra Pure-gene DNA Extraction kit (QIAGEN (Venlo, Limburg, The Netherlands)) according to the manufacturer's instructions.

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA) was amplified with the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). The large subunit region of rDNA was amplified with LR0R and LR6 (Vilgalys & Hester 1990). Part of the translation elongation factor 1- α (EF-1 α) gene was amplified with the primers EF1-728 F (Carbone & Kohn 1999) and EF2 (O'Donnell et al. 1998). All PCRs were performed with high fidelity Phusion enzyme (New England Biolabs) according to the manufacturer's instructions. The PCRs were performed with the following annealing temperatures: ITS and LSU at 62 °C, and EF-1 α at 60 °C. PCR products were sent to Macrogen Korea for purification and direct sequencing.

The systematic placement of the new taxa described herein is based on the phylogeny of the LSU region recovered by Crous et al. (2013). The 14 clades recovered by Crous et al. (2013) were represented by 49 species. Type species were used when available. The four newly described cercosporoid fungi and three previously described species from Australia were also included. *Saccharomyces cerevisiae* was selected as an outgroup taxon as used by Crous et al. (2013). The LSU sequences were aligned in MAFFT (Katoh et al. 2009) (available: <http://www.ebi.ac.uk/Tools/msa/mafft/>), and analysed with two phylogenetic criteria, maximum likelihood (ML) and Bayesian inference. Maximum likelihood was implemented as a search criterion in RAxML (Stamatakis 2006). GTRGAMMA was specified as the model of evolution for nucleotide sequence data. The RAxML analyses were run with a rapid Bootstrap analysis (command -f a) using a random starting tree and 1000 ML bootstrap replicates. A Markov Chain Monte Carlo (MCMC) search in a Bayesian analysis was conducted with MrBayes (Ronquist & Huelsenbeck 2003). Four runs, each consisting of four chains, were implemented for 5 million generations. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 500 generations and trees were saved every 500 generations. Convergence of the Bayesian analysis was confirmed using

AWTY (Nylander et al. 2008) (available at: ceb.csit.fsu.edu/awty/) and used to calculate a burnin. The final topology was summarised from 4200 trees generated in four runs. The ML and Bayesian analyses were run three times to test accuracy.

Taxonomy

Morphological descriptions are based on fungal structures in *plantae*. Fungal structures were mounted on glass slides in clear lactic acid (100 % v/v). At least 30 measurements ($\times 1000$) were made if possible, with values expressed as ranges and outliers given in parentheses. Microscopic observations were made with a Leica DM 2500 compound microscope using differential interference contrast illumination and a Leica DFC 550 camera. Colony colours were determined on PDA and/or oatmeal agar (OMA) at 25 °C in the dark after 4 wk, using the colour charts of Rayner (1970). Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org).

Results

Phylogeny

The tree topologies recovered by ML and Bayesian inference were identical (Fig 1) and represented the 14 major clades defined by Crous et al. (2013). *Cercospora eremochloae* (HM235406) on *Eremochloa* (Poaceae) and one isolate (BRIP 56010) on *Ischaemum* (Poaceae), were sister to several species of *Cercospora*, which was non-monophyletic in the study by Crous et al. (2013). The other three isolates (BRIP 58545, BRIP 58549 and BRIP 58550) belonged to a well-supported clade (90 % bootstrap; 1.0 posterior probability) that corresponded to *Pseudocercospora* s. str. (Crous et al. 2013) and contained the type species, *Pseudocercospora vitis*.

Taxonomy

Based on the phylogenetic analysis and morphological characteristics, four novel species are described.

Cercospora ischaemi R.G. Shivas, Marney & McTaggart, sp. nov. Fig 2.

MycoBank MB 809257.

Etymology. Named after the host plant genus, *Ischaemum*.

