Cryptic diversity found in Didymellaceae from Australian native legumes

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Abstract

Ascochyta koolunga (Didymellaceae, Pleosporales) was first described in 2009 (as Phoma koolunga) and identified as the causal agent of Ascochyta blight of Pisum sativum (field pea) in South Australia. Since then A. koolunga has not been reported anywhere else in the world, and its origins and occurrence on other legume (Fabaceae) species remains unknown. Blight and leaf spot diseases of Australian native, pasture and naturalised legumes were studied to investigate a possible native origin of A. koolunga.

Ascochyta koolunga was not detected on native, naturalised or pasture legumes that had leaf spot symptoms, in any of the studied regions in southern Australia, and only one isolate was recovered from P. sativum. However, we isolated five novel species in the Didymellaceae from leaf spots of Australian native legumes from commercial field pea regions throughout southern Australia. The novel species were classified on the basis of morphology and phylogenetic analyses of the internal transcribed spacer region and part of the RNA polymerase II subunit B gene region. Three of these species, Nothophoma garlbiwalawarda sp. nov., Nothophoma naiawu sp. nov. and Nothophoma ngayawang sp. nov., were isolated from Senna artemisioides. The other species described here are Epicoccum djirangnandiri sp. nov. from Swainsona galegifolia and Neodidymellipsoidis tinkyukuku sp. nov. from Hardenbergia violacea. In addition, we report three new host-pathogen associations in Australia, namely Didymella pinodes on S. artemisioides and Vicia cracca, and D. lethalis on Lathyrus tingitanus. This is also the first report of Didymella prosopidis in Australia.

* These authors contributed equally to this paper.
Keywords
Alternative host, multilocus phylogeny, pathogen reservoir

Introduction

The Didymellaceae was established to accommodate Ascochyta, Didymella, and other allied Phoma-like genera (de Gruyter et al. 2009). To date, more than 5,400 species from 31 genera have been recorded, including recently established genera such as Dimorphoma and Macroascochyta (Hou et al. 2020). Species of Didymellaceae are cosmopolitan and occupy a broad range of environments. Many species are plant pathogens that cause leaf and stem lesions, often with a broad host range (Aveskamp et al. 2009; Aveskamp et al. 2010; Chen et al. 2015b). Multilocus phylogenetics and a polyphasic approach to classify species have helped to revise taxa and refine systematic relationships in the Didymellaceae (Aveskamp et al. 2009, de Gruyter et al. 2009; Aveskamp et al. 2010; Chen et al. 2015a, de Gruyter 2012; Hou et al. 2020).

In Australia, reports of taxa in the Didymellaceae mostly refer to plant pathogenic species, particularly on crop and pasture legumes (Fabaceae). In Australia, the disease Ascochyta blight of Pisum sativum (field pea) is typically caused by three fungal species, Ascochyta koolunga, Didymella pinodella, and D. pinodes. A fourth species, Ascochyta pisi, is very rarely isolated. One species in particular, A. koolunga, is an important part of the Ascochyta blight disease complex of field pea in South Australia (Davidson et al. 2009a). First described in 2009, A. koolunga (syn. Phoma koolunga) had spread across southern Australia and had been detected in Victoria and Western Australia by 2015 (Davidson et al. 2011; Tran et al. 2015a).

Molecular techniques are now routinely used to understand the genetic diversity and population structure of Didymellaceae (Aveskamp et al. 2010; Salam et al. 2011, de Gruyter 2012; Chen et al. 2015a, Hou et al. 2020). To date, there has not been a systematic inventory of leaf spot pathogens associated with Australian native legume species despite international reports from a diversity of countries on Ascochyta blight since 2009 (Le May et al. 2009; Mathew et al. 2010; Panicker and Ramraj 2010; Skoglund et al. 2011; Soylu and Dervis 2011; Gaurilcikiene and Viciene 2013; Liu et al. 2013; Ahmed et al. 2015; Liu et al. 2016). Ascochyta koolunga is only known to occur in Australia, which suggests an Australasian origin, with perhaps an association with native legume species. The aim of this study was to determine the species of Didymellaceae associated with leaf spot diseases, and to investigate possible native sources of A. koolunga. To this end we collected legume specimens from both cultivated and neighbouring natural ecosystems. In particular, we collected specimens from Australian native, pasture and naturalised legumes in the field pea growing regions of eastern and southern Australia.
Materials and methods

Sample collection and culturing

Samples of leaf tissue displaying leaf spot disease symptoms on legumes were obtained from 22 field pea trial sites, from the immediate surrounds of experimental and commercial crops and roadsides around crops in field pea growing regions of southern Australia. In total, 124 samples (stems with multiple leaves and more rarely seed pods and flowers) were collected during four separate 4–5 day (d) periods in August, September and October 2017. In addition to trial sites, local agronomists were contacted to obtain approval to allow access to growers’ properties in Eyre Peninsula (South Australia) and Horsham (Victoria).

The national parks, or conservation areas, nearest to the field pea sampling sites were identified prior to field trips and permits were obtained to enable collections of samples from native plants that exhibited leaf disease symptoms within these neighbouring natural ecosystems. Leaf disease samples were also collected from two botanic gardens, Adelaide Botanic Garden, Adelaide, South Australia and the Australian Botanic Garden, Mount Annan, New South Wales. Plants with leaf spots were photographed in the field with a Samsung galaxy S5 or S8 mobile phone camera and the GPS locations recorded. Representative leaf samples were placed in plastic bags, labelled and stored at 4 °C.

