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ORIGINAL PAPER

Re-classification of the causal agent of white grain disorder on wheat as three separate species of *Eutiarosporella*

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Abstract In the late 1990s, a novel *Botryosphaeria*-like fungal pathogen was observed causing a disease on wheat in Queensland, characterised as white grain disorder (WGD). In recent years, this disease has sporadically appeared across the eastern states of Australia. In this study, internal transcribed spacer (ITS) region sequences were used to compare these fungi to other *Botryosphaeriaceae* spp. to show that they should be reclassified as members of the *Eutiarosporella* genus. Using a small population of WGD isolates, we built a three-loci maximum likelihood tree, using ITS, β-tubulin, and Elongation Factor1-α sequences to show that there are three separate *Eutiarosporella* spp. found in infected grain. This multigene tree, with the support of phenotypic differences between clades observed in vitro, show that that the causal agents of WGD should be delimited into three divergent species; *Eutiarosporella tritici-australis, Eutiarosporella darliae,* and *Eutiarosporella pseudodarliae*.

Keywords *Botryosphaeriaceae* · *Botryosphaeria zeae* · Phylogeny · *Eutiarosporella* · White grain

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Introduction

In Australia, wheat is an economically significant industry that is worth over $4.5 billion dollars per year (Murray and Brennan 2009), a significant amount of which is exported to foreign markets. As an important export crop, understanding the abiotic and biotic factors that result in yield loss is critical. In the Australian wheat industry the major source of financial loss from disease is attributed to fungal pathogens (Murray and Brennan 2009). These losses are caused by reduction in overall yield due to disease or by reduction of overall grain quality and thus final sale price of the harvested grain.

In the late 1990s, a fungal pathogen of wheat was observed causing mummification of wheat grain in Queensland (Platz 2011). This fungus was initially identified as *Botryosphaeria zeae* (Platz 2011; Wildermuth et al. 2001), the pathogen responsible for grey ear rot in maize (De Leon et al. 2004). This disease, which shrivels and discolours wheat grain, became known as white grain disorder (WGD) (Platz 2011). Toxicity assays performed, showed this grain poses no detectable human health risk (Kopinski and Blaney 2010). Symptoms in wheat consistent with those of WGD were first recorded in Victoria (Vic) and South Australia (SA) in 2010 (Evans 2013; Penfold 2011). Since 2010, this disease has appeared sporadically across the Eastern Australian states.

To determine the causal agent(s) of WGD, the ITS region was sequenced from collected isolates and phylogenetic trees constructed. This analysis revealed two distinct clades (Stephen Neate, unpublished data). Most of the isolates from QLD and NSW were identified and labelled as *B. zeae*. *B. zeae* was also found in infected grain from VIC and SA,

however the majority of isolates collected from these states were differentiable from *B. zeae* and were designated as Clade 1 (Stephen Neate, unpublished data). The geographic localisation of Clade 1 in the Southern states and presence of *B. zeae* across all Eastern states, suggested that there was not a single species causing mummification of wheat grains in Australia, but instead a complex of closely related species. To date, however, no comprehensive analysis has been conducted that would enable growers or pathologists to accurately identify this disease in their harvested grain.

Species of *Botryosphaeriaceae* are widespread across the world, and are observed as both pathogens and endophytes (or potential pathogens) on a wide range of plant hosts (Sakalidis et al. 2011a, 2013; Alvarez-Loayza et al. 2011; Slippers and Wingfield 2007). In Australasia, a selection of these includes conifers (Slippers et al. 2005), eucalypts (Barber et al. 2005; Slippers et al. 2004b), mangoes (Sakalidis et al. 2011a), grapes (Taylor et al. 2005) and baobabs (Sakalidis et al. 2011a). Closely related fungal species, or recently diverged species, can remain morphologically similar for a long period of time (Luque et al. 2005; Slippers et al. 2004c, 2009; Sakalidis et al. 2011b). Accordingly, it can be a flawed exercise to delimit species of *Botryosphaeriaceae* with the use of morphological observations alone (Phillips et al. 2013). Analyses within the fungal kingdom, and more specifically within the *Botryosphaeriaceae* family, have shown the power of using multiple gene loci comparisons, complemented with morphological and observations, to perform species delimitation (Aoki et al. 2014; Dettman et al. 2003; McDonald et al. 2012; Jami et al. 2014; Slippers et al. 2004a, c; Zhou et al. 2014).

Modern genetic data is vital for continued examination and differentiation of fungal pathogens (Hyde et al. 2010). Currently, there are no sequenced genes of *B. zeae* present on GenBank or similar publicly available gene databases. However, since the original observation of wheat-infecting *B. zeae* in the late 1990s, there has been a significant increase in the available gene sequence data for various *Botryosphaeriaceae* species. Many genera of this family have been described with the support of multiple genetic loci. This has lead to a significant refinement in the resolution of these genera (Phillips et al. 2013; Slippers et al. 2013). Previously, genera that were poorly resolved, such as the genus of *Tiarosporella* (now *Tiarosporella* and *Eutiarosporella* (Crous et al. 2015)), have now been characterised and their genetic relationship to other *Botryosphaeriaceae* described (Phillips et al. 2013; Slippers et al. 2013; Crous et al. 2006, 2015).

Using this increase in the available genetic data to perform phylogenetic analyses, we sought to verify the validity of the initial placement of these fungi within the *Botryosphaeriaceae* genera. In addition, we used phylogenetic analyses and morphological evidence displaying differences between isolates to assess whether a complex of separate but closely related species cause WGD.

Materials and methods

Isolate sampling and primary culturing

Isolates were provided by the Queensland Department of Primary Industries (QLD DPI) and the South Australian Research and Development Institute (SARDI). Additional isolates were isolated on tap water agar (12 g agar/L) (TWA) from WGD grain samples provided by the QLD DPI and SARDI.

All isolates were sub-cultured on petri dishes of potato dextrose agar (PDA) (Difco, USA) with mycelia-covered plugs, and incubated at 23 °C under 12 h light/dark cycles. When isolates showed growth fatigue after numerous sub-culturing events, mycelia from 20 % glycerol stocks, stored at −80 °C, were plated onto PDA. Morphological characterisation on PDA was performed by sub-culturing mycelia-covered plugs from single-conidia cultures. For agar media inoculation, pycnidia were crushed and diluted in 1 mL of sterile water. This spore solution was filtered through cotton wool in a 1 mL syringe to decrease the amount of superfluous mycelial fragments. The filtered spore solution was inoculated over the surface of an agar plate. Plates were left overnight for conidia to germinate. Germinating conidia, forming single colonies, were observed under a dissecting microscope and picked from the agar with a toothpick and sub-cultured to new PDA plates. Mycelial measurements were performed at 400× magnification.

