Persoonia 27, 2011: 80 – 89
www.ingentaconnect.com/content/nhn/pimj

RESEARCH ARTICLE

http://dx.doi.org/10.3767/003158511X617110

Stem cankers on sunflower (*Helianthus annuus*) in Australia reveal a complex of pathogenic *Diaporthe* (*Phomopsis*) species

S.M. Thompson[1,3], Y.P. Tan[2], A.J. Young[2], S.M. Neate[1], E.A.B. Aitken[3], R.G. Shivas[2]

Key words

Diaporthe gulyae
Diaporthe kochmanii
Diaporthe kongii
ITS
phylogeny
sunflower taxonomy
TEF-1α

Abstract The identification of *Diaporthe* (anamorph *Phomopsis*) species associated with stem canker of sunflower (*Helianthus annuus*) in Australia was studied using morphology, DNA sequence analysis and pathology. Phylogenetic analysis revealed three clades that did not correspond with known taxa, and these are believed to represent novel species. *Diaporthe gulyae* sp. nov. is described for isolates that caused a severe stem canker, specifically pale brown to dark brown, irregularly shaped lesions centred at the stem nodes with pith deterioration and mid-stem lodging. This pathogenicity of *D. gulyae* was confirmed by satisfying Koch's Postulates. These symptoms are almost identical to those of sunflower stem canker caused by *D. helianthi* that can cause yield reductions of up to 40 % in Europe and the USA, although it has not been found in Australia. We show that there has been broad misapplication of the name *D. helianthi* to many isolates of *Diaporthe* (*Phomopsis*) found causing, or associated with, stem cankers on sunflower. In GenBank, a number of isolates had been identified as *D. helianthi*, which were accommodated in several clades by molecular phylogenetic analysis. Two less damaging species, *D. kochmanii* sp. nov. and *D. kongii* sp. nov., are also described from cankers on sunflower in Australia.

Article info Received: 4 July 2011; Accepted: 11 November 2011; Published: 2 December 2011.

INTRODUCTION

Phomopsis species are widespread and occur on a diverse range of host plants as pathogens, endophytes or saprobes (Uecker 1988). The morphological characters that define *Phomopsis* are dark eustromatic or pycnidial conidiomata containing elongated phialides with cylindrical, well-developed collarettes that form two types of hyaline conidia: 1-celled α-conidia that are biguttulate, fusiform, and easily germinate on artificial media, and ß-conidia that are filiform and rarely germinate (Wehmeyer 1933, Sutton 1980). Species of *Phomopsis* represent anamorphs of *Diaporthe* (*Ascomycota*, *Diaporthales*, *Valsaceae*) with at least 180 connections given by Uecker (1988), which represents about 80 % of named *Phomopsis* species. The name *Diaporthe* Nitschke (1870) precedes *Phomopsis* Sacc. & Roum. in Saccardo (1884).

Host association has often been the basis for species identification in *Diaporthe* and *Phomopsis*, as morphological and culture characteristics are inadequate or unreliable for species differentiation (van Rensburg et al. 2006). Recent studies have demonstrated that a number of *Phomopsis* species have wide host ranges (van Niekerk et al. 2005, Santos & Phillips 2009, Ash et al. 2010), and more than one species can occur on a single host (Mostert et al. 2001, Santos & Phillips 2009).

Molecular phylogenies, especially those derived from DNA sequence analyses of the ribosomal internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA genes and translation elongation factor-1α (TEF-1α) have been used to identify species (Mostert et al. 2001, van Niekerk et al. 2005, van Rensburg et al. 2006, Santos & Phillips 2009, Ash et al. 2010). The polyphyletic status of *D. helianthi* has been recognised by Rekab et al. (2004). Hyde et al. (2010) suggested that discarding the host-based species concept was the first step in the development of a useful and reliable classification for *Phomopsis* and highlighted that there had been much confusion around the application of species names, drawing particular attention to the name *D. helianthi*.

Stem canker attributed to *D. helianthi* (anamorph *P. helianthi*) has become one of the most important diseases of sunflower (*Helianthus annuus*) worldwide since first described from the former Yugoslavia (Muntañola-Cvetković et al. 1981). Yield reductions of up to 40 % have been recorded in Europe (Masirevic & Gulya 1992) including the former Yugoslavia as well as France where it was considered a major pathogen of sunflower (Battilani et al. 2003, Debaeke et al. 2003). *Diaporthe helianthi* is also widespread in the sunflower growing regions of the USA (Gulya et al. 1997) but has not been reported from Australia.

Muntañola-Cvetković et al. (1985) found that multiple *Phomopsis* species were associated with cankers on sunflower in the former Yugoslavia, although only *P. helianthi* was responsible for the serious disease outbreaks. Gulya et al. (1997) suggested that pathogenic *Phomopsis* species on sunflower might consist of more than one species or biotype with apparent biological differences between the isolates from Europe and the USA. Miric et al. (2001) raised the possibility that several pathogenic *Phomopsis* species occurred on sunflower in Australia.

In 2009, lodging and premature senescence caused significant damage to sunflower crops in New South Wales (NSW), and to a lesser extent in Queensland (Qld), Australia, after extended periods of wet weather. The symptoms included pith damage behind elongated, brown to brown-black lesions, which weakened stems and led to mid-stem lodging as the heads filled. The aim of this study was to use morphological, molecular and pathogenicity studies to clarify the identity of the *Diaporthe* (*Phomopsis*) species occurring on sunflower in Australia.

[1] Agri-Science Queensland, Department of Employment, Economic Development and Innovation, P.O. Box 102, Toowoomba, Qld 4350, Australia; corresponding author e-mail: sue.thompson@deedi.qld.gov.au.
[2] Plant Pathology Herbarium, Ecosciences Precinct, Department of Employment, Economic Development and Innovation, 41 Boggo Road, Dutton Park, Qld 4102, Australia.
[3] School of Agriculture and Food Science, The University of Queensland, St Lucia, Qld 4072, Australia.

Table 1 *Diaporthe* cultures isolated from sunflower investigated in this study.

Species	Isolate number (BRIP)[1]	Locality	Source	Sunflower Hybrid/Wild	Virulence Rating[2]	GenBank Accession numbers	
						ITS	TEF-1α
Diaporthe gulyae	53158	Goran Lake, NSW	stem	Wild *H. annuus*	4	JF431284	JN645799
	53166	Premer, NSW	seed	Ausigold 62	4	JF431289	JN645801
	53172	Premer, NSW	seed	Hyoleic 41	5	JF431290	JN645802
	53159	Premer, NSW	seed	Advantage	5	JF431291	JN645800
	54030	Nobby, Qld	stem	Sunbird 7	5	JF431292	JN645808
	54029	Hermitage, Qld	stem	Hyoleic 41	4	JF431293	JN645807
	54028	Hermitage, Qld	stem	Hyoleic 41	5	JF431294	JN645806
	54027	Ryeford, Qld	leaf	Sunbird 7	5	JF431297	JN645805
	54026	Ryeford, Qld	leaf	Sunbird 7	5	JF431298	JN645804
	54025	Ryeford, Qld	leaf	Sunbird 7	4	JF431299	JN645803
Diaporthe kochmanii	**54033**	Gatton, Qld	stem	Experimental	2	JF431295	JN645809
	54034	Gatton, Qld	stem	Experimental	3	JF431296	JN645810
Diaporthe kongii	54032	Childers, Qld	stem	Female	3	JF431300	JN645798
	54031	Childers Qld	stem	Female	3	JF431301	JN645797

[1] Ex-type cultures are in **bold**.
[2] At 14 d after inoculation where 0 = no discolouration or very slight discolouration or scarring at site of inoculation; 1 = low level discolouration at site of inoculation; 2 = very small lesion or slight discolouration 1–2 mm diam; 3 = necrotic lesions 2–5 mm, some light stem streaking, leaf wilting and twisting; 4 = lesions 5–10 mm diam, significant necrosis and dark stem streaking, leaf and plant wilting, stunting, and some lodging; 5 = very severe necrosis and lesions, dark streaking, leaf necrosis, twisting and wilting, stunting, lodging or plant death.