Within 5 d of collection, leaf specimens were surface disinfected by spraying with 70% v/v ethanol and blotted dry with fresh, non-sterilised tissue paper. Excised leaf pieces were placed on plates of potato dextrose agar (PDA) (Oxoid) acidified by supplementation with 1 ml of 85% v/v lactic acid per litre (APDA) to minimise bacterial contamination. Incubation was under a 12 hour (h) black and fluorescent light /12 h dark cycle at 22 °C for 7–10 d, when fungal colonies were examined microscopically for pycnidia and conidia. Representative isolates were subcultured onto PDA using hyphal tips and deposited in the culture collection of the Queensland Plant Pathology Herbarium (BRIP).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from 7 d old mycelium grown on PDA from the subculture isolates using the FastDNA Kit (Q-biogene Inc. Irvine, California, USA) according to the manufacturer’s instructions. A section of DNA from the internal transcribed spacer (ITS) region was amplified with the primers ITS1 and ITS4 (White et al. 1990), and the partial region of the RNA polymerase II subunit B (rpb2) gene was amplified with the primers RPB2-5F2 (Sung et al. 2007) and RPB2-7cR (Liu et al. 1999). The PCR conditions were as described by White et al. (1990) for ITS and O’Donnell et al. (2007) for rpb2. All PCRs were undertaken
in 25 μl reaction volumes containing the final concentrations; 1 unit of PCR 5X buffer (Promega Corporation, Madison, Wisconsin, USA), 1.6 mM of 25 mM MgCl₂ (Sigma-Aldrich Corporation, Louis, Missouri, USA), 0.025 U/μl of GoTaq™ (Promega), 0.6 mM of primer 1 and primer 2 and 1.6 mM of each dNTP (Promega). The PCR amplicons were purified using ExoSAP-IT (USB Corporation) following the manufacturer’s instructions. The purified amplicons were sent to the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Kensington, NSW), where DNA sequences were determined using an ABI PRISM 3700 DNA Analyser (Applied Biosystems Inc).

Phylogenetic analysis

Forward and reverse sequences were assembled using Geneious v. 11.1.5 (Biomatters Ltd) and deposited in GenBank (Table 1, in bold). The sequences were aligned with selected reference sequences of Didymellaceae (Table 1) using the multiple alignment MAFFT algorithm (Katoh et al. 2009) in Geneious. Neoascochyta desmazieri strain CBS 267.69 was included as the outgroup. The sequences of each locus were aligned separately and manually adjusted where necessary.

Maximum likelihood (ML) analysis was run using the RAxML v. 7.2.8 (Stamatakis and Alachiotis 2010) plug-in in Geneious v. 11.1.5 starting from a random tree topology. The nucleotide substitution model used was general time-reversible (GTR) with a gamma-distributed rate variation. The Bayesian analysis was performed using the MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003) plug-in in Geneious v. 11.1.5. To remove the need for a priori model testing, the Markov chain Monte Carlo (MCMC) analysis was set to sample across the entire GTR model space with a gamma-distributed rate variation across the nucleotide sites. Ten million random trees were generated using the MCMC procedure with four chains. The sample frequency was set at 2000 and the temperature of the heated chain was 0.1. “Burn-in” was set at 25%, after which the log-likelihood values were stationary.

Morphology

Fungal isolates were cultured on four media types; PDA, oatmeal agar (OA), malt extract agar (MEA) (Boerema et al. 2004; Chen et al. 2015a), and carnation leaf agar (CLA). The colonies were measured at 7 d, and morphology examined after 12–14 d incubation in the same light and temperature conditions described above. Images of the colonies were captured by an Epson Perfection V700 scanner at a 300 dpi resolution. Colony colour was determined on surface and reverse using the colour charts of Rayner (1970). Isolates were characterised microscopically from the PDA plates. Lactic acid (100 % v/v) was used as the mounting fluid. Specimens were examined using a Leica DM5500B compound microscope with a Leica DFC 500 camera fitted to capture images under Nomarski differential interference contrast illumination. Micromorphological measurements and descriptions of pycnidia,
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Pycnidial wall cells and conidia were taken from up to 20 samples, and septation and colour recorded. Images of pycnidia were taken from CLA plates using a Leica M165C stereo microscope and Leica DFC 500 camera. The NaOH spot test on MEA culture plates helped distinguish taxa (Boerema et al. 2004).

Results

From 124 samples of legumes collected at 22 locations, 194 isolates were obtained of which 54 isolates were identified as Didymellaceae by ITS sequences. Of these, 36 isolates were further sequenced (rpb2 locus). Duplicate isolates were excluded where they were from the same host species, which left 18 isolates for multilocus sequence analysis and inclusion in the phylogenetic analysis.

Phylogeny

A multilocus sequence analysis based on the ITS region and partial region of the rpb2 gene was used to infer the relationship of the 18 isolates and recognised species in Didymellaceae (Table 1). The resulting concatenated aligned dataset comprised 124

Table 1. Didymellaceae isolates examined in this study. Novel taxa and newly generated sequences are indicated in bold.