Induction of asexual reproduction and microscopy of conidia

Asexual reproduction was induced on oat media agar (OMA) as described by Mead et al. (2013) and on recommendation from SARDI for WGD fungi (without wheat-extract) (Mead et al. 2013). OMA has been previously shown to be appropriate for the induction of pycnidia in *Botryosphaeriaceae* spp. (Vasconcelos et al. 2001). Plugs of mycelia-covered PDA were transferred to petri dishes of OMA and left to grow in the dark at 23 °C for 5 days. The surface mycelia were scraped from these plates with a scalpel and 2 mL of sterile water was scraped over the surface. The plates were incubated at 23 °C under constant near u.v. light for 5–10 days until conidia-bearing pycnidia developed. Individual pycnidia were cut out from the agar surface and crushed in a drop of water between a microscope slide and coverslip. The conidia were photographed under 400× magnification with an EOS Rebel T3i camera (Canon, Japan). Fifty conidia of each type specimen were analysed to obtain the conidia dimension

measurements. To allow conidia to germinate, pycnidia were taken from the agar plate with a sterile toothpick, crushed with a pestle in a 1.5 ml tube (Eppendorf, Germany) with 20 μL of sterile water, and incubated at room temperature for 3 h.

Secondary culturing and growth analyses

Phenotypic variation was observed on six minimal media (MM) and four OMA media. The base minimal media (25 mMol of a carbon source, 10 mM of a nitrogen source, 10 mL/L of 100× trace element stock) was altered to incorporate varied carbon and nitrogen sources. Glucose, fructose, sucrose, and sorbitol were interchanged as the carbon source (with glutamate as the nitrogen source) and glutamate, glutamine and sodium nitrate were interchanged as the nitrogen source (with sucrose as the carbon source). In addition to standard OMA, OMA with 60 mL of V8 juice (V8-OMA) and OMA with 10 mMol gamma-aminobutyric acid (GABA) (GABA-OMA). All media contained 12 g Bacto agar/L (Difco, AUS).

Plugs of mycelia from single-conidia colonies (described above) were sub-cultured to appropriate media for growth analyses. Inoculated MM and OMA plates were grown under 12 h light/dark cycles at 23 °C. 150 mL of liquid MM was inoculated in a 250 mL flask, and shaken at 140 rpm, under 12 h light/dark cycles at 23 °C. Inoculated PDA plates were grown in full light for 15 h at 23 °C to ensure all isolates were growing healthily. Isolates were then rapped in metal-foil and grown in the dark at 16, 23, 28 and 30 °C, respectively, for effect of temperature on growth to be studied.

Genome sequencing and assembly

DNA was extracted from potato dextrose broth (PDB) (Edwards, USA) inoculated separately with isolate 153 (Clade 1, South Australia), isolate V4B6 (*B. zeae,* South Australia), and isolate 2G6 (*B. zeae,* Queensland). DNA was extracted using a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Netherlands). Illumina paired-end sequencing was performed using a HiSeq™ 2000 (Illumina, USA) at the John Curtain School of Medical Research, Australian National University (ANU). The resulting sequenced reads were quality trimmed using Trimmomatic v0.27 (Lohse et al. 2012) and assembled de novo with SPAdes v2.5.0, with –k mer values 21, 33, 55, and 77 (Bankevich et al. 2012).

DNA extraction and PCR

DNA was extracted from mycelia scraped off previously inoculated PDA plates. A QIAGEN DNeasy Plant Mini Kit (QIAGEN, Netherlands) was used to extract DNA. Universal fungal ITS1 and ITS4 primers were used to amplify the ITS region (White et al. 1990). Primers for β-tubulin and elongation-factor 1-α (EF1-α) regions were designed using Vector NTI® (Life Technologies, USA) (Supplementary Table 1). A local BLAST+ tools search using homologous sequences from *Botryosphaeria dothidea* (Table 1) (Altschul et al. 1990) was performed to locate the homologous ITS, β-tubulin, and EF1-α regions on the genomes of the four sequenced isolates.

Sequence alignment and phylogenetic analyses

All sequences (ITS, β-*tubulin*, EF1-α) were aligned with MEGA 5.2.2 (Tamura et al. 2011) and Geneious 7.0.6 (Biomatters, New Zealand). Regions of poor alignment were trimmed manually where necessary. Congruence between the three individual gene trees was determined using Concaterpiller 1.8a (Leigh et al. 2008). Phylogenetic analyses were performed using RaxML version 7.2.8 (Stamatakis 2006) with the '-f a' rapid bootstrap function and 10,000 bootstrap replicates. A complete list of all species and accession numbers are listed in (Table 2). The resulting trees were plotted with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Isolate sampling and phylogenetic analyses

Eleven isolates of white grain disorder fungi sourced from Queensland and South Australia were used in this study. ITS phylogenies performed by the QLD DPI have previously shown that there were likely two clades of WGD fungi (data not shown). The genomes of three isolates, isolate 153 (Clade 1), isolate V4B6 (*B. zeae*), and isolate 2G6 (*B. zeae*), were sequenced using Illumina paired-end sequencing. ITS sequences in these genomes were used as templates to assign a further eight isolates of WGD fungi into Clade 1 or *B. zeae* (Supplementary Fig. 1). The ITS region used was 367 bp in length and a maximum likelihood tree was generated using RAXML. This analysis was rooted with ITS sequences of *Botryosphaeria agaves.* According to this phylogeny, a total of four isolates resolved in Clade 1, and seven into *B. zeae. B. zeae* isolates separated into two independent clades, and will be referred to throughout the text as *B. zeae* and *B. zeae* Clade 2.

Phylogenetic analyses

A type specimen was chosen to represent each separate clade of WGD fungi; Isolate 153 for Clade 1, Isolate 2E2 for *B. zeae*, and Isolate HR599118 for *B. zeae* Clade 2. The ITS sequences of these isolates of WGD fungi were compared to members of *Botryosphaeriaceae.* The resulting maximum

Table 1 Details for the isolates of wheat-infecting *Eutiarosporella* spp. used in this study

Isolate	Collector	State	Species	Collection number	ITS GenBank accession No.	β-tubulin GenBank accession No.	EF1-α GenBank accession No.
153	SARDI	SA	*Eutiarosporella tritici-australis*	DAR 82485	KP309788	KP282462	KP309799
V6	SARDI	SA	*Eutiarosporella tritici-australis*	DAR 82486	KP309787	KP282463	KP309804
V26	SARDI	SA	*Eutiarosporella tritici-australis*	DAR 82487	KP309790	KP282461	KP309798
WG022	QLD DPI	VIC	*Eutiarosporella tritici-australis*	DAR 82488	KP309791	KP282460	KP309801
WG016	QLD DPI	QLD	*Euiarosporella darliae*	DAR 82493	KP309786	KP282457	KP309807
WG034	QLD DPI	QLD	*Eutiarosporella darliae*	DAR 82494	KP309792	KP282456	KP309806
2E2	QLD DPI	QLD	*Eutiarosporella darliae*	DAR 82491	KP309793	KP282454	KP309805
2G6	QLD DPI	QLD	*Eutiarosporella darliae*	DAR 82492	KP309795	KP282459	KP309803
HR511105	QLD DPI	QLD	*Eutiarosporella darliae*	DAR 82495	KP309789	KP282458	KP309802
HR599118	QLD DPI	QLD	*Eutiarosporella pseudodarliae*	DAR 82489	KP309796	KP282455	KP309797
V4B6	SARDI	SA	*Eutiarosporella pseudodarliae*	DAR 82490	KP309794	KP282453	KP309800

likelihood tree was rooted to *Endomelanconiopsis microspora*, a close relative to the *Botryosphaeriaeceae* (Slippers et al. 2013). The partial ITS sequence, 381 bp, was used to construct the phylogenetic tree (Fig. 1). Of the sampled *Botryosphaeriaceae* spp., the WGD fungi form a well-supported clade with the *Eutiarosporella* genus. Based on this single locus, WGD fungi resolve closely with *E. urbis-rosarum, E. tritici, T. madreeya*. The genus of *Tiarosporella* was recently resolved into four genera (*Tiarosporella, Marasasiomyces, Eutiarosporella,* and *Mucoharknessia*) (Crous et al. 2015). Accordingly, a subsequent tree was performed comparing the ITS sequences (369 bp) from these new genera (and species) with all of the WGD isolates (Supplementary Fig. 2). All of the isolates still resolve between the *Eutiarosporella spp.*. Based on ITS sequence comparison, *B. zeae* Clade 2 isolates resolve closely with *Mucoharknessia cortaderiae*. However, the morphological descriptions of *M. cartaderiae* (Crous et al. 2015) are in stark contrast to those of the WGD isolates (see below), and so it is reasonable to conclude that these WGD isolates are still members of the *Eutiarosporella* and not *Mucoharknessia*.