MATERIAL AND METHODS

Isolates

Over 300 isolates of *Diaporthe* (*Phomopsis*) were obtained from stems, leaves and seed of both cultivated and wild sunflower plants exhibiting symptoms of stem canker across NSW and Qld. Small excised stem and leaf pieces with brown or brownish black lesions were surface-sterilised by dipping into 90 % ethanol and flaming briefly prior to placement on 1.5 % water agar amended with 100 µg/mL streptomycin sulphate (WAS) in 9 cm diam Petri dishes. Cultures that grew from this tissue were incubated for up to 3 wk to induce pycnidial formation. For seed isolations, seeds harvested from infected crops and individual plants were incubated without surface sterilisation on WAS in Petri dishes for up to 14 d to allow pycnidia to develop.

For all isolations, conidia oozing from pycnidia were streaked onto potato-dextrose agar (Oxoid) (PDA) amended with 100 µg/mL streptomycin sulphate (PDAS). Hyphal tips were then taken from all isolates and grown on PDAS to establish pure isolates. Cultures were incubated for 7 d under ambient light at 23–25 °C. For pathogenicity experiments, 7 d old cultures were used to provide inocula. Fourteen selected isolates representing a range of virulence symptoms and morphological characteristics were deposited in the Plant Pathology Herbarium (BRIP), Brisbane, Australia as both living and dried cultures (Table 1).

Morphology

For fungal morphology, isolates were grown on PDA with pieces of sterilised wheat stems placed on the surface and incubated under 12 h near-ultraviolet light / 12 h dark (Smith 2002) at 25 °C. Fungal structures were mounted on glass slides in lactic acid (100 % v/v) for microscopic examination after 28 d of incubation. Means and standard deviations (SD) of selected structures were made from at least 20 measurements. Ranges were expressed as (min.–) mean-SD – mean+SD (–max.) with values rounded to 0.5 µm. Images were captured with a Leica DFC 500 camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

For colony morphology, 3 d old cultures on 9 cm diam plates of PDA and oatmeal agar (OA) (Oxoid) that had been grown in the dark at 23 °C were grown for a further 7 d under 12 h near-ultraviolet light / 12 h dark. Colony colours (surface and reverse) were rated according to the colour charts of Rayner (1970).

DNA isolation, amplification and analyses

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 Gentra Puregene DNA Extraction kit (QIAGEN) according to the manufacturer's instructions.

The primers ITS1 and ITS4 (White et al. 1990) were used to amplify the ITS region of the ribosome genes. To further differentiate *D. angelicae*, *D. stewartii*, *D. gulyae* and *P. dauci*, the primers EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell et al. 1998) were used to amplify part of the translation elongation factor-1alpha (TEF-1α) gene. Both the ITS and TEF loci were amplified with the Phusion High-Fidelity PCR Master Mix (Finnzymes). The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) and sequenced on the 3730*xl* DNA Analyzer (Applied Biosystems) using the amplifying primers.

The sequences generated in this study were assembled using Vector NTi Advance v. 11.0 (Invitrogen) and deposited in GenBank (Table 2). These sequences were aligned with sequences from representative *Diaporthe*/*Phomopsis* species from GenBank (Table 2) in MEGA v. 5.05 (Tamura et al. 2011).

The sequences of *Leucostoma persoonii* and *Valsa ceratosperma* were used as outgroups in the ITS dataset, whilst sequences of *Leucostoma niveum* and *Valsa ambiens* were used as outgroups in the TEF-1α dataset. Alignment gaps were treated as missing character states and all characters were unordered and of equal weight.

The ITS and TEF-1α phylogenetic trees were inferred in MEGA v. 5.05 by Maximum Likelihood (ML). Modeltest in MEGA v. 5.05 determined that the K2+G and HKY+G models were the most suitable nucleotide substitution models for ITS and TEF-1α, respectively. Bootstrap support values with 1 000 replications were calculated for tree branches. The sequences obtained from GenBank are listed by their taxon names followed by strain numbers in the trees (Fig. 1, 2). Nomenclatural novelties were deposited in MycoBank (www. MycoBank.org) (Crous et al. 2004).

Pathogenicity

Pathogenicity was determined by inoculating plants of the sunflower hybrid Hyoleic 41 at the V6–V8 (Schneiter & Miller 1981) growth stage and grown in a cabinet under a 25 °C 12 h light / 20 °C 12 h dark cycle using two methods, wound inoculation and mycelium contact. The wound inoculation method (adapted

Table 2 Reference isolates used in the phylogenetic analyses.

Species	Isolate no. [1, 5]	Host	GenBank accession numbers		Reference
			ITS[2]	TEF-1α[3]	
Diaporthe alleghaniensis	**CBS 495.72**	*Betula alleghaniensis*	FJ889444	GQ250298	Santos et al. 2010
Diaporthe ambigua	**CBS 114015**	*Pyrus communis*	AF230767	GQ250299	Mostert et al. 2001 Santos et al. 2010
Diaporthe angelicae	**CBS 111592** **AR3776**	*Heracleum sphondylium*	AY196779	GQ250302	Santos et al. 2010
Diaporthe aspalathi	**CBS 117169**	*Aspalathus linearis*	DQ286275	DQ286249	van Rensburg et al. 2006
Diaporthe australafricana	STE-U 2655	*Vitis vinifera*	AF230744		Mostert et al. 2001 van Niekerk et al. 2005
Diaporthe crotalariae	**CBS 162.33**	*Crotalaria spectabilis*	FJ889445	GQ250307	Santos et al. 2010
Diaporthe helianthi	Ar3	*Arctium lappa*	FJ841859		Vrandecic et al. 2010
	CBS 592.81	*Helianthus annuus*	AY705842	GQ250308	Santos et al. 2010
	Su 5/04		FJ841854		Vrandecic et al. 2010
	Su 20/05		FJ841855		
	Su 3/04		FJ841856		
	Su 11/04		FJ841861		
	Su 3/06		FJ841863		
	Su 12/04		FJ841864		
	Su 8/05		FJ841865		
	Su 18/06		FJ841866		
	Su 12/05		FJ841867		
	Su 25/05		FJ841868		
	Dh95016		AF358435		Says-Lesage et al. 2002
	Dh95048		AF358436		
	Dh95057		AF358437		
	Dh95004		AF358438		
	Dh95045		AF358439		
	Dh95049		AF358440		
	Dh95099		AF358441		
	G23 A1-62	*Luehea divaricata*	EU878427		Bernardi-Wenzel et al. 2010
	STE-U 5355	*V. vinifera*	AY485745		van Niekerk et al. 2005
	STE-U 5353		AY485746		
	STE-U 5344		AY485747		
	STE-U 5356		AY485748		
	STE-U 5354		AY485749		
	Xa 2	*Xanthium italicum*	FJ841860		Vrandecic et al. 2010
	Xa 3		FJ841857		
	Xa 5		FJ841852		
	Xa 9	*Xanthium strumarium*	FJ841858		
	Xa 12		FJ841853		
	Xa 13	*Xanthium* sp.	FJ841862		
Diaporthe hickoriae	**CBS 145.26**	*Carya glabra*	FJ889446	GQ250309	
Diaporthe lusitanicae	**CBS 123212** **Di-C001/5**	*Foeniculum vulgare*	EU814477	GQ250310	Santos & Phillips 2009 Santos et al. 2010
Diaporthe melonis	**CBS 507.78**	*Cucumis melo*	FJ889447	GQ250314	Santos et al. 2010
Diaporthe neotheicola	**CBS123208**[4] **Di-C004/5**	*F. vulgare*	EU814480	GQ250315	Santos & Phillips 2009 Santos et al. 2010
Diaporthe perjuncta	**CBS 109745**	*Ulmus glabra*	AY485785	GQ250323	van Niekerk et al. 2005 Santos et al. 2010
Diaporthe stewartii	**CBS 193.36**	*Cosmos bipinnatus*	FJ889448	GQ250324	Santos et al. 2010
Diaporthe strumella var. *longispora*	**CBS 194.36**	*Ribes* sp.	FJ889449	GQ250325	
Diaporthe vaccinii	**CBS 160.32**	*Oxycoccus macrocarpus*	AY952141	GQ250326	
Diaporthe viticola	**CBS 113201** **STE-U 5683**	*V. vinifera*	AY485750	GQ250327	van Niekerk et al. 2005 Santos et al. 2010
Diaporthe sp.	DAR 73811	*Carthamus lanatus*	EU311607	FJ389003	Ash et al. 2010
Phomopsis amygdali	**CBS 126679**	*Prunus dulcis*	GQ281791		Diogo et al. 2010
	11A		GQ281792	GQ250339	Santos et al. 2010
Phomopsis cotoneastri	**CBS 439.82**	*Cotoneaster* sp.	FJ889450	GQ250341	Santos et al. 2010
Phomopsis cuppatea	**CBS 117499**	*Aspalathus linearis*	AY339322	AY339354	van Rensburg et al. 2006
Phomopsis dauci	**CBS 315.49**	*Daucus carota*	FJ889451	GQ250348	Santos et al. 2010
Phomopsis longicolla	SSLP-1	*Glycine max*	HQ333500	HQ333505	unpublished
Phomopsis phoenicicola	**CBS 161.64**	*Areca catechu*	FJ889452	GQ250349	Santos et al. 2010
Phomopsis sclerotioides	**CBS 296.67**	*Cucumis sativus*	AF439626	GQ250350	Farr et al. 2002 Santos et al. 2010
Phomopsis subordinaria	CBS 104.84	*Plantago lanceolata*	GQ922519		unpublished
Phomopsis viticola	**CBS 114016**	*V. vinifera*	AF230751	GQ250351	Mostert et al. 2001 Santos et al. 2010

