THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Plant pathogenic *Magnaporthales* in Australia, with particular reference to *Pyricularia oryzae* on wild and cultivated rice

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Master of Science (Plant Pathology)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

Queensland Alliance for Agriculture and Food Innovation

Abstract

The *Magnaporthales* is an order of fungi that contains plant pathogens and saprobes. This order consists of three families, *Pyriculariaceae*, *Magnaporthaceae* and *Ophioceraceae,* which are phylogenetically, morphologically and ecologically distinct. To date, about 200 species have been described in *Magnaporthales*, of which approximately 50% are plant pathogens. Some species are important pathogens of grasses and cereals such as the rice blast fungus *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) and the take-all pathogen of cereals *Gaeumannomyces graminis*. The study of classification and identification of *Magnaporthales* in Australia and pathogenicity of *Pyricularia oryzae* are reported in this thesis.

The genus *Pyricularia* comprises species that cause blast diseases on various hosts, especially grasses (*Poaceae*) that include crops such as rice, wheat, barley and grasses. This study used morphology, phylogenetic concordance, ecology and pathogenicity, to study *Pyricularia* and allied genera in the *Magnaporthales*. The fungi associated with blast diseases on eleven monocot hosts in Australia were identified by morphological characters and DNA sequence analysis in Chapter 2. Three species of *Pyricularia*, namely, *Pyricularia angulata*, *Pyricularia pennisetigena* and *Pyricularia oryzae*, were confirmed as present in Australia. Another species, *Pyricularia rabaulensis* was found to belong to a recently established genus *Barretomyces*. A formal transfer of this fungus to *Barretomyces* has been proposed.

In Chapter 3, the phylogenetic relationships of some other Australian *Magnaporthe*-like fungi were investigated based on morphology and DNA sequence analysis of multiple genes. The root pathogenic fungi of *Cynodon dactylon* and *Agrostis stolonifera* as well as holotype specimens of *Magnaporthe garrettii* and *Magnaporthe griffinii* were examined. Two novel genera, *Budhanggurabania* and *Wongia*, were described as well as a new species, *Magnaporthiopsis agrostidis*.

Rice (*Oryza sativa* L., *Poaceae*) is the most important cereal crop globally and a primary source of starch for more than half the world’s population. The major constrain to rice production worldwide is rice blast disease, which is caused by the fungus *Pyricularia oryzae* Cavara (syn. *Magnaporthe oryzae* Hebert). Rice blast disease is a problem in over 85 countries in all continents where rice is cultivated in both paddy and upland situations. Unpredictable epidemics can cause significant economic losses.

Foliar disease surveys of wild and cultivated rice in northern Queensland were completed in 2014 and 2015. The results presented in Chapter 4 show that a total of 109 fungal isolates was recovered and classified into seven genera, viz. *Bipolaris*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Phoma*, *Pestalotiopsis* and *Pyricularia*. Isolates from three genera, *Pyricularia*, *Bipolaris* and *Curvularia* were identified to species level by multigenes analysis because of their potential to cause important diseases on rice. The rice blast pathogen, *Pyricularia oryzae*, brown spot pathogen, *Bipolaris oryzae* and a large diversity of *Curvularia* species were recovered. To remove uncertainty about the identity of the host plants from which the fungi were isolated, a DNA barcoding strategy was developed using regions of the chloroplast genome.

In Chapter 5, pathogenicity and cross infectivity of *Pyricularia* species are investigated. Four Australian isolates of *Pyricularia oryzae*, two isolates from domesticated rice and two isolates from wild rice, were tested onto nine commercial rice cultivars namely, Doongara, langi, Kyeema, Illabong, Sherpa, Reiziq, Koshi, Opus and Quest. The results showed that infection occurred on all rice varieties with Quest the most susceptible and Sherpa the least susceptible. The host ranges of four Australian isolates of *Pyricularia oryzae* from rice and one isolate from barley, were tested on barley, oats, rice and wheat. None of the isolates infected wheat, and one isolate from rice infected oats. Isolates of *Pyricularia oryzae* from *Setaria italica* and *Pennisetum clandestinum* were shown to cause blast disease on their original hosts and barley but did not infect rice and wheat.

The research described in this thesis presents the identification and classification of plant pathogenic fungi in order *Magnaporthales* in Australia. Three species of *Pyricularia* were identified in this study based on a phylogenetic species concept as well as two novel species of root pathogens in family *Magnaporthaceae*. Phylogenetic analysis placed two Australian root pathogen species, *Magnaporthe garrettii* and *Magnaporthe griffinii*, in the new genus, *Wongia*, *Diaporthomycetidae*. Conidial morphology is a primary character to distinguish genera. The *Magnaporthales* is a diverse order and more novel species certaintly await discovery. Future researches that might provide better understanding of *Pyricularia oryzae* and other closely related fungi in the *Magnaporthales* are discussed in Chapter 6.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Khemmuk, W., Roger G. Shivas, Robert J. Henry and Andrew D.W. Geering. 2016. Fungi associated with foliar disease of wild rice and cultivated rice (*Oryza* spp.) in northern Queensland. *Australasian Plant Pathology* **45**, 297-308. doi:10.1007/s13313-016-0418-3

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. 2016. *Wongia* gen. nov. (*Papulosaceae*, *Sordariomycetes*), a new generic name for two root-infecting fungi from Australia. *IMA Fungus* **7(2)**, 247–252. doi:10.5598/imafungus.2016.07.02.04

Peer-reviewed papers (ancillary papers)

Wong, P.T.W**., Khemmuk, W.**, Geering, A. D. W. and Shivas, R.G. (2015). *Budhanggurabania cynodonticola* Fungal Planet No. 359. *Persoonia* **34**, 240–241.

Wong, P.T.W., **Khemmuk, W.**, Geering, A. D. W. and Shivas, R.G. (2015). *Magnaporthiopsis agrostidis*. Fungal Planet No.398. *Persoonia* **35**, 322–323.

Conference abstracts

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2014) The diversity of *Pyricularia* in Australia. The 10th International Mycological Congress, 3rd–8th August 2014, Bangkok, Thailand.

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2014) The co-evolution of wild rice and its pathogens, especially *Pyriculaira* spp. Science Exchange 2014, Plant Biosecurity Cooperative Research Centre, 11th–14th May 2014, Twin Waters, Queensland, Australia.

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2015) The co-evolution of wild rice and its pathogens, especially *Pyriculaira* spp. Science Exchange 2015, Plant Biosecurity Cooperative Research Centre, 11th–14th August 2015, Twin Waters, Queensland, Australia.

Khemmuk, W., Geering, A. D. W., Robert, J. Henry and Shivas, R.G. (2015) Wild rice pathogens in northern Queensland. Tropical Agriculture Conference 2015, 16th–18th November 2015, Brisbane Convention and Exhibition Center, Brisbane, Australia.

Publications included in this thesis

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. 2016. *Wongia* gen. nov. (*Papulosaceae*, *Sordariomycetes*), a new generic name for two root-infecting fungi from Australia. *IMA Fungus* **7(2)**, 247–252.

— incorporated as Chapter 3

Contributor	**Statement of contribution**
Wanporn Khemmuk	- Designed experiments (50%) - Performed the experiments, analysed and interpreted data (80%) - Discussion of taxonomy and phylogeny (50%) - Wrote the paper (50%)
Andrew D.W. Geering	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (10%) - Critically reviewed and edited the manuscript (20%)
Roger G. Shivas	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (20%) - Critically reviewed and edited the manuscript (80%)

Wong, P.T.W**., Khemmuk, W.**, Geering, A. D. W. and Shivas, R.G. (2015). *Budhanggurabania cynodonticola* Fungal Planet No. 359. *Persoonia* **34**, 240–241.

— incorporated as Chapter 3

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Wanporn Khemmuk	- Designed experiments (50%) - Performed the experiments, analysed and interpreted data (80%) - Discussion of taxonomy and phylogeny (50%) - Wrote the paper (50%)
Andrew D.W. Geering	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (10%) - Critically reviewed and edited the manuscript (20%)
Roger G. Shivas	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (20%) - Critically reviewed and edited the manuscript (80%)
Percy T. Wong	- Designed experiments (10%) - Collected *Wongia garrettii*, *Wongia griffinii*, *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis* (100%) - Discussion of taxonomy and phylogeny (20%) - Performed the morphology experiment, analysed and interpreted data (20%) - Wrote the papers (50%)

Wong, P.T.W., **Khemmuk, W.**, Geering, A. D. W. and Shivas, R.G. (2015). *Magnaporthiopsis agrostidis.* Fungal Planet No. 398. *Persoonia* **35**, 322–323.

— incorporated as Chapter 3

Contributor	**Statement of contribution**
Wanporn Khemmuk	- Designed experiments (50%) - Performed the experiments, analysed and interpreted data (80%) - Discussion of taxonomy and phylogeny (50%) - Wrote the paper (50%)
Andrew D.W. Geering	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (10%) - Critically reviewed and edited the manuscript (20%)
Roger G. Shivas	- Designed experiments (20%) - Discussion of taxonomy and phylogeny (20%) - Critically reviewed and edited the manuscript (80%)
Percy T. Wong	- Designed experiments (10%) - Collected *Wongia garrettii*, *Wongia griffinii*, *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis* (100%) - Discussion of taxonomy and phylogeny (20%) - Performed the morphology experiment, analysed and interpreted data (20%) - Wrote the papers (50%)

Khemmuk, W., Roger G. Shivas, Robert J. Henry and Geering A. D.W. (2016). Fungi associated with foliar disease of wild rice and cultivated rice (*Oryza* spp.) in northern Queensland — incorporated as Chapter 4.

Contributor	**Statement of contribution**
Wanporn Khemmuk	- Designed experiments (30%) - Performed the experiments, analysed and interpreted data (100%) - Discussion of taxonomy and phylogeny (50%) - Wrote the paper (60%)
Robert J. Henry	- Designed experiments (20%) - Critically reviewed and edited the manuscript (20%)
Roger G. Shivas	- Discussion of taxonomy and phylogeny (25%) - Critically reviewed and edited the manuscript (30%)
Andrew D.W. Geering	- Designed experiments (50%) - Wrote the paper (40%) - Critically reviewed and edited the manuscript (50%)

Contributions by others to the thesis

Andrew Geering, Roger Shivas and Robert Henry contributed to the conception and design of the project, specimens, field surveys, provided advice, guided analysis, critically reviewed and assisted with preparation of the whole thesis.

For Chapter 2, isolates of *Pyricularia* were sourced from the Plant Pathology Herbarium (BRIP), Department of Agriculture and Forestry (DAF), Queensland.

For Chapter 3, Percy Wong (University of Sydney) provided specimens of *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis*. New South Wales Plant Pathology Herbarium (DAR), Department of Primary Industries provided two holotype specimens of *Magnaporthe garrettii* and *Magnaporthe griffinii*.

For Chapter 5, Mike Hedditch (SunRice Co. Ltd.) provided rice seed and Greg Platz provided seeds of barley, oats and wheat.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

I would like to thank the Royal Thai government through the Office of the Civil Service Commission and the Rice Department, Ministry of Agriculture and Cooperatives, who provided this opportunity and supported me for the duration of my PhD study in Australia. I am so grateful to the Plant Pathology Herbarium (BRIP), Department of Agriculture and Forestry, Queensland and the Queensland Alliance for Agriculture and Food Innovation, who supported aspects of my project.

I thank to my supervisory team, led by Dr Andrew Geering, who was the first person I met in Australia, for his support, guidance and developing the idea of my PhD. I would like to thank Dr Roger Shivas for his huge support and encouragement during my time in Australia and hopefully in the future, and to Prof Robert Henry for his advice about wild rice in Australia. I would like to thank Dr Alistair McTaggart for his guidance in molecular biological techniques and phylogenetic analyses. I had a great time with everyone at the Ecosciences Precinct, Dean Beasley, Yu Pei Tan, Thomas Marney, Nga Tran, Merran Neilson, Marjan Shivas, Margaret Geering, Jill and Ross McTaggart. I would like to give special thanks to Chanintorn Doungsa-ard, who has helped me for over fifteen years since we first met in academic and non-academic things, thank you to introduce me to these fantastic people.

Finally, I would like to thank my parents, my aunt, Nu, for their unconditional love and support, my big brother, Boom, who always supported and listened to me. I could not have completed my PhD without my lovely husband, Gu, who came to Australia for me. He has always supported and encouraged me in every single day in Australia.

Keywords

Pyricularia, *Pyriculariaceae*, *Magnaporthaceae*, *Magnaporthales*, phylogenetic, wild rice

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060309, Phylogeny and Comparative Analysis, 60%

ANZSRC code: 060704, Plant Pathology, 20%

ANZSRC code: 060307, Host-Parasite Interactions, 20%

Fields of Research (FoR) Classification

FoR code: 0604, Evolution Biology, 40%

FoR code: 0605, Microbiology, 30%

FoR code: 0699, Plant pathology, 30%

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Chapter 1 General introduction and Literature review

1.1 Introduction

The *Magnaporthales* is an order of fungi that contains plant pathogens and saprobes that are found mostly on grasses as well on submerged wood (Thongkantha et al., 2009). This order contains three families, *Magnaporthaceae*, *Ophioceraceae* and *Pyriculariaceae*, which are phylogenetically, morphologically and ecologically distinct (Klaubauf et al., 2014). The *Ophioceraceae* is mainly comprised of aquatic or wood associated saprobic species (Klaubauf et al., 2014). The *Magnaporthaceae* and *Pyriculariaceae* contain mainly plant pathogenic species. In particular, the genera *Pyricularia* (*Pyriculariaceae*) and *Gaeumannomyces* (*Magnaporthaceae*) contain several species that are significant plant pathogens of economic and agriculture importance (Luo and Zhang, 2013). The most widely studied species in the *Pyriculariaceae* is certainly *Pyricularia oryzae* (synonym *Magnaporthe oryzae*). This fungus is part of a species complex that can cause blast diseases on about 50 grass (*Poaceae*) and sedge (*Cyperaceae*) species, including rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), maize (*Zea mays*), oats (*Avena sativa*), rye (*Secale cereale*), finger millet (*Eleusine corocana*) and perennial ryegrass (*Lolium perenne*) (Skamnioti and Gurr, 2009).

Rice blast disease, caused by *Pyricularia oryzae*, is one of the major contributing factors that threatens rice production worldwide (Divya et al., 2014). This disease is a serious problem in over 85 countries where rice is cultivated in both paddy and upland situations, with losses of between 10 % and 30 % of the annual rice harvest (Skamnioti and Gurr, 2009). Carozzi (2013) modelled the impact of climate change on crop production and predicted that in the next 30 years, *Pyricularia oryzae* could spread to all rice growing areas and might double in severity. The most effective management of *Pyricularia oryzae* is the cultivation of resistant rice varieties (Ou, 1985; Namai, 2011). Resistance to infection by *Pyricularia oryzae* can be short-lived because of the high genetic variability of the fungus. The breakdown of resistance to *Pyricularia oryzae* results from evolution of genetic variants (races) in the pathogen populations (Liu et al., 2011). The extensive use of *Pyricularia oryzae* resistant cultivars has led to the subsequent breakdown of resistance as new pathogenic variants arise (Noguchi et al., 2007). A focus on the genetic structure and population biology of *Pyricularia oryzae* is a prerequisite for successful disease management (Taheri and Tarighi, 2011).

In the past two decades, *Pyricularia oryzae* has been developed as a model species for the study of plant-pathogen interactions (Xue et al., 2012). *Pyricularia oryzae* was the first plant pathogenic fungus to have its genome sequenced and made publically available in 1991 (Valent and Chumley, 1991). *Pyricularia oryzae* was ranked number one in a list of plant parasitic fungi based on perceived scientific or economic importance (Dean et al., 2012).

1.1.1 The order *Magnaporthales*

The *Magnaporthales* (Ascomycota) consists of three families, 28 genera and over 200 species (Kirk et al., 2008; Thongkantha et al., 2009; Zhang et al., 2016). The *Magnaporthales* is morphologically defined by perithecial ascomata with long necks, cylindrical and unitunicate asci, with 8 ascospores that are mostly filiform, septate and pigmented (Thongkantha et al., 2009). Members of the *Magnaporthales* are morphologically distinct. Anamorphic states have phialophora or phialophora-like conidia and form hyphopodia that penetrate host cells (Luo et al., 2015). Molecular phylogenetic studies indicated that the *Magnaporthales* is monophyletic and belongs to the *Sordariomycetes*. The *Magnaporthales* is sister to the *Diaporthales* and *Ophiostomatales* (Zhang et al., 2006; Thongkantha et al., 2009; Luo et al., 2015). Results of a five-gene phylogenetic analysis found three distinct clades within the *Magnaporthales* that corresponded to three families, the *Magnaporthaceae*, *Ophioceraceae* and *Pyriculariaceae* (Klaubauf et al., 2014), which have different ecological niches (Luo et al., 2015).

The *Magnaporthaceae*, typified by *Nakataea oryza* (syn. *Magnaporthe salvinii*), comprise of root-infecting fungi. This family contains important pathogens of grasses (*Poaceae*) and other monocots, e.g. *Gaeumannomyces graminis* var. *tritici*, which causes take-all of wheat; *Gaeumannomyces graminis* var. *avenae*, which causes take-all of oats (Walker, 1972); *Magnaporthiopsis poae*, which causes summer patch disease (Luo and Zhang, 2013) and *Magnaporthiopsis agrostidis*, which causes root rot of couch grass (Wong et al., 2015b).