| Species                        | Strain   | Host           | Locality     | GenBank accessions |
|--------------------------------|----------|----------------|--------------|--------------------|
|                                |          |                |              | ITS               | rpb2               |
| Ascochyta astragalina          | CBS 113797 | Lathyrus vernus | Sweden       | KT389482           | MT018257           |
| Ascochyta breutingeriorum      | CBS 144957T | Soil         | The Netherlands | MN823581           | MN824606           |
| Ascochyta coronillar-emeri     | MFLUCC 13-0820T | Hippocrepis emerus | Italy       | MH069661           | MH069679           |
| Ascochyta fabae                | CBS 524.77 | Pisum sativus  | Belgium      | GU237880           | MT018241           |
| Ascochyta herbaria             | CBS 629.97 | Water         | USA, Montana, Missoula | GU237898 | KP330421 |
| Ascochyta koolanga             | DAR 78535T | Pisum sativum | Australia, SA, Minnipa | EU338416 | EU874849 |
|                                | BRIP 70265 | Pisum sativum | Australia, SA, Riverton | MN567671 | MG094922 |
|                                | BRIP 69590 | Pisum sativum | Australia, SA, Mundulla | MN567672 | MG094923 |
| Ascochyta lentis               | CBS 370.84 | Lens culinaris | Unknown      | KT389474           | MT018246           |
| Ascochyta medicagincola        | CBS 112.53T | Medicago sativa | USA        | GU237749           | MT018251           |
| Ascochyta nigripycnidia        | CBS 116.96T | Vicia cracca  | Russia       | GU237756           | MT018253           |
| Ascochyta phaeae               | CBS 184.55T | Phoca alpina  | Switzerland  | KT389475           | MT018255           |
| Ascochyta pilosella            | CBS 583.97T | Clintonia uniflora | Canada     | MN973590           | MT018258           |
| Ascochyta pisii                | CBS 122785 | Pisum sativum | The Netherlands | GU237763 | MT018244 |
| Ascochyta rabiei               | CBS 237.37T | Cicer arietinum | Bulgaria    | KT389479           | MT018256           |
| Ascochyta rosea                | MFLUCC 13-0063T | Rubus ulmifolius | Italy      | KY406751           | KY514409           |
| Ascochyta syringae             | CBS 545.72T | Syringa vulgaris | The Netherlands | KT389483 | MT018245 |
| Ascochyta verubelis            | CBS 876.97 | Silene sp.     | The Netherlands, Wageningen | GU237909 | KT389561 |
| Ascochyta viciae               | CBS 451.68 | Vicia sepium  | The Netherlands, Baarn, Prangmacht | KT389484 | KT389562 |
| Ascochyta viciae-pannonicae    | CBS 254.92 | Vicia pannonica | Czechoslovakia | KT389485 | MT018250 |
| Ascochyta viciae-villoae       | CBS 255.92 | Vicia villoa  | Czechoslovakia | MN973584 | MT018249 |
| Didymella americana            | CBS 185.85 | Zea mays       | USA, Georgia | FJ426972 | KT389594 |
| Didymella arrientina           | CBS 253.80 |            | Germany      | KT389498           | KT389595           |
| Didymella arachidisola         | CBS 333.75T | Anachis hypogaea | South Africa, Cape Province | GU237833 | KT389598 |
| Didymella aurita               | CBS 269.93T | Medicago polymorpha | New Zealand, Auckland | GU237818 | KT389599 |
| Didymella chlamydospora        | YW23-14T |            | South Korea  | MK836111           | LC480708           |
| Species                          | Strain      | Host                          | Locality  | GenBank accessions | ITS       | rpb2         |
|---------------------------------|-------------|-------------------------------|-----------|--------------------|-----------|--------------|
| **Didymella coffeae-arabicae**   | CBS 123380  | Coffea Arabica                | Ethiopia  | FJ420993           | KT389603  |              |
| **Didymella comerti**           | CBS 137982  | Combreum musambiacens        | Zambia    | MN973525           | MT018139  |              |
| **Didymella cartiisi**          | CBS 251.92  | Nerine sp.                    | The Netherlands | FJ427038   | MT018131  |              |
| **Didymella degnissaiae**       | CBS 144956  | Soil                          | The Netherlands | MN832444   | MN832440  |              |
| **Didymella eucalyptica**       | CBS 377.91  | Eucalyptus sp.                | Australia, WA | GU237886   | KT389605  |              |
| **Didymella gardeniae**         | CBS 620.68  | Gardenia jasminoides          | India     | FJ420703           | KT389606  |              |
| **Didymella glomerata**         | CBS 528.66  | Chrysanthemum sp.             | The Netherlands | FJ427013   | GU371781  |              |
| **Didymella gnnataula**         | CBS 127976  | Soil                          | Zimbabwe  | MN973524           | MT018138  |              |
| **Didymella heterobasidens**    | CBS 109.92  | Unidentified food material    | The Netherlands | FJ420983   | KT389601  |              |
| **Didymella keratinophila**     | UTHSC DI16-200 | Hono sapiens                      | USA       | UT592901           | UT593039  |              |
| **Didymella lelalii**           | CBS 103.25  |                               |           | GU377729           | KT389607  |              |
| **Didymella magnoliae**         | MFLUCC 18-1560 | Magnolia grandiflora              | China     | MK347814           | MK434852  |              |
| **Didymella majalis**           | CBS 588.96  | Zea mays                      | USA, Wisconsin, Hancock | FJ420786   | GU371782  |              |
| **Didymella mitii**             | CBS 443.72  | Soil                          | South Africa | MN973523   | MT018137  |              |
| **Didymella mucinea**           | CBS 463.69  | Mangifera indica              | India     | FJ427026           | MT018148  |              |
| **Didymella nagricola**         | CBS 444.81  | Acer palmatrum                | Japan     | KY742075           | KY742158  |              |
| **Didymella pinodes**           | CBS 318.90  | Pisum sativum                 | The Netherlands | FJ427051   | MN939533  |              |
|                                | BRIP 69589  | Pisum sativum                 | Australia, VIC, Rainbow | MN667675   | MN604926  |              |
| **Didymella pinodes**           | CBS 525.77  | Pisum sativum                 | Belgium   | GU377883           | KT389614  |              |
|                                | BRIP 69581  | Senna artemisioide            | Australia, SA, Blanchetown | MN66766   | MN604927  |              |