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Ph- & Di-: culture collection housed at Centro de Recursos Microbiolo´ gicos, Caparica, Portugal.
[2] ITS: internal transcribed spacer.
[3] TEF-1α: translation elongation factor-1alpha.
[4] Di-C004/5 is also recorded as CBS 123208.
[5] Ex-type cultures are in **bold**.

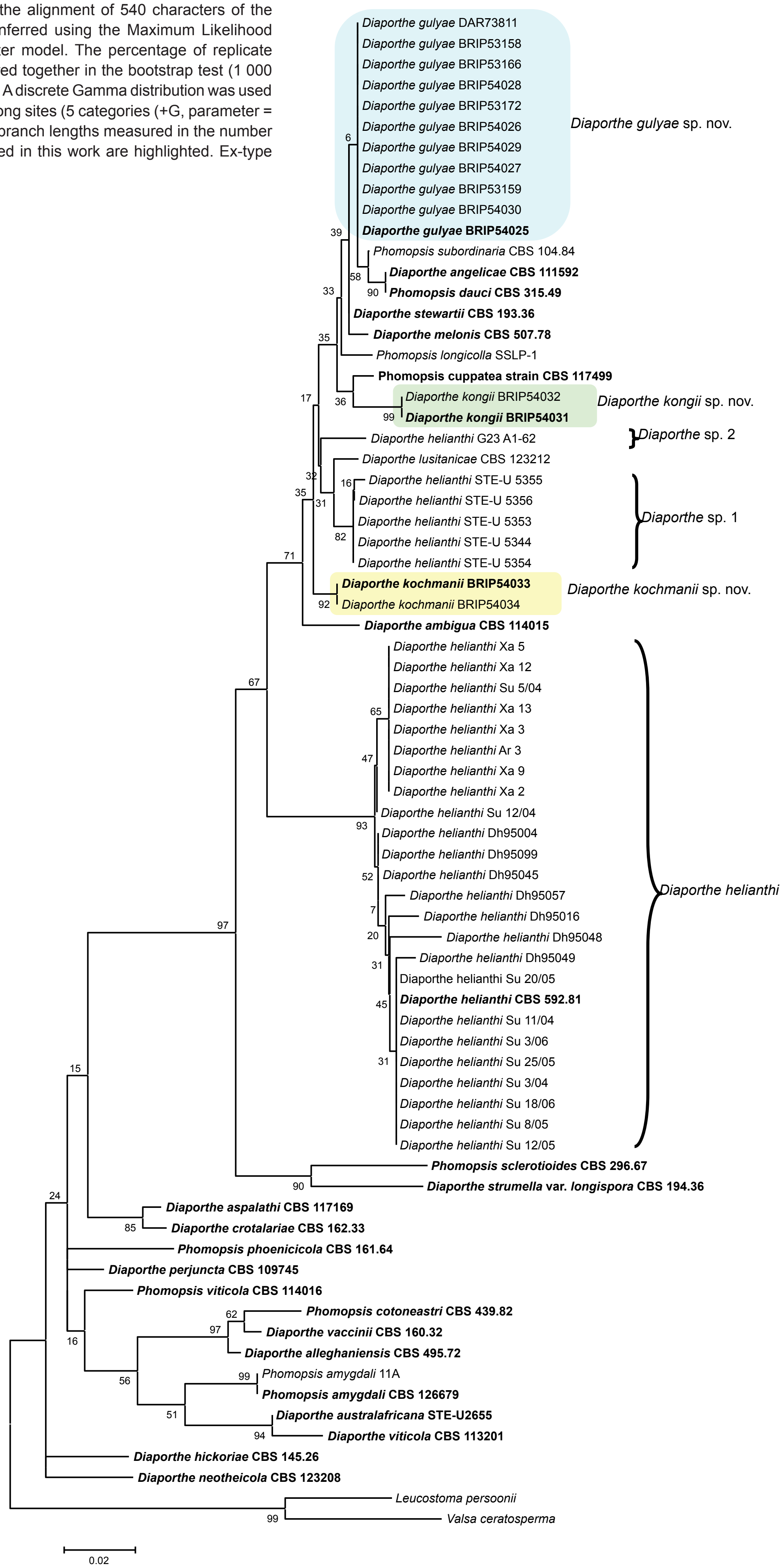
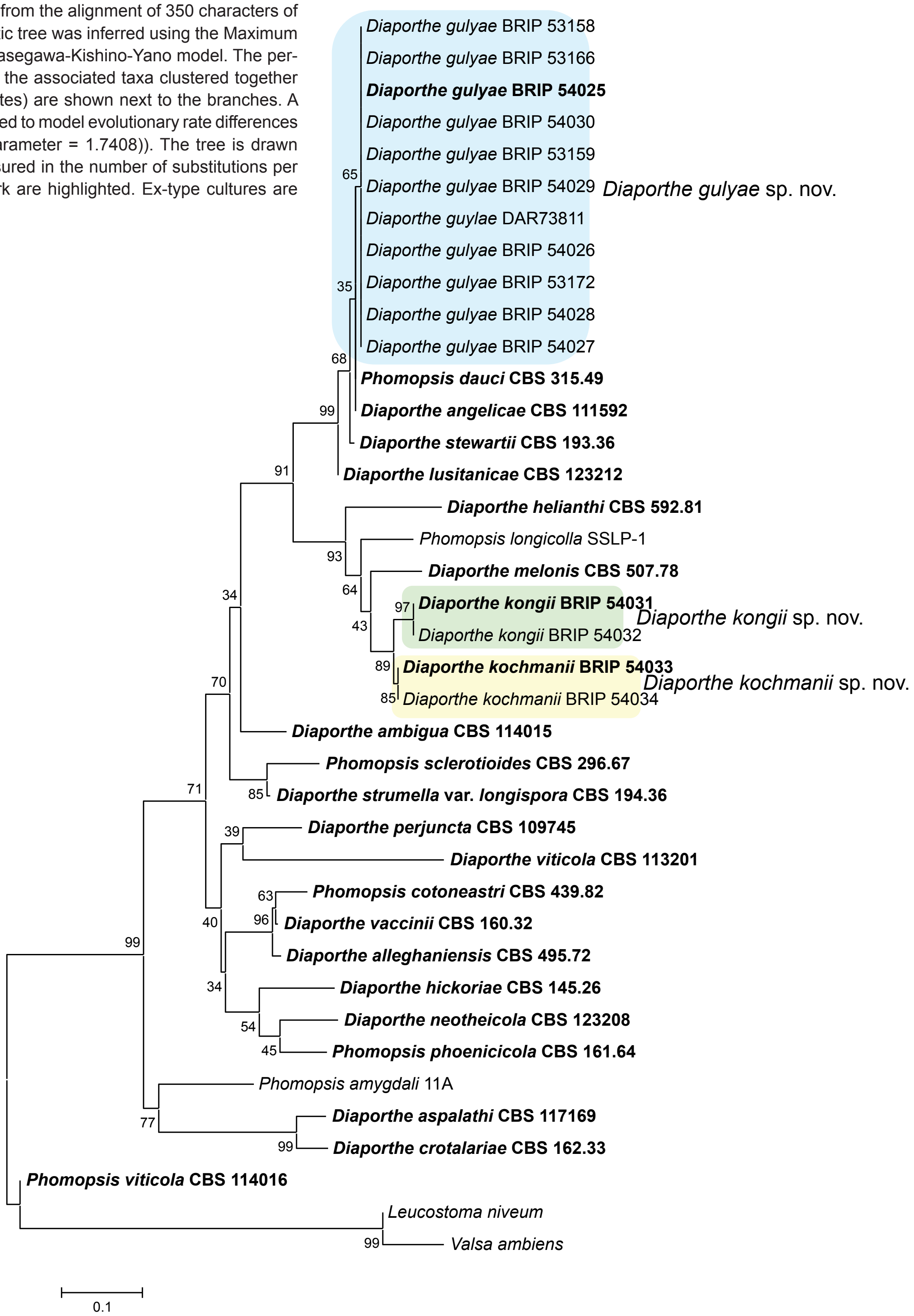