Leaf spots amphigenous, linear to narrowly ellipsoidal, bordered by parallel leaf veins, up to 1 cm long and 1 mm wide, dark reddish brown to dark brown, with a narrow yellowish diffuse halo up to 0.5 mm wide, scattered, of similar appearance on upper and lower leaf surfaces. *Caespituli* epiphyllous, inconspicuous. *Mycelium* internal. *Stromata* absent. *Conidiophores* erumpent through the cuticle, in loose fascicles of 2–5, erect, subcylindrical, 10–60 \times 3–5 µm, sometimes geniculate, 0–1 septate, pale brown to subhyaline, thin-walled, smooth; conidiogenous cells, terminal, subcylindrical, 10–60 µm, sympodial, hyaline to subhyaline, smooth, polyblastic; conidiogenous loci conspicuous, flat, circular, thickened, and darkened, 1.5–2.0 µm wide. *Conidia* obclavate,

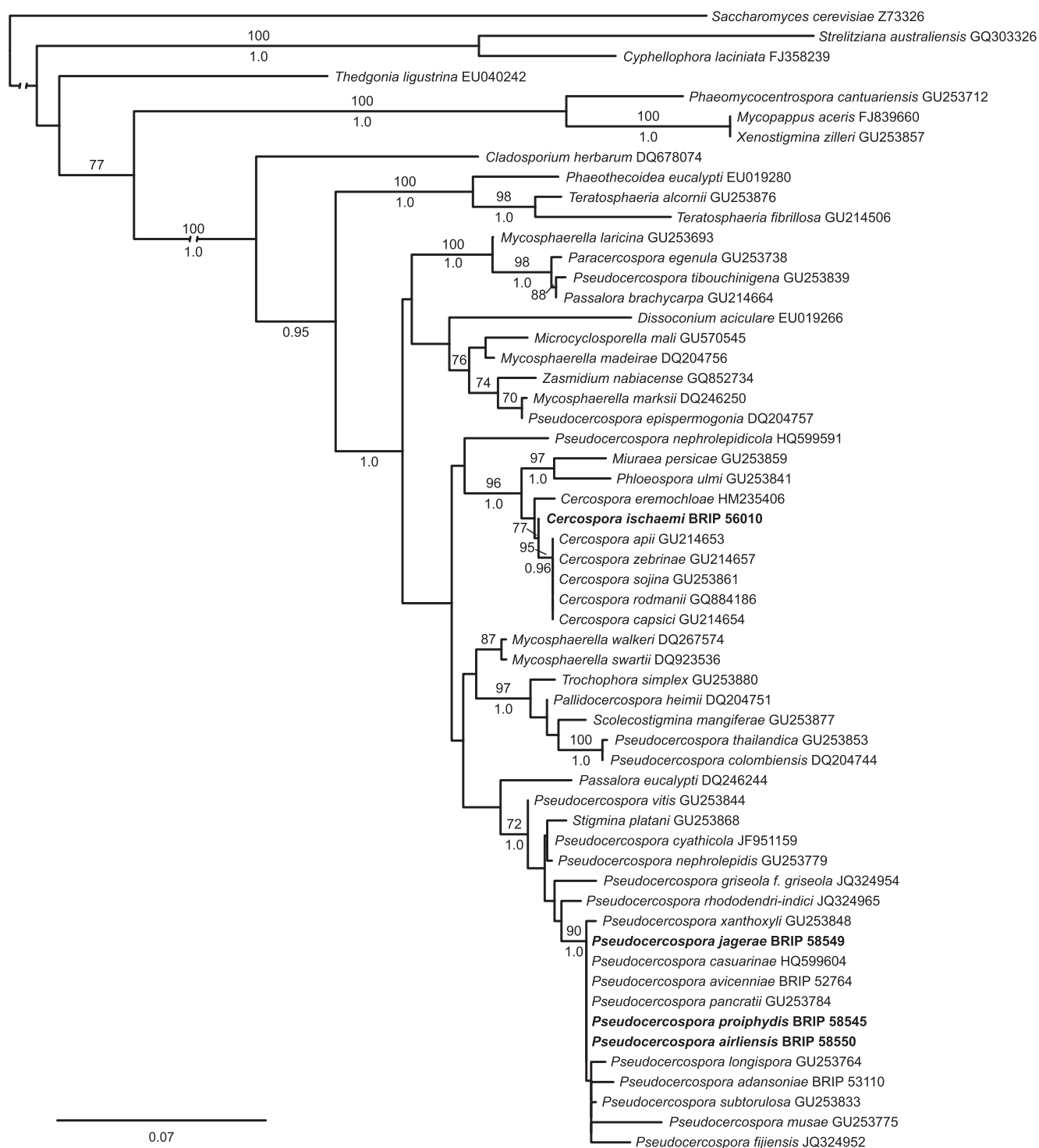


Fig 1 – Phylogram obtained from a maximum likelihood search in RAxML based on the nuclear LSU region of rDNA. Bootstrap support (≥70 %) values from 1000 replicates above nodes. Posterior probabilities (≥0.95) from 4200 trees in a Bayesian search are shown below nodes.