Multi-loci phylogenetic analyses

To further clarify the phylogenetic relationship both within clades of WGD fungi, and between WGD fungi and other *Eutiarosporella* spp., a phylogenetic analysis using ITS, β-tubulin, and EF1-α sequences was performed (Fig. 2). These loci were selected due to their prevalence in other studies that compared *Botryosphaeriaceae* spp. (Slippers et al. 2004a, b, c, 2005; Luque et al. 2005; Jami et al. 2012, 2013). Publically available sequence data for each of the three loci from *Eutiarosporella* spp., *E. urbis-rosarum, T. graminis* (now *Marasasiomyces karoo*), and *E. tritici* were used for comparison. Congruence between the three individual gene trees was determined using Concaterpiller 1.8a (Leigh et al. 2008), which produced a single concatenated set of the three loci, 1016 bp in length. The concatenated multi-gene phylogeny showed WGD fungi resolve most closely with *E. tritici*. Wheat-infecting *B. zeae* and *B. zeae* Clade 2 were separated both from each other and from Clade 1 with strong support (bootstrap values of 100). On the basis of these three loci, *E. tritici, B. zeae* and *B. zeae* Clade 2 branch separately to Clade 1, which all resolve on a monophyletic branch. A notable difference in the alignment of *B. zeae*, and *B. zeae* Clade 2 with the other species, is a 37–38 bp deletion in the EF1-α gene (Supplementary Fig. 3).

Morphological analyses

In accordance with the phylogenetic results, the three clades displayed morphological characteristics reminiscent of the *Eutiarosporella spp.* described in the literature (Figs. 3, 4, 5, and 6). On nutrient rich media (such as PDA or V8-OMA), each clade displayed rapid white growth (Fig. 6, Supplementary Fig. 7), similar to that observed in other members of the *Eutiarosporella* genus (Jami et al. 2014; Phillips et al. 2013). Each of the clades also produced hyaline, thin-

Table 2 The Accession numbers of the *Botryosphaeriaceae* spp. loci used in the phylogenetic analyses

Genus	Species	Collection number	NCBI accession numbers		
			ITS	Beta-tubulin	EF-1α
Botryosphaeria	*agaves*	MFLUCC 11-0125	JX646791.1		
	corticis	CBS 119048	DQ299246.1		
	dothidea	BD080705002	FJ493245.1		
Barriopsis	*fusca*	CBS 122538	NR_119698.1		
Cophinforma	*eucalypti*	MFLUCC 11-0655	JX646801.1		
Diplodia	*sapinea*	CMW 190	KF766159.1		
	seriata	CBS 112555	NR_111151.1		
Dothiorella	*dulcispinae*	CMW 36460	NR_111702.1		
	thailandica	MFLUCC 11-0438	NR_111794.1		
Endomelanconiopsis	*microspora*	CBS 186.97	KF766165.1		
Lasiodiplodia	*venezuelensis*	CBS 174.26	KF766194.1		
	crassispora	CBS 353.97	KJ774059.1		
Macrophomina	*phaseolina*	CMM 3650	KF234552.1		
Neodeightonia	*phoenicum*	CMW 38428	NR_111325.1		
Neofusicoccum	*parvum*	CMW 37263	KF432951.1		
	ribis	CBS 115475	KF766205.1		
Neoscytalidium	*dimidiatum*	CMM 3979	JX513637.1		
Phaeobotryon	*mamane*	CMW 7060	NR_111324.1		
Phaeobotryosphaeria	*visci*	CAP 339	KF766211.1		
Phyllosticta	*podocarpi*	CPC 12440	KF766217.1		
Pseudofusicoccum	*adansoniae*	PD252	GU251155.1		
Eutiarosporella	***africana***	CMW 38428	KC769959.1		
	tritici	CBS 118719	KF531830.1	KF531810.1	KF531809.1
	urbis-rosarum	CMW 36479	JQ239409.1	JQ239383.1	JQ239396.1
	tritici	CBS 118719	KF531830.1	KF531810.1	KF531809.1
Marasasiomyces	***graminis***	CBS 118718	KC769962.1	KF531808.1	KF531807.1
Mucoharknessia	***cortaderiae***	CPC 19974	KM108374.1		
		CPC 22208	KM108375.1		
Tiarosporella	***madreeya***	CBS 532.76	KC769960.1		

walled and aseptate conidia, some having mucosal appendages, which is a key feature of the *Eutiarosporella* spp. amongst the *Botryosphaeriaceae* (Phillips et al. 2013). The conidia from both Clade 1 and *B. zeae* Clade 2 had conidial dimensions similar to *E. tritici*, as opposed to the other *Eutiarosporella* spp. (Supplementary Fig. 5) (Jami et al. 2012, 2014; Phillips et al. 2013; Sutton and Marasas 1976). Surprisingly though, *B. zeae* had smaller conidia than the other two clades.

On defined minimal media (MM), the extent to which growth was affected by supplied nutrients and the resulting growth morphology was different between the clades (Fig. 6). In general, growth of *B. zeae* was diminished on all defined media in comparison with the other clades. This is in contrast to growth on nutrient-rich media where the difference in healthy growth appearance was less distinct between the clades. As expected, sorbitol was the poorest carbon source for all of the clades. Each responded more positively to the remaining carbon sources. The responses of *B. zeae* to these were similar to each other, with wispy, white mycelial growth. *B. zeae* Clade 2 grew in a thick, striated pattern on glucose and fructose, but was more wispy and floccuous on sucrose. Clade 1 displayed thicker growth on glucose and sucrose, with the appearance of thinner growth on fructose. Growth for Clade 1 on all of the carbon sources was floccuous and white. For the nitrogen sources, glutamate appeared to provide the thickest growth, followed by glutamine. Interestingly, none of the clades responded well to nitrate as the carbon source, a commonly used nitrogen source in defined fungal media. On liquid MM (with sucrose as the carbon source and glutamine as the carbon source) (Supplementary Fig. 6) Clade 1 produced an iron-ore coloured pigment 2-3 dpi. *B. zeae* produced a similar pigment 5–7 dpi. No pigment formation was observed in this analysis for *B. zeae* Clade 2.