Fig. 1 Phylogenetic tree resulting from the alignment of 540 characters of the ITS region. The phylogenetic tree was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3209)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Species described in this work are highlighted. Ex-type cultures are in **bold.**

Fig. 2 Phylogenetic tree resulting from the alignment of 350 characters of the TEF-1α region. The phylogenetic tree was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.7408)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Species described in this work are highlighted. Ex-type cultures are in **bold.**



from Herr et al. 1983 and van Rensburg et al. 2006) required the placement of a 5 mm cube of colonised WAS into a 5–10 mm long slit made in the stem at a node. This wound was then sprayed with distilled water and wrapped with permeable film (Parafilm™). Control plants were wounded with a 5–10 mm long slit at the nodes as for the treated plants, then wrapped with permeable film without placing an agar cube in the wound. Both inoculated and control plants were sprayed with distilled water, placed in a dew chamber and incubated at 25 °C 12 h light / 20 °C 12 h dark for 48 h then returned to a growth cabinet under the light and temperature regime described above. This test was replicated five times for each isolate.

The less invasive mycelium contact method (Miric 2002) was used as a secondary test for pathogenicity of selected isolates. A 5 mm cube of inoculated agar was placed in contact with the stem at a node, sprayed with distilled water, wrapped with permeable film and incubated as described above. Plants were assessed for lesion development at 14 d after inoculation on a scale of 1 to 5 (Table 1).

RESULTS

Phylogenetic analysis

For the ITS region, approximately 540 bases were sequenced for the isolates in this study and added to the alignment. The alignment included sequences from 58 *Diaporthe*/*Phomopsis* species (including two outgroups), of which 23 were from ex-type cultures.

For the TEF-1α region, approximately 580 bases were sequenced for the isolates in this study. However, only 350 bases could be used to compare with the GenBank-retrieved sequences. The alignment included sequences from 24 *Diaporthe/ Phomopsis* species (including two outgroups), of which 20 were from ex-type cultures. Evolutionary relationships of these sequences were analysed using the ML method based on a K2+G model for ITS, and a HKY+G model for TEF-1α, as determined by Modeltest in MEGA v. 5.05.

The phylogramme of the ITS region showed that the Australian isolates of *Diaporthe* from stem cankers on sunflower formed three well-supported clades, which indicate novel species (Fig. 1). One of these clades was close to ex-type strains of three species, namely *D. angelicae*, *D. stewartii* and *P. dauci*, as well as an isolate of *P. subordinaria*. Furthermore, this clade included an isolate (DAR 73811) identified by Ash et al. (2010) as *Phomopsis* sp. that was pathogenic on *Carthamus lanatus* (saffron thistle, *Asteraceae*). To improve the resolution between this clade and *D. angelicae*, *D. stewartii* and *P. dauci*, an ML analysis was conducted on the TEF-1α dataset, which is consistent with the ITS phylogramme, but with a stronger bootstrap value (65 %) (Fig. 2).

The phylogenetic analysis of the ITS dataset included 31 isolates of *D. helianthi* sourced from five publications (Says-Lesage et al. 2002, van Niekerk et al. 2005, Bernardi-Wenzel et al. 2010, Santos et al. 2010, Vrandecic et al. 2010) and formed three distinct clades (Fig. 1). One clade included the ex-type culture of *D. helianthi* (CBS 592.81), while two other clades appeared to represent novel *Diaporthe* species (Fig. 1, *Diaporthe* sp. 1 and 2).

Pathogenicity

The 14 selected isolates inoculated onto sunflower caused a range of symptoms (Table 1), which divided them into two main groups. Ten isolates causing the most severe symptoms, rated 4 or 5 for virulence, originated from stems, seeds and leaves of infected sunflower plants from both NSW and Qld. Four isolates, causing less severe symptoms and rated 2 or 3 were collected from stems of infected plants in Queensland.

Using the wound inoculation method, tan to brown elongated lesions were evident above and below the point of inoculation after 3–7 d for the most virulent isolates, (those rated 4 or 5) with lesions expanding rapidly upwards causing plant death after 7–14 d. Earliest symptoms at 1–3 d after inoculation for the most virulent isolates (rated 4 or 5) included brownish streaks moving upwards from the inoculation site, wilting of leaves at the node closest to the site of inoculation as well as leaves directly above the site. At times, wilting of leaves above the site of inoculation occurred without obvious stem streaking. Generally, affected leaves developed a water-soaked appearance sometimes associated with twisting.

Two to four weeks after inoculation, stem pieces above and below the site of the wound were excised from all plants with lesions, surface sterilized as previously described, and incubated on WAS at 23–25 °C for up to 3 wk. Pycnidia developed between 7–21 d. Conidia oozing from pycnidia were streaked onto PDAS and the cultures compared with those of the original isolates. Isolates were re-inoculated onto sunflower plants to confirm their pathogenicity and to complete Koch's Postulates. A comparison of wound and mycelium contact inoculation methods showed similar results for pathogenicity for individual isolates after 14 d, although wound inoculated plants displayed symptoms 1–7 d earlier than those inoculated by the mycelium contact method.