|                                | BRIP 69593  | Senna artemisioide            | Australia, SA, Blyth  | MN667677  | MN604928  |              |
|                                | BRIP 69596  | Senna artemisioide            | Australia, SA, Wudinna | MN66768   | MN604929  |              |
| **Didymella pomorum**           | CBS 539.66  | Polygonum tataricum           | The Netherlands | FJ427056   | KT389618  |              |
| **Didymella prodactiisola**     | CBS 126182  | Soil                          | Namibia   | MN973533           | MT018157  |              |
|                                | CBS 136414  | Prosopis sp.                  | South Africa | KP777180  | MT018149  |              |
| **Didymella protobragnii**      | BRIP 69579  | Gastrolobium celosanum        | Australia, SA, Adelaide | MN667680  | MN604931  |              |
| **Didymella protobragnii**      | CBS 381.96  | Lycium balsilicolon           | The Netherlands | GU237853  | KT389620  |              |
| **Didymella sancta**            | CBS 281.83  | Atlanticus utilissima         | South Africa | FJ427063   | KT389623  |              |
| **Didymella sinensis**          | CBS 364.91  | Ananas sativus                |           | MN973531           | MT018153  |              |
| **Didymella subglobispora**     | CBS 110.92  | Triticum sp.                  | USA, North Dakota | FJ427080  | KT389626  |              |
| **Didymella subglomerata**      | CBS 990.95  | Soil                          | Papua New Guinea | MN973513   | MT018119  |              |
| **Didymella brasiliens**        | CBS 120105  | Anamnesus sp.                 | Brazil    | GU237760           | KT389627  |              |
| **Didymella carnelliae**        | CGMCC 3.18343 | Camellia sinensis              | China     | KY742091           | KY742170  |              |
| **Didymella cateniporum**       | CBS 181.80  | Orzzy sativa                  | Guinea-Bissau | FJ427069   | LT325323  |              |
| **Didymella dentrobi**          | CGMCC 3.18359 | Dendrobiopsis fendriatrum     | China     | KY742093           | MT018084  |              |
| **Didymella dickmani**          | CBS 124671  | Aeropora Formosa              | Australia  | MN973509           | MT018113  |              |
| **Didymella djirangandri**      | BRIP 69585  | Swainsonia galgelfolia        | Australia, NSW, Mount Annan | MN667673  | MN604924  |              |
| spp.nov.                        | CBS 186.83  | Dnanema sp.                   | Rwanda    | GU237795           | KT389628  |              |
| **Didymella dossenhae**         | CGMCC 3.18345 | Duchsensia indica             | China     | KY742095           | MT018115  |              |
| **Didymella hensengsi**         | CBS 104.80  | Acanth mansieni               | Kenya     | GU237731           | KT389629  |              |
| **Didymella harderi**           | CGMCC 3.18360 | Hordeum vulgare               | Australia  | KY742097           | MT018102  |              |
| **Didymella immortu**           | CBS 105.80  | Sorerus sp.                   | Pera      | GU237732           | KT389630  |              |
| **Didymella italicus**          | CGMCC 3.18361 | Acer sellowiana              | Italy     | KY742099           | KY742172  |              |
| **Didymella keratinophilum**    | UTHSC DI16-271 | Homo sapiens                | USA       | LT592930           | LT930608  |              |
| **Didymella latiosidoides**     | CGMCC 3.18346 | Sorghum bicolor              | China     | KY742101           | KY742174  |              |
| **Didymella langistablettum**   | CBS 886.95  | Setaria sp.                   | Papua New Guinea | FJ427074  | MT018108  |              |
| **Didymella macchani**          | MFLUCC 16-033 | Ononis spinosa               | Italy     | KX698039           | KX698035  |              |
| **Didymella mezzettii**         | CBS 173.38  | Pupulsp pulp                  | Italy     | MN973496           | MT018095  |              |
| **Didymella nigromes**          | CBS 173.73  | Dactylis glomerata            | USA       | FJ426996           | KT389632  |              |
| **Didymella osiporum**          | CBS 180.80  | Zea mays                      | South Africa | FJ427068   | LT232523  |              |
| **Didymella phragmopora**       | CGMCC 3.19139 | Saccharum officinarum        | China     | MN215619           | MN255460  |              |
| **Didymella pinipinnia**        | CBS 246.60  | Soil                          | India     | FJ427049           | MT018100  |              |
| **Didymella plurivorum**        | CBS 558.81  | Setaria sp.                   | New Zealand | GU237888  | KT389634  |              |
| Species                                 | Strain ¹ | Host                  | Locality       | GenBank accessions ³ |
|-----------------------------------------|----------|-----------------------|----------------|---------------------|
| **Epicoccum pneumoniae**                 |          |                       |                | **ITS**             |
| MFLUCC 18-1593                          |          | Prunus avium          | China          | MH827002            |
| **Epicoccum pseudokeratinophilum**       |          |                       |                | **rbp2**            |
| MFLUCC 18-1593                          |          | Prunus avium          | China          | MH853659            |
| **Epicoccum purpurascens**               |          |                       |                |                    |
| CBS 128906                               |          | Soil                  | USA            | MN973488            |
| **Epicoccum spirotrichum**               |          | Sargassum bicolore     | Puerto Rico    | KT389635            |
| CBS 179.80                               |          | Soil                  | Indonesia      | MN973493            |
| **Epicoccum subaureum**                  |          |                       |                |                    |
| CBS 2056.77                              |          | Acalypha triphylla     | Canada         | KT389508            |
| **Epicoccum variae**                     |          |                       |                |                    |
| CBS 142853                               |          | Conocarpus erectus    | Iran           | KY449009            |