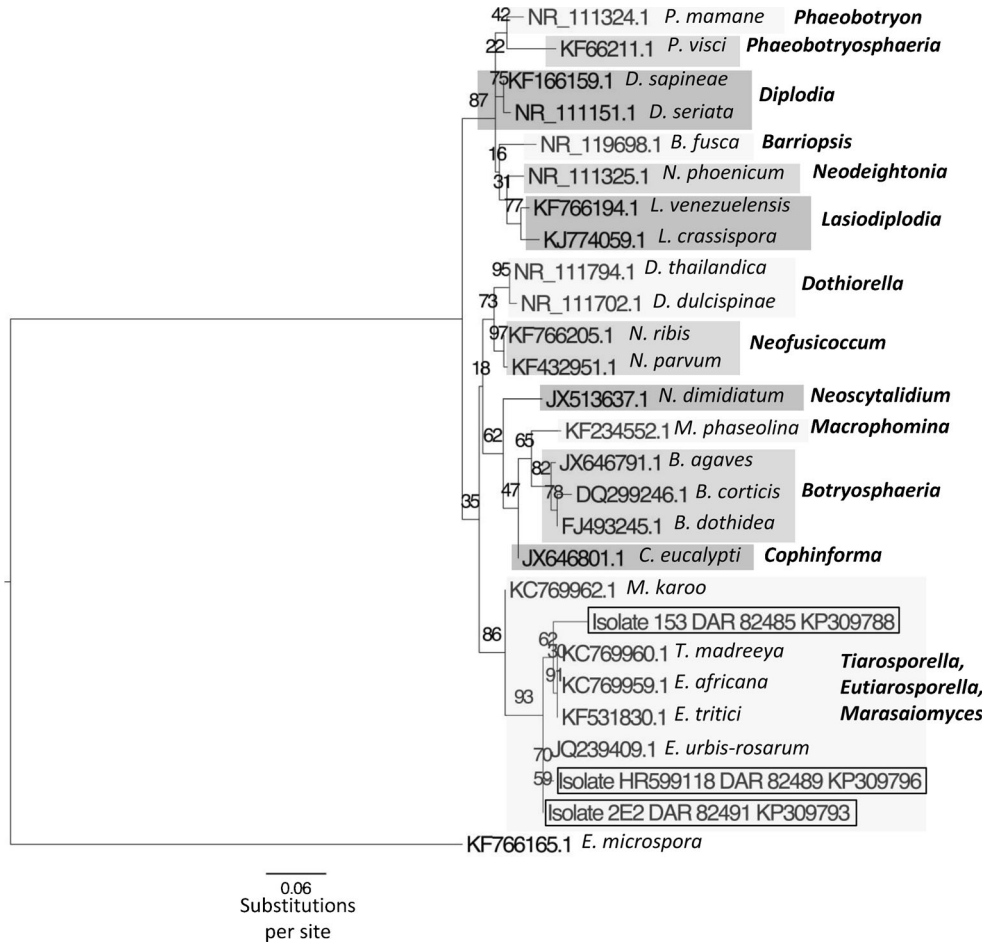

Fig. 1 Comparison of ITS sequences showed that WGD fungi resolved most closely with members of the *Tiarosporella* genus. A Maximum likelihood (ML) tree was performed comparing ITS sequences of the holotype isolates of WGD fungi to other *Botryosphaeriaceae.* The ML tree had 10,000 supporting bootstrap replicates, and was rooted to *Endomelanconiopsis microspora*. WGD fungi (*black* outlines) grouped within the *Tiarosporella* genus with high bootstrap support. *Grey* translucent boxes delineate the genera listed on the right

On PDA, all three clades responded similarly to each other under different growth temperatures (Supplementary Fig. 4). At 16 °C, growth was inhibited for all isolates. At 23 °C, radial growth was rapid (see below for more detailed descriptions of growth at this temperature). At 28 °C, radial growth was rapid, with thick mycelial growth for all isolates. At 3dpi at this temperature, Clade 1 mycelial growth was thick, white, and floccuous, becoming more appressed toward the site of inoculation; *B. zeae* mycelial growth was bright white with thick growth around the edge of the petri dish; *B. zeae.* Clade 2 growth was thick white at the border of the petri dish with a segregating band of thin-growth separating thick, white-to-olivaceous growth towards the site of inoculation. At 30 °C, radial growth was inhibited for each of the clades, however, mycelial growth was thick. According to this preliminary analysis, the optimum temperature for each of the clades is <23 °C but >30 °C.

Growth on OMA was observed as this is the base sporulation media used for these fungi (Supplementary Fig. 7). Each of the clades displayed thick, white mycelial growth. *B. zeae* and Clade 2 *B. zeae* were more floccuous in appearance than when observed on PDA. GABA-OMA was used as a media source in order to determine if GABA as a supplement may alter the growth of the fungi. GABA has previously been shown to alter sporulation in *Parastagonospora nodorum* and Clade 1 (previously acknowledged as "*Botryosphaeria spp.*") (Mead et al. 2013) when added as a media supplement. Growth was not altered by the addition of GABA for any of the clades.

V8-OMA was used to observe growth on a more nutrient rich, complex media. Each of the clades responded well to the V8 juice with thicker mycelial growth, covering more of the plate's surface.

We describe the type species of three novel species of WGD fungi (*Eutiarosporella tritici-australis* (HOLOTYPE: DAR 82485) (formerly Clade 1) (Fig. 3), *Eutiarosporella darliae* (HOLOTYPE: DAR 82491) (formerly *B. zeae*) (Fig. 4), and *Eutiarosporella pseudodarliae* (HOLOTYPE: DAR 82489)(formerly *B. zeae* Clade 2) (Fig. 5)) and continue to develop upon their specific morphology, in detail below.

Eutiarosporella tritici-australis E. Thynne, M.C. McDonald, M. Evans, H. Wallwork, S. Neate, P.S. Solomon sp. nov. **(formerly Clade 1)**

MycoBank no.: MB 811115

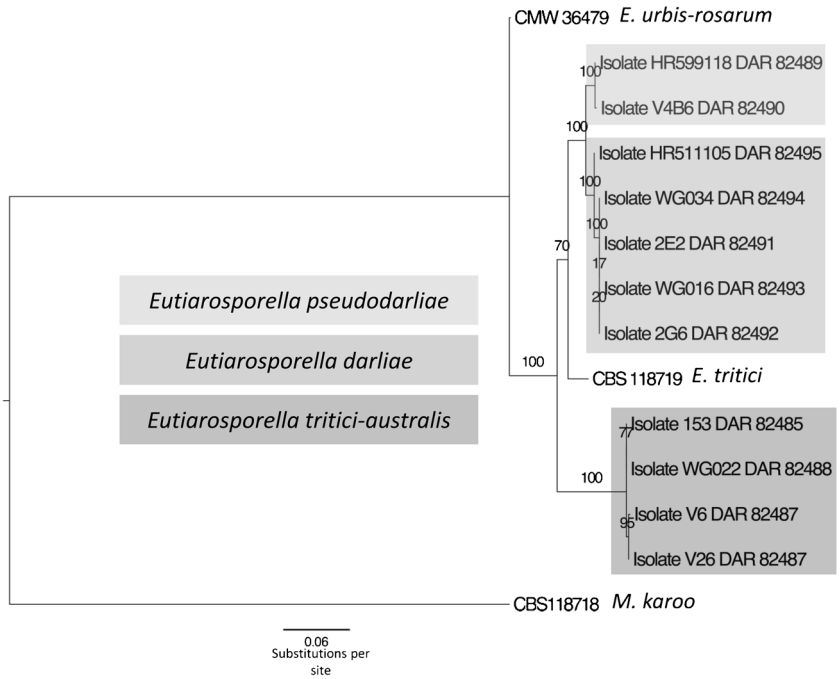

Fig. 2 A comparison of ITS, β-tubulin, EF1-α from WGD fungi and *Tiarosporella* spp. distinctly separates the three clades of WGD fungi. A Maximum likelihood (ML) tree shows the genetic relationship between the clades of WGD fungi and with other *Tiarosporella* spp. The tree was rooted with *Marasasiomyces karoo*

Etymology: Named after a close relative, *Eutiarosporella tritici*, (and because it infects wheat (*Triticum aestivum*)) in conjunction with the country in which it causes disease.