Taxonomy

Based on morphology, pathogenicity and DNA sequence analysis, three undescribed species of *Diaporthe* were recognised. Although two of the new fungi only produced an anamorphic stage, all have been described in *Diaporthe* (1870), which has priority over *Phomopsis* (1884).

Diaporthe gulyae R.G. Shivas, S.M. Thompson & A.J. Young, *sp. nov.* — MycoBank MB561569; Fig. 3

Conidiomata pycnidialia, sparsa in PDA, subglobosa, usque ad 3 mm diametro, interdum rostris ostiolatis usque ad 1 mm longis, cinctis ectostromate nigro. Conidiophora facta e strato interiore parietis locularis, interdum ramosa et septata, subhyalina, usque ad 6 μm diametro. Cellulae conidiogenae cylindraceae, hyalinae, 7–18 × 1.5–2.5 μm. Alpha conidia globosa, subglobosa, ellipsoidea, ovalia vel obovoidea, hyalina, (6–)6.5–9.0(–10) × 2.5–3.5 μm. Beta conidia haud conspecta.

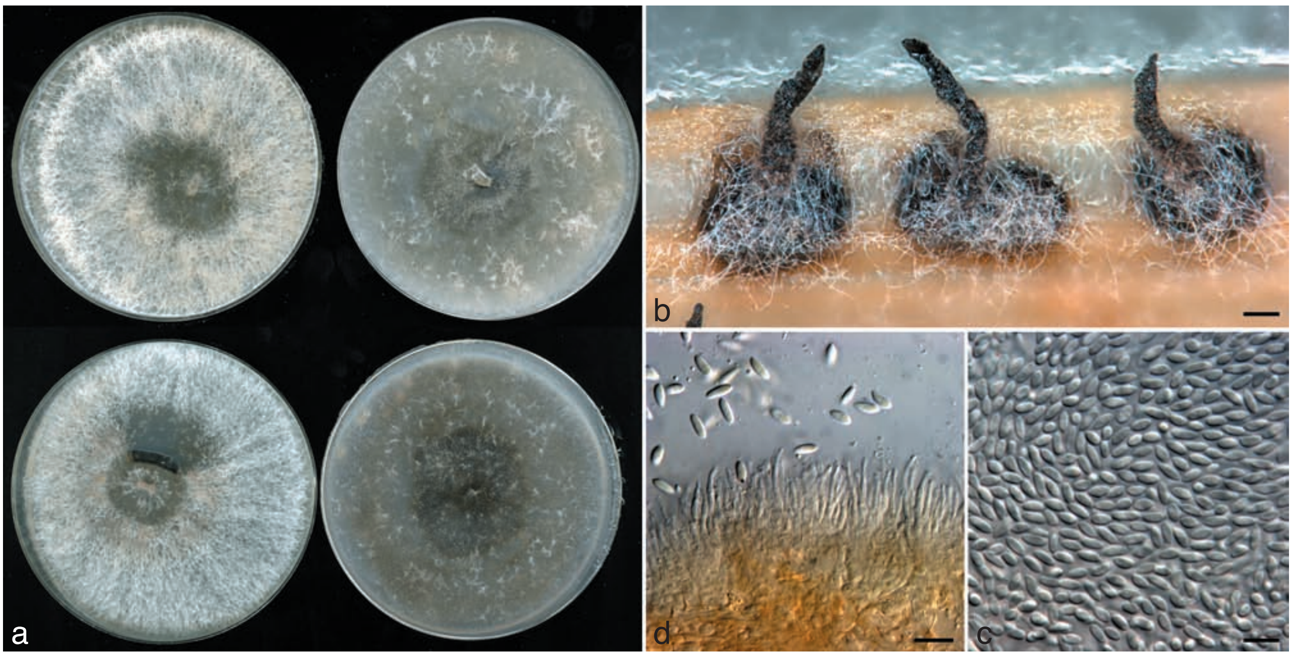


Fig. 3 *Diaporthe gulyae* (ex-type BRIP 54025). a. Cultures on PDA (left), OA (right) after 7 d (top) and 28 d (bottom); b. pycnidial beaks on sterilised wheat straw; c. alpha conidia; d. conidia and conidiophores. — Scale bars: b = 100 μm; c, d = 10 μm.

Etymology. In recognition of Dr Tom Gulya for his outstanding contributions to sunflower pathology research and enduring mentoring roles in the USA, Europe and Australia.

Conidiomata pycnidial, scattered on PDA, subglobose, up to 3 mm diam, occasionally with ostiolate beaks up to 1 mm long, surrounded by a black ectostroma. *Conidiophores* formed from the inner layer of the locular wall, sometimes branched and septate, subhyaline, up to 6 µm diam, *Conidiogenous cells* cylindrical, hyaline, 7–18 × 1.5–2.5 µm. *Alpha conidia* globose, subglobose, ellipsoidal, oval or obovoid, hyaline, (6–)6.5–9.0 (–10) × 2.5–3.5 µm. *Beta conidia* not seen.

Culture characteristics — Colonies on PDA covering entire plate after 10 d, buff, ropey near the margin and adpressed in the centre, scant aerial mycelium, reverse buff with a slightly darker centre; on OA covering the entire plate after 10 d, adpressed with scattered tufts of greyish mycelium, greyish sepia, with a fuscous black central zone 3 cm diam, reverse greyish sepia with a fuscous black central zone.

Specimens examined. Australia, Queensland, Ryeford near Clifton, on *Helianthus annuus* hybrid Sunbird 7, 29 Nov. 2010, *S.M. Thompson* (holotype BRIP 54025, includes ex-type culture); Ryeford near Clifton, on *Helianthus annuus* hybrid Sunbird 7, 29 Nov. 2010, *S.M. Thompson*, paratypes BRIP 54026, 54027.

Notes — Based on molecular phylogenetic inference, *D. gulyae* was placed near to the ex-type specimens of *D. angelicae*, *D. stewartii* and *P. dauci*, as well as a strain of *P. subordinaria* (Fig. 1, 2). Morphologically there is little difference between these species but unique fixed nucleotides accurately differentiate *D. gulyae*. *Diaporthe gulyae* differs from *D. stewartii* in two loci: ITS position 24 (T) and 98 (A); TEF-1α position 19 (A), 324 (T), 30 (T), 46 (T), 47 (A) and 315 (T). *Diaporthe gulyae* differs from *D. angelicae* and *P. dauci* in two loci: ITS position 59 (C), 90 (T), 136 (A), 158 (A) and 457 (A); TEF-1α position 30 (T) and 47 (A).

Diaporthe gulyae causes a severe stem canker on sunflower and saffron thistle. On the basis of pathology and substrate preference *D. gulyae* differs from *D. angelicae*, which is found on the decaying stems of hosts in the *Apiaceae* (Castlebury et al. 2003); *D. stewartii*, which causes stem blight of *Cosmos bipinnatus* (*Asteraceae*) (Harrison 1935); *P. dauci*, which causes inflorescence blight of *Daucus carota* (carrot, *Umbelliferae*) (von Arx 1951); and *D. adunca* (*P. subordinaria*), which attacks the scapes of *Plantago lanceolata* (*Plantaginaceae*) (Meijer et al. 1994).