with a narrowly obconically truncate base and attenuated towards the apex, 60–120 × 4–5 μm, 1–3-septate, hyaline, smooth, thin-walled; hila thickened, and darkened-refractive, 1.5–2.0 μm wide.

Culture characteristics — (in the dark, 23 °C, after 4 wk): Colonies on PDA agar 5 cm diam., flat with scarce aerial mycelium, pale mouse grey with irregular pale, and darker patches, margin irregularly crenate; reverse fuscous black, and paler

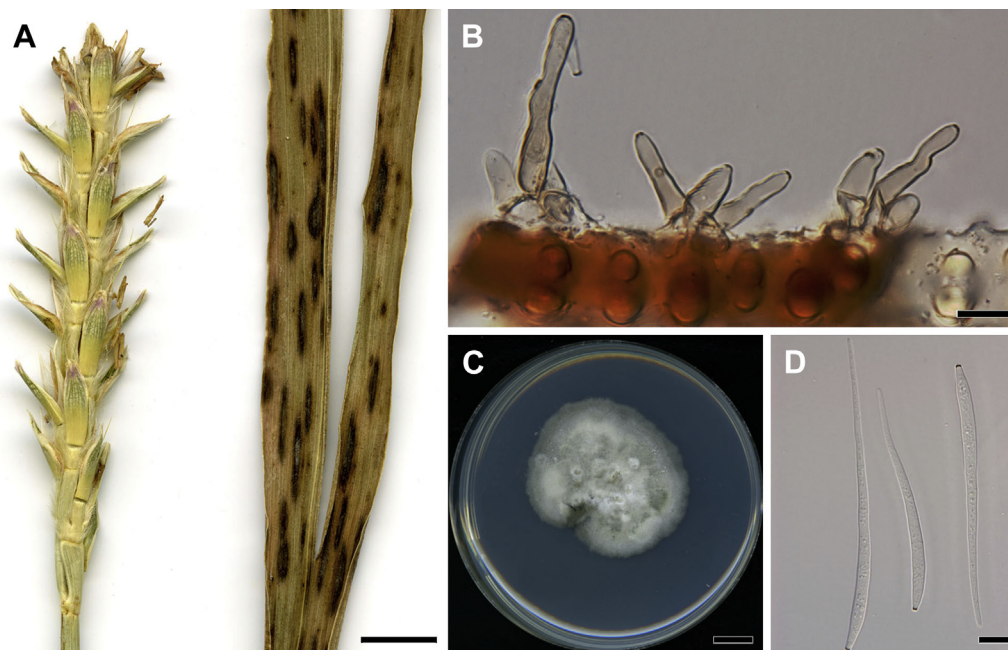


Fig 2 – *Cercospora ischaemi* (from holotype BRIP 56010). (A). Leaf spots on *Ischaemum australe* (right) and healthy inflorescence (left); (B). Conidiophores; (C). Colony on PDA after 4 weeks; (D). Conidia. Bars: (A) = 1 cm; (B, D) = 10 μm; (C) = 1 cm.

towards the margin. On OMA 2.5 cm diam., flat with scarce aerial mycelium, pale mouse grey, zonate, faintly rosy vinaceous towards the margin, reverse fuscous black; on malt extract agar 3 cm diam., flat, radially wrinkled, margin entire, grey olivaceous, reverse black.