On PDA media, growth is floccuous with thick aerial mycelia. Radial growth is rapid with 5.5–5.9 cm growth 2 days post inoculation (dpi) and the plate is covered by 3 dpi, with mycelia extending up the plate walls by 4 dpi. The mycelia, initially white, discolour with age to white-grey, dark grey, and olivaceous grey/black. Mycelia remain generally floccuous with age, however, are slightly more appressed close to the site of inoculation. Microscopically, healthy growing mycelia are hyaline and septate, with a granular surface appearance. Hyaline mycelia range from 3 to 8 μm in diameter. Pigmented mycelia range from 7 to 16 μm in diameter. Pycnidia form on PDA after an extended period of growth (beyond 4 weeks post inoculation (WPI)). However, pycnidia formation is more rapid under near u.v. lights and on OM. Pycnidia were observed both solitarily and/or grouped and were semi-immersed. Initially globular, a stem grows above surface mycelia (0.26(±0.06)mm × 0.93(±0.14)mm). Pycnidia can remain single stemmed or become highly branched. Pycnidia can become erumpent with maturity, exuding conidia bearing cirrus. Conidiogenous cells (7–12 μm × 3–5 μm) are hyaline, thin walled and holoblastic. Conidia are hyaline, thin-walled, aseptate, and range from clavate to fusiform in shape (12.25(±0.37) μm × 37.8(±0.65) μm). Some conidia bear a faint mucosal appendage reminiscent of a halo. Conidia were observed germinating from both the apical and basal ends.

Typification*:* AUSTRALIA, South Australia, isolated from *Triticum aestivum* (bread wheat), 2012, SARDI

(HOLOTYPE and EX-TYPE: DAR 82485)

Host and distribution: Individuals have been observed in infected wheat and wheat-stubble in South Australia and Victoria.

Optimum temperature range: >23 °C but <30 °C

Eutiarosporella darliae E. Thynne, M.C. McDonald, M. Evans, H. Wallwork, S. Neate, P.S. Solomon sp. nov. **(Formerly *B. zeae*)**

MycoBank no.: MB 811116

Etymology: Named after the location where it was first observed infecting wheat, Darling Downs, Queensland.

On PDA media, initially, mycelia are floccuous close to the site of inoculation with limited aerial growth. However, radially out, the mycelia become dense and close-knit. Microscopically, healthy growing mycelia are hyaline and septate, with a granular surface appearance. Viewed from beneath the media plate, the fungus darkens in a piebald pattern. On occasion, a secreted pigment stains the media a violaceous colour. Viewed from the surface, the mycelia appear to remain vividly white with age. These mycelia are highly appressed into a dense mat that can be pealed back from the agar surface in a single layer. However, light brown to olivaceous-gray mycelia can be present. Hyaline mycelia range from 1 to 5 μm in diameter. Pigmented mycelia range from 4 to 8 μm in diameter. Conidia bearing pycnidia form sporadically on OMA under near u.v. light. Conidia are semi-immersed to not immersed. They appear to remain small and predominantly globular, with some skewed to the cylindrical, ranging from 80 to 170 μm in diameter. Conidiogenous cells (7–16 μm × 3–7 μm) are hyaline, thin walled and holoblastic. Conidia are hyaline, thin-walled, aseptate, and range from clavate to