The *Pyriculariaceae* contains mostly pathogens of grasses, and causes foliar blast diseases. The *Pyriculariaceae* produce perithecia with long cylindrical necks, unitunicate short stipitate asci with large apical rings, and ascospores similar to other *Magnaporthaceae*. The asexual morph produced hyaline or brown conidia on simple branched conidiophores and also formed appressoria (Klaubauf et al., 2014; Luo et al., 2015). The most studied species is *Pyricularia oryzae*, which cause blast disease on rice and other cereal crops worldwide. The fungi in this group have the ability to infect all aerial parts of host plants, and sometimes roots as well (Sesma and Osbourn, 2004).

The *Ophioceraceae* is the smallest family in the *Magnaporthales* with a single genus, *Ophioceras*, comprising about 39 species (www.indexfungorum.org). Although *Ophioceras* is morphologically similar to *Gaeumannomyces*, the two genera can be distinguished by the aquatic habit of *Ophioceras*, occurring as saprobes on wood and herbaceous material. Species of *Ophioceras* formed a clade that was phylogenetically distinct from other genera in the *Magnaporthaceae* and *Pyriculariaceae* (Klaubauf et al., 2014).

1.2 The genus *Pyricularia*

Pyricularia belongs to the family *Pyriculariaceae* in the order *Magnaporthales*, (Maharachchikumbura et al., 2015). *Pyricularia grisea* (Cooke) Saccardo (1880) is the type species of the genus, having been described from grey leaf spot disease of *Digitaria sanguinalis* (originally as *Panicum sanguinale*) in the USA. The basionym is *Trichothecium griseum* Cooke in Cooke & Ellis (1879) and the original publication did not include a description of the fungus (Rossman et al., 1990). Currently, there are 82 species recognised in the genus *Pyricularia* (http://www.indexfungorum.org/Names/Names.asp). Species of *Pyricularia* have been reported on over 100 plant species, with most recorded from the monocotyledonous families *Poaceae*, *Cannaceae*, *Commelinaceae*, *Cyperaceae*, *Musaceae*, and *Zingiberaceae* (Park and Shin, 2009). There are five species that have been described from dicotyledonous plants, namely *Pyricularia caffera* on *Acer* sp. (Matsushima, 1996), *Pyricularia lourinae* on *Aniba bruchellii* (de Albuquerque and Duarte, 1971), *Pyricularia peruamazonica* on *Theobroma cacao* (Matsushima, 1993), *Pyricularia subsigmoidea* on *Lasiascis divaricata* (Castañeda Ruiz and Kendrick, 1991) and *Pyricularia vandalurensis* on *Ficus benghalensis* (Subramanian and Vittal, 1974)

1.2.1 Rice blast disease caused by *Pyricularia oryzae*

Cavara (1891) described a fungus found on rice in Italy as *Pyricularia oryzae* with similar morphology to *Pyricularia grisea*. Until recently, there was confusion as to the correct name of the rice blast pathogen, as both names *Pyricularia oryzae* and *Pyricularia grisea* were often used synonymously (Choi et al., 2013). Studies in the pre-molecular era (before about 1995) used morphological characters, cross-pathogenicity and sexual incompatibility to distinguish *Pyricularia grisea* and *Pyricularia oryzae*. For example, Thomas (1940) found that *Pyricularia oryzae* from rice could infect wheat (*Triticum aestivum*), oats (*Avena sativa*), barley (*Hordeum vulgare*), maize (*Zea mays*), Italian millet (*Setaria italica*) and Egyptian crowfoot grass (*Dactylotenium aegyptiacum*), while *Pyricularia oryzae* isolates from Italian millet and *Eleusine coracana* failed to infect rice (*Oryza sativa*). In the 1970s, teleomorphic states of *Pyricularia grisea* and *Pyricularia oryzae* were discovered in several laboratories. At that time, multiples names could be applied to a single fungal species and Hebert (1971) gave the name *Ceratosphearia grisea* to the teleomorphic state of *Pyricularia grisea*. *Ceratosphearia grisea* produced non-stromatic black perithecia with long necks and four-celled, spindle-shaped ascospores. *Ceratosphearia grisea* was subsequently transferred to *Magnaporthe grisea* (Barr, 1977), and both of these names are now considered synonyms of *Pyricularia grisea*.

Kato (2000) compared the pathogenicity, mating ability and restriction fragment length polymorphism with single-copy DNA probes of 85 *Pyricularia* isolates from 29 hosts. The results showed that isolates of *Pyricularia* from *Digitaria* spp. were genetically distinct from isolates from rice and other crop species. Couch and Kohn (2002) analysed the DNA sequence data of three genetic loci (actin, beta-tubulin and calmodulin) and found that isolates of *Pyricularia grisea* from *Digitaria* spp. were in a clade that was phylogenetically distinct from isolates from rice and other grasses. They further concluded that the rice blast pathogen was *Pyricularia oryzae*, while *Pyricularia grisea* was restricted to *Digitaria*.

Since 1 January 2013, one fungus can only have one correct name (Hawksworth et al., 2013) and other names are considered synonyms. Based on the current nomenclatural code for algae, fungi, and plants (McNeill et al., 2012), all legitimate fungal names are treated equally for the purpose of establishing taxonomic precedence (Wingfield et al., 2012). The correct name of the rice blast fungus has been argued for more than thirty years. Luo and Zhang (2013) analysed DNA sequences of multiple genes including SSU, LSU, ITS, *MCM7*, *RPB1* and *TEF1* of fungi in the order *Magnaporthales* including *Magnaporthe*, *Gaeumannomyces* and *Pyricularia*. The results showed that *Magnaporthe salvinii*, the type species of *Magnaporthe* (Krause and Webster, 1972), resided in a clade distinct from the rice blast pathogen, *Pyricularia oryzae*. Consequently, *Magnaporthe salvinii*, which causes of rice stem rot, was synonymized with *Nakataea oryzae* (Cattaneo) J. Luo& Zhang, based on priority.

1.2.2 Life cycle and infection process of *Pyricularia oryzae*

Pyricularia oryzae produces airborne conidia that infect leaves and panicles of host plants (Murata et al., 2014). Its life cycle comprises a series of discrete developmental steps (Fig. 1.1): conidia attachment, spore germination, germ tube development, appressorium formation, penetration peg emergence and invasive growth in the host plant (Galhano and Talbot, 2011). Lesions appear after 5–7 days, and conidiophores and conidia may be produced after 20 days (Saleh et al., 2012). Under favourable moisture and temperature conditions (long periods of plant surface wetness, high humidity, little or no wind at night and night temperatures between 12–32 °C), the infection cycle repeats because the newly developed leaves act as receptors, with 20,000–60,000 spores potentially produced from one lesion in one night (Kato, 2001).

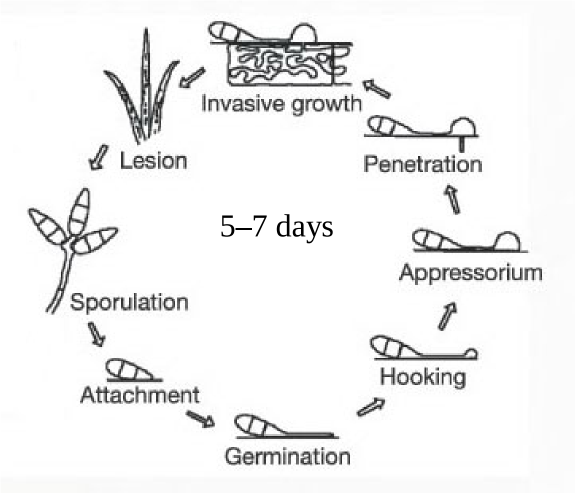

Figure 1.1 Life cycle of *Pyricularia oryzae*. (from Dean et al., 2012)

Initiation process

Pyricularia oryzae requires favourable conditions, specifically surface moisture, humidity, temperature and light, during the early stage of the infection process (Struck, 2006). Infection is initiated by conidia that land on the surface of the leaves. Conidia germinate by forming a polarised germ tube within two hours (Talbot, 2003). During germ tube elongation, a cluster of cytoplasmic vesicles forms near the apex cell of the conidium (Bourett and Howard, 1990). The germ tube swells over the next 6–10 hours and differentiates into an appressorium (Galhano and Talbot, 2011). Appressoria are structures that pathogenic species use for attachment to the plant surface in preparation for infection. Appressoria are unicellular, dome-shaped and generate cellular turgor, which is translated into mechanical force that rupture the leaf cuticle, enabling penetration of the plant tissue (Talbot, 2003; Ebbole, 2007). Once appressoria have differentiated, they often become darkly pigmented due to the formation of a distinct layer of melanin in the cell wall (Talbot, 2003; Ebbole, 2007). Appressorial turgor is generated by the accumulation of glycerol to a high concentration within the infected cell, leading to increased pressure and rupturing of the host plant cuticle (de Jong et al., 1997; Dixon et al., 1999).

Penetration and invasive growth

Penetration of host tissue occurs within 10–14 hours of production of the appressorium (Zeigler and Robert, 1998). Invasive hyphae develop from the penetration peg and grow within the plant host, moving from cell to cell without causing symptoms in susceptible rice cultivars in the first 4–5 days after penetration (Kankanala et al., 2007). Necrotic lesions then develop on the surface of the leaf, from which spores are produced to continue the life cycle. The filamentous invasive hyphae enlarge into bulbous hyphae. Invaded cells are alive as the fungus enters them but later die as they become full of hyphae (Fernandez and Wilson, 2014). Kankanala et al. (2007) showed that the bulbous invasive hyphae filled the first cell after 8–12 hours post-inoculation, before spreading into neighbouring cells after 32–36 hours. Subsequently, the invasive hyphae grew more rapidly from one cell to the next, requiring only 2–3 hours to move into neighbouring cells.

1.2.3 Symptoms of rice blast disease

Symptoms of blast disease occur on all aerial parts of rice, including the leaf, leaf collar, culm and nodes, panicle, neck, node, panicle and seeds (TeBeest et al., 2007). Symptoms may be either lesions or spots; the shape, colour and size vary depending on varietal resistance, environmental conditions and the age of the lesions (Ou, 1985). On leaves, the spots initially appear as water-soaked, white to grey pinhead dots. These develop into elliptical or diamond-shaped lesions, with grey centres, surrounded by a reddish brown margin. Lesion size is commonly 1–1.5 cm long and 0.3–0.5 cm wide. Lesion enlargement depends on availability of moisture, rice cultivar and other factors such as fertilizer application (Ou, 1985). Symptoms of collar rot are necrosis of the tissues at the union of the leaf blade and leaf sheath (Fig. 1.2). Collar infections can kill the entire leaf and may extend a few mm. into and around the leaf sheath. The fungus may produce spores on these lesions (TeBeest et al., 2007).

The neck of the rice plant refers to that portion of the stem that rises above the leaves and supports the seed head or panicle. The neck is often infected at the node by *Pyricularia oryzae* and infection leads to a condition called rotten neck or neck blast (Fig. 1.2). Infection of the neck can be very destructive, causing failure of the seeds to fill (a condition called blanking) or causing the entire panicle to fall over as if rotted. *Pyricularia oryzae* can also infect the panicles as the seeds form. Lesions may be found on the panicle branches, spikes, and spikelets. The lesions are often grey brown, and over time, the branches of the panicle may break at the lesion (TeBeest et al., 2007). Infected nodes appear blackish brown and often occur in a banded pattern. This infection often causes the culm to break, resulting in death of the rice plant (Fig. 1.2). Symptoms on seeds consist of brown spots, blotches (Fig.1.2), and occasionally the classic diamond-shaped lesion as seen on leaves. The process by which seed infection occurs has not been fully described. Recent studies show that *Pyricularia oryzae* may infect seeds via the florets as they mature into seeds (TeBeest et al., 2007).

Rice blast pathogens can infect roots under laboratory conditions and root infection can lead to the appearance of blast symptoms on the aerial parts of the plant (Sesma and Osbourn, 2004).

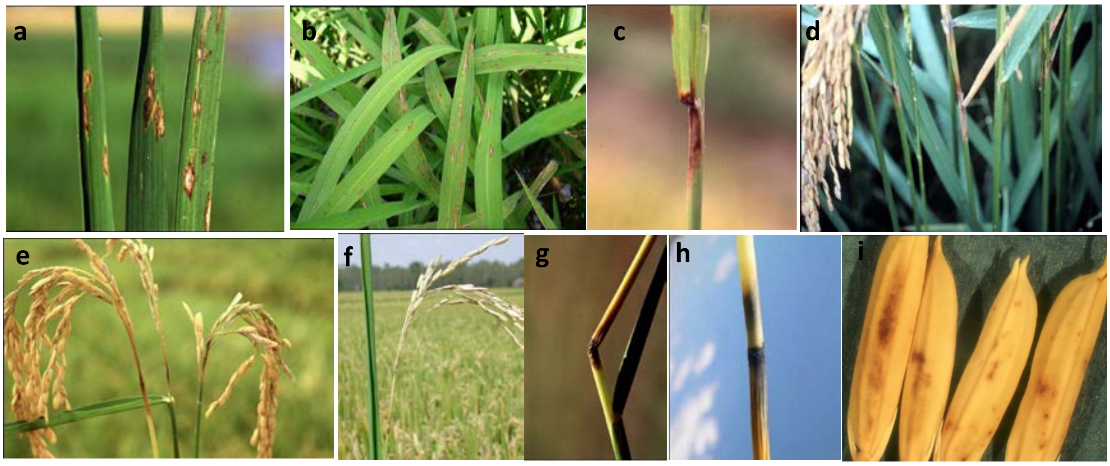

Figure 1.2 Rice blast symptoms on different parts of plant; a–b, leaf blast; c–d, collar rot; e–f, neck rot; g–h, node infection; and I, seed infection (Reproduced from National Diagnostic Protocol, 2015; images a, c–e, g by Groth; b, f, h by Cartwright; i by TeBeest).

1.2.4 Host range, pathotype and genetic diversity of *Pyricularia oryzae*

Pyricularia oryzae is a species complex (Couch and Kohn, 2002) that causes blast disease in many cultivated crops around the world (Urashima et al., 1993), including wheat (Igarashi et al., 1986; Castroagudin et al., 2016), corn (Bailey and Eijnatten, 1961), millet (Kato et al., 1976), rice (Cavara, 1891) and barley (Okada and Yaegashi, 1985). Currently, over 50 species of *Poaceae* have been reported as hosts of *Pyricularia oryzae*, with most being monocotyledonous plants, especially in those form the families *Commelinaceae*, *Cyperaceae*, *Poaceae* and *Zingiberaceae* (Park and Shin, 2009).

On rice, differential sets of cultivars have been used to identify rice pathotypes in *Pyricularia oryzae* populations. Sets of rice cultivars with different resistance genes have been developed to characterise *Pyricularia oryzae* populations in several countries (Correa-Victoria and Zeigler, 1993; Mekwatanakarn et al., 2000; Zhu et al., 2000; Chen et al., 2001). Recently, Ghaley (2012) evaluated the Bhutan rice landraces for genetic diversity in blast resistance by genetic analysis of the 352 landraces with 27 microsatellite markers. The results of landraces cluster analysis revealed two distinct groups of rice cultivars. The resistant land races were genetically diverse originating from different rice cultivation zones. Zeigler et al. (1995) studied the relationship between phylogeny and pathotype of *Pyricularia oryzae* among isolates collected from two sites in the Philippines and tested 15 rice cultivars and six near-isogeneic lines. They concluded that there was a very high degree of diversity in the virulence of *Pyricularia oryzae*. Although reactions were variable for some host-lineage combinations, consistent incompatibility was observed for the majority of host-lineage interactions.

Kato (2000) classified pathotypes of *Pyricularia oryzae* from nine different hosts into several pathotypes based on pathogenicity, mating ability and genetic characters. The representative pathotypes were (i) *Oryza* pathotype that was pathogenic on rice; (ii) *Setaria* pathotype that was pathogenic on foxtail millet (*Setaria italica*); (iii) *Panicum* pathotype that was pathogenic on common millet (*Panicum miliaceum*); (iv) *Eleusine* pathotype that was pathogenic on finger millet (*Eleusine coracana*); and (v) *Digitaria* pathotype that was pathogenic on *Digitaria sanguinalis*. Molecular analyses of wheat (*Triticum aestivum*) isolates showed them to be distinct from *Eleusine* isolates and belong to a separate, single lineage (Urashima and Kato, 1998).

Wheat blast disease, caused by *Pyricularia oryzae* pathotype *Triticum* and the recently described *Pyricularia graminis-tritici*, emerged in South America in 1985 with the first epidemics in southern Brazil, then Paraguay and Bolivia (Castroagudin et al., 2016). Wheat blast is a serious threat to wheat production and has spread to other countries in South America. Yield losses range from 10 to 100 % under favourable conditions (Tagle et al., 2015). Nunes et al. (2014) used 11 microsatellite loci to elucidate the population structure of *Pyricularia oryzae* in Brazil, and found that the pathogen had a mixed reproductive system and displayed a high degree of gene flow across large spatial scales and exhibited significant pathotype diversity. Urashima (1993) suggested that the wheat blast pathogen in Brazil could be divided into two populations, one that may have originated from rice and another that originated from an unidentified source. Recently, Castroagudin et al. (2016) studied *Pyricularia* species causing blast on several hosts in Brazil including wheat, rice and grasses. They concluded that wheat blast disease in Brazil was caused by multiple *Pyricularia* species, including *Pyricularia oryzae* pathotype *Triticum* and *Pyricularia graminis-tritici*, based on DNA sequence analyses and inoculation tests. The phylogeny of the combined dataset of 10 housekeeping genes showed three distinct clades. The first group, *Pyricularia oryzae* pathotype *Triticum* (PoT), contained isolates from wheat and grasses. The second group, *Pyricularia graminis-tritici* (Pgt), contained wheat isolates and the last group, *Pyricularia oryza*e pathotype *Oyrza* (PoO), contained rice isolates. Pgt and PoT were pathogenic on *Triticum aestivum* (wheat), *Hordeum vulgare* (barley), *Urochloa brizantha* (signal grass) and *Avena sativa* (oats), while PoO was virulent on the original host (*Oryza sativa*), and also on wheat, barley, and oats, but not on signal grass. Castroagudin et al. (2016) concluded that Pgt represented a serious threat to wheat cultivation globally.