Diaporthe kongii R.G. Shivas, S.M. Thompson & A.J. Young, *sp. nov.* — MycoBank MB561570; Fig. 4a, c, e

Conidiamata pycnidialia, sparsa in PDA, subglobosa, usque ad 2 mm diametro, rostris ostiolatis levibus ad apicem et saepe tectis hyphis brevibus inramosis usque ad 200 µm, cinctis ectostromate nigro. Conidiophora facta e strato interiore parietis locularis, polyangularia, interdum ramosa et septata, subhyalina ad brunneola olivacea, usque ad 6 µm diametro. Cellulae conidiogenae cylindraceae ad obclavatas, hyalinae, 6–12 × 1.5–4 µm. Alpha conidia ovalia ad cylindracea, biguttulata, hyalina, 5.5–7(–7.5) × 2–2.5(–3) µm. Beta conidia sigmoidea vel lunata, plerumque curvata per 90–180°, hyalina, 13–23 × 1–1.5 µm.

Etymology. In recognition of Dr Gary Kong for his innovative contributions to sunflower pathology in Australia, specifically his investigation of the genetics of resistance to *Puccinia helianthi* and *Alternaria helianthi*.

Conidiomata pycnidial, scattered on PDA, subglobose, up to 2 mm diam, with short (less than 0.5 mm) ostiolate beaks smooth towards apex and often covered with short unbranched hyphae up to 200 µm, surrounded by a black ectostroma. *Conidiophores* formed from the inner layer of the locular wall, polyangular, sometimes branched and septate, subhyaline to pale olivaceous brown, up to 6 µm diam. *Conidiogenous cells* cylindrical to obclavate, hyaline, 6–12 × 1.5–4 µm. *Alpha conidia* oval to cylindrical, biguttulate, hyaline, 5.5–7(–7.5) × 2–2.5(–3) µm. *Beta conidia* sigmoid to lunate, mostly curved through 90–180°, hyaline, 13–23 × 1–1.5 µm.

Culture characteristics — Colonies on PDA covering entire plate after 10 d, ropey with a conspicuous ring 2.5 cm in diam of tufted aerial mycelium and abundant tufts towards the margin, white to greyish white with scattered amber patches, with several scattered minute black stroma, reverse with an isabelline ring, paler towards the margin; on OA covering the entire plate after 10 d, adpressed, rosy-buff, with an irregular grey olivaceous central zone about 4.5 cm diam and smaller irregular grey olivaceous patches towards the margin containing a few minute black stroma, the central zone and patches have yellowish margins, reverse rosy buff with irregular isabelline patches.

Specimens examined. Australia, Queensland, Childers, on *Helianthus annuus* hybrid PDAS, 1 Dec. 2010, *S.M. Thompson* (holotype BRIP 54031, includes ex-type culture); Childers, on *Helianthus annuus* hybrid PDAS, 1 Dec. 2010, *S.M. Thompson*, paratype BRIP 54032.

Notes — Based on phylogenetic inference from the ITS sequence data (Fig. 1), *D. kongii* is closely related to *P. cuppatea*, which was isolated from plants of *Aspalanthus linearis* (rooibos, *Fabaceae*) with die-back (van Rensburg et al. 2006). Morphologically *D. kongii* has smaller conidia than those of *P. cuppatea*, which measure (10–)12–13(–14) µm.

Diaporthe kochmanii R.G. Shivas, S.M. Thompson & A.J. Young, *sp. nov.* — MycoBank MB561571; Fig. 4b, d, f–h

Perithecia formata in PDA et in caulibus sterilifactis apricifloris post octo hebdomades, subglobosa, usque ad 350 µm diametro, plerumque solitaria in agaro vel aggregata in fasciculis in caulibus, cincta ectostromate nigro, uno vel pluribus collis cylindraceis nigris ostiolatis usque ad 2 mm haud distinctis ab eis in pycnidiis. Asci unitunicati, cylindracei, 33–41 × 5–7 µm, hyalini, octospori, biseriati, annulo conspicuo refractivo apicali. Ascosporae hyalinae, mediane septatae, ovales ad cylindraceas, haud constrictae ad septum, guttula in quaque cellula, 9–10 × 2.5–3.5 µm, leves. Conidiomata pycnidialia, sparsa in PDA, nigra, subglobosa, usque ad 2 mm diametro, uno vel pluribus collis cylindraceis nigris ostiolatis usque ad 2 mm. Conidiophora facta e strato interiore parietis locularis, polyangularia, interdum ramosa et septata, subhyalina ad brunneola olivacea, usque ad 6 µm diametro. Cellulae conidiogenae cylindraceae ad obclavatas, hyalinae, 5–10 × 1.5–3 µm. Alpha conidia ovalia ad cylindracea, (5–)5.5–7(–7.5) × 2–3 µm. Beta conidia flexuosa ad lunata, plerumque curvata per 45–90°, hyalina, 11–17 × 1–1.5 µm.

Etymology. In recognition of Dr Joe Kochman who pioneered the investigation of rust races on sunflower in Australia and his widely recognised contributions to sunflower pathology.

Perithecia formed on PDA and on sterilised stems of sunflower after 8 wk, subglobose, up to 350 µm diam, usually solitary in the agar or aggregated in clusters on the stems, surrounded by a black ectostroma, with 1 or more cylindrical, black, ostiolate necks up to 2 mm, indistinguishable from those on pycnidia. *Asci* unitunicate, cylindrical, 33–41 × 5–7 (av. = 37 × 6 µm), hyaline, 8-spored, biseriate, with conspicuous refractive apical ring. *Ascospores* hyaline, medially septate, oval to cylindrical, not constricted at the septum, with a guttule in each cell, 9–10 × 2.5–3.5 µm (av. = 9.5 × 3 µm), smooth. *Conidiomata* pycnidial, scattered on PDA, black, subglobose, up to 2 mm diam, with 1 or more cylindrical black ostiolate necks up to 2 mm long. *Conidiophores* formed from the inner layer of the locular wall, polyangular, sometimes branched and septate, subhyaline to pale olivaceous brown, up to 6 µm diam. *Conidiogenous cells* cylindrical to obclavate, hyaline, 5–10 µm × 1.5–3 µm. *Alpha conidia* oval to cylindrical, (5–)5.5–7(–7.5) × 2–3 µm. *Beta conidia* flexuous to lunate mostly curved through 45–90°, hyaline, 11–17 × 1–1.5 µm.