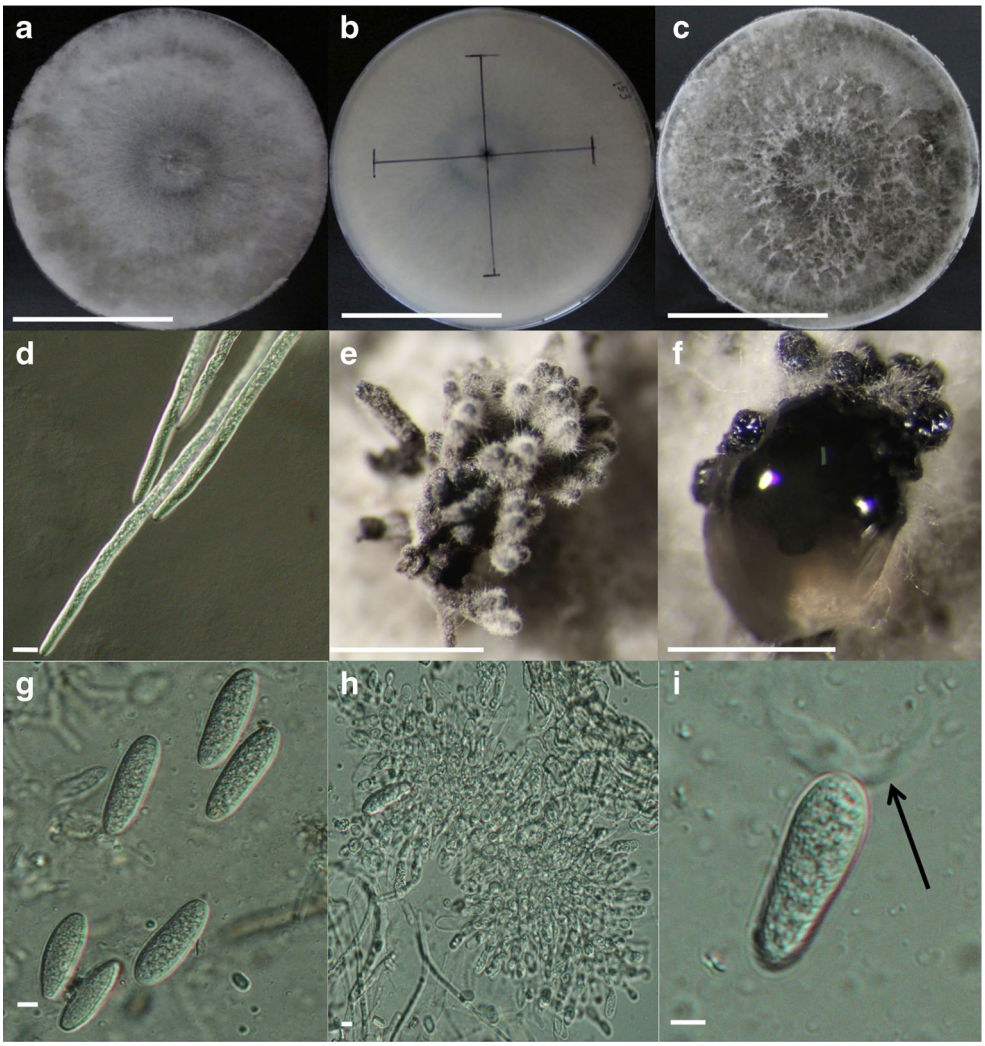

Fig. 3 Images of different aspects of *Tiarosporella tritici-australis* sp. nov.. Mycelial growth of *E. tritici-australis* on PDA. **a**–**c**. Mycelial growth 4DPI, bar=4.5 cm (**a**). Underside growth of mycelia 4DPI (drawn lines indicate radial growth point at 2DPI), bar=4.5 cm (**b**). Mycelial appearance after 4 WPI, bar=4.5 cm (**c**). Growing hyphal tips, bar=10 μm (**d**). Multi-branched pycnidium, bar=1 mm (**e**). Erumpent pycnidium, bar= 1 mm (**f**). Conidia, bar=10 μm (**g**) Conidiogenous cells, bar=10 μm. Conidium, arrow indicates mucosal appendage, bar=10 μm (H)

fusiform in shape. The observed conidia were surprisingly small in comparison to *E. tritici-australis* and *E. pseudodarliae* (9.26(±0.33) μm×27.37(±0.49) μm). Some conidia bear a faint mucosal appendage reminiscent of a halo. Conidia were observed germinating from both the apical and basal ends.

Typification: AUSTRALIA, Queensland, isolated from *Triticum aestivum* (bread wheat), 2012, QLD DPI

(HOLOTYPE and EX-TYPE: DAR 82491)

Host and Distribution: Individuals have been observed in infected wheat and wheat-stubble in Queensland and Northern New South Wales.

Optimum temperature range: >23 °C but <30 °C

Eutiarosporella pseudodarliae E. Thynne, M.C. McDonald, M. Evans, H. Wallwork, S. Neate, P.S. Solomon sp. nov. **(Formerly *B. zeae* Clade 2)**

MycoBank no.: MB 811117

***Etymology*:** Named after its closest known relative *Eutiarosporella darliae*, with which it shares very close growth characteristics.

Individuals have been observed in infected wheat and wheat-stubble across the Eastern Australian states. On PDA media, mycelial growth is thick with a limited amount of dense aerial growth. Radial growth is rapid with 5.5–6 cm of growth, 2 days post inoculation (dpi) and the plate is covered by 3 dpi. Initial mycelial growth is white, but discolours. Viewed from the surface of the media plate, the mycelia darken to grey-white, olivaceous-grey/black, and light brown. Viewed from beneath the media plate, the fungus darkens rapidly from the site of inoculation. The agar beneath the surface mycelia becomes tar-like in appearance. Microscopically, healthy growing mycelia are hyaline and septate, with a granular surface appearance. Hyaline mycelia range from 1 to 7 μm in diameter. Pigmented mycelia range from 3 to 7 μm in diameter. On PDA and OMA, pycnidia form readily and profusely. Pycnidia were observed as semi-immersed, and single stemmed. Stems are smaller than observed on *E. tritici-australis* (0.11(±0.04)mm× 0.37(±0.15)mm). Pycnidia can become erumpent with maturity, exuding conidia bearing cirrus. Conidiogenous cells (7–

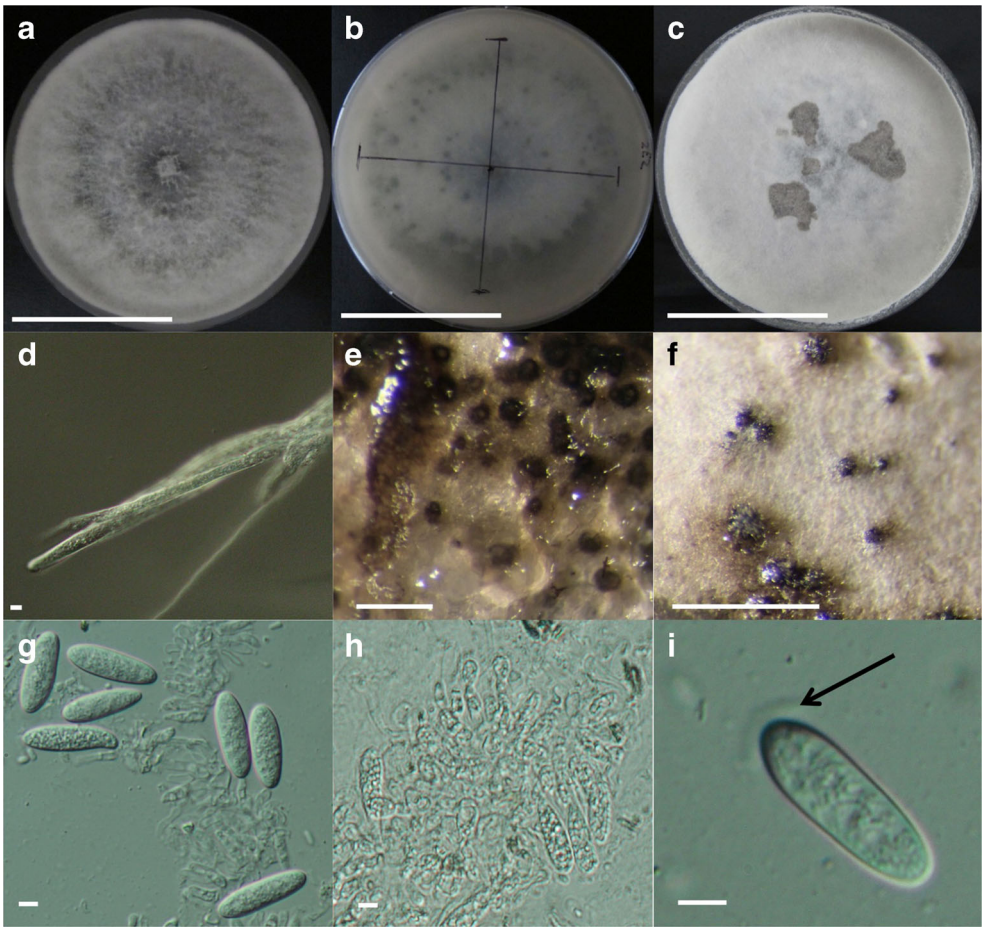

Fig. 4 Images of different aspects of *Tiarosporella darliae* sp. nov.. Mycelial growth of *E. darliae* on PDA. **a**–**c**. Mycelial growth 4DPI, bar=4.5 cm (**a**). Underside growth of mycelia 4DPI (*drawn lines* indicate radial growth point at 2DPI), bar= 4.5 cm (**b**). Mycelial appearance after 4 WPI, bar=4.5 cm (**c**). Growing hyphal tips, bar=10 μm (**d**). Pycnidia immersed in agar, bar=0.5 mm (**e**). Pycnidia through mycelia, bar=0.5 mm (**f**). Conidia, bar=10 μm (**g**) Conidiogenous cells, bar=10 μm. Conidium, arrow indicates mucosal appendage, bar=10 μm (**h**)

16 μm×3–7 μm) are hyaline, thin walled and holoblastic Conidia are hyaline, thin-walled, aseptate, and range from clavate to fusiform in shape (12.39(±0.47) μm× 35.84(±0.60) μm). Some conidia bear a faint mucosal appendage reminiscent of a halo. Conidia were observed germinating from both the apical and basal ends.