Typus. AUSTRALIA, Northern Territory, Victoria River Downs, S 15° 36' 4.7356" E 131° 12' 48.5903", on leaves of *Ischaemum australe* (Poaceae), 20 Apr. 2012, R.G. Shivas (holotype BRIP 56010; includes ex-type culture), ITS sequence GenBank KM055428, LSU sequence GenBank KM055432.

Other specimens examined. AUSTRALIA, Western Australia, unnamed swamp about 140 km northwest of Kununurra, S 14° 41.698', E 128° 02.344', M.P. Weinert (BRIP 51367).

Notes — *Cercospora ischaemi* is known from two collections in northern Australia. A specimen (BRIP 4473) on *Ischaemum australe* from Queensland had comparable symptoms to *C. ischaemi*, but morphological examination indicated the fungus differed and warrants further study. *Cercospora ischaemi*, together with *C. eremochloae*, are recently described species from native Australian tropical grasses (Crous et al. 2011). *Cercospora coniogrammes* (JX143583) on *Coniogramme* (Pteridaceae) from Australia had the highest identity (96%, 496/518 identical base pairs) to *C. ischaemi* in a BLAST search of the ITS region of rDNA.

Pseudocercospora airliensis R.G. Shivas, Marney & Y.P. Tan, sp. nov. Fig 3. MycoBank MB 809258.

Etymology. Named after Airlie Beach.

Leaf spots amphigenous, irregular, sometimes bordered by leaf veins, 1–4 mm diam., with pale tan to dark grey centres

and irregular black borders up to 0.5 mm wide, often surrounded by a diffuse pale yellowish irregular halo that extends for 2–15 mm, scattered, of similar appearance on upper and lower leaf surfaces. *Caespituli* mostly hypophyllous, dense, dark, punctiform. *Mycelium* internal. *Stromata* erumpent from centres of leaf spots, reddish brown, up to 80 μm diam. and 70 μm high. *Conidiophores* in loose fascicles of 5–30, erect, subcylindrical, geniculate to sinuous, 20–70 × 3–4 μm, sometimes with one or more branches, 0–8-septate, pale to medium brown, smooth, thin-walled; conidiogenous cells, terminal, integrated, subcylindrical to geniculate, 10–20 μm, hyaline, smooth, monoblastic or sometimes polyblastic, proliferating percurrently; conidiogenous loci inconspicuous. *Conidia* cylindrical, straight or curved to flexuous, with a narrowly obconically truncate base and attenuated towards the apex, 25–60 × 2–4 μm, 2–7-septate, subhyaline to pale brown, smooth, thin-walled; hila truncate, 1–2 μm wide, unthickened, and not darkened.

Culture characteristics — (in the dark, 23 °C, after 4 wk): Colonies on PDA up to 2 cm diam., flat with scarce aerial mycelium, cream to faintly rosy vinaceous; reverse dark brown, paler towards the margin.

Typus. AUSTRALIA, Queensland, Airlie Beach, Mount Conway Range, S 20° 20' E 148° 46', on leaves of *Polyalthia nitidissima* (Annonaceae), 4 Apr. 2013, R.G. & M.D.E. Shivas (holotype BRIP 58550; includes ex-type culture), ITS sequence GenBank KM055429, LSU sequence GenBank KM055433, EF-1 α sequence GenBank KM055436.