1.2.5 Epidemiology

Dispersal

Conidia of *Pyricularia oryzae* are usually distributed by air currents. There are two different types of conidial dispersal, local dispersal of about 1–5 m or long distance dispersal (Tharreau et al., 2009). Air-borne conidia are the most important means of dissemination, although this fungus may also spread by infected seed, straw and in irrigation water (Ou, 1972). Long distance spread is possible through the transport of infected seeds or travellers. Phillips et al. (1992) estimated 240,000 conidia of *Pyricularia oryzae* have been introduced in Australia on the clothes of international travellers. There are several Australian specimens identified as *Pyricularia oryzae* from *Brachiaria mutica*, *Digitaria* sp., *Echinochloa colona* and *Eleusine indica* in the Plant Pathology Herbarium (BRIP), Department of Agriculture and Forestry, Queensland but the ability of these isolates to infect rice has never been determined.

Environmental influences on rice blast disease

In general, long periods of leaf wetness, high relative humidity, and temperatures of 17–28 °C favour the development of *Pyricularia oryzae* (Greer and Webster, 2001). The critical range of temperature for penetration and establishment of infection is around 25–26 °C, whereas germination of spores and appressoria formation occurs within 6–10 h at 20–30 °C in the presence of water on the surface of the leaf (Asuyama, 1965; Ou, 1985). High temperatures (around 32 ºC) increase lesion expansion, especially in the first eight days after infection. Lesion enlargement is slow and constant over a 20 d period at lower temperatures (16 ºC). High nitrogen supply is another factor that enhances the disease susceptibility (Ballini et al., 2013). *Pyricularia oryzae* survives in living plants from one crop season to another in the tropics, where there may be up to three overlapping crops per year. In the temperate regions, *Pyricularia oryzae* survives on residues of diseased plants or seeds, or on ratoons or stubble (Kato, 2001). The mycelium of *Pyricularia oryzae* may remain viable at 18–32 °C for up to three years. Conidia were reported as viable after 1 year at 8 °C and 20 % relative humidity (Zeigler, 1995).

Severity and yield loss of rice blast

Rice blast disease is a major constraint in rice production in all rice growing countries (Khush and Jena, 2009). Yield loss from this disease may reach 80 % under favourable conditions of heavy dew, high mean temperatures, high humidity, drought and excessive nitrogen fertilizer (Piotti et al., 2005). In China, during the last 30 years, the area with damaged by rice blast disease was more than 3.8 million hectares with yield losses of several million tons (Sun et al., 1999) in eastern India yield losses reached to 50 % in upland conditions in 2006 (Variar, 2007). The climate in Japan is highly conducive to the epidemic of rice blast disease and yield losses ranged from 20-100 % (Khush and Jena, 2009). In 2004 in Indonesia, upland rice production in West Java was reduced by 70 % of total yield by rice blast (Sobrizal and Anggiani, 2007). In 2009, the rainy summer in Arkansas, USA was favourable for the disease, which caused up to 80 % yield loss in some fields (www.detlafarmpress.com/arkansas-2009-crop-loss-397-million).

1.2.6 Genetics of resistance

The major resistance (*R*) genes are considered to be the most effective and economical ways to control rice blast disease (Bonman, 1992). The rice blast pathosystem follows the classic gene-for-gene hypothesis involving specific interaction of the products of the *R* gene in the host and of an avirulence (*AVR*) gene in the pathogen (Flor, 1971). The *R* genes may show complete resistant to certain pathotypes of *Pyricularia oryzae*, or minor genes may act in a polyphyletic fashion to provide quantitative or partial resistance (Bonman, 1992). Currently, at least 85 gene loci (Pi genes) and 350 QTLs (quantitative trait loci) have been identified (McCouch et al., 1994; Ballini et al., 2008), these *R* genes are distributed throughout 11 rice chromosomes, reported as the *R* genes: *Pib, Pita, Pi9, Pi2, Piz-t, Pid2, Pi36, Pi37, Pik-m, Pit, Pi5, Pid3, pi21, Pb1, Pish, Pik, Pik-p, Pi54, Pia, NLS1,* and *Pi25* (Liu et al., 2011; Yang et al., 2009). Genetic characterisation of rice blast resistance has indicated that rice variety *indica* has more complicated resistance patterns than variety *japonica* (Mackill and Bonman, 1986; Mackill and Bonman, 1992; Pan et al., 1996; Imbe et al., 1997; Yu et al., 2002).

1.3 Rice in Australia

1.3.1 History of rice cultivation in Australia

Rice was first introduced into Australia *c*. 1850 by Chinese prospectors who worked in the gold fields of Victoria and northern Queensland. In 1905, Isaburo Takasuka, a former Japanese parliamentarian, moved to Melbourne and grew rice at Swan Hill on 200 acres of flood plain along the Murray River. About this time, the State Government of New South Wales developed a large scale irrigation scheme along the Murrumbidgee River and created the Yanco Experimental Station. Ten years later, Takasuka successfully delivered cultivated rice to the government, harvested at Yanco. By 1930, rice production was sufficient for the small domestic Australian market (Sharma, 2010).

During World War II (1939–1945), rice was declared an essential commodity. All rice products were controlled by the Commonwealth Government. This stimulated the expansion of rice to other production areas, such as Wakool in the Murray Valley and later Deniliquin, NSW. In 1988, rice production expanded to Hay, Carathool, Hillston, Forbes and Echuca in

NSW (Sharma, 2010). Currently, about 800,000 ha operated by about 2,000 family farm businesses grow rice in NSW and Victoria (www.daff.qld.gov.au). The vast majority of Australian rice production is located in the Murray Darling Basin (Fig. 1.3) where the availability of irrigation water is likely to decline as a result of climate change and government policies to increase environmental flows (ABARES, 2013).

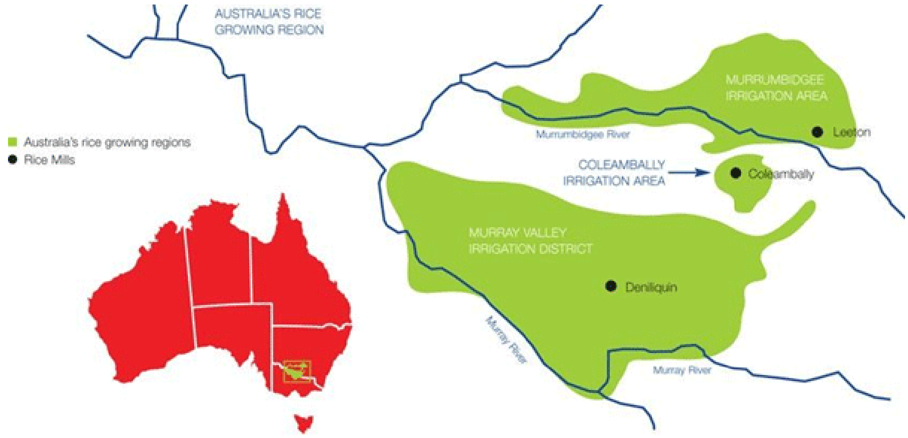

Figure 1.3 Rice growing areas in Australia (Illustration credit: Rice Grower's Association of Australia)

Australian rice production from 1999 to 2002 averaged more than 1.3 million tons per year. However, in the following seven years (2003–2009), the average was less than 360,000 tons. The 2008 harvest saw the lowest production since the industry began in Australia, at 19,000 tons. The drought average was skewed by a million-ton crop in 2006, partly the result of an advance of growers' future water allocations (ABARES, 2013).

Rice production and exports have increased sharply in more recent wetter years. Australia exports rice to around 60 countries mostly in the Middle East, North America and Asia. Australia exported up to 85 % of total rice yield, around 460,000 tons in 2012−13, up about 17 % from the 390,000 tons of rice exports in 2011−12 (Fig. 1.4). The rice growing season in Australia starts in October and ends with harvesting in March–May. The planting methods are by sowing dry rice or transplanting. Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES) estimated the area under rice cultivation in 2012–2013 at around 101,000 hectares, which was down about 11 % from the previous crop. The average yield of Australian's rice production is 9 tonnes per hectare (ABARES, 2013).

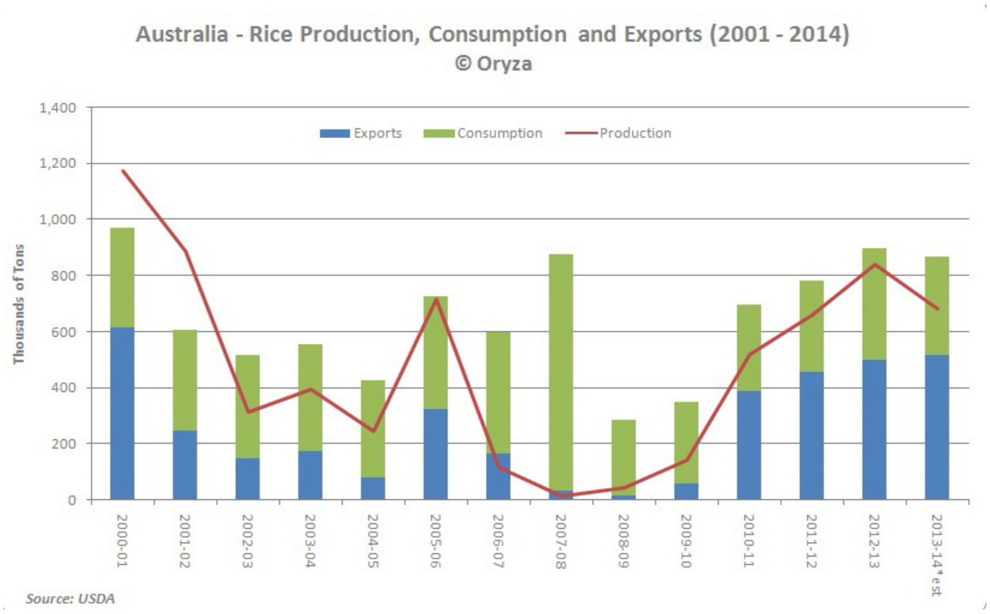

Figure 1.4 Australian rice production, consumption and exports between 2001 and 2014 (USDA, 2012; http://usda.mannlib.cornell.edu/MannUsda/)

1.3.2 Australian wild rice species

Australian wild rice species (*Oryza* spp.) are distributed in the northern regions, monsoonal of Western Australia, the Northern Territory and Queensland (Henry et al., 2010). Four *Oryza* species are indigenous to Australia, namely *Oryza australiensis*, *Oryza meridionalis, Oryza rufipogon,* and *Oryza officinalis* (Henry et al., 2010). Although a single herbarium specimen of *Oryza nivara* was collected in Queensland (Vaughan, 1994), its status is uncertain because the normal distribution of *Oryza nivara* is in India, Sri Lanka, Nepal and Thailand (Wilson, 2009). *Oryza minuta*, a wild species with BBCC-genome, has been taxonomically confused with *Oryza officinalis* and molecular analysis is needed to clarify this species (Henry et al., 2010).

Complete chloroplast genome sequences showed that Australian and Asian perennial wild rice were distinct from each other (Waters et al., 2012). Sotowa et al. (2013) found two distinct types within Australian perennial wild rice. One of the populations was referred as the m-type (Taxon B), which is morphologically similar to *Oryza meridionalis* and shared two loci in the chloroplast genome. The other population, referred as r-type (Taxon A), was morphologically similar to *Oryza rufipogon*. Recently, the whole chloroplast genome of Taxon A was analysed (Brozynska et al., 2014).

Wild *Oryza* species, including endemic Australian species, have been a source of both biotic and abiotic resistant genes (Brar and Khush, 1997). *Oryza officinalis* and *Oryza australiensis* have provided resistance genes for *Pyricularia oryzae* (Jeung et al., 2007), bacterial blight (*Xanthomonas oryzae*), whitebacked planthopper (*Sogatella furcifera*), and brown planthopper (*Nilaparvata lugens)* (Brar and Khush, 1997), through introgression into rice cultivars (Jena et al., 2006). *Oryza meridionalis* and *Oryza australiensis* have been proposed as sources of drought tolerance genes (Sundaramoorthi et al., 2009). *Oryza rufipogon* has been used as a source of both tungro resistance and tolerance to acid sulfate and aluminum in soil (Brar and Khush, 1997; Nguyen et al., 2003; Ram et al., 2007). The grains of *Oryza rufipogon*, *Oryza meridionalis* and *Oryza australiensis* are of sufficient quality to have potential as whole grain food that could offer an alternative choice for consumers (Kasem et al., 2010). Their seeds are similar in size but with a darker pericarp when compared to domestic rice (Shapter et al., 2008). Australian wild rice species are diverse genetic resources, with potential for use in genetic improvement of domestic rice for future pests, diseases and abiotic stresses especially the AA-genome group, which shares the same genome with the cultivated rice and can be useful for domesticated rice breeding program through sexual hybridization (Henry et al., 2010).

1.3.3 Rice pathogens in Australia

Diseases in rice crops may be caused by fungi, bacteria, viruses and nematodes. The rice industry in southern Australia remains free of many of the major pathogens (You et al., 2012). There are some reports of rice diseases and their pathogens in Australia, which are discussed below.

Fungal pathogens

Pythium and *Achlya* spp.

Seedling damping-off is caused by *Pythium* and *Achlya* spp. It is the most significant seedling disease for rice growers in south-eastern Australia (Cother and Gilbert, 1993). Seeds may fail to germinate and rot before emergence. Typically, infected seeds are covered with a whitish mycelium. Death of the coleoptile may occur soon after aerial sowing or within a few days of emergence for drill sown crops. This disease occurs every year, although severity increases in cooler seasonal conditions (Anon., 2014).

Tilletia spp.

Rice kernel smut (*Tilletia horrida*) is noticeable at crop maturity and affects both yield and grain quality of cultivated rice. Teliospores push through the glumes, especially if they swell from overnight dew or if rain occurs at the flowering stage (Slaton et al., 2007). The teliospores can survive for at least 3 years on grains and even survive the passage through the digestive track of animals (Ou, 1985). Kernel smut was reported in Queensland on rice at Mareeba, Ayr and Brendon in 1980 (Tonello, 1980). Second-hand farm equipment from overseas, transportation of rice material and farmers travelling to other rice growing areas, pose the greatest threats of the spread of kernel smut. Kernel smut has never been reported in NSW or Victoria but an accidental introduction in the rice growing regions would probably lead to its establishment. Vánky and Shivas (2008) reported anther smut, *Tilletia australiensis*, which was found on wild rice (*Oryza rufipogon*) in the Northern Territory.

Nakataea oryzae

Stem rot is a fungal disease that is considered serious in many rice growing regions throughout the world. The cause of this disease is the fungus *Nakataea oryzae* (syn. *Magnaporthe salvinii*). Stem rot was found in rice in south-eastern Australia in 1994−95 (Cother and Nicol, 1999). Initially the disease appears as small irregular black lesions on leaf sheaths at the waterline then enlarge and infect to rice stem. Sclerotia and mycelia are produced and destroy the tillers, usually the secondary tillers (Krause and Webster, 1972). Damage ranges from unfilled grain to the death of the tiller. Yield losses in Australia are less than 10 %, but overseas can be as high as 80 % (Anon., 2014).

Sclerophthora macrospora

Downy mildew is a minor fungal disease, which is occasionally seen on the leaves of rice and is caused by the fungus *Sclerophthora macrospora* (Shiratsuchi and Ohdaira, 2014). The rice seedling can be readily infected but symptoms generally are more noticeable near the flowering stage. Infected leaves have white to yellow spots and can be twisted if infection is severe. Panicles are unable to completely emerge; they remain green and generally fail to produce grain. Although downy mildew has been recorded in Yanco in 1930 (Noble et al., 1935) the hot dry climate does not generally favour its development. Downy mildew is favoured by cool temperatures (18–23 °C) and high humidity. The fungus survives as oospores in infected plant material. These oospores then become the primary inoculum for new infections (Anon., 2014).

Bipolaris oryzae

Brown spot disease is a minor fungal disease of rice caused by *Bipolaria oryzae* (syn. *Cochliobolus miyabeanus*). The disease is correlated with soil nutrient deficiency. Dark brown spots occur on the leaves, ranging in size from minute flecks to elongated spots (2 × 10 mm). Occasionally, larger spots and brown streaks appear. Heavily infected seeds cause blight of seedlings. On older plants, the disease reduces the number of grains per panicle and also reduces kernel weight (Anon., 2014).