Typification: AUSTRALIA, Queensland, isolated from *Triticum aestivum* (bread wheat), 2011, QLD DPI

(HOLOTYPE and EX-TYPE: DAR 82489)

Host and distribution*:* Individuals have been observed in infected wheat and wheat-stubble across the Eastern Australian states.

Notes: *Eutiarosporella pseudodarliae* and *Eutiarosporella darliae* are closely related genetically, and can appear very similar in culture.

Optimum temperature range: >23 °C but <30 °C

Discussion

In this study fungal specimens isolated from diseased wheat-grain, previously described as *Botryosphaeria zeae,* were reclassified as members of the genus *Eutiarosporella* based on comparison of ITS sequences. Although originally grouped as a single species, our results revealed a species complex within the isolates analysed. With the support of a multigene phylogeny (ITS, β-tubulin and EF1-α), three novel species of *Eutiarosporella* are described; *Eutiarosporella tritici-australis*, *Eutiarosporella darliae*, and *Eutiarosporella pseudodarliae*.

Wheat is an economically significant industry in Australia that is under constant threat from fungal diseases (Murray and Brennan 2009). Accordingly, correct identification of the pathogen species that affect this industry is vital for growers and pathologists. Due to lack of sequence data, we were unable to compare these unknown wheat-infecting isolates to their original species classification, *B. zeae*. Fortunately, however, since the first observation of WGD fungi in 1999, there has been a significant expansion in the number of *Botryosphaeriaceae* spp. recognised. In various cases, the genera and species have been greatly refined (Crous et al. 2006, 2015; Slippers et al. 2013) and the species within described in detail (Phillips et al. 2013). This allows rapid identification of other *Botryosphaeriaceae* spp., as was shown in this study.

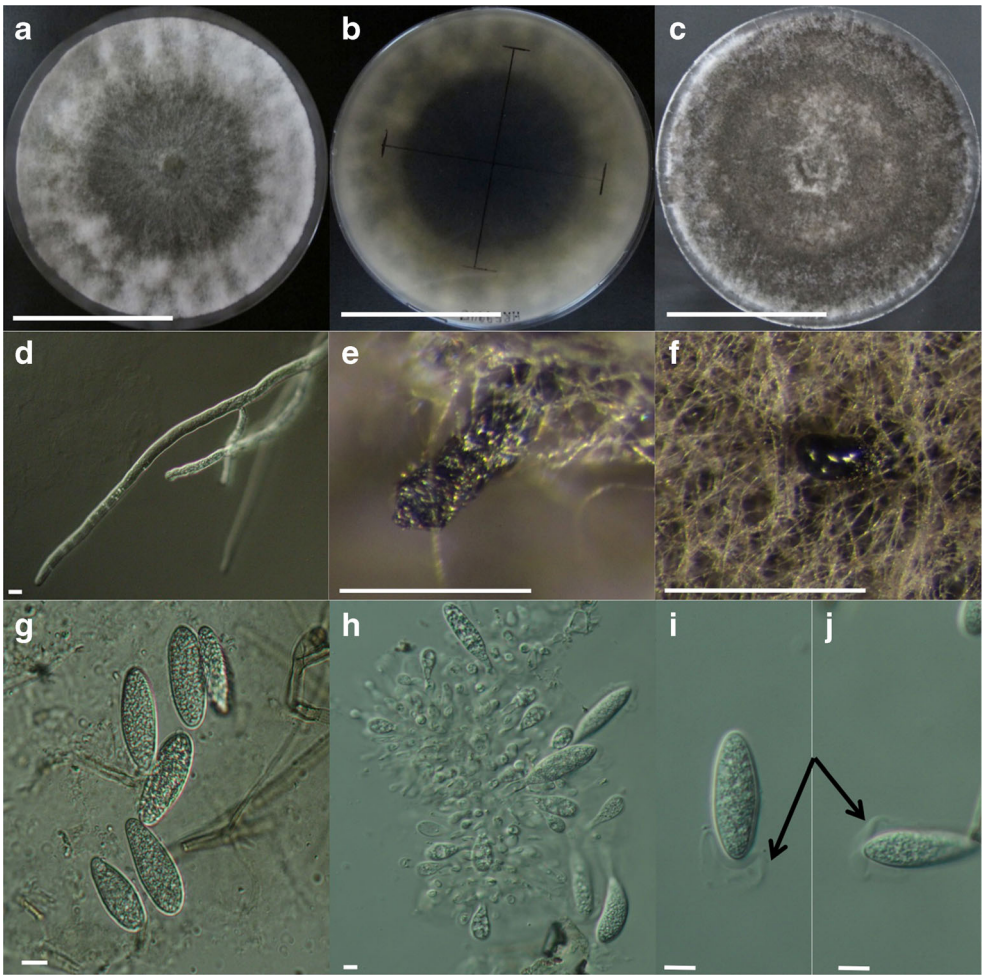

Fig. 5 Images of different aspects of *Tiarosporella pseudodarliae* sp. nov.. Mycelial growth of *E. pseudodarliae* on PDA. **a**–**c**. Mycelial growth 4DPI, bar=4.5 cm (**a**). Underside growth of mycelia 4DPI (*drawn lines* indicate radial growth point at 2DPI), bar=4.5 cm (**b**). Mycelial appearance after 4 WPI, bar=4.5 cm (**c**). Growing hyphal tips, bar=10 μm (**d**). Pycnidium, bar=0.5 mm (**e**). Erumpent pycnidium, bar=0.5 mm (**f**). Conidia, bar=10 μm (**g**) Conidiogenous cells, bar=10 μm. Conidium, *arrow* indicates mucosal appendage, bar=10 μm (**h**)

Herein, we re-designate WGD *Botryosphaeriaceae* species to now be contained within the genus *Eutiarosporella*. This is interesting because, to the best of our knowledge, the only species of the former *Tiarosporella* (now four generea) identified in Australia previously were saprophytic isolates, isolated from submerged trees in Queensland and described as *T. paludosa* (Hyde 1993). Unfortunately, gene sequence data is currently not available for these isolates. For many years, sequences confirming the identity of *Tiarosporella and Eutiarosporella* spp. have been limited to isolates collected from South Africa (Jami et al. 2014). The South African species *T. madreeya, T. tritici* (now *Eutiarosporella tritici),* and *T. graminis* (now *Marasasiomyces karoo*) (Crous et al. 2015)) were documented early in the literature (Sutton and Marasas 1976), but only had genes sequenced more recently (Crous et al. 2006; Phillips et al. 2013). Other species, *E. urbis-rosarum* and *E. africana* were only recently identified and characterised (Jami et al. 2012, 2014). Of all of these South African *Eutiarosporella* spp., the only species documented to have been interacting with wheat is *E. tritici*. Sutton and Marasas (1976) described *E. tritici* from wheat-straw in South Africa. The morphological descriptions of *E. tritici* from South Africa (Sutton and Marasas 1976) resemble those of the three, novel Australian wheat-infecting *Eutiarosporella* spp. described in this study. For example, the described conidia size for all species. We have also shown that *E. tritici* resolves closely with these three species, and is one of the roots for the etymology of *E. tritici-australis.*

This analysis does not yet shed light on the definitive origin of these pathogen species. Jami et al. discussed the overrepresentation of *Tiarosporella spp.* from South Africa, but note this is probably due to lack of more widespread sampling (Jami et al. 2014). From the perspective of WGD, a greater survey into potential alternative hosts for these pathogens may be required in order to determine the presence of other *Eutiarosporella* spp. in Australia.