| **Epicoccum vitis**                      |          |                       |                | KY469422            |
| **Neoascocoryne aschidi**                |          |                       |                |                    |
| CBS 286.72                               |          | Acalypha triphylla     | Canada, British Columbia, Vancouver Island | KT389531 |
| **Neoascocoryne cannabis**               |          |                       |                |                    |
| CBS 234.37                               |          | Castanea sativa       | Unknown        | GU237804            |
| **Neoascocoryne farahkhiiefal**          |          |                       |                | KP329403            |
| **Neoascocoryne longicolla**             |          |                       |                |                    |
| CBS 382.96                               |          | Soil                  | Israel, En Avdat, Negev desert | KT389632 |
| **Neoascocoryne mucedora**               |          |                       |                |                    |
| MFLUCC 17-1063                           |          | Morus alba            | Russia         | KY684939            |
| **Neoascocoryne negundini**              |          |                       |                | MG564164            |
| JZB380011                                |          | Acer negundo          | Germany, Hohenleith | KT389644 |
| **Neoascocoryne polemonii**              |          |                       |                |                    |
| CBS 109181.¹                            |          | Polemonium caeruleum  | The Netherlands | GU237746            |
| **Neoascocoryne ranunculi**              |          |                       |                | KP330427            |
| CBS 286.72                               |          | Citrus limonum        | Italy          | MN973612            |
| **Neoascocoryne tillae**                 |          |                       |                | MT019283            |
| CBS 519.95.¹                             |          | Tilia sp.             | Italy          | MN973610            |
| **Neoascocoryne trinukuku sp. nov.**     | BRIP 69592 ¹ | Hardenbergia violacea | Australia, SA, Clare | MN6676781 |
| **Nothophoma achelid-mitidgal**          |          |                       |                | MN604932            |
| CBS 308.68.¹                             |          | Delphinium sp.        | The Netherlands, Baarn | GU237855 |
| **Nothophoma acidae**                    |          |                       | Australia       | MG386056            |
| CBS 143404.¹                             |          | Acacia melanoxylon    | Australia       | MG386144            |
| **Nothophoma anigozanthi**               |          |                       |                |                    |
| CBS 381.91.¹                             |          | Anigozanthus magellanicus | The Netherlands | GU237852            |
| **Nothophoma arachidis-lygo-gangi**      |          |                       |                | KT389655            |
| CBS 125.93                               |          | Anachis hypogaecae     | India, Madras  | GU237771            |
| **Nothophoma brennandiae**               |          |                       |                | KT389656            |
| CBS 145912.¹                             |          | Tilia sp.             | Italy          | MN823579            |
| **Nothophoma garliwalawarda sp. nov.**   |          |                       |                | MN604934            |
| BRIP 69580                               |          | Senna artemisioides    | Australia, SA, Adelaide | MN6676782 |
| BRIP 69586                               |          | Senna artemisioides    | Australia, SA, Berri | MN6676783 |
| BRIP 69587                               |          | Senna artemisioides    | Australia, SA, Berri | MN6676784 |
| BRIP 69594                               |          | Senna artemisioides    | Australia, SA, Kimba | MN6676785 |
| BRIP 69595.¹                             |          | Senna artemisioides    | Australia, SA, Wadinda | MN667686 |
| **Nothophoma eclypticgina**              |          |                       |                | MN604937            |
| CBS 142535.¹                             |          | Eclypticgina sp.       | Australia      | KY797771            |
| **Nothophoma genpinicia**                |          |                       |                | KY797852            |
| CBS 377.67                               |          | Gynophila sp.         | USA, Texas     | GU237845            |
| **Nothophoma infusa**                    |          | Fuchsia pensylvanica   | Argentina, Buenos Aires Province, La Plata | KT389659 |
| CBS 123395.¹                             |          |                       |                | KT389659            |
| **Nothophoma infuscula**                 |          | Acacia longifolia     | New Zealand    | MN973559            |
| CBS 121931.¹                             |          |                       |                | MN973559            |
| **Nothophoma macropora**                 | UTHSC DI16-199 ¹ | Hono sapieru         | USA, Arizona   | LN880536            |
| **Nothophoma natawsp. sp. nov.**         |          |                       |                | LT39073             |
| BRIP 69578.¹                             |          | Sena artemisioides     | Australia, SA, Blanchean | MN667687 |
| BRIP 69578.¹                             |          | Sena artemisioides     | Australia, SA, Blanchean | MN667688 |
| **Nothophoma sillicana**                 |          |                       |                | MN604938            |
| CPC 32330.¹                              |          | Acacia falciformis    | Australia      | NR_156665            |
| **Nothophoma prati**                     |          |                       |                | MG386143            |
| MFLUCC 18-1600                           |          | Prunus avium          | China          | MH827005            |
| **Nothophoma quercina**                  |          | Microsporid alphaoides from Quercus sp. | Ukraine | GU237900 |
| CBS 633.92                               |          |                       |                | KT389657            |
| **Nothophoma variabilis**                | UTHSC DI16-257 ¹ | Hono sapieru         | USA            | LT592939            |