Notes — *Polyalthia nitidissima* is a tree species found in dry rainforests and along watercourses in eastern and northern Australia, extending to New Caledonia and New Guinea. One other species of *Pseudocercospora*, *P. polyalthiae*, has

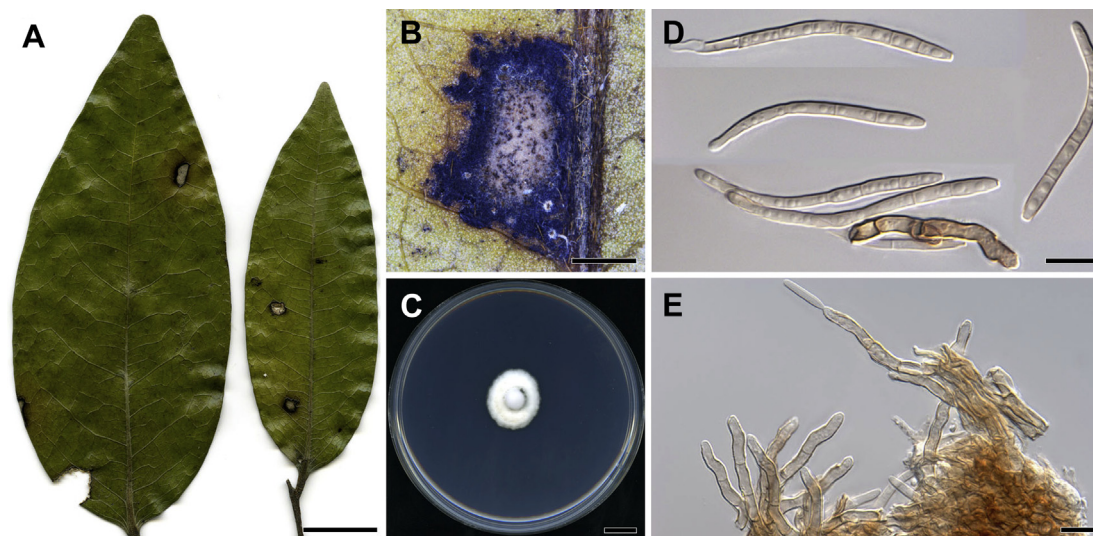


Fig 3 – *Pseudocercospora airliensis* (from holotype BRIP 58550). (A, B). Leaf spots on *Polyalthia nitidissima*; (C). Colony on PDA after 4 weeks; (D). Conidia; (E). Conidiophores. Bars: (A, C) = 1 cm; (B) = 1 mm; (D, E) = 10 μ m.

been recorded on *Polythia* (type on *Polythia suberosa*, India) (Yen et al. 1982). *Pseudocercospora polyalthiae* lacks stromata and has simple conidiophores not in fascicles, which differ from *P. airliensis*. Braun & Crous (2008) provided a key for the cercosporoid fungi on hosts in the *Annonaceae*. Using this key led to *P. aethiopicae* (type on *Xylopia aethiopicae*, Sierra Leone), which differs from *P. airliensis* by having simple, continuous or 1–2 septate conidiophores. *Pseudocercospora tereticornis* (GQ852770 and GQ85277) on *Eucalyptus* (*Myrtaceae*) from Australia had the highest identity (99 %, 570/577 identical base pairs) to *P. airliensis* in a BLAST search of the ITS region of rDNA.

Pseudocercospora proiphydis R.G. Shivas, Marney & Y.P. Tan, sp. nov. Fig 4.
Mycobank MB 809297.

Etymology. Named after the host plant genus, *Proiphys*.

Leaf spots amphigenous, circular to ellipsoidal or irregular, up to 3 cm diam., pale brown with diffuse irregular halo that extends for up to 1 cm, scattered, of similar appearance on both leaf surfaces. **Caespituli** mostly hypophyllous, abundant on leaf spots, dense, dark, punctiform. **Mycelium** internal. **Stromata** erumpent from stomata, dark brown, up to 60 μ m high and 50 μ m wide. **Conidiophores** in fascicles of up to 60, arising from stomata, erect, subcylindrical, slightly tapered towards the rounded apex, straight or slightly curved to geniculate, 10–70 \times 3–6 μ m, unbranched, 0–5-septate, subhyaline to pale brown, smooth, thin-walled; conidiogenous cells, terminal, integrated, subcylindrical, 20–60 μ m, hyaline, smooth, monoblastic or polyblastic, proliferating percurrently; conidiogenous loci slightly thickened, 1–2 μ m. **Conidia** cylindrical to narrowly lageniform, straight, obconically truncate at the base and rounded at the apex, 25–120 \times 2–6.5 μ m, 5–10-septate, subhyaline to pale brown, smooth, thin-walled; hila sometimes slightly thickened, 1–2 μ m wide.