Waitea circinata

Sheath spot, caused by *Waitea circinata* (syn. *Rhizoctonia oryzae*), was first reported in the southern NSW during the 1995−96 (Lanoiselet et al., 2001). Sheath spot disease can cause lodging on very susceptible cultivars but it is generally considered of minor importance. Yield losses have been shown to be as high as 10 % (Lanoiselet et al., 2005). It may become more serious under intensive rice crop rotations. The fungus survives the overwintering period as sclerotia or mycelia present in the soil or in rice crop debris (Anon., 2014). The disease causes spot type lesions on the leaf sheath midway up the tiller. Typical lesions are oval and 5–30 mm long. The spot is generally grey-white surrounded with a reddish-brown border. In contrast to the very similar aggregate sheath spot disease, the lesions are usually separated and do not coalesce (join up). Lesions are generally found near the water line, later progressing up the leaf sheaths. Favourable conditions for the fungus can cause the death of the infected leaves (Anon., 2014).

Rhizoctonia oryzae-sativae

Aggregate sheath spot, caused by *Rhizoctonia oryzae-sativae*, was discovered in 2000−01 in several rice crops of southern NSW (Lanoiselet et al. 2007). This disease leads to grain sterility, reduced grain filling and grain yield loss. It has been shown to reduce yield by as much as 20 %. The fungus survives the overwintering period as sclerotia or mycelia present in the soil or in rice crop debris. The first symptoms of aggregate sheath spot appear during the tillering stage as small black lesions on leaf sheaths near the water line. Lesions are oval and can be grey-green or yellowish coloured surrounded by a brown margin and range in size from 5–40 mm in length. The disease then moves upwards and reaches the upper leaf sheaths. Secondary infections progress up the stem and may spread to the flag leaf and panicle. Leaves of infected sheaths turn yellow and generally die. The culm may also be infected. Young sclerotia appear whitish as they are covered with mycelium, becoming dark-brown colour overtime. The size of sclerotia can vary greatly (Anon., 2014).

Bacterial pathogens

Pseudomonas syringae pv. s*yringae*

Glume blotch is a bacterial disease, caused by *Pseudomonas syringae* pv. s*yringae* (Kazempour et al., 2010). In severe cases, 75 % of the panicle may be affected, although florets at the base of the panicle are rarely completely discoloured. Some florets may be sterile but this may be caused by factors other than the disease. Glume blotch is favoured by any factor causing stress to the plant before panicle emergence. Small dark spots (1–2 mm) are found on the glumes or outer parts of the developing grain. The spots are surrounded by green to light brown tissue. Florets, the individual grain on the panicle (also called a spikelet) can be totally discoloured. Light to dark brown lesions without definite margins occur on the flag leaf sheath, and veins are darker than the interveinal tissue (Anon., 2014).

Pseudomonas fuscovaginae

Sheath brown rot, caused by *Pseudomonas fuscovaginae*, is a sporadic bacterial disease affecting stems and panicles (Patel et al., 2014). Sheath and panicle symptoms were observed in rice plants at the NSW Department of Primary Industries Leeton Field Station in 2005 (Cother et al., 2009). The bacteria can be seed borne but also can be epiphytic. The disease is more severe in cooler climates. Low temperatures at night during panicle extension would be favourable to the pathogen (Anon., 2014).

Pantoea ananus

This disease is of minor importance and is caused by *Pantoea ananus* (Cother et al., 2004). This bacterium may be epiphytic and cause disease only under certain environmental conditions. It was first reported in 2003 at Leeton Field Station, NSW and the predisposing conditions for the epidemic were thought to be the severe dust storms that crops experienced that year; these storms may have abraded tissue to allow pathogen entry. The disease appears as browning of the neck region of the panicle. The discoloration then may extend down the stem to the flag leaf and even down to the second node. The node may turn black often leading to a weakening of the stem compared to plants that are not affected (Anon., 2014).

Xanthomonas oryzae pv. *oryzae*

This is an important rice disease worldwide that is caused by the bacterium *Xanthomonas oryzae* pv. *oryzae.* The disease was first reported in Australia in 1972 in the Northern Territory and occurred on cultivated rice and the indigenous wild rice species, *Oryza rufipogon* and *Oryza australiensis* (Aldrick et al., 1973). The pathogen was isolated, characterized, and tested in Hawaii on a differential set of rice cultivars, demonstrating that the bacterial strain was likely indigenous to northern Australia (Aldrick et al., 1973).

Viruses

There are now over 30 viruses reported to infect rice through experimental tests and in nature. However, only 25 are of any direct economic impact to rice production (Abo and Sy, 1997). The most important of these is rice tungro, which is caused by a mixed infection of *Rice tungro bacilliform virus* and *Rice tungro spherical virus* (Hibino, 1996). For the vast majority of these diseases, the virus requires tropical insect vectors. Virus diseases are therefore mostly a problem in tropical or sub-tropical regions (Ou, 1985). Some of the vectors can be found in Northern Australia but would be unable to survive the climate of south-eastern Australia (Lanoiselet, 2007).

1.4 Rationale and aims of the thesis

The *Magnaporthales* contain important plant pathogens but have never been comprehensively studied in Australia. The best known fungus in this order is *Pyricularia oryzae,* which causes rice blast disease. This disease has been known in Australia since 1955 on cultivated rice in the Northern Territory, and subsequently in Queensland and Western Australia in 2011. The fungus was also recorded in the Northern Territory on a wild rice species in 1964. The biology of *Pyricularia oryzae* in northern Australia has not been previously studied, despite that the fungus is considered as one of the major limiting factors for further expansion of the rice industry. Very little is known about the threats that pests and diseases of wild rices pose to that the newly emerging northern Australian rice industry. This project addresses five research questions that focus on the taxonomy and epidemiology of *Pyricularia oryzae* and other plant pathogenic *Magnaporthales* in Australia.

1. How many species of *Pyricularia* and related fungi occur in Australia?
2. Does *Pyricularia oryzae* occur on native Australian wild rice species?
3. Are there other pathogenic fungi on wild rice species that might threaten rice production in Australia?
4. Are there Australian rice varieties resistant to *Pyricularia oryzae*?
5. Are there other *Poaceae* that serve as hosts of *Pyricularia oryzae*?

The objectives of the project are:

1. To identify Australian isolates of *Pyricularia* and related fungi in the *Magnaporthales* using morphological, molecular and biological characters.
2. To survey for leaf spotting fungi on wild rice in northern Queensland.
3. To examine the pathogenicity of Australian isolates of *Pyricularia oryzae* on domesticated rice cultivars and host range in *Poaceae.*

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Chapter 2 Identification and classification of *Pyricularia* spp. (*Magnaporthales*, *Sordariomycetes*) and allied species in Australia

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To be submitted to *Australasian Plant Pathology*

Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2016). *Pyriculariaceae* (*Sordariomycetes*, *Ascomycota*) in Australia.

(manuscript in preparation)

Abstract

Phylogenetic analysis of DNA sequence data from eight loci including internal transcribed spacer (ITS) and large subunit (LSU) of rDNA, actin (*ACT*), beta-tubulin (*BT*), calmodulin (*CAL*), the largest subunit of RNA polymerase II (*RPB1*), translation elongation factor 1-alpha (*TEF1*) and DNA replication licensing factor (*MCM7*), was used to classify and identify 24 Australian isolates of *Pyricularia*-like fungi from eleven host plants. Three species, *Pyricularia angulata*, *Pyricularia pennisetigena* and *Pyricularia oryzae* were identified amongst the isolates. Another isolate, previously identified as *Pyricularia rabaulensis* was shown to belong to *Barretomyces*. Furthermore, a teleomorphic state was found for *Pyricularia rabaulensis* and an amended description is provided for the proposed new combination *Barretomyces rabaulensis*.

2.1 Introduction

The genus *Pyricularia* is classified in the family *Pyriculariaceae,* order *Magnaporthales*, subclass *Diaportheomycetidae*, class *Sordariomycetes*, subphylum *Pezizomycotina*, phylum *Ascomycota* (Klaubauf et al., 2014; Maharachchikumbura et al., 2015). The *Pyriculariaceae* currently contains about 70 species distributed in the following nine genera, namely, *Bambusicularia*, *Barretomyces*, *Deightoniella*, *Macgarvieomyces*, *Neopyricularia*, *Proxipyricularia*, *Pseudopyricularia*, *Pyricularia* and *Xenopyricularia* (Klaubauf et al., 2014).

The most widely studied species in the *Magnaporthales* is *Pyricularia oryzae* (synonym *Magnaporthe oryzae*), which was ranked first in a list of the top 10 fungal plant pathogens in the world based on scientific and economic importance (Dean et al., 2012). *Pyricularia oryzae* causes blast disease on a range of plants, mostly on species in the monocotyledonous families *Poaceae*, *Cannaceae*, *Commelinaceae*, *Cyperaceae*, *Musaceae*, and *Zingiberaceae* (Park and Shin, 2009). *Pyricularia oryzae* is best known as the cause of rice blast disease, causing yield losses in cultivated rice (*Oryza sativa*) of up to 100 % under favourable conditions (Divya et al., 2014). *Pyricularia oryzae* can infect leaves, stems and inflorescences as well as roots under laboratory conditions (Sesma and Osbourn, 2004). Not all species of *Pyricularia* are plant pathogens, as some species are saprobic on leaf litter (McKenzie et al., 2010). Species of *Pyricularia* are characterized by having 3-celled conidia that are obpyriform or obclavate, hyaline and have a protuberant hilum (Cavara, 1891). The classification of species of *Pyricularia* has often been based on host genera. Some examples are, (i) *Pyricularia oryzae* named because it was a pathogen of *Oryza sativa* (Cavara, 1891); (ii) *Pyricularia zingiberi* on *Zingiber officinale* (Kotani and Kurata, 1992); (iii) *Pyricularia zizaniaecola* on *Zizania* sp. (Hashioka, 1973); and (iv) *Pyricularia commelinicola* on *Commelina communis* (Park and Shin, 2009). Klaubauf et al. (2014) studied the phylogenetic relationships of *Pyriculariaceae* and found that the host genus was not useful as a taxonomic criterion because many species on *Pyriculariaceae* had broad host ranges across different host genera.

Pyricularia oryzae was first reported in Australia near Darwin in the Northern Territory, where the fungus was isolated from three domesticated rice cultivars, namely, Caloro, Kentjana and Radin-China, (Stahl, 1955 as *Piricularia oryzae*). Moreover, *Pyricularia oryzae* was also reported on *Panicum* sp. (barnyard millet), *Digitaria adsendens* and *Echinochloa esculenta* (Japanese millet) but not on native rices (Stahl, 1955). A decade later, Heaton (1964) surveyed two rice fields at Humpty Doo and Tortilla Flats, in the Northern Territory and found rice blast disease caused by *Pyricularia oryzae* of four rice cultivars Kentjana, Gendjah Beton, A36-3 and Sirena. The isolate represented Race 18 (United States series) of *Pyricularia oryzae*, which was equivalent to Japanese Race N5 (Heaton, 1964). Recently, *Pyricularia oryzae* was recorded in Western Australia for the first time, where it was found on *Oryza sativa* cv. Quest with symptoms of rice blast disease (You et al., 2012).

Two other species of *Pyricularia* have been recorded in Australia. Firstly, *Pyricularia grisea* was isolated from blast-like lesions on *Cenchrus ciliaris* (buffel grass) in central Queensland, identified by morphological methods and its pathogenicity confirmed by host inoculation tests (Perrott and Chakraborty, 1999). Secondly, *Pyricularia angulata* was identified by morphological and molecular methods from *Musa acuminata* cv. Dwarf Cavendish in northern Queensland, where it was associated with leaf blast disease on banana (Male et al., 2011). *Pyricularia angulate*, while morphologically similar to *Pyricularia grisea*, is only pathogenic on banana plants (Kim et al., 1987).

In this study, the species identification of *Pyricularia*-like species from Australian plant species has been examined using a combined morphological and molecular approach. We analysed eight gene regions from genomic DNA, together with morphological characters, of twenty four specimens of *Pyricularia*-like species from around Australia.

2.2 Materials and methods

2.2.1 Culture preparation

There were 146 herbarium specimens of *Pyricularia* accessioned in the Plant Pathology Herbarium (BRIP), Department of Agriculture and Forestry, Queensland (Appendix 8). These accessions represented six species, *Pyricularia angulata*, *Pyricularia caricis*, *Pyricularia grisea*, *Pyricularia penniseti*, *Pyricularia rabaulensis* and *Pyricularia oryzae*, all from host species in several different monocotyledonous families. Of these specimens, 24 had associated living cultures available for study (Table 2.1). These *Pyricularia* isolates were collected from 11 different host plant species and cultures were stored at -20 °C under 15 % glycerol.

Subcultures were plated onto potato dextrose agar (PDA, 39 g Potato Dextrose Agar Difco./L) and incubated at 25 °C for 7−14 d. Hyphal tips were transferred to plates of ½ strength PDA (19.5g Becton Dickenson Potato Dextrose Powder/L, 3.75 Amresco Agar/L, 200 ppm streptomycin sulphate Sigma) or wheat straw agar (WSA) for inducing sporulation then incubated at 25 °C for 14 d prior to examination. Conidia were mounted on glass slide with 100 % lactic acid and gently heated before examination. Length and width of the conidia were measured with the mean and standard deviations (SD) calculated from at least 20 conidia per isolate.

2.2.2 DNA extraction, amplification and sequencing

Mycelium was scraped off from PDA plates and placed in a 2 ml snap-lock tube containing 300 µl cell lysis solution (10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5 % SDS), 5 µl of proteinase K (20 mg/ml) and 0.5 mm diameter glass beads (BioSpec Product Inc.), then incubated at 65 °C for 1 h. The tissue was shaken at 20,000 rpm for 3 min using a TissueLyser (QIAGEN), centrifuged at 1000 g for 5 min, then the supernatant transferred into a new tube that contained 100 µl of 5 M ammonium acetate and incubated at 4 ºC for 10 min. DNA was precipitated with isopropanol and centrifuged for 10 min at 1000 g before discarding the supernatant. The DNA pellet was treated with 70 % ethanol centrifuged at 1000 g for 5 min and the solution discarded before air-drying for 20 min and resuspended in 50 µl of DNAse-free water and stored at -20 °C.

Table 2.1 GenBank accession number used in this study

				GenBank Accession no.							
Species	**Voucher**	**Host**	**Country**	**ITS**	**LSU**	**RPB1**	**ACT**	**CAL**	**BT**	**TEF1**	**MCM7**
Bambusicularia brunnea	CBS 133599 (ex-type)	*Sasa* sp.	Japan	KM484830	KM484948	KM485043	AB274449	AB274482	-	-	-
Barretomyces calatheae	CBMAI 1060 (ex-type)	*Calathea longifolia*	Brazil	GU294490	-	-	-	-	-	-	-
Barretomyces rabaulensis	BRIP 22632 b	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 23325 a	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 23858 a	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 23904 a	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 23906 a	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 23928 a	*Alpinia caerulea*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
Deightoniella roumeguerei	CBS 128780 (ex-type)	*Phragmites australis*	Netherlands	JF951153	JF951176	KM485047	KM485163	KM485232			
Macgarvieomyces borealis	CBS 461.65 (ex-type)	*Juncus effusus*	Scotland	KM484854	DQ341511	KM485070	KM485170	KM485239	-	-	-
Nakataea oryzae	ATCC 44754	*Oryza sativa*	Japan	JF414838	JF414887	JF710441	-	-	-	JF710406	JF710382
Neopyricularia commelinicola	CBS 128308 (ex-type)	*Commelina communis*	South Korea	FJ850122	KM484985	KM485087	KM485175	-	-	-	-
Proxipyricularia zingiberis	CBS 132355	*Zingiber mioga*	Japan	AB274433	KM484987	KM485090	KM485176	AB274481	-	-	-
Pseudohalonectria lignicola	YMF1.01288	rotten wood	China	JX134679	JX134691	JX134733	-	-	-	JX134705	JX134719

Pseudopyricularia cyperi	CBS 133595 (ex-type)	*Cyperus iria*	Japan	KM484872	KM484990	AB818013	AB274453	AB274485	-	-	-
Pseudopyricularia higginsii	CBS 121934	*Typha orientalis,*	New Zealand	KM484875	KM484991	KM485095	KM485180	KM485250	-	-	-
Pseudopyricularia kyllingae	CBS 133597 (ex-type)	*Kyllinga brevifolia*	Japan	KM484876	KM484992	KM485096	AB274451	AB274484	-	-	-
Pyricularia angulata	BRIP 53746 a	*Musa* sp.	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 53869 a	*Musa* sp.	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
Pyricularia ctenantheicola	GR0002 (ex-type)	*Ctenanthe oppenheimiana*	Greece	KM484879	-	KM485099	KM485183	KM485253	-	-	-
Pyricularia grisea	M82	*Digitaria* sp.	USA	JX134670	JX134682	JX134724	-	-	-	JX134696	JX134710
	M83	*Digitaria* sp.	USA	JX134671	JX134683	JX134725	-	-	-	JX134697	JX134711
Pyricularia oryzae	70-15		USA	Genome[a]	Genome[a]	Genome[a]	Genome[a]	Genome[a]	Genome[a]	Genome[a]	Genome[a]
	BRIP 5636	*Oryza sativa*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 14879	*Pennisetum clandestinum*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 15666 a	*Pennisetum clandestinum*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 15748 a	*Oryza rufipogon*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 15815 a	*Brachiaria mutica*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 15828 a	*Melinis repens*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 15855 a	*Pennisetum clandestinum*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 39772 a	*Hordeum vulgare*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 53870 a	*Phalaris canariensis*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 55372 a	*Oryza sativa*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 55376 a	*Oryza sativa*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 58447 a	*Oryza sativa*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx

	BRIP 59311a	*Setaria sp.*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 61930	*Stenotaphrum secundatum*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
	BRIP 62336a	*Setaria italica*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
Pyricularia penniseticola	ML0031 (ex-type)	*Pennisetum typhoides*	Mali	KM484929	-	KM485148	KM485220	-	-	-	-
Pyricularia pennisetigena	ML0036 (ex-type)	*Pennisetum sp.*	Mali	KM484935	-	KM485153	KM485225	KM485294	-	-	-
	BRIP 25865 a	*Cenchrus ciliaris*	Australia	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
Pyricularia zingibericola	RN0001	*Zingiber officinale*	R union	KM484941	KM485037	KM485157	KM485229	KM485297	-	-	-
Xenopyricularia zizaniicola	CBS 133593	*Zizania latifolia*	Japan	KM484947	-	KM485161	KM485230	AB274479	-	-	-

ATCC: American Type Culture Collection, Manassas, Virginia, USA; BRIP: Plant pathology herbarium, Department of Agriculture, Fisheries and Forestry, Queensland, Australia; CBMAI: the Coleção Brasileira de Microrganismos para Ambiente e Indústria, Brazil ; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands;YMF: Yunnan Microbiological Fermentation Culture Collection Center, China; Genome[a]: Broad Institute, USA.