¹ BRIP, Queensland Plant Pathology Herbarium, Brisbane, QLD, Australia; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC, China General Microbiological Culture Collection, Beijing, China; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA.
² NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia.
³ ITS, internal transcribed spacer region; *rbp2*, RNA polymerase II second subunit.
⁴ ex-type strain.
Figure 1. Phylogenetic tree based on maximum likelihood analysis of the combined multilocus (rpb2 and ITS) alignment. RAxML bootstrap values (bs) greater than 70 % and Bayesian posterior probabilities (pp) greater than 0.95 are given at the nodes (bs/pp). Genera are delimited in coloured boxes, with the genus name indicated to the right. Isolates identified in this study are in bold, and novel taxa are in red bold. Ex-type isolates are marked with T. The outgroup is Neuroscoychta desmaziieri (CBS 297.69).
Cryptic diversity found in Didymellaceae from Australian native legumes

Figure 1. Continued.

ingroup isolates from 111 taxa, and consisted of 1,090 characters (493 for ITS, and 596 for rpb2, including alignment gaps). The ML tree based on the combined dataset is presented, with bootstrap support values (BS) greater than 70% and Bayesian posterior probabilities (PP) greater than 0.95 indicating four well-supported clades, and limited support for Nothophoma (Fig. 1). The ITS phylogeny, using either ML or Bayesian analysis, provided poor resolution at the genus and species level (data not shown). The phylogenetic tree based on the concatenated alignment of ITS and rpb2 indicates the placement of the 18 isolates (Fig. 1), five of which represent novel species (Figs 2–6).

We identified three new host-pathogen associations, and one new record for Australia Didymella pinodes (strains BRIP 69581, 69593, and 69596) was isolated from native S. artemisioides from three locations in South Australia separated by over 400 km. Didymella pinodes (strain BRIP 69578) was also isolated from naturalised Vicia cracca (tufted vetch) in New South Wales from an area which did not cultivate P. sativum.
Didymella lethalis (strain BRIP 69584) was isolated from the naturalised Lathyrus tingitanus (tangier pea) from a recreational walking area within an urban environment. Didymella prosopidis (strain BRIP 69579) was isolated from Gastrolobium celsianum from the botanic gardens in the capital city of South Australia, Adelaide.

Taxonomy

Multilocus sequence analysis and morphological comparisons classified nine fungal isolates from legumes in southern Australia into five novel species from three Didymellaceae genera. The novel species are described and illustrated in Figs 2–6. Nomenclatural novelties are registered in MycoBank.

The species epithets were derived from Indigenous Australian Peoples’ language groups to provide a uniquely Australian theme. Permission to use words from the local language of the area in which the fungi were collected was granted by elders or community representatives.

Epicoccum djiranguandiri E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.
MycoBank No: 833689
Fig. 2

Type. AUSTRALIA, New South Wales, Mount Annan, Swainsona galegifolia, 19 Jan. 2017, E.C. Keirnan (holotype BRIP 69585, includes culture ex-type).

Description. Colonies on OA, 76–80 mm diam. after 7 d, covered in dense aerial mycelium, variable shades of grey, pale cinnamon towards centre; reverse dark vinaceous; on MEA, 70–72 mm after 7 d, margin entire, covered in low dense aerial mycelium, pale mouse grey with lighter patches; reverse olivaceous with radiating spokes; on PDA, 73–80 mm after 7 d, margin entire, mycelia felty, mouse grey becoming vinaceous buff towards centre; reverse fuscous black. NaOH spot test: negative. Conidiomata on CLA, pycnidial, globose 100–200 μm diam., pale brown becoming black, solitary, glabrous, non-papillate; pycnidial wall composed of textura globulosa, pale brown, cells 5–15 μm diam. Conidiogenous cells phialidic, cylindrical, thin-walled, hyaline, rounded ends. Conidia aseptate, 5–7 × 2–3 μm.

Etymology. From the language of the Indigenous Australian Dharawal people, meaning leaf spot. The Dharawal people are from the western Sydney region in New South Wales, which includes Mount Annan, where the holotype was collected.

Notes. Epicoccum djiranguandiri is phylogenetically close to E. pneumoniae ex-type strain UTHSC DI16-257 (Fig. 1) and is distinguished in rpb2 sequences with 99% identity. Morphological comparisons could not be made as E. pneumoniae was sterile in culture (Valenzuela-Lopez et al. 2018). Epicoccum djiranguandiri is only known from one specimen on Swainsona galegifolia.
Cryptic diversity found in Didymellaceae from Australian native legumes

Figure 2. Epicoccum djirangnandiri: a leaf lesions on Swainsona galegifolia b 14-d old colonies on PDA, MEA, OA (left, top to bottom) and lower surface (right) c upper surface d pycnidia on CLA e conidia. Scale bars: 200 μm (d); 7 μm (e).

Neodidymelliopsis tinkyukuku E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.
Mycobank No: 833692
Fig. 3

Type. AUSTRALIA, South Australia, Clare, Hardenbergia violacea, 17 Sep. 2017, E.C. Keirnan (holotype BRIP 69592, includes culture ex-type).

Description. Colonies on OA, 26–28 mm diam. after 7 d, dense low aerial mycelium, buff with numerous grey patches, darker with abundant pycnidia at centre; reverse buff to rosy buff with darker concentric rings towards centre; on MEA, 28–30 mm after 7 d, margin entire, dense low aerial mycelium, vinaceous buff paler at margin; reverse rosy buff to buff at margin with abundant scattered pycnidia; on PDA, 35–38 mm after 7 d, margin entire, dense low aerial mycelium, pale mouse grey lighter at margin; reverse cinnamon with concentric dark rings, darker at centre. NaOH spot test: light yellow. Conidiomata on CLA pycnidial, globose to ampulliform, 250–350 μm diam., brown becoming black, solitary, abundant in centre of colony, zonate, glabrous, non-papillate; ostiole c. 25 μm diam.; pycnidial wall composed of textura angularis, pale brown, cells 5–8 μm diam. Conidiogenous cells phialidic, cylindrical, thin-walled, hyaline. Conidia occasionally septate, 6–9 × 2–3 μm, cylindrical, hyaline, thin-walled.
**Etymology.** From the language of the Indigenous Australian Kaurna people, meaning leaf disease. The Kaurna people are from the Adelaide plains region, which includes Clare, the locality where the holotype was collected.