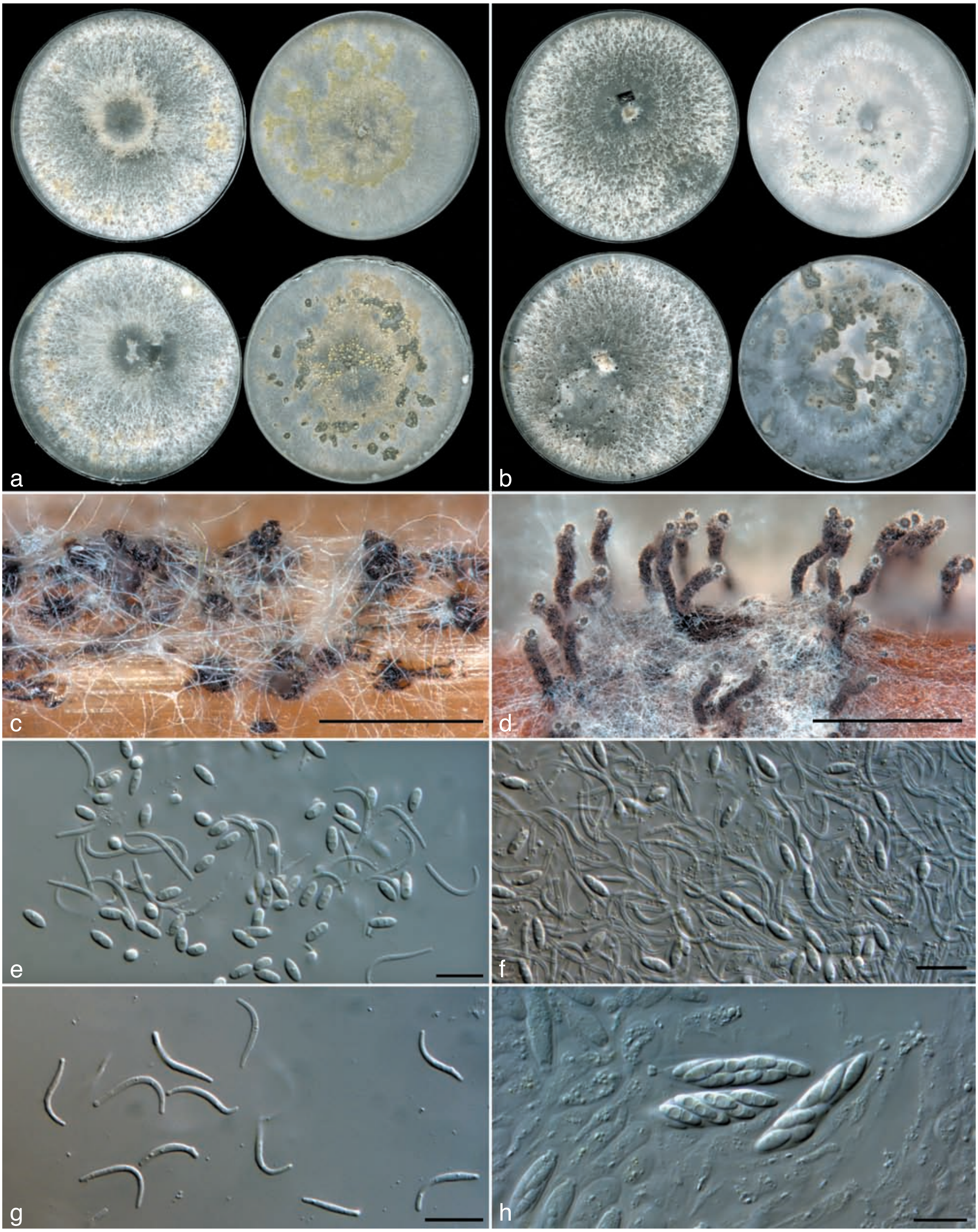


Fig. 4 *Diaporthe kongii* (ex-type BRIP 54031) and *D. kochmanii* (ex-type BRIP 54033). a. *Diaporthe kongii* cultures on PDA (left), OA (right) after 7 d (top) and 28 d (bottom); b. *Diaporthe kochmanii* cultures on PDA (left), OA (right) after 7 d (top) and 28 d (bottom); c. pycnidial beaks of *D. kongii* on sterilised wheat straw; d. perithecial necks of *D. kochmanii* on sterilised wheat straw; e. alpha and beta conidia of *D. kongii*; f. alpha and beta conidia of *D. kochmanii*; g. beta conidia of *D. kochmanii*; h. asci and ascospores of *D. kochmanii*. — Scale bars: c, d = 1 mm; e–h = 10 µm.

Culture characteristics — Colonies on PDA covering entire plate after 10 d, ropey with abundant tufts of mycelium, pale mouse grey, lighter towards the margin, with abundant scattered minute black stroma, reverse smoke grey with a darker central zone 5 cm diam; on OA covering the entire plate after 10 d, adpressed with scant tufted aerial mycelium, pale rosy vinaceous, with irregular pale olivaceous grey patches up to 1 cm wide containing minute black stroma, reverse pale rosy vinaceous with pale greyish areas where stroma form.

Specimens examined. Australia, Queensland, Lawes, on *Helianthus annuus* Experimental Line, 25 Nov. 2010, *S.M. Thompson* (holotype BRIP 54033, includes ex-type culture); Lawes, on *Helianthus annuus* hybrid PDAS, 25 Nov. 2010, *S.M. Thompson*, paratype BRIP 54034.

Notes — Based on phylogenetic inference of the TEF-1α sequence data *D. kochmanii* is closest to *D. kongii*. Morphologically these two species cannot be reliably separated. *Diaporthe kochmanii* differs from *D. kongii* in the TEF-1α locus position 60 (A), 83 (C), 184 (C), 219 (C), 240 (T), 260 (G), 266 (C), 268 (T), 280 (G), 284 (T), and 288 (T).

DISCUSSION

In this study, pathogenic *Diaporthe* species have been identified from wild, inbred and hybrid sunflowers grown throughout NSW and Qld. We have demonstrated that there are at least three previously unrecognised and novel species, namely *D. gulyae*, *D. kongii* and *D. kochmanii*, associated with stem cankers on sunflower in Australia. The most virulent of these species, *D. gulyae*, also contained an isolate identified by Ash et al. (2010) as pathogenic to saffron thistle. Symptoms caused by *D. gulyae* on sunflower closely resembled those of *D. helianthi.*

Unfavourable dry environmental conditions and low pathogen populations may explain the previous low frequency of sunflower stem cankers attributed to *Diaporthe* species in Australia. It is possible that severe outbreaks in Australia will remain sporadic, as has been found in Italy, despite climatic conditions appearing to be conducive to the disease (Battilani et al. 2003, Vergara et al. 2004). We consider it likely that outbreaks caused by these new *Diaporthe* species will become more widespread in the current cycles of wet summer weather, especially with the tendency towards minimum tillage practices that appear to increase pathogen inoculum in unprocessed stubble.

The molecular phylogenetic analysis showed that authentic *D. helianthi* derived from an ex-type isolate, clustered in a clade with isolates from the former Yugoslavia and France (Fig. 1). *Diaporthe helianthi* has also been recorded from hosts other than sunflower in Croatia (Vrandecic et al. 2010), which was part of the former Yugoslavia. All records of *D. helianthi* from hosts other than sunflower, without comparison to sequence data from ex-type cultures, should be treated with caution (e.g. van Niekerk et al. 2005, Bernardi-Wenzel et al. 2010). Unintentional misapplications of the name *D. helianthi* have resulted from the absence and inaccessibility of cultures derived from type material, which are needed for molecular comparison.

Based on the localities of previous *Diaporthe* collections in Australia from sunflower, soybean (*Glycine max*), Noogoora burr (*Xanthium pungens*) (Miric 2002), saffron thistle (Ash et al. 2010) plus herbarium records, we expect that future surveys will broaden the host and distribution ranges of these newly described species. We also anticipate that more species associated with stem cankers on sunflower in Australia will be identified.

The results of our study highlight the need for the re-evaluation of the identification and classification of *Diaporthe* (*Phomopsis*) species (Farr et al. 2002, Hyde et al. 2010, Santos et al. 2010, Udayanga et al. 2011). Accurate and reliable methods of identification for *Diaporthe* species is a major concern for biosecurity agencies in many countries, including Australia. In this regard, *D. helianthi* has not been identified from sunflower in Australia and remains a biosecurity threat.

Advances in molecular identification techniques are helping to further define species boundaries by providing more specific genetic evidence in support of taxonomic differences (Udayanga et al. 2011). The combination of pathology (host range and pathogenicity), taxonomic descriptions and molecular analyses will certainly result in the identification and description of more *Diaporthe* species from a range of host plants worldwide.

Acknowledgements We thank Dr Tom Gulya, USDA-ARS Northern Crops Laboratory ND, USA, and Dr Malcolm Ryley (Department of Employment, Economic Development and Innovation QLD, Australia) for their pathology advice, Loretta Serafin (NSW Department of Primary Industries, NSW, Australia) for agronomic expertise, the Australian Oilseeds Federation (AOF), the Australian Sunflower Association (ASA), Pacific Seeds, Nuseed and HSR Seeds and Grains Research Development Corporation (GRDC) for financial support. We also acknowledge the support of the University of Queensland. Don Barrett (University of Queensland) is thanked for translating the species diagnoses into Latin.

REFERENCES

Arx JA von. 1951. De phomopsisziekte van Zaadwortelen. European Journal of Plant Pathology 57: 44–51.