Hyde et al. (2010) emphasised the need for up-to-date cataloguing of Australian fungal plant-pathogens (Hyde et al. 2010). They illustrate this need by highlighting discrepancies between gene-sequence and morphological data attributed to a single *Botryosphaeriaceae* isolate (Hyde et al. 2010). Our study supports Hyde's assertion about the importance of

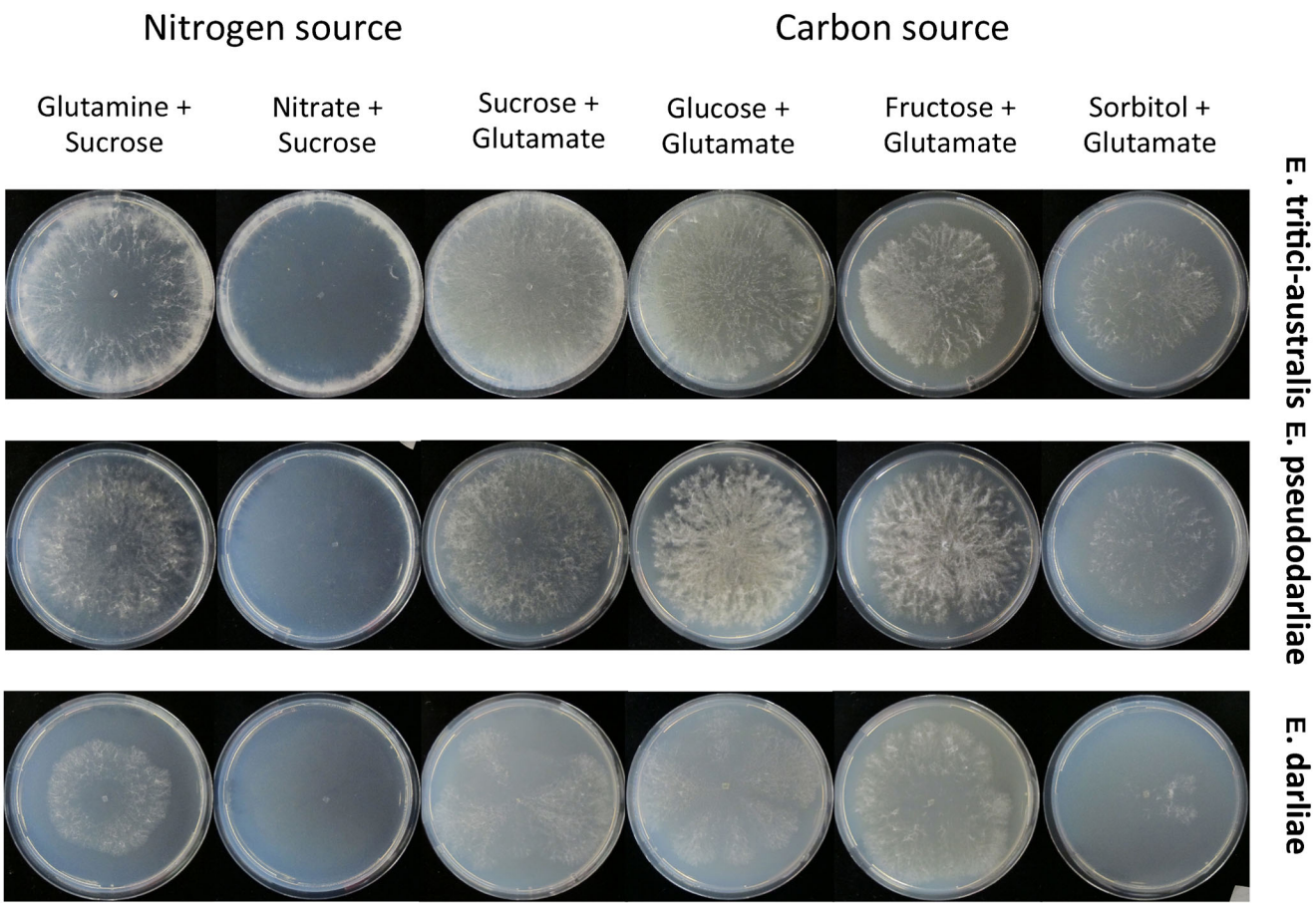

Fig. 6 Images displaying the growth of each of the clades on defined minimal media with interchanged carbon or nitrogen sources. Images taken 4 days post inoculation

performing a "re-inventory" of Australian fungal plant-pathogens (Hyde et al. 2010), as we too have highlighted nomenclature discrepancies that could only be resolved with genetic comparisons. To date, there are no published genetic studies analysing the maize pathogen, *B. zeae*, although morphologically they are believed to be similar to wheat-infecting *Eutiarosporella* spp. (Platz 2011). As a speculative side note, perhaps the initial morphology based comparison between these species was in fact accurate, but rather it is actually *B. zeae* that that is classified incorrectly, and should instead be of the *Eutiarosporella*. This further emphasises the need for continual gene and whole-genome sequencing to better understand the existing diversity of fungal species, particularly when they are found in close association with plants.

For the purposes of this study, we have merely utilised the genome sequences available to us to retrieve the ITS, β-tubulin and EF1-α from the three sequenced isolates. Our delimitation of the three novel species of wheat-infecting *Eutiarosporella* spp. was strongly supported by the multigene phylogeny of these three genes. These sequences have been used successfully in studies defining the boundaries of particular *Botryosphaeriaceae* spp. and so we felt that it was appropriate to utilise these sequences. We will now endeavour to use these genome sequences to assist in better understanding these recently emerged pathogens.

The ability to obtain nearly complete fungal genomes is becoming ever easier. This ease of access has made it feasible to rapidly compare multiple genomes and has led to major improvements in our understanding of the lifestyles, niche specialisation, and emergence of fungal phytopathogens (Gardiner et al. 2012; Klosterman et al. 2011; Thynne et al. 2015). Genomes of *Botryosphaeriaceae* family are now being exploited to identify basic biological processes such as homothalism vs. heterothallism, which once took years of careful experiments to correctly assign (Islam et al. (2012); Blanco-Ulate et al. (2013); Bihon et al. (2014)). For example, Bihon et al. (2014) used the genome sequences of *Diplodia pinea* to describe the mating type idiomorphs for this species, and in doing so described a novel putative MAT gene, characterised as *MAT1-2-5* (Bihon et al. 2014).

Currently, very little is known about wheat-infecting *Eutiarosporella* spp. and their emergence in the Australian grain industry have yet to cause significant enough damage to warrant large-scale studies. We expect, however, that the

sequenced genomes of wheat-infecting *Eutiarosporella* spp. will greatly assist in answering remaining questions about their lifecycle, especially related towards host specificity. Until this point, it is our hope that the three loci sequences provided in this study will contribute valuable data for the community, adding to the knowledge about the distribution and host-range of members of the *Botryosphaeriaceae* family.

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