**Notes.** *Neodidymelliopsis tinkyukuku* (strain BRIP 69592) is sister to a clade that includes *N. farokhinejadii* (strain CBS 142853), *N. longicolla* (ex-type strain CBS 382.96) and *N. ranunculi* (strain CBS 286.72) (Fig. 1). *Neodidymelliopsis* conidial dimensions are distinct from *N. farokhinejadii* (4.6–7.5 × 2.4–3.9 μm), *N. longicolla* (12–15 × 4–7 μm), and *N. ranunculi* (3–5 × 7.5–10 μm). *Neodidymelliopsis tinkyukuku* can be easily distinguished from these three species by DNA sequences of the *rpb2* locus.

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![Image](image.png)

**Figure 3.** *Neodidymelliopsis tinkyukuku* **a** leaf lesions on *Hardenbergia violacea* **b** 12-d old colonies top to bottom on PDA, MEA, OA (left, top to bottom) and lower surface (right) **c** upper surface **d** pycnidia on CLA **e** pycnidia **f** pycnidial wall **g** conidia. Scale bars: 300 μm (**d, e**); 10 μm (**f**); 7 μm (**g**).

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**Nothophoma garlbiwalawarda** E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.
MycoBank No: 833693
Fig. 4

**Type.** AUSTRALIA, South Australia, Wudinna, *Senna artemisioides*, 19 Aug. 2017, E.C. Keirnan (holotype BRIP 69595, includes culture ex-type).

**Description.** Colonies on OA, 27–30 mm diam. after 7 d, flat with scant aerial mycelia with a few zonate rings, vinaceous to dark vinaceous; vinaceous to dark vinaceous; on MEA, 23–25 mm after 7 d, margin entire, flat, scant aerial mycelium towards centre, amber with abundant pycnidia; reverse amber darker towards centre; on PDA, 28–30 mm after 7 d, margin irregular, flat with aerial mycelia tufted in centre, dark with abundant pycnidia in concentric rings, buff at margin; reverse dark becoming buff at margin. *NaOH spot test*: reddish. *Conidiomata* pycnidial, globose to
subglobose, 130–320 μm diam., pale brown, scattered, abundant, zonate, glabrous, non-papillate; ostiole c. 25 μm diam.; pycnidial wall composed of textura angularus, pale to medium brown, cells 5–12 μm diam. Conidiogenous cells phialidic, cylindrical, thin-walled, hyaline 5–12 × 2–4 μm long, narrower at the apex. Conidia aseptate, 5–7.0 × 2.0–3.0 μm, parallel to narrowly ellipsoidal, hyaline, wall c. 0.5 μm.

**Etymology.** From the native language of the Indigenous Australian Barngarla people, meaning leaf-fun-guy. The Barngarla people are from the Eyre Peninsula region, which includes Wudinna, the locality where the holotype was collected.

**Additional material examined.** AUSTRALIA, South Australia, Adelaide, *Senna artemisiodioides*, 26 Oct. 2016, E.C. Keirnan (BRIP 69580); Berri, *Senna artemisiodioides*, 01 Jul. 2017, E.C. Keirnan (BRIP 69586); ibid, 01 Jul. 2017, E.C. Keirnan (BRIP 69587); Kimba, *Senna artemisiodioides*, 17 Sep. 2017, E.C. Keirnan (BRIP 69594).

**Notes.** *Nothophoma garlbiwalawarda* is phylogenetically closest to *No. anigozanthi* and two novel species (see below for notes) (Fig. 2). *Nothophoma garlbiwalawarda* is distinguished from *No. anigozanthi* by its larger conidia (cf. 3.5–5 × 1.5–2.5 μm), *rpb2* sequence (93% identity), and its reaction to *NaOH spot test* on MEA (dull green then black).

**Figure 4.** *Nothophoma garlbiwalawarda*: a pin-prick leaf spots on *Senna artemisiodioides* from Wudinna SA b 12-d old colonies top to bottom on PDA, MEA, OA (left, top to bottom) and lower surface (right) c upper surface d pycnidia on CLA e pycnidia and pycnidial ooze on OA f pycnidia on PDA g conidia. Scale bars: 300 μm (d, e, f); 7 μm (g).

*Nothophoma naiawu* E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov. MycoBank No: 833694
Fig. 5

**Type.** AUSTRALIA, South Australia, Blanchetown, from *Senna artemisiodioides*, 22 Oct. 2016, E.C. Keirnan, holotype BRIP 69583 (includes culture ex-type).
Description. Colonies on OA, 21–25 mm diam. after 7 d, flat with scant aerial mycelia, rosy vinaceous, dark at centre; reverse rosy buff, dark at centre, with a few dark radiating fissures; on MEA, 27–30 mm after 7 d, margin entire, flat, with sparse aerial mycelium towards centre rosy vinaceous; reverse peach, darker at centre; on PDA, 27–30 mm after 7 d, margin entire, flat felty, rosy buff; reverse peach, dark at centre. NaOH spot test: slightly yellow. Conidiomata pycnidial, globose to subglobose, 200–300 μm diam., pale brown becoming black, semi-immersed, confluent on MEA, glabrous, non-papillate; ostiole c. 25 μm diam.; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 μm diam.. Conidiogenous cells phialidic, cylindrical, very thin-walled, hyaline. Conidia aseptate or 1-septate, 8–12 × 4–6 μm, cylindrical to narrow ellipsoidal, pale yellow.