Ash GJ, Sakuanrungsirikul S, Anschaw E, Stodart BJ, Crump N, Hailstones D, Harper JDI. 2010. Genetic diversity and phylogeny of a Phomopsis sp., a putative biocontrol agent for Carthamus lanatus. Mycologia 102: 54–61.

Battilani P, Rossi V, Girometta B, Delos M, Rouzet J, Andre N, Esposito S. 2003. OEPP/EPPO Bulletin 33: 427–431.

Bernardi-Wenzel J, Garcia A, Filho CJR, Prioli AJ, Pamphile JA. 2010. Evaluation of foliar fungal endophyte diversity and colonisation of medicinal plant Luehea divericata (Martius et Zuccarini). Biological Research 43: 375–384.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553–556.

Castlebury LA, Farr DF, Rossman AY, Jaklitsch W. 2003. Diaporthe angelicae comb. nov., a modern description and placement of Diaporthopsis in Diaporthe. Mycoscience 44: 203–208.

Crous PW, Gams W, Stalpers JA, Robert V, Stegehuis G. 2004. MycoBank: an online initiative to launch mycology into the 21st century. Studies in Mycology 50: 19–22.

Debaeke P, Estragnat A, Reau R. 2003. Influence of crop management on sunflower stem canker (Diaporthe helianthi). Agronomie 23: 581–592.

Diogo ELF, Santos JM, Phillips AJL. 2010. Phylogeny, morphology and pathogenicity of Diaporthe and Phomopsis species on almond in Portugal. Fungal Diversity 44: 107–115.

Farr DF, Castlebury LA, Rossman AY. 2002. Morphological and molecular characterization of Phomopsis vaccinni and additional isolates of Phomopsis from blueberry and cranberry in the eastern United States. Mycologia 94: 494–504.

Gulya TJ, Rashid KY, Masirevic SM. 1997. Sunflower diseases. In: Schneiter AA (ed), Sunflower technology and production: 313–319. American Society of Agronomy, Madison USA.

Harrison AL. 1935. The perfect stage of Phomopsis stewartii on Cosmos. Mycologia 27: 1935.

Herr LJ, Lipps PE, Watters BI. 1983. Diaporthe stem canker of sunflower. Plant Disease 67: 911–913.

Hyde KD, Chomnunti P, Crous PW, Groenewald JZ, Damm U, KoKo TW, Shivas RG, Summerell BA, Tan YP. 2010. A case for re-inventory of Australia's plant pathogens. Persoonia 25: 50–60.

Masirevic S, Gulya TJ. 1992. Sclerotinia and Phomopsis – two devastating sunflower pathogens. Field Crop Research 30: 271–300.

Meijer G, Megnegneau, Linders EGA. 1994. Variability for isozyme, vegetative compatibility and RAPD markers in natural populations of Phomopsis subordinaria. Mycological Research 98: 267–276.

Miric E. 2002. Pathological, morphological and molecular studies of a worldwide collection of the sunflower pathogens Phomopsis helianthi and Phoma macdonaldii. PhD thesis, University of Queensland, Australia.

Miric E, Aitken EAB, Goulter KC. 2001. Phomopsis pathogenic on sunflower (Helianthus annuus L.) in Australia is not Phomopsis helianthi Munt.-Cvet et al. In: Proceedings of the 13th Australasian Plant Pathology Conference. Department of Primary Industries, Brisbane, Australia: 29.

Mostert L, Crous PW, Kang C-J, Phillips AJL. 2001. Species of Phomopsis and a Libbertella sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterisation. Mycologia 93: 146–167.

Muntañola-Cvetković M, Mihaljčević M, Petrov M. 1981. On the identity of the causative agent of a serious Phomopsis-Diaporthe disease in sunflower plants. Nova Hedwigia 34: 417–435.

Muntañola-Cvetković M, Mihaljčević M, Vukojević J, Petrov M. 1985. Comparisons of Phomopsis isolates obtained from sunflower plants and debris in Yugoslavia. Transactions of the British Mycological Society 85: 477–483.

Niekerk JM van, Groenewald JZ, Farr DF, Fourie PH, Halleen F, Crous PW. 2005. Reassessment of Phomopsis species on grapevines. Australasian Plant Pathology 34: 27–39.

Nitschke TRJ. 1870. Pyrenomycetes Germanici. Die Kernpilze Deutschlands Bearbeitet von Dr. Th. Nitschke 2: 161–320. Breslau, Eduard Trewent.

O'Donnell K, Kistler HC, Cigelink E, Ploetz RC. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proceedings of the National Academy of Sciences of the United States of America 95: 2044–2049.

Rayner RW. 1970. A mycological colour chart. Commonwealth Mycological Institute, Kew, UK.

Rekab D, Sorbo G del, Reggio C, Zoina A, Firrao G. 2004. Polymorphisms in nuclear rDNA and mtDNA reveal the polyphyletic nature of isolates of Phomopsis pathogenic to sunflower and a tight monophyletic clade of defined geographic origin. Mycological Research 108: 393–402.

Rensburg JCJ van, Lamprecht SC, Groenewald JZ, Castlebury LA, Crous PW. 2006. Characterisation of Phomopsis spp. associated with die-back of rooibos (Aspalathus linearis) in South Africa. Studies in Mycology 55: 65–74.

Saccardo PA. 1884. Reliquiae mycologicae Libertianae. IV. Revue Mycologique, Toulouse 6: 26–39.

Santos JM, Correia VG, Phillips AJ. 2010. Primers for mating-type diagnosis in Diaporthe and Phomopsis: their use in teleomorph induction in vitro and biological species definition. Fungal Biology 114: 255–270.

Santos JM, Phillips AJL. 2009. Resolving the complex of Diaporthe (Phomopsis) species occurring on Foeniculum vulgare in Portugal. Fungal Diversity 34: 111–125.

Says-Lesage V, Roeckel-Drevet P, Viguié A, Tourvielle J, Nicolas P, Tourvieille de Labrouhe D. 2002. Molecular variability within Diaporthe/Phomopsis helianthi from France. Phytopathology 92: 308–313.

Schneiter AA, Miller JF. 1981. Description of sunflower growth stages. Crop Science 21: 901–903.

Smith D. 2002. Culturing, preservation and maintenance of fungi. In: Waller JM, Lenné JM, Waller SJ (eds), Plant pathologist's pocketbook. 3rd ed: 384–409. CAB Publishing, UK.

Sutton BC. 1980. The Coelomycetes. CMI, Kew, Surrey, England.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar G. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution. doi: 10.1093/molbev/msr121.

Udayanga D, Xingzhong L, McKenzie EHC, Chukeatirote E, Bahkali AHA, Hyde KD. 2011. The genus Phomopsis: biology, applications, species concepts and names of common pathogens. Fungal Diversity: doi: 10.1007/s13225-011-0126-9.

Uecker FA. 1988. A world list of Phomopsis names with notes on nomenclature, morphology and biology. Contributions from the U.S. National Fungus Collection. Mycologia Memoir 13: 9–12.

Vergara M, Cristani C, Regis C, Vannacci G. 2004. A coding region in Diaporthe helianthi reveals genetic variability among isolates of different geographic origin. Mycopathologia 158: 123–130.

Vrandecic K, Jurkovic D, Riccioni L, Cosic J, Duvnjak T. 2010. Xanthium italicum, Xanthium strumarium and Arctium lappa as new hosts for Diaporthe helianthi. Mycopathologia 170: 51–60.

Wehmeyer LE. 1933. The genus Diaporthe Nitschke and its segregates. University of Michigan Studies 9: 1–161.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), PCR Protocols: A guide to methods and applications: 315–322. Academic Press, San Diego, USA.