Polymerase chain reaction amplifications (PCR) of eight loci including the internal transcribed spacer (ITS) and large subunit (LSU) of rDNA and six protein coding genes; actin (*ACT*), beta-tubulin (*BT*), calmodulin (*CAL*), the largest subunit of RNA polymerase II (*RPB1*), translation elongation factor 1-alpha (*TEF1*) and DNA replication licensing factor (*MCM7*) were conducted in 20 µl reactions with 10 µl of high fidelity Phusion® DNA Polymerase (New England Biolabs), forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl and distill water 7 µl, using the manufacturer-specified cycling and reaction conditions. PCR products were sent to Macrogen Ltd., South Korea, for sequencing. Primers used in this study are shown in Table 2.2.

Table 2.2 PCR amplification and sequencing primers used in this study

Locus	**Primers**	**Annealing Temp (ºC)**	**Sequence (5’-3’)**	**References**
ITS	ITS1	62	TCCGTAGGTGAACCTGCGG	(White et al., 1990)
	ITS4		TCCTCCGCTTATTGATATGC	
LSU	LROR	55	ACCCGCTGAACTTAAGC	(Rehner and Samuels, 1995)
	LR5		TCCTGAGGGAAACTTCG	
Actin	Act-512F	62	ATGTGCAAGGCCGGTTTCGC	(Carbone and Kohn, 1999)
	Act-783R		TACGAGTCCTTCTGGCCCAT	
TUB	Bt1a	60	TTCCCCCGTCTCCACTTCTTCATG	(Glass and Donaldson, 1995)
	Bt1b		GACGAGATCGTTCATGTTGAACTC	
CAL	CAL-228F	64	GAGTTCAAGGAGGCCTTCTCCC	(Carbone and Kohn, 1999)
	CAL-737R		CATCTTTCTGGCCATCATGG	
RPB1	RPB1-Ac	59	GARTGYCCDGGDCAYTTYGG	(Matheny et al., 2002; Castlebury et al., 2004)
	RPB1-Cr		CCNGCDATNTCRTTRTCCATRTA	
TEF1	EF1-983F	65	GCYCCYGGHCAYCGTGAYTT	(Zhang et al., 2011)
	EF1-2218R		ATGACACCRACRGCRACRGTYTGYAT	
MCM7	MCM7-709	60	ACIMGIGTITCVGAYGTHAARCC	(Schmitt et al., 2009)
	MCM7-1348		GAYTTDGCIACICCIGGRTCWCCCAT	

2.2.3 Phylogenetic analysis

Sequencher ver. 5.1 (Gene Codes Crop., Ann Arbor, Michigan) was used to make contigs. The ITS, LSU, actin, beta-tubulin, calmodulin, *RPB1*, *TEF1* and *MCM7* were aligned separately in MAFFT 6.611 (http://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh and Toh, 2008) and viewed using the MEGA6 software package (Kumar et al., 2008). The sequences from each locus were concatenated in a supermatrix and analysed as patitioned datasets with maximun likelihood (ML) and Bayesian inferences (BI) as phylogenetic criteria. The maximum likelihood was conducted with RAxML ver. 7.2.6 (Stamatakis, 2006) using GTRGAMMA as the model of evolution. The RAxML analyses were run with a rapid Bootstrap analysis (command -f a) using a random starting tree and 1 000 maximum likelihood bootstrap replicates. A Markov Chain Monte Carlo (MCMC) search in a Bayesian analysis was conducted with MrBayes ver. 3.1.2 (Ronquist et al., 2012). Four runs, each consisting of four chains, were implemented for 10 million generations. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 1000 generations and trees were saved every 5000 generations then visualized in FigTree (Morariu et al., 2008). The ML and Bayesian analyses were run four times to test accuracy.

2.3 Results

2.3.1 Phylogenetic relationships obtained with nuclear rDNA

Most of the 24 isolates examined were morphologically characteristic of *Pyricularia*, having 3-celled, obpyriform or obclavate, hyaline conidia, each with a protuberant hilum. However, isolates from *Alpinia caerulea* had cylindrical to obclavate, pale brown to sybhyaline conidia with a tapered apical cell that was clearly distinct from *Pyricularia*. The concatenated sequence matrix had 4,877 nucleotide characters including 304 nucleotide characters in the ITS, 773 in the LSU, 750 in the *RPB1*, 387 in the *ACT*, 657 in the *BT*, 629 in the *CAL*, 813 in the *TEF1* and 564 in the *MCM7*. The phylogenetic trees recovered from ML and BI analyses were congruent (Fig. 2.1). *Pyricularia* species formed a monophyletic clade (100 % bootstrap/1.0 posterior probability) that contained three species, *Pyricularia angulata, Pyricularia oryzae* and *Pyricularia pennisetigena*.

Pyricularia oryzae was found on eight host species, namely, *Oryza sativa*, *Oryza rufipogon*, *Hordeum vulgare*, *Brachiaria mutica*, *Pennisetum clandestinum*, *Phalaris canariensis*, *Setaria italica* and *Melinis repens*. The records on *Hordeum*, *Brachiaria*, *Pennisetum*, *Phalaris*, *Setaria* and *Melinis* confirm host genera for *Pyricularia oryzae* in Australia. The two species, *Pyricularia pennisetigena* and *Pyricularia angulata*, were phylogenetically distinct from *Pyricularia oryzae* and each was restricted to a single host species, *Cenchrus ciliaris* and *Musa acuminata*, respectively (Fig. 2.1). The phylogenetic analysis also showed that *Pyricularia rabaulensis* was sister to *Barretomyces calatheae*, which is the type species of *Barretomyces* (*Pyriculariaceae*, *Magnaporthales*). This necessitates the transfer of *Pyricularia rabaulensis* to *Barretomyces*. Further, a teleomorphic state was found for *Pyricularia rabaulensis* and an amended description of this fungal species follows.

2.3.2 Taxonomy

Barretomyces rabaulensis (Matsushima) Khemmuk, Geering & R.G. Shivas, **comb. nov. et. ememd. nov.**

MycoBank MB******

Basionym. *Pyricularia rabaulensis* Matsushima, Bull. Nat. Sci. Mus. Tokyo, 14 (3): 473. 1971.

Classification — *Pyriculariaceae*, *Magnaporthales*, *Diaportheomycetidae*

On sterilised leaf pieces of *Alpinia caerulea* on Sach's agar, cross between BRIP 22632b and BRIP 23325a after 35 d with near ultraviolet light. *Ascomata* perithecial, single, scattered, immersed, globose to subglobose, 125–200 µm diam, without necks, dark brown. *Asci* unitunicate, 8-spored, cylindrical to fusiform, 70–110 x 8–15 µm, deliquescent, spores biseriate in ascus. *Ascospores* 2- or 3-sepate, fusiform, 20–25 x 5–7 µm, slightly curved, hyaline, smooth.

Description of anamorphic state and illustrations: (Kobayasi, 1971)

Material examined: Australia, Queensland, on blighted leaves of *Alpinia caerulea*, Maiala National Park, 26 Feb. 1995, *J.M. Stanton*, BRIP 22632b (IMI 367259); The Knoll National Park, Mount Tamborine, on blighted leaves of *Alpinia caerulea*, 26 Apr. 1996, *J.L. Alcorn*, BRIP 23325a (**proposed neotype to be designated elsewhere when published in accordance with the Act 9.7 of the International Code of Nomenclature for algae, fungi and plants (Melbourne Code)**); on leaves, 18 Jul. 1996, *J.L. Alcorn*, BRIP 23520;

Indooroopilly, 26 Jul. 1996, *J.L. Alcorn*, BRIP 23904, BRIP 23906a; Indooroopilly, 22 Aug. 1996, *J.L. Alcorn*, BRIP 23928; Indooroopilly, 26 Aug. 1996, *J.L. Alcorn*, BRIP 23939; Brisbane, 23 Sep. 1996, *J.L. Alcorn*, BRIP 23988; Brisbane, 23 Sep. 1996, *J.L. Alcorn*, BRIP 23989; Indooroopilly, 26 Aug. 1996, *J.L. Alcorn*, BRIP 23940. On *Alpinia arctiflora*: Mount Lewis, near Julatten, on leaves, 9 Nov. 1997, *C.A. Pearce*, BRIP 24847; New South Wales, on leaves of *Alpinia caerulea*: Levers Plateau, 1996, *J.M. Stanton*, BRIP 23858.

Hosts: Alpinia caerulea and *Alpinia arctiflora*

Notes — The holotype (MFC-2903) of *Pyricularia rabaulensis* was held in Dr. Takashi Matsushima's private collection (Matsushima Fungal Collection (MFC), Kobe, Japan), which was destroyed in the 1995 Kobe earthquake as stated in a letter to Dr John Alcorn signed by Matsushima on 26 Nov. 2001. The letter further indicates that Matsushima had examined a slide preparation of BRIP 23325a and considered it represented *Pyricularia rabaulensis*. The conidial and conidiophore morphology of the type description (Matsushima, 1971) matches that seen in BRIP 23325a. Consequently BRIP 23325a has been designated here as the neotype of *Pyricularia rabaulensis*. Differences between the destroyed holotype and the neotype are that the former was collected on *Musa paradisiaca* (*Musaceae*) in Papua New Guinea and the later occurred on *Alpinia caerulea* (*Zingiberaceae*), which is endemic to sub-tropical and tropical regions of eastern Australia. The phylogenetic analysis indicated that *Pyricularia rabaulensis* was sister to *Barretomyce calatheae*, which is the type species of *Barretomyces*, and occurs on *Calathea longifolia* (*Marantaceae*) in Brazil (Klaubauf et al., 2014). A teleomorphic state for *Barretomyces* is described here for the first time.

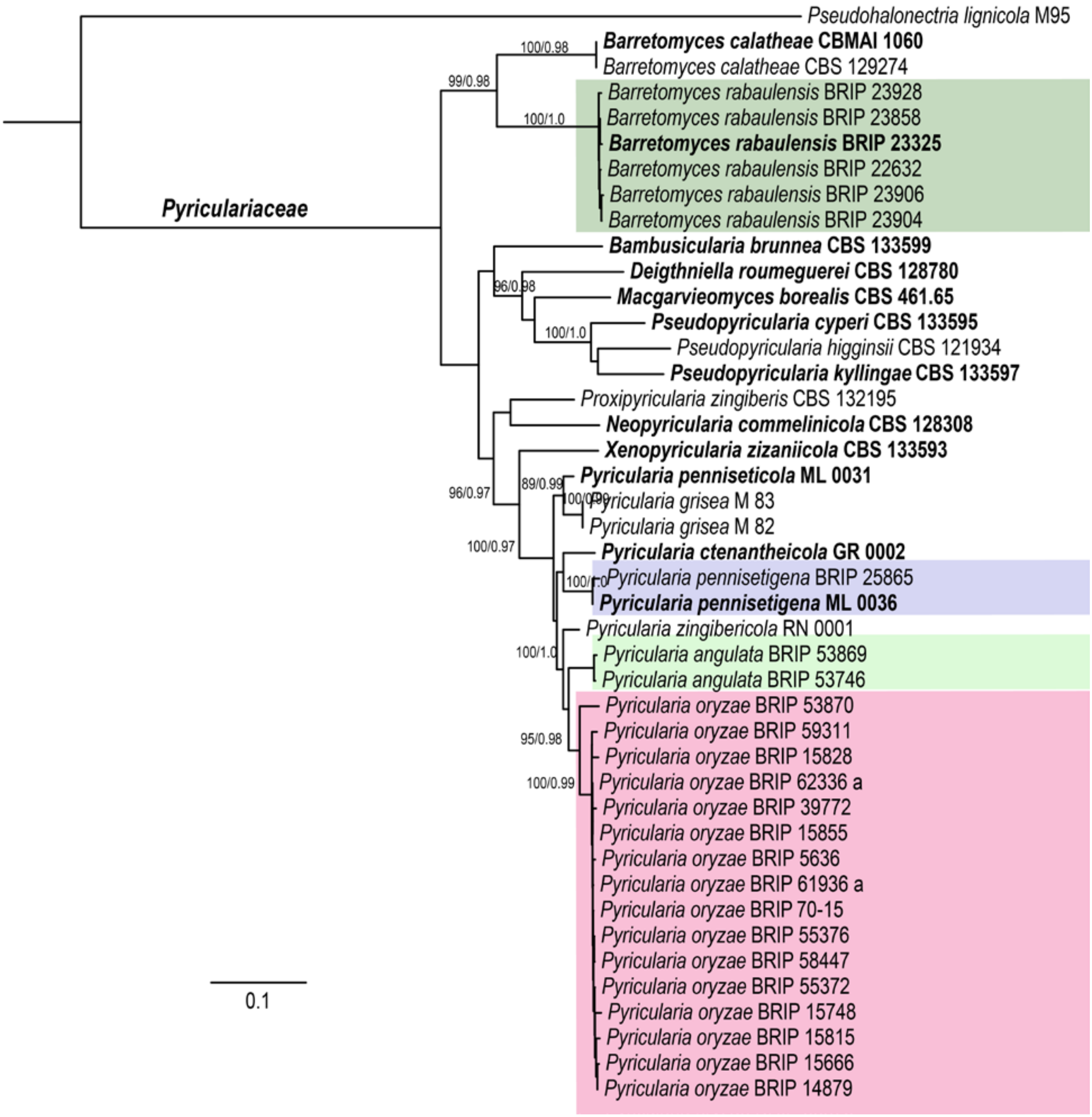

Figure 2.1 Phylogenetic relationships of *Pyriculariaceae*, inferred using DNA sequence dataset of ITS, LSU, *RPB1*, *ACT*, *BT*, *CAL*, *TEF1* and *MCM7*. The analysis was done using the maximum likelihood (ML) method, as implemented in RaxML 7.2.6. Bootstrap values (>70%) from 1000 replicates in an ML search, and posterior probabilities (>0.95) from Bayesian inference are shown on nodes respectively. Ex-type cultures are written in **bold** and *Pseudohalonectria lignicola* was included as an outgroup.

2.4 Discussion

The phylogenetic analysis showed that *Pyricularia* spp. formed a monophyletic clade with strong bootstrap support that was distinct from other genera in the *Pyriculariaceae* as recently described by Klaubauf et al. (2014). *Pyricularia oryzae* is the best known and most widespread species affecting family *Poaceae* (Park and Shin, 2009). *Pyricularia oryzae* has been known in Australia for over 60 years on *Oryza sativa*, *Panicum* sp., *Digitaria adsendens* and *Echinochloa esculenta* (Stahl, 1955). *Pyricularia oryzae* is reported here to comfirm that *Hordeum vulgare, Brachiaria mutica, Melinis repens, Pennisetum clandestinum, Phalaris canariensis* and *Setaria italica* can be infected by this pathogen.

Most of the known blast pathogens of cultivated grasses belong to *Pyricularia oryzae,* which is a species complex (Castroagudin et al., 2016) that also includes several pathotypes defined by their restricted ranges of host species (Tosa and Chuma, 2014). The specificity between plant genera and the fungal pathotype is under the control of gene-for-gene interactions (Murakami et al., 2000; Takabayashi et al., 2002; Tosa et al., 2006; Tosa and Chuma, 2011).

Pyricularia graminis-tritici and *Pyricularia oryzae* pathotype *Triticum* have particular importance as they demonstrate the potential threat by posed to cultivated cereals other than rice, by the *Pyricularia oryzae* species complex (Castroagudin et al., 2016). The *Pyricularia oryzae* pathotype *Triticum* was discovered as a new pathogen on wheat in Brazil in the 1980s, which then spread to other countries in South America and was also later found on *Hordeum vulgare* (Anjos and Charchar, 2000; Urashima et al., 2004). The first occurrence of wheat blast fungus outside of South America was in Kentucky, USA (Pratt, 2012). However isolates of the wheat blast fungus in the USA were genetically different from the wheat blast isolates in South America. The USA isolates were more similar to the *Lolium* (annual ryegrass) pathotype, which is a common pasture crop in North America. This is important evidence that shows the potential of *Pyricularia oryzae* to jump hosts (Pratt, 2012). Taxonomic resolution of the *Pyricularia oryzae* species complex, especially the wheat infecting pathotypes and species, will undoubtedly be a focus of global research in coming years.