Etymology. A variation of the Indigenous Australian Ngayawang people’s language group, who lived in the Murray River region of South Australia, which includes Blanchetown, the locality where this specimen was collected.

Notes. Nothophoma naiawu is phylogenetically close to No. eucalyptigena and No. infuscata (Fig. 2). Nothophoma naiawu is easily distinguished from No. eucalyptigena and No. infuscata by the ITS region (98 % identity to both) and the rpb2 locus (95%, and 94% identity, respectively). Nothophoma infuscata produce a pale red discolouration in response to NaOH spot test on MEA media, which is distinct from the slightly yellow response by No. naiawu.

Nothophoma ngayawang  E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.
MycoBank No: 833695
Fig. 6

Type. AUSTRALIA, South Australia, Blanchetown, Senna artemisioides, 22 Oct. 2016, E.C. Keirnan, holotype BRIP 69582 (includes culture ex-type).
**Description.** Colonies on OA, 18–20 mm diam. after 7 d, covered by scant tufted aerial mycelia at centre becoming abundant and floccose towards margin, rosy buff becoming darker towards centre; reverse salmon with centre and margins pale isabelline; on MEA, 15–20 mm after 7 d, margin irregular, felty buff becoming white towards the margin; reverse pale rosy buff, darker at centre becoming paler near margin; on PDA, 18–21 mm after 7 d, margin regular, aerial mycelia tufted in centre becoming floccose toward the margin, white to pale rosy buff; reverse pale rosy buff with few scattered vinaceous spots. *NaOH spot test:* slightly yellow. *Conidiomata* pycnidial, globose to subglobose, 200–300 μm diam., pale brown becoming black, solitary, abundant in centre of colony, glabrous, non-papillate; ostiole c. 25 μm diam.; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 μm diam. *Conidiogenous* cells phialidic, cylindrical, thin-walled, hyaline. *Conidia* aseptate, 2.5–4.0 × 1.0–2.0 μm, cylindrical to narrow ellipsoidal, hyaline, thin-walled.

**Etymology.** Named after the Indigenous Australian Ngayawang people’s language group, who existed in the Murray River region of South Australia, which includes Blanchetown, the locality where this specimen was collected.

**Notes.** *Nothophoma ngayawang* is phylogenetically close to *No. anigozanthi* ex-type strain CBS 381.91 (Fig. 2). *Nothophoma ngayawang* is distinguished from *No. variabilis* by the ITS region (98 % identity) and the *rpb2* locus (93% identity). The *NaOH spot test* of *No. variabilis* was negative on MEA, which is distinguished from the slightly yellow reaction of *No. ngayawang*.

**Discussion**

Our investigations did not identify *A. koolunga* from native Australian legumes. In fact, the incidence was low in that only one isolate (BRIP 69590) was collected from...
P. sativum in South Australia. It is difficult to make an association between the low incidence of A. koolunga on P. sativum and the absence of A. koolunga on other legumes. While the current evidence suggests that A. koolunga is unlikely to have originated from Australian native legumes, additional field surveys may be required to investigate the possible source of A. koolunga.

Our investigations instead uncovered five novel Didymellaceae species not yet known to science. Epicoccum djirangnandiri on S. galegifolia was collected from the botanic garden in New South Wales, where the host is endemic. Neodidymelliopsis tinkyukuku on H. violacea was collected from a public garden in South Australia. Growing in the same garden is V. sativa from which D. pinodes (strain BRIP 69578), a known Ascochyta blight pathogen, was isolated. Hardenbergia violacea has a wide distribution in southern and eastern Australia. These three native Australian legume species were found in a cultivated environment rather than in a natural environment. Further studies are warranted to understand how widespread these fungal species may be in cultivated or natural environments, and if they are host specific.

Leaf spots were commonly seen on the native legume S. artemisioides throughout the regions sampled in South Australia. Three novel Nothophoma species were isolated from S. artemisioides. Nothophoma garlbiwalawarda was collected from five locations across South Australia, separated by over 400 km, in field pea and non-field pea growing regions. Nothophoma naiawu and No. ngayawang were collected from the South Australian Murray River region on the roadside of a main highway. The leaf spot symptoms for the three Nothophoma species were similar (small pin-prick lesions), with some larger spots on the seed pods caused by No. ngayawang.

Our investigations also identified new host-pathogen associations, namely D. pinodes on S. artemisioides and V. cracca, and D. lethalis on L. tingitanus. These hosts could be a reservoir of Ascochyta blight inoculum if found growing adjacent to field pea crops. The discovery of an alternative host has implications for disease epidemiology and management. The symptoms of D. pinodes on S. artemisioides are indistinguishable from the pin-prick leaf spot symptoms caused by the three Nothophoma species described in this study. Didymella pinodes was isolated from five locations. Four of these locations also yielded a novel Nothophoma species. Didymella prosopidis was isolated from the Australian native G. celsianum, a species first described as associated with stem disease of Prosopis sp. (also a member of the Fabaceae family) in South Africa (Crous et al. 2013). This is the first report of D. prosopidis outside of South Africa.

At the outset, our study sought to identify if any A. koolunga could be isolated from Australian native legumes causing leaf spot disease. This study uncovered five novel isolates in the Didymellaceae from Australian native legumes, and identified three new legume host-pathogen associations for Australia. Ascochyta koolunga was not isolated from hosts other than field pea, which might be an artefact of the low incidence of the fungus during the collection period. Further investigations using a longitudinal systematic survey are needed to identify any native hosts of A. koolunga.
and to further investigate the diversity and prevalence of Didymellaceae species on Australian native, pasture and naturalised legumes, to classify novel isolates and to identify new Australian hosts for known species.

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