Culture characteristics — (in the dark, 23 °C, after 4 wk): Colonies on PDA up to 1 cm diam., domed with scarce aerial mycelium, olivaceous grey.

Typus. AUSTRALIA, Queensland, Shute Harbour, in deep shade near forest walk lookout, 20° 16' 44" S, 148° 47' 42" W, on leaves of *Proiphys amboinensis* (*Amaryllidaceae*), 3 Apr. 2013, R.G. & M.D.E. Shivas (holotype BRIP 58545; includes ex-type culture), ITS sequence GenBank KM055430, LSU sequence GenBank KM055434, EF-1 α sequence GenBank KM055437.

Other specimens examined. AUSTRALIA, Queensland, Mt Nebo, on leaf spots on *Proiphys cunninghamii*, J.H. Simmonds, 15 Apr. 1972, J.H. Simmonds (BRIP 8746); Mt Nebo, on leaf spots on *P. cunninghamii*, R.G. Shivas, 1 Mar. 1972, J.H. Simmonds (BRIP 39565).

Notes — *Proiphys amboinensis* is a perennial herbaceous bulbous plant found in rainforests of subtropical eastern Australia and Southeast Asia. *Pseudocercospora proiphydis* is morphologically similar to *P. pancratii* (Braun & Castañeda 1991), which has been reported on several genera in the *Amaryllidaceae* (Crous & Braun 2003). The molecular sequence data showed that *P. proiphydis* and *P. pancratii* differ genetically in the ITS region (GU269759: 88 % identical over 97 % query coverage) and the EF-1 α region (GU384470: 82 % identical over 97 % query coverage). Moreover, slightly thickened conidiogenous loci are not present in *P. pancratii*. These results indicate that *P. pancratii* may represent a complex of closely related species. *Pseudocercospora jagerae* R.G. Shivas, Marney & Y.P. Tan, sp. nov. Fig 5.
Mycobank MB 809298.

Etymology. Named after the host plant genus, *Jagera*.

Leaf spots amphigenous, of similar appearance on both leaf surfaces, irregular, bordered by veins, 1–4 mm diam., with grey centres and purplish brown borders c. 0.5 mm wide,