Two other species of *Pyricularia* were found in this study. *Pyricularia angulata* is a leaf spot pathogen on banana that has been reported from Australia (Male et al., 2011) and Korea (Kim et al., 1987). *Pyricularia angulata* is similar in morphology to *Pyricularia grisea* but is pathogenic only on banana (Kim et al., 1987). A fungus causing pitting disease on banana, was found in Queensland, Australia and identified as *Pyricularia grisea* based on morphological characters (Hoette, 1936). More recently, molecular research has demonstrated the differences between *Pyricularia angulata* and *Pyricularia grisea* and 39 other *Pyricularia* species and allied genera (Bussaban et al., 2005). This indicates that the original identification of *Pyricularia grisea* in Australia (Hoette, 1936) was most likely *Pyricularia angulata.*

Pyricularia pennisetigena is reported on *Cenchrus ciliaris* for the first time in Australia. This species has been reported on different hosts such as *Cenchrus echinatus* and *Echinochloa colonum* in Brazil, *Cenchrus ciliaris* in Japan, *Pennisetum* sp. in Mali, *Pennisetum glaucum* in USA (Klaubauf et al., 2014). *Pyricularia pennisetigena* is morphologically similar to typical *Pyricularia* with 3-celled conidia and obpyriform but differ from others by pathogenicity test (Kato et al., 2000). Multigene analysis indicated that *Pyricularia pennisetigena* is a distinct species in the *Pyricularia* clade with strong bootstrap support (Klaubauf et al., 2014).

The leaf spot pathogen on *Alpinia caerulea* that was originally described as *Pyricularia rabaulensis* will be formally transferred to *Barretomyces rabaulensis* based on phylogenetic analysis. *Barretomyces rabaulensis* differed morphologically from other *Pyricularia* species by having tapered apices conidia. *Barretomyces calathea* was original described as *Pyriculariopsis calatheae* based on its versicoloured conidia and schizolytic secession (Soares et al., 2011). Recent molecular analysis showed that the genus *Pyriculariopsis* is placed in *Magnaporthaceae* while *Barretomyces* is grouped in *Pyriculariceae* (Klaubauf et al., 2014).

This study used multigene phylogenetic analysis to show that three species of *Pyricularia* occur in Australia on nine different host species. Typical morphology has been shown to be a primary character to distinguish the genus *Pyricularia. Pyricularia* is a diverse genus and more novel species are likely to occur in nature.

2.5 References

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Chapter 3 *Magnaporthe*-like fungi (*Diaportheomycetidae, Sordariomycetes, Ascomycota*) in Australia

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Published in *Persoonia*

1. Wong, P.T.W., Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2015). *Budhanggurabania cynodonticola* Fungal Planet Description 359. *Persoonia* **34**, 240–241.
2. Wong, P.T.W., Khemmuk, W., Geering, A. D. W. and Shivas, R.G. (2015). *Magnaporthiopsis agrostidis.* Fungal Planet Description 398. *Persoonia* **35**, 322–323.

Published in *IMA Fungus*

3. Khemmuk, W., Geering, A. D. W. and Shivas, R.G. 2016. *Wongia* gen. nov. (*Papulosaceae, Sordariomycetes*), a new generic name for two root-infecting fungi from Australia. *IMA Fungus* **7(2)**, 247–252.

Abstract

The phylogenetic relationships of some Australian fungi belonging to the *Diaportheomycetidae* (*Sordariomycetes*, *Ascomycota*) were investigated based on morphology and DNA sequences of multiple genes. Two novel genera, *Budhanggurabania* and *Wongia*, were described, as well as two new species, *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis*. Two species of *Magnaporthe* were transferred to *Wongia*.

3.1 Introduction

The *Sordariomycetes* is the largest class of *Ascomycota*, which all members are characterised by having perithecial ascomata and inoperculate unitunicate asci including many important plant pathogens, endophytes, saprobes, epiphytes, fungicolous and lichenicolous taxa in freshwater, marine and terrestrial environments (Kirk et al., 2008; Maharachchikumbura et al., 2015). Anamorph and teleomorph of taxa in the *Sordariomycetes* often occur separately, or only one morph is formed, and it is consequently difficult to determine links between the different morphs (Wijayawardene et al., 2014). However, utilization of PCR and DNA sequencing in the fungi, can confirm incontestable link between asexual and sexual morphs. Since 1 January 2013, the International Code of Botanical Nomenclature (ICBN) has radical modification of Article 59 establishing the principle of “one fungus, one name” (Norvell, 2011). Current classification, based on phylogeny and morphology, clusters the *Sordariomycetes* into six subclasses, *Sordariomycetidae*, *Hypocreomycetida*, *Xylariomycetidae* (Lumbsch and Huhndorf, 2010), *Meliotomycetidae* (Kirk et al., 2001), *Diaportheomycetidae* and *Lulworthiomycetidae* (Maharachchikumbura et al., 2015).

The subclass *Diaportheomycetidae* comprises 10 orders, 26 families and 273 genera which include pathogens, parasites, endophytes and saprobes (Maharachchikumbura et al., 2015). In Australia, the *Diaportheomycetidae* are represented by six orders (Table 3.1). Of these, the *Magnaporthales* is best known by plant pathologists as it contains mostly necrotrophic or hemibiotrophic parasites that infect stems or roots of *Poaceae* (grasses) and *Cyperaceae* (sedges) (Thongkantha et al., 2009; Zhang et al., 2011; Luo and Zhang, 2013). A recent phylogenetic study proposed that the *Magnaporthales* contained three families, *Magnaporthaceae*, *Pyriculariaceae* and *Ophioceraceae* (Klaubauf et al., 2014). Members of the *Magnaporthaceae* are mostly root associated fungi that infect hosts plants through the formation of hyphopodia or appressoria, producing long-necked perithecia immersed in host tissue. The asexual morphs of *Magnaporthaceae* are usually phialophora-like (Luo et al., 2015). The best known fungus in the *Magnaporthaceae* in Australia is *Gaeumannomyces graminis* var. *tritici*, which causes the highly destructive disease known as take-all of wheat and other cereals (Walker, 1972; MacNish, 1988). *Magnaporthe griffinii* and *Magnaporthe garrettii* caused a serious root-infecting disease on turf grasses in South Australia, New South Wales and Queensland (Wong et al., 2012). Occasionally, some members of the *Magnaporthaceae* are associated with leaf and stem diseases, for example, *Buergenerula zelandica*, which is associated with a leaf spot on *Carex* in New Zealand (McKenzie, 1991). Endophytes are also found in the *Magnaporthaceae*, for example, *Bussabanomyces longisporus*, which has been reported from healthy leaves of *Amomum siamense* in Thailand (Bussaban et al., 2003).

In this Chapter, isolates of Australian *Magnaporthe*-like fungi in culture collection were studied. Two unidentified root pathogens of grasses in Australia were found to represent novel species using a combined morphological and molecular approach. The phylogenetic relationships of the two Australian species, *Magnaporthe garrettii* and *Magnaporthe griffinii*, were determined by analysing of DNA sequences from type specimens of both species.

Table 3.1 Representative species in *Diaporthomycetidae* in Australia

Order	**Species**	**Substrates**	**References**
Annulatascales	*Annulatascus velatisporus*	decaying wood	Hyde 1992
	A. bipolaris	decaying wood	
Calosphaeriales	*Calosphaeria pulchella*	*Prunus avium*	Trouillas et al. 2011
Coniochaetales	*Coniochaeta fodinicola*	uranium mining	Vázquez-Campos et al. 2014
Diaporthales	*Diaporthe gulyae*	*Helianthus annuus*	Thompson et al. 2011
	Phomopsis viticola	grapevine	Merrin et al. 1995
Magnaporthales	*Gaeumannomyces graminis* var. *tritici*,	*Triticum astivum*	Walker, 1972; MacNish, 1988
	Budhanggurabania cynodonticola	*Cynodon dactylon*	Wong et al. 2015a
	Magnaporthiopsis agrostidis	*Agrostis stolonifera*	Wong et al. 2015b

Table 3.1 Continued

Order	Species	Substrates	References
	Magnaporthe garrettii	*Cynodon dactylon* cv. Greenlees	Wong et al. 2012
	Magnaporthe griffinii	*Cynodon dactylon* × *C. transvaalensis,*	Wong et al. 2012
Ophiostomatales	*Ophiostoma tasmaniense*	*Eucalyptus globulus*	Nkuekam et al. 2011
		E. nitens	
	O. undulatum	*E. grandis*	
	O. quercus	*Corymbia variegata*	
		Eucalyptus dunii	
		E. agglomerata	
		E. grandis	
		E. nitens	
		E. globulus	
	O. tsotsi	*E. grandis*	
		E. pilularis	

3.2 Material and methods

3.2.1 Isolates

Living cultures of *Magnaporthales* were obtained from the Plant Pathology Herbarium, Department of Agriculture and Fisheries (BRIP) (Table 3.2). Dried specimens of the holotypes of *Magnaporthe garrettii* (DAR 76937) and *Magnaporthe griffinii* (DAR 80512) were borrowed from the Plant Pathology Herbarium, New South Wales Agriculture (DAR). Cultures were grown on half potato dextrose agar (½PDA) and incubated at 25 ºC for 14 d.

3.2.2 Morphology

Morphological characters including colony on PDA and conidial shape were observed. Fungal conidia were mounted on glass slides in 100 % lactic acid and gently heated to boiling before microscopic examination. Observations were made with a Leica DFC 500, images were captured with camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

3.2.3 DNA extraction, amplification and sequencing

DNA from fungal cultures was extracted as described in Chapter 2. Dried perithecia of *Magnaporthe garrettii* and *M. griffinii* were excised from the dried specimens and soaked in extraction buffer overnight at 65 ºC before DNA was extracted with an UltraClean® Microbial DNA Isolation Kit (MoBIO Laboratories, Inc.), according to the manufacturer's instructions. The primer pairs which used to amplify *internal transcribed spacer* (ITS), *the largest subunit of RNA polymerase II* (RPB1), *large subunit* (LSU) of rDNA, and *translation elongation factor 1-alpha* (TEF1) showed in Table 3.2. PCR amplifications were conducted in a 20 µl reaction with 10 µl of high fidelity Phusion® DNA Polymerase (New England Biolabs), forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl and distill water 7 µl, using the manufacturer-specified cycling and reaction conditions. Annealing temperatures of each primer are shown in Table 3.2. PCR products were sent to Macrogen, South Korea for direct sequencing using the amplification primers.

Table 3.2 PCR amplification and sequencing primers used in this study

Locus	**Primers**	**Annealing Temp (ºC)**	**Sequence (5'-3')**	**References**
ITS	ITS1	62	TCCGTAGGTGAACCTGCGG	(White et al., 1990)
	ITS4		TCCTCCGCTTATTGATATGC	
LSU	LROR	55	ACCCGCTGAACTTAAGC	(Rehner and Samuels, 1995)
	LR5		TCCTGAGGGAAACTTCG	
RPB1	RPB1-Ac	59	GARTGYCCDGGDCAYTTYGG	(Matheny et al., 2002; Castlebury et al., 2004)
	RPB1-Cr		CCNGCDATNTCRTTRTCCATRTA	
TEF1	EF1-983F	65	GCYCCYGGHCAYCGTGAYTT	(Zhang et al., 2011)
	EF1-2218R		ATGACACCRACRGCRACRGTYTGYAT	

Table 3.3 GenBank accession numbers of isolate includes in this study

				GenBank accession no.			
Species	**Voucher**	**substrate**	**Locality**	**ITS**	**LSU**	**RPB1**	**TEF1**
Annulusmagnus triseptatus	CBS 128831	Decayed wood	France	-	GQ996540	-	-
Bambusicularia brunnea	CBS 133599	*Sasa* sp.	Japan	KM484830	KM484948	KM485043	-
Barretomyces clalatheae	CBMAI 1060	*Calathea longifolia*	Brazil	GU294490	-	-	-
Brunneosporella aquatica	HKUCC 3708	submerged wood	Hong Kong	-	AF132326	-	-
Budhanggurabania cynodonticola	BRIP 59305	*Cynodon dactylon*	Australia	KP162134	KP162140	KP162143	KP162138
Buergenerula spartinae	ATCC22848	*Spartina alterniflora*	unknown	JX134666	DQ341492	JX134720	JX134692
Calosphaeria pulchella	CBS 115999	*Prunus avium*	France	-	AY761075	-	-
Cordana pauciseptata	CBS 121804	unknown	Spain	-	HE672160	-	-
Coniochaeta ligniaria	C8	unknown	unknown	-	AY198388	-	-
Cornipulvina ellipsoides	SMH1378	On decorticated wood	Puerto Rico	-	DQ231441	-	-
Cryphonectria havanensis	CBS 505.63	*Eucalyptus saligna*	Russia		AF408339		
Cryphonectria parasitica	ATCC38755	*Castanea dentata*	USA	Genome[a]	Genome[a]	Genome[a]	Genome[a]
Diaporthe phaseolorum	FAU458 (SMBL, BPI)	unknown	unknown	-	AY346279	-	-
Diaporthe eres	CBS 109767	*Acer campestre* L.	Austria	-	AF408350	-	-
Fluminicola coronata	HKUCC 3717	unknown	Hong Kong	-	AF132332		
Gaeumannomyces oryzinus	CBS 235.32	*Oryza sativa*	USA	JX134669	JX134681	JX134723	JX134695
Harknessia eucalypti	CBS 342.97	*Eucalyptus regnans*	Australia	-	AF408363	-	-
Lecythophora luteoviridis	CBS 206.38	unknown	Switzerland	-	FR691987	-	-
Magnaporthiopsis agrostidis	BRIP 59300	*Agrostis stolonifera*	Australia	KT364753	KT364754	KT364755	KT689623
M. poae	ATCC 64411	*Triticum aestivum*	USA	JF414836	JF414885	JF710433	JF710415
Nakataea oryzae	ATCC 44754	*Oryza sativa*	Japan	JF414838	JF414887	JF710441	JF701406
Neurospora crassa	MUCL 19026	unknown	unknown	-	AF286411	-	-
Ophioceras leptosporum	CBS 894.70	dead stem of dicot plant	UK	JX134678	JX134690	JX134732	JX134704
O. dolichostomum	CBS 114926	rotten wood	China	JX134677	JX134689	JX134731	JX134703
O. commune	YMF1.00980	rotten wood	China	JX134675	JX134687	JX134729	JX134701
Ophiostoma floccosum	AU55-6	*Pinus* sp.	unknown	-	AF234836	-	-
O. stenoceras	AFTOL-ID 1038	unknown	unknown	-	DQ836904	-	-

Table 3.3 Continued

Species	Voucher	substrate	Locality	GenBank accession no. ITS	 LSU	 RPB1	 TEF1
Papulosa amerospora	AFTOL-ID 748	unknown	unknown	-	DQ470950	-	DQ471069
Pseudophialophora eragrostis	RUTTP-CM12m9	*Eragrostis* sp.	USA	KF689648	KF689638	KF689618	KF689628
Pseudopyricularia kyllingae	CBS 133597	*Kyllinga brevifolia*	Japan	KM484876	KM484992	KM485096	-
Pyricularia oryzae	70-15		USA	Genome[b]	Genome[b]	Genome[b]	Genome[b]
Pyricularia grisea	M83	*Digitaria* sp.	USA	JX134671	JX134683	JX134725	JX134697
Togniniella acerosa	CBS 113648	*Decayed wood*	New Zealand	-	AY761076	-	-
Wongia garrettii comb. nov.	DAR76937	*Cynodon dactylon*	Australia	KU850474	-	KU850469	KU850467
Wongia griffinii comb. nov.	DAR80512	*Cynodon dactylon* × *C. transvaalensis*	Australia	KU850473	KU850471	-	-
	BRIP60377	*Cynodon dactylon* × *C. transvaalensis*	Australia	KU850472	KU850470	KU850468	KU850466

AFTOL: Assembling the Fungal Tree of Life; ATCC: American Type Culture Collection, Manassas, Virginia, USA; BRIP: Plant pathology herbarium, Department of Agriculture, Fisheries and Forestry, Queensland, Australia; CBMAI: the Coleção Brasileira de Microrganismos para Ambiente e Indústria, Brazil ; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DAR: Plant Pathology Herbarium, Orange Agriculture Institute, New South Wales, Australia; IMI: International Mycological Institute, CBI-Bioscience, Egham, Bakeham Lane, United Kingdom; HKUCC: Hong Kong University Culture Collection; MUCL: Universite Catholique de Louvain, Louvain-la-Neuve, Belgium; RUTPP = Rutgers Mycological Herbarium, New Jersey, USA; SMBL, BPI: Systematic Botany and Mycology Laboratory, USDA, Beltsville, Maryland, USA; SMH = S.M. Huhndorf collection; YMF: Yunnan Microbiological Fermentation Culture Collection Center, China;

Genome[a]: Joint Genome Institute;

Genome[b]: Broad Institute, USA.