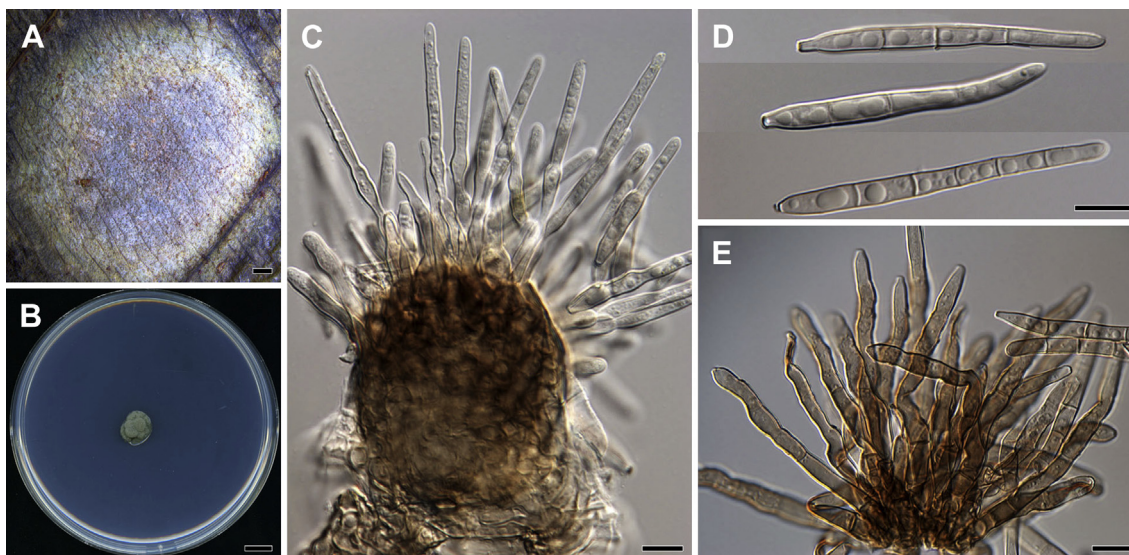


Fig 4 – *Pseudocercospora proiphydis* (from holotype BRIP 58545). (A). Leaf spot on *Poiraphys amboinensis*; (B). Colony on PDA after 4 weeks; (C, E). Stromata and conidiophores; (D). Conidia. Bars: (A) = 1 mm; (B) = 1 cm; (C–E) = 10 μ m.

with a diffuse pale yellowish green irregular halo that extends for up to 1 cm. *Caespituli* hypophyllous, abundant on leaf spots, dense, dark, punctiform. Mycelium internal. *Stromata* erumpent from stomata, dark brown, up to 60 diam. *Conidiophores* in dense fascicles, arising from stromata, erect, subcylindrical to geniculate-sinuous, slightly tapered towards the apex, 10–30 \times 2–3 μ m, unbranched, 0–3-septate, subhyaline to pale brown, smooth, thin-walled; conidiogenous cells integrated, terminal, subcylindrical, 10–25 μ m, polyblastic, proliferating sympodially, conidiogenous loci inconspicuous. *Conidia* formed singly, cylindrical to narrowly obclavate, straight

curved to flexuous, truncate at the base and rounded at the apex, 35–75 \times 3–6.5 μ m, 1–6-septate, subhyaline to pale brown, smooth, thin-walled; hila unthickened and not darkened, 1–2 μ m wide.

Culture characteristics — (in the dark, 23 °C, after 4 wk): Colonies on PDA up to 2.5 cm diam., flat with scarce aerial mycelium, grey olivaceous (becoming olivaceous with age).

Typus. AUSTRALIA, Queensland, Mt Conway, 20° 16' 44" S, 148° 47' 42" W, on leaves of *Jagera pseudorhus* var. *pseudorhus* (*Sapindaceae*), 3 Apr. 2013, R.G. & M.D.E. Shivas (holotype BRIP 58549;

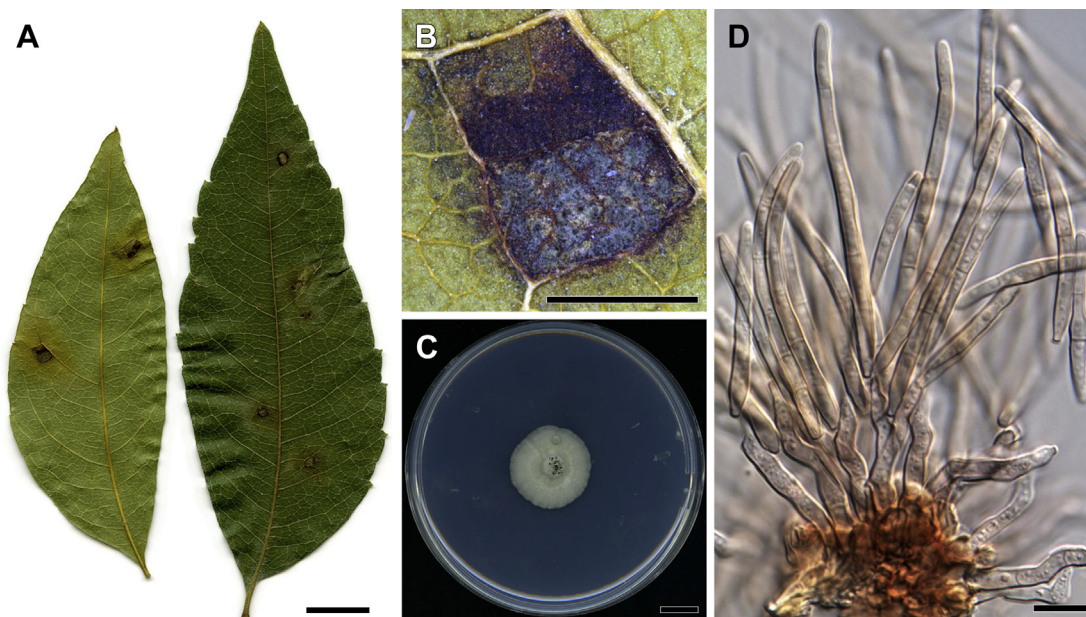


Fig 5 – *Pseudocercospora jagerae* (from holotype BRIP 58549). (A). Leaf spot on *Jagera pseudorhus* var. *pseudorhus* (abaxial leaf surface at left, adaxial leaf surface at right); (B). Leaf spot; (C). Colony on PDA after 4 weeks; (D). Conidiophores and conidia. Bars: (A, C) = 1 cm; (B) = 1 mm; (D) = 10 μ m.

includes ex-type culture), ITS sequence GenBank KM055431, LSU sequence GenBank KM055435, EF-1 α sequence GenBank KM055438.

Notes — The host of *P. jagerae* is an endemic rainforest tree species that occurs in eastern Australia and the island of New Guinea. Only one species of *Pseudocercospora*, *P. dodonaeae*, has been reported from Australia on a host in the Sapindaceae (Beilharz & Pascoe 2004). Chupp (1954) listed nine species of *Cercospora* (including two species as homonyms under *C. alophylis*), on hosts in the Sapindaceae. Six of these species, *P. alophylis*, *P. allophylina*, *P. cupaniae*, *P. mitteriana*, *P. sapindi-emarginati*, and *P. thouinia*, were subsequently transferred to *Pseudocercospora* (Crous & Braun 2003). None of these species occurs on *Jagera*, and none have been reported from Australia or New Guinea. *Pseudocercospora fuligena* (GU214675), *P. chengtzensis* (GU214672) and *P. atomarginalis* (GU214671) had the highest identities (99 %, 522/523 identical base pairs) to *P. jagerae* in a BLAST search of the ITS region of rDNA. These three taxa, which differed by one base-pair in the ITS region occur on hosts in the Solanaceae.

Discussion

The newly described species of *Pseudocercospora* from Australian native plants were recovered in a monophyletic group considered *Pseudocercospora* s. str. by Crous et al. (2013). This clade, typified by *Pseudocercospora vitis*, is morphologically characterised by pale to dark brown conidiophores, with an inconspicuous conidial scar, and conidia that are usually scoliosporous with an unthickened and not darkened hilum. Crous et al. (2013) determined species of *Pseudocercospora* were mostly specific to a single host species and rarely pathogenic on several hosts. The Australian species of *Pseudocercospora* appear also to be host specific, and are differentiated by morphology and the ITS region of rDNA. The LSU region of rDNA was identical for the Australian species, *Pseudocercospora arieliensis*, *Pseudocercospora proiphydis*, *Pseudocercospora jagerae*, *Pseudocercospora avicenniae* and *Pseudocercospora casuarinae*, which were recovered in a polytomy. One interpretation of the apparent close relationship between Australian species of *Pseudocercospora*, on diverse and unrelated native plant species, is that these fungi diverged as opportunistic pathogens from a recent common ancestor. Groenewald et al. (2013) discussed this possibility as an explanation for the rapid radiation of *Cercospora*, which was enabled by the production of cercosporin.

Future taxonomic studies on *Pseudocercospora* and other cercosporoid fungi in Australia and elsewhere should be based on morphology, sequence data and host association (Groenewald et al. 2013). Cultures of these fungi are essential for molecular based studies, yet unfortunately these are rarely established and preserved in collections. The Australian flora boasts over 30 000 species of vascular plants (Orchard 1999) of which about 89 % are native or endemic (Hnatiuk 1990). It is possible that the number of undiscovered and unidentified species of *Pseudocercospora* and other cercosporoid fungi in Australia will be substantial.

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