3.2.4 Phylogenetic analysis

Contigs were made from sequence trace files with Sequencher 5.1 (Gene Codes Crop., Ann Arbor, Michigan). The individual genes were aligned separately in MAFFT (available: http://www.ebi.ac.uk/Tools/msa/mafft) (Katoh and Toh, 2008) and viewed using the MEGA6 software package (Tamura et al., 2013). Phylogenetic trees were reconstructed by Maximum likelihood (ML) and Bayesian inference (BI). ML was performed by RAxML v7.2.6 using GTRGAMMA as a model of evolution (Stamatakis, 2006). The analyses were run with a rapid bootstrap analysis (command –f a) using a random starting tree and 1000 maximum likelihood bootstrap replications. BI was done in MrBayes 3.1.2 (Ronquist et al., 2012) with four runs of Markov Chain Monte Carlo (MCMC). Each run consisted of four chains that were analysed with 10 000 000 generations, sampled every 1 000 generations and trees were saved every 5 000 generations then visualized the phylogenetic tree by FigTree (Morariu et al., 2008). The analyses consisted of 35 taxa belonging to seven orders and one family *incertae sedis* in the *Diaporthomycetidae* (*Sordariomycetes*). *Camarops ustulinoides*, a representative of *Boniliales*, was used as the out group.

3.3 Results

3.3.1 Phylogenetic analysis

Phylogenetic trees recovered from ML and BI were congruent and provided strong bootstrap support values (Fig. 3.1). The results showed that *Magnaporthe garrettii* and *Magnaporthe griffinii* formed a separate well-supported clade of uncertain familial placement that sat outside the *Magnaporthales* in the subclass *Diaportheomycetidae. Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis* were shown to be located inside a clade that represented *Magnaporthales* (Fig. 3.1). Based on this analysis and combined with morphological characters, two novel genera and one new species are established as follows.

3.3.2 Taxonomy

Wongia Khemmuk, Geering & R.G. Shivas, **gen. nov. (submitted)**

Etymology: Named after the eminent Australian mycologist and plant pathologist Dr Percy T.W. Wong, who first studied these specimens.

Classification – *Diaportheomycetidae, Sordariomycetes*

Perithecia superficial and immersed, globose, black, mostly single, separated or aggregated, perithecial wall pseudoparenchymatous, external cell much darker. *Asci* unitunicate, numerous, cylindrical, mostly straight, 8-spored, short stalked, tapering but rounded apex and a light refractive, non-amyloid apical ring. *Ascospores* uniseriate, cylindrical to fusiform, straight or slightly curved with rounded ends, with dark brown middle cells and hyaline shorter distal cells. Paraphyses delicate, hyaline, filamentous, septate and shrivelling in mature perithecia. Hyphae brown, straight or flexuous. Appressoria simple and unlobed.

MycoBank: MB 564204

Type species: Magnaporthe garrettii P.T.W. Wong & M.L. Dickinson

Wongia garrettii (P.T.W. Wong & M.L. Dickinson) Khemmuk, Geering & R.G. Shivas, **comb. nov. (submitted)**

≡*Magnaporthe garrettii* P.T.W. Wong & M.L. Dickinson. *Australasian Plant Pathology* 41: 326. 2012.

Description and illustrations: Wong et al., 2012; Fig. 3.2

Type: Australia: South Australia, Adelaide, Colonel Light Gardens Bowling Club, from rotted roots of *Cynodon dactylon* cv. Greenlees, Sep. 2001, *P.T.W. Wong* (DAR 76937 – holotype; ITS sequence GenBank KU850474, TEF sequence GenBank KU850467 and RPB1 sequence GenBank KU850469).

Wongia griffinii (P.T.W. Wong & A.M. Stirling) Khemmuk, Geering & R.G. Shivas, **comb. nov. (submitted)**

MycoBank MB 564205.

≡*Magnaporthe griffinii* P.T.W. Wong & A.M. Stirling. *Australasian Plant Pathology* 41: 327. 2012.

Description and illustrations: Wong et al., 2012; Fig. 3.2

Type: Australia, Queensland, Hyatt Coolum Golf Club, from rotted roots of *Cynodon dactylon* × *Cynodon transvaalensis*, Mar. 2008, *M. Whatman* (DAR 80512– holotype; ITS sequence GenBank KU850473, LSU sequence GenBank KU850471).

Additional specimens examined: Australia, South Australia, Adelaide, from rotted roots of *Cynodon dactylon* × *Cynodon transvaalensis*, Jan. 2000, *A.M. Stirling* (BRIP 60377; ITS sequence GenBank KU850472, LSU sequence GenBank KU850470, TEF sequence GenBank KU850466, RPB1 sequence GenBank KU850468); Adelaide, from rotted roots of *Cynodon dactylon* × *C. transvaalensis*, Dec. 2010, *P. Toy* (BRIP 60379). New South Wales, Cobitty, from rotted root of *Cynodon dactylon*, 19 Apr. 2013, *G. Beehag* (BRIP 60378).

Notes — The long perithecial necks of *Wongia garrettii* differentiate it from *Wongia griffinii*. Further, *Wongia griffinii* has longer and wider ascospores (24–35 x 6–9 µm) than *Wongia garrettii* (19–25 x 5–7 µm) (Wong et al., 2012). *Wongia garrettii* normally has 3-septate, rarely 4–7 septate ascospores.

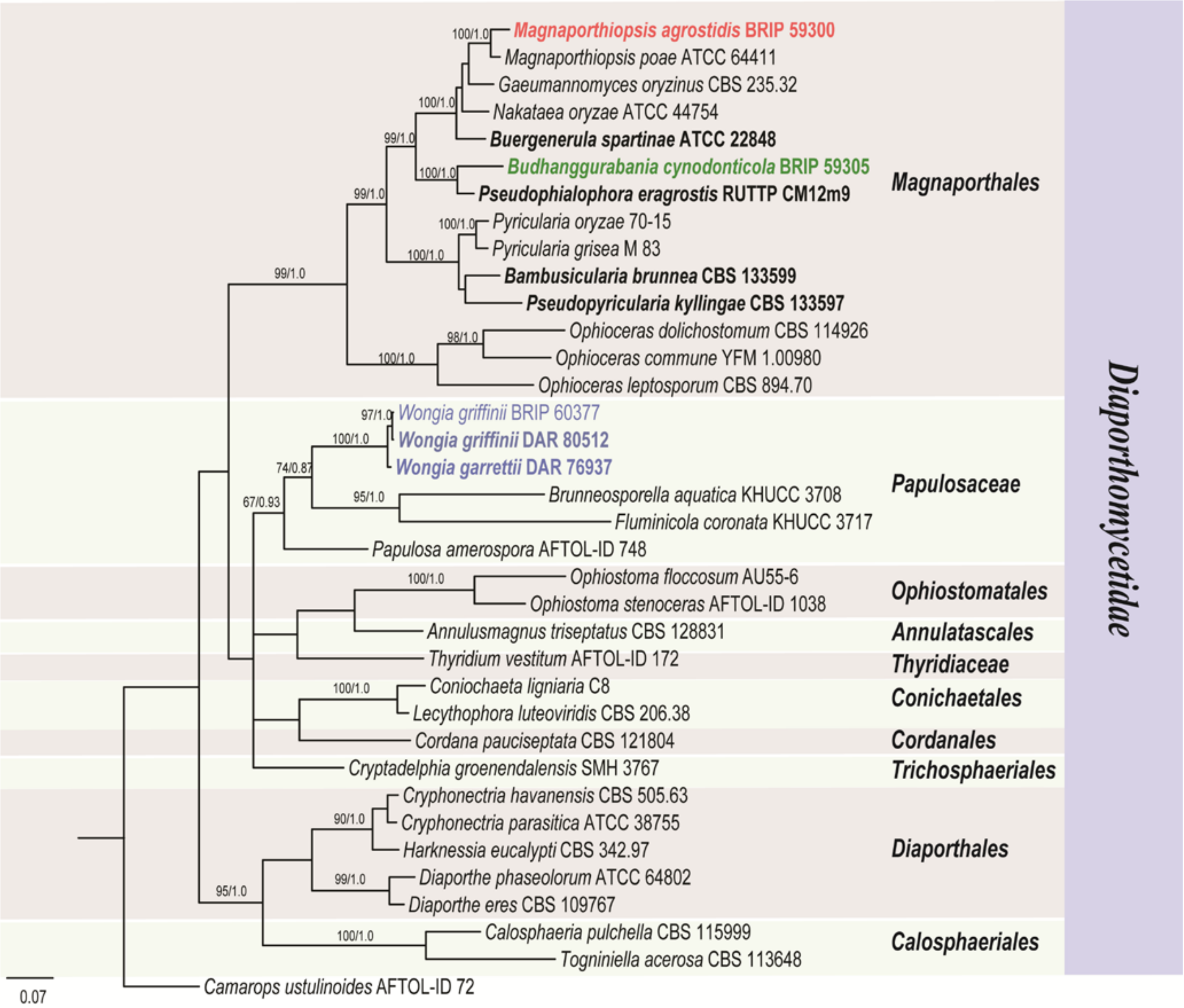

Figure 3.1 Multilocus phylogenetic tree inferred using maximum likelihood methods, as implemented with RAxML v7.2.6 using GTRGAMMA as a model of evolution. Bootstrap support values are indicated at the nodes. The scale bar indicates the expected changes per site. Ex-type species are in **bold**.

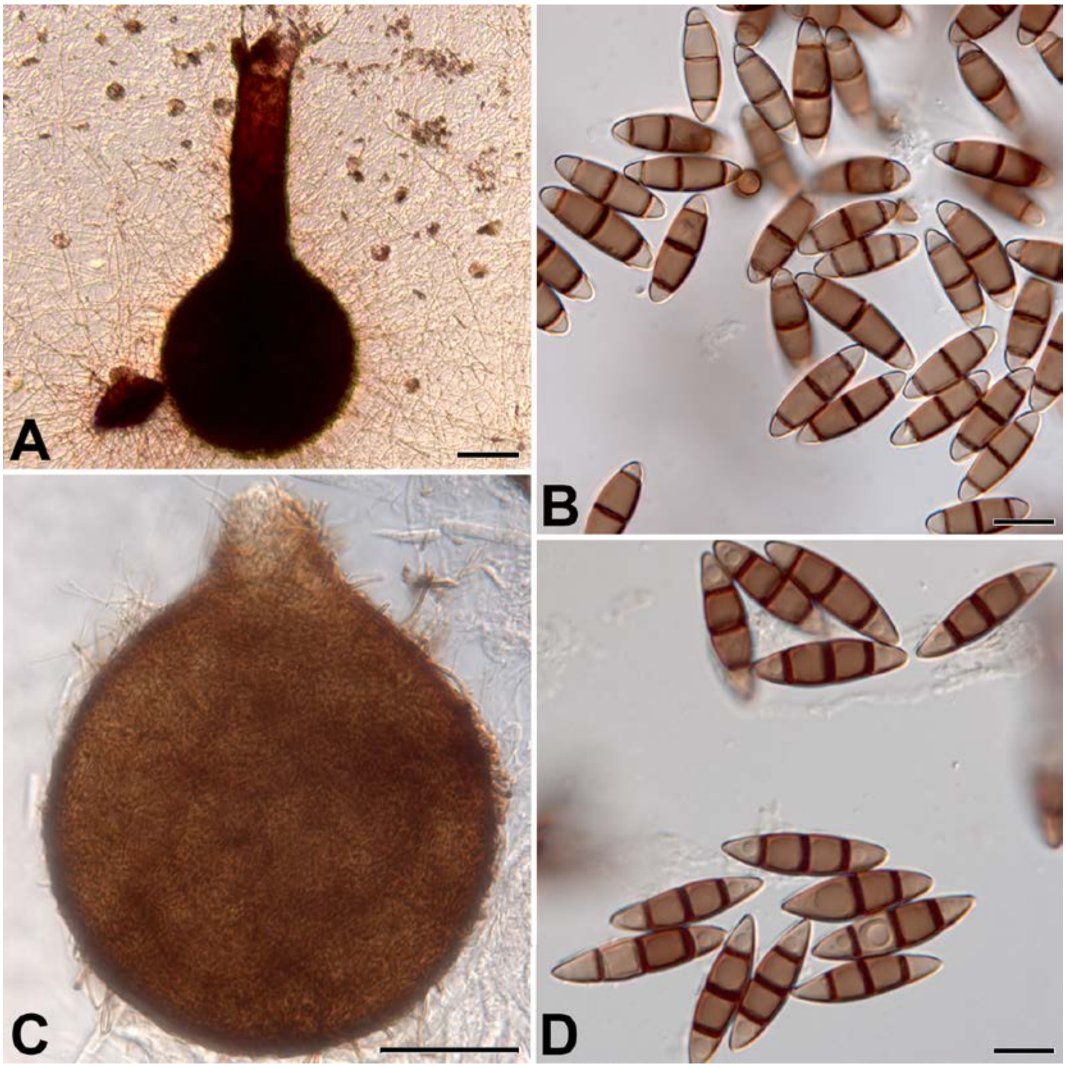

Figure 3.2 *Wongia*, A. B. Perithecium and ascospores of *Wongia garrettii* (DAR 76937) C. D. Perithecium and ascospores of *Wongia griffinii* (BRIP 60378). Scale bars; (A, C) = 100 µm; (B, D) = 10 µm (Reproduced from Khemmuk et al., 2016)

Budhanggurabania P.T.W. Wong, Khemmuk & R.G. Shivas, *Persoonia* 34: 241. 2015.

Etymology: Name derived from the combination of *budhang* and *guraban*, meaning black and fungus, respectively, in the traditional language of Wiradjuri people of Australia, on whose country the fungus was first discovered.

Classification – *Magnaporthaceae, Magnaporthales, Sordariomycetes.*

Mycelium hyaline, becoming dark grey to black with age, hyphae septate, branched, smooth. *Conidiophores* hyaline, single or branched. *Conidiogenous cells* hyaline, phialidic, straight to slightly curved, narrowed at the base. *Conidia* hyaline, aggregated in slimy heads, cylindrical or slightly curved, apex rounded, base acute, aseptate, hyaline, smooth. *Ascomata* on infected stolons, rhizomes and roots, ostiolate perithecia, ampulliform, includes a short apical neck, periphysate, dark brown to black, superficial, solitary. *Asci* obovoid to saccate, 8-spored, ascus wall deliquescent at maturity within the ascomata releasing the ascospores that extrude from the ostiole. *Ascospores* multi-seriate, ellipsoidal with rounded ends, 3 dark brown septa, slightly constricted at each of the septa; central cells brown, with 4–6 oblique striations in lateral view; apical cells subhyaline to pale brown, smooth.

MycoBank: MB 811696

Type species. Budhanggurabania cynodonticola

Figure 3.3 (a) Conidiophore and conidia from ex-holotype culture of *Budhanggurabania cynodonticola* (BRIP 59305), (b) ascomata, (c) asci with 8 ascospores from infected roots of *Cynodon dactylon* (d) ascospores of *B. cynodonticola*. Scale bars; (a,c,d) = 10 µm; (b) = 100 µm. (Reproduced from Wong et al., 2015a).

Budhanggurabania cynodonticola P.T.W. Wong, Khemmuk & R.G. Shivas, *Persoonia* 34: 241. 2015

Type: Australia, New South Wales, Deniliquin, Deniliquin Golf Club, from rotted roots of *Cynodon dactylon*, 1 Mar. 2012, *P.T.W. Wong* (holotype BRIP 59305, includes ex-type culture, ITS sequence GenBank KP162134, LSU sequence GenBank KP162140, SSU sequence GenBank KP162130, TEF1 sequence GenBank KP162138, *mcm7* sequence GenBank KP162131 and RPB1 sequence GenBank KP162143, MycoBank MB811697).

Etymology. Named after the host genus, *Cynodon*, from which it was isolated.

Mycelium hyaline becoming dark grey to black with age; hyphae septate, branched, smooth, 1–3 µm wide. *Conidiophores* hyaline, single or branched. *Conidiogenous cells* hyaline, phialidic, straight to slightly curved, 5–29 × 1.5–3 µm, narrowed at the base. *Conidia* hyaline, aggregated in slimy heads, cylindrical or slightly curved, 5.5–9(–12) × 1.5–2 µm, apex rounded, base acute, aseptate, hyaline, smooth. *Ascomata* occasionally formed on agar, present on infected stolons, rhizomes and roots, ostiolate perithecia, ampulliform, 300–400 µm high × 200–350 µm diam, includes a short apical neck 75–100 µm high × 70–80 µm wide, periphysate, dark brown to black, superficial, solitary. *Asci* obovoid to saccate, 50–75 × 25–35 µm, 8-spored, ascus wall deliquescent at maturity within the ascomata releasing the ascospores that extrude from the ostiole. *Ascospores* multi-seriate, ellipsoidal with rounded ends, 25–38 × 10–15 µm, with 3 dark brown septa, slightly constricted at each of the septa, middle septum 3.5–4.5 µm wide, apical and basal septa 1–2.5 µm wide; central cells brown, with 4–6 oblique striations in lateral view; apical cells 3–6 µm long, subhyaline to pale brown, smooth.

Culture characteristics: On quarter-strength potato dextrose agar (QPDA), colonies reaching 3.5–4 cm diam after 1 wk at 25 °C in the dark, mostly adpressed with sparse to moderately abundant gray aerial mycelium, becoming darker with age; reverse dark grey to black, paler at the margin. On PDA, colonies grow more slowly, reaching 3 cm after 1 wk. (Fig 3.3).

Additional material examined: Australia, New South Wales, from rotted roots of *Cynodon dactylon*, 1 Mar. 2012, *P.T.W. Wong* (BRIP 59306, BRIP 59307); Northern Territory, Darwin Bowling Club, from rotted roots and stolons of *Cynodon dactylon*, Feb. 2013, *P.T.W. Wong*, BRIP 61692 (PW13051); Queensland, Townsville Golf Club, from rotted roots and stolons of *Cynodon dactylon*, Oct. 2014, *P.T.W. Wong*, BRIP 61818 (PW14042).

Notes — Phylogenetic analysis places *Budhanggurabania* in the *Magnaporthaceae* close to other genera with similar phialidic conidial states, including *Buergenerula, Gaeumannomyces*, *Magnaporthiopsis* and *Pseudophialophora* (Klaubauf et al., 2014; Luo et al., 2014). *Budhanggurabania* differs from these genera by producing distinctive 3-septate ascospores, with the larger central cells dark brown and striate; and the smaller end cells pale brown and smooth. *Budhanggurabania cynodonticola* was isolated from diseased roots and stolons of *Cynodon dactylon* from bowling greens and golf course fairways and greens with a serious patch disease in New South Wales, Queensland and the Northern Territory. Pathogenicity tests in the glasshouse have shown that *Budhanggurabania cynodonticola* is highly pathogenic to *Cynodon dactylon*, causing extensive root and stolon rotting (Wong et al., 2015a). Ascomata of *Budhanggurabania cynodonticola* are occasionally formed in culture and often occur on diseased roots, stolons and rhizomes of *Cynodon dactylon* when incubated in humid chambers at 20–30 °C in diffuse daylight for several weeks. Ascospores from these ascomata germinate at 25 °C on QPDA + 100 mg of novobiocin/L of medium, to form colonies that produce the asexual state within a few days. The conidia also germinate readily on QPDA or PDA.

Magnaporthiopsis agrostidis P.T.W. Wong, Khemmuk & R.G. Shivas, *Persoonia* 35: 323. 2015

Etymology: Named after the host genus, *Agrostis*, from which this fungus was isolated.

Classification — *Magnaporthaceae*, *Magnaporthales*, *Sordariomycetes*.

MycoBank: MB 814222

Type: Australia, New South Wales, Little Bay, New South Wales Golf Club, from rotted roots of *Agrostis stolonifera*, May 2013, *P.T.W. Wong*, PW13010 (holotype BRIP 59300, includes ex-type culture, ITS sequence GenBank KT364753, LSU sequence GenBank KT364754, RPB1 sequence GenBank KT364755, TEF sequence GenBank KT364756).

Mycelium hyaline, becoming dark grey to dark brown with age; hyphae septate, branched, smooth, 1–4 µm wide, forming mycelial strands and fans at the margins. *Conidiophores* brown, single and terminal or penicillate and integrated. *Conidiogenous cells* brown or slightly pigmented, phialidic, cylindrical to lageniform, 5–20 × 1.5–3 µm, tapering to a conspicuous flared collarette c. 3 µm high × 1.5 µm wide. *Conidia* hyaline, aseptate, smooth, filiform, rounded at the apex and narrowed towards the base, curved to lunate, 4–6 × 1 µm, aggregated in slimy heads. *Ascomata* not observed in culture.

Culture characteristics: On PDA, colonies reaching 7.5 cm diam after 1 wk at 25 °C in the dark; moderately abundant grey aerial mycelium, becoming olivaceous brown with age and forming dark grey to dark brown crust-like mycelial aggregations on the agar surface in older cultures (> 4 wk); reverse dark grey to olivaceous brown, paler at the margin. The crust-like mycelial aggregations were formed more commonly on quarter-strength PDA amended with novobiocin (100 mg/L) (Fig 3.4).

Notes — Phylogenetic analysis placed *Magnaporthiopsis agrostidis* in a monophyletic clade that includes the type species of the genus, *Magnaporthiopsis poae*. All species of

Magnaporthiopsis are root-inhabiting fungi or root-infecting pathogens of grasses (Luo and Zhang, 2013). *Magnaporthiopsis agrostidis* produces harpophora-like conidia (Gams 2000), which differ from other *Magnaporthiopsis* species by being smaller, filiform, and more curved. The conidia of *Magnaporthiopsis agrostidis* did not germinate on PDA and may have a role as spermatia in sexual reproduction (Wong and Walker, 1975). *Magnaporthiopsis agrostidis* produces superficial crust-like mycelial aggregations that become dark grey to dark brown in older cultures, similar to those of *Magnaporthiopsis incrustans* (Landschoot and Jackson 1989). *Magnaporthiopsis agrostidis* was isolated from diseased roots of *Agrostis stolonifera* from a golf green with symptoms of a patch disease. It has been shown to be pathogenic to *Agrostis stolonifera* in glasshouse pathogenicity tests.

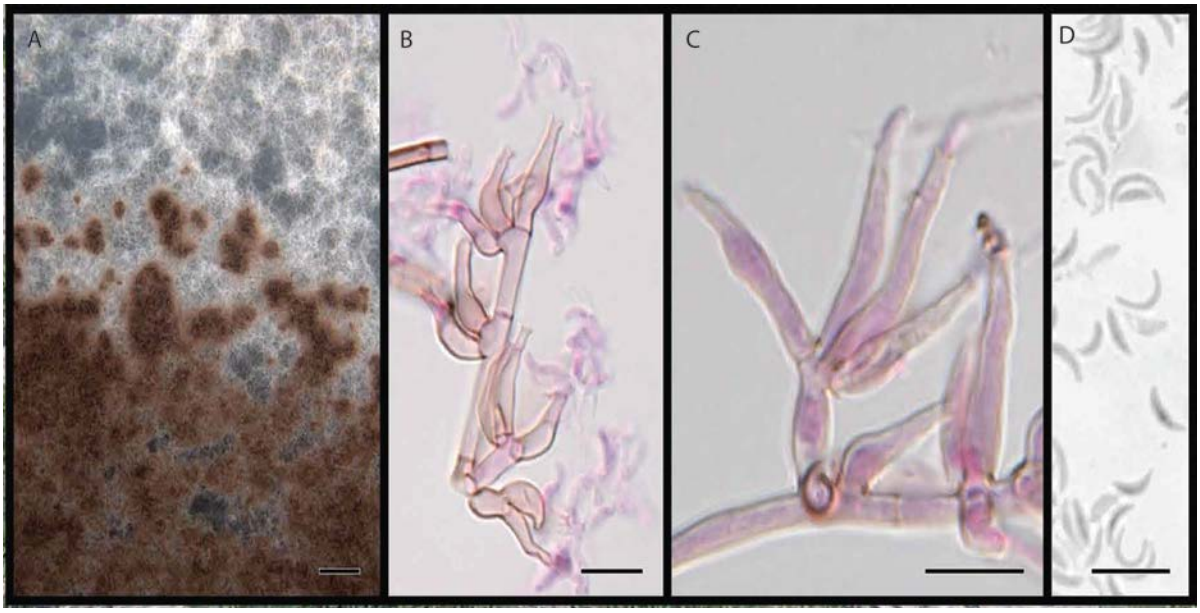

Figure 3.4 (a) Crust-like mycelial aggregations, (b, c, d) conidiophores and conidia from ex-holotype culture of *Magnaporthiopsis agrostidis* (BRIP 59300). Scale bars (a) = 1 mm; (b, c, d) = 10 μm. (Reproduced from Wong et al., 2015b)

3.4 Discussion

This Chapter reports the discovery of two novel Australian genera, *Budhanggurabania* and *Wongia*, as well as two new species in the subclass *Diaportheomycetidae* (*Sordariomycetes*) based on morphology and phylogenetic analysis. The genus *Wongia* accommodates two species, *Wongia garrettii* and *Wongia griffinii*, originally classified in *Magnaporthe* (Wong et al., 2012). *Wongia griffinii* was shown to have uncertain ordinal placement on the *Sordariomycetes* based on ITS analysis (Klaubauf et al., 2014 as *Magnaporthe griffinii*). In this Chapter, DNA from the holotypes of *Magnaporthe garrettii* and *Magnaporthe griffinii* was used to resolve the classification of these fungi. Multigene analyses confirmed that these two species were sister and clustered in *Papulosaceae*, the uncertain family in *Diaportheomycetidae*, *Sordariomycetes* (Maharachchikumbura et al., 2015). The two species of *Wongia* were sister to taxa in the *Papulosaceae* with moderate statistical support (Fig. 3). Morphologically, *Wongia garrettii* and *Wongia griffinii* both have non-amyloid apical rings in the asci, which differentiate them from the three genera in the *Papulosaceae*, namely *Brunneosporella* (Ranghoo et al. 2001), *Fluminicola* (Wong et al., 1999) and *Papulosa* (Kohlmeyer and Volkmann-Kohlmeyer, 1993) which all have amyloid apical rings. An anamorphic state has not been found in nature or in cultures of either *Wongia garrettii* and *Wongia griffinii* grown on artificial media in the laboratory (Wong et al., 2012). Morphology has been shown to be an unreliable character for classifying members of the *Magnaporthaceae* (Luo and Zhang, 2013; Klaubauf et al., 2014).

The phylogenetic analyses of *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis* presented in this Chapter indicated that both species belong to the family *Magnaporthaceae*. The former species shared several morphology characteristics with *Pseudophialophora* such as phialidic conidia and relatively slow growth. The sexual morph was also examined for *Budhanggurabania cynodonticola* and found to have distinctive 3-septate ascospores with the larger central cells dark brown and striate unlike any other known fungal genera (Wong et al., 2015a). *Budhanggurabania cynodonticola* and *Pseudophialophora* are poorly differentiated by morphology but highly divergent based on molecular phylogenetic analysis (Wong et al., 2015a). A root pathogen of *Agrostis stolonifera*, *Magnaporthiopsis agrostidis*, was reported as a new species base on DNA sequence analysis (Wong et al., 2015b). Morphologically, the colonies of *Magnaporthiopsis agrostidis* are similar to those of *Magnaporthiopsis incrustans* on PDA (Landschoot and

Jackson 1989) but differs from others species by having smaller, filiform, and more curved of harpophora-like conidia (Gams, 2000).

Acknowledgements The authors would like to acknowledge the support of the Australian Government's Cooperative Research Centres Program (Project No. PBCRC62082). We would also like to thank Dr. Percy T.W. Wong for his helpful advice and providing specimens. Dr Chris Anderson (New South Wales Plant Pathology Herbarium, DAR) is thanked for the loan of the holotype specimens of *Magnaporthe garrettii* and *Magnaporthe griffinii*.

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Chapter 4 Fungi associated with foliar diseases of wild and cultivated rice (*Oryza* spp.) in northern Queensland

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Published in *Australasian Plant Pathology*

Khemmuk, W., Roger G. Shivas, Robert J. Henry and Andrew D.W. Geering. 2016. Fungi associated with foliar disease of wild rice and cultivated rice (*Oryza* spp.) in northern Queensland. *Australasian Plant Pathology* **45**, 297-308. doi:10.1007/s13313-016-0418-3

Abstract

Surveys for fungi associated with foliar diseases of wild and cultivated rice in northern Queensland in 2014 and 2015, yielded a total of 109 isolates. The isolates were identified by morphology of conidial characters as belonging to *Bipolaris, Colletotrichum*, *Curvularia*, *Nigrospora*, *Phoma*, *Pestalotiopsis* and *Pyricularia*. Isolates of *Pyricularia*, *Bipolaris* and *Curvularia* were identified to species level by multigene analyses of the internal transcribed spacer (ITS), the largest subunit of RNA polymerase II (*RPB1*), translation elongation factor 1-alpha (*TEF1*) and glyceraldehyde-3-phosphate dehydrogenase (*GPDH*). The most commonly identified fungus was *Bipolaris oryzae*, which was associated with brown spot disease. *Bipolaris oryzae* accounted for 16.5 % of the isolates and was found on *Oryza australiensis*, *Oryza rufipogon*-like and *Oryza rufipogon meridionalis*-like. The next most frequently identified fungus was *Pyricularia oryzae*, which accounted for 10.1 % of the isolates and was found on *Oryza sativa*, *Oryza* Taxon A and *Oryza* Taxon B. Species of *Curvularia* made up 7.3 % of the isolates, including *Curvularia aeria, Curvularia alcornii*, *Curvularia asianensis, Curvularia clavata, Curvularia lunata*, *Curvularia muehlenbeckiae* and an unidentified species. To remove uncertainty of the host plants from which the fungi were isolated, a DNA barcoding strategy was developed using six loci in the chloroplast genome for plant identification.

4.1 Introduction

The genus *Oryza* includes 21 wild species and two cultivated species. The domesticated rice, *Oryza sativa*, is cultivated throughout tropical and subtropical regions, while *Oryza glaberrima* is restricted to western Africa, (Londo et al., 2006). The genus *Oryza* comprises six diploid genomes, AA, BB, CC, DD, EE and GG, as well as four allotetraploid genomes, BBCC, CCDD, HHKK and HHJJ (Khush, 1997; Lu et al., 2009). The AA genome group contains cultivated rice and wild species, and some of its closest relatives are endemic to Australia. The wild progenitors provide a broad gene pool of potentially useful genetic resources for improved rice cultivars (Hajjar and Hodgkin, 2007).

In Australia, the largest rice growing area is located between the Murray and Murrumbidgee River in southern New South Wales (RIRDC, 2011). However, the greatest diversity of wild rices is found in northern Australia. Four native *Oryza* species were reported, *Oryza meridionalis* N.Q.Ng, *Oryza rufipogon* Griff., *Oryza australiensis* Domin, and *Oryza* *officinalis* Wall. ex G.Watt (Henry et al., 2010). *Oryza meridionalis* is an annual species that grows in wet areas and survives as seed in the dry season (Henry et al., 2010). *Oryza rufipogon* is a native perennial found in wet or swamp areas, recent molecular evidence confirms that Australian species is genetically distinct from Asian population (Waters et al., 2012). Moreover, Sotowa et al., (2013) described two undescribed taxa of Australian perennial wild rice were informally classified as wild rice "Taxon A" and "Taxon B" which corresponded to populations of perennial *Oryza rufipogon*-like (r-type) and *Oryza meridionalis*-like (m-type), respectively. Both species are primarily distinguished by anther size and grow in close proximity to each other (Bronzska et al., 2014). *Oryza officinalis* and *Oryza australiensis* are representatives of CC and EE genome types respectively. The first species has been recorded only twice, from Moa Island in the Torres Strait and from eastern Arnhem Land (Henry et al., 2010). The remaining wild rice species are likely to be broadly distributed across northern Australia, but with slightly different habitat preferences. Wild species represent virtually untapped gene pools for the improvement of domesticated rice. The two wild rice species, *Oryza officinalis* and *Oryza australiensis*, have provided resistance genes for *Pyricularia oryzae* (Jeung et al., 2007). Further, *Oryza meridionalis* and *Oryza australiensis* have been suggested as sources of drought tolerance (Sundaramoorthi et al., 2009). *Oryza rufipogon* has been used as a source of rice tungro disease and tolerance to acid sulfate and aluminum in soil (Brar and Khush, 1997; Nguyen et al., 2003; Ram et al., 2007).

The physical separation of cultivated and wild rice populations in Australia means that wild rice populations are unlikely to have become contaminated by genes from domesticated rice. Consequently wild rice populations offer a potential reservoir of resistance genes to pests and diseases which could be exploited for the improvement of cultivated rice (Henry et al., 2010; Water et al., 2012). In Queensland and north Western Australia, there have been renewed efforts to cultivate rice in anticipation of future water shortages in the Riverina region of NSW (Cockfield et al., 2012). The relocation of the rice industry to northern Australia raises the prospect of crossover of pathogens between wild and cultivated species of rices. Among the foliar fungal pathogens that have been reported from cultivated rice in northern Australia, *Pyricularia oryzae*, the cause of rice blast disease, is of greatest concern to the industry (You et al., 2012).

The research presented in this chapter aimed to determine the diversity of fungi associated with foliar diseases on wild rice in northern Queensland. Fungi were identified using morphological characters and DNA barcoding methods. Four loci of genomic DNA were targeted for amplification; the internal transcribed spacer (ITS), the largest subunit of RNA polymerase II (*RPB1*), translation elongation factor 1-alpha (*TEF1*) and glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) genes. During the disease surveys of wild and cultivated rice conducted in 2014 and 2015, host identification became a problem due to the lack of appropriate morphological keys. Consequently, a DNA barcoding strategy for identification was devised. Six genes of the chloroplast genome were targeted for host identification including maturase K (*matK*), intergenic region (*interG*)-cytochrome b6/f complex subunit VIII (petN), DNA-directed RNA polymerase subunit β (*rpoC2*), ATP synthase subunit α–30S ribosomal protein S14 (*rps14*), 50S ribosomal protein L14–50S ribosomal protein L16 (*rpl16*) and photosystem II reaction center protein Z–tRNA-Gly (*psbZ*).

4.2 Materials and methods

4.2.1 Fungal and host specimens

Leaf spots of wild rice were collected during two surveys in northern Queensland during 12–14 May 2014 and 27–29 March 2015. Thirteen sites were surveyed from the Atherton Tableland in the south to Rinyirru National park in the north (Fig 4.1). Sites were chosen based on known and easily accessible locations for wild rice as determined by recent surveys in northern Queensland (Brozynska et al., 2014). Samples of leaves with disease symptoms, together with whole plants for host determination, were collected. For comparative purposes, three cultivated rice crops (cv. Doongara) growing on the Atherton Tablelands were also surveyed on 15 May 2014.

Leaf lesions (0.5–1.0 cm) were surface-sterilised with 70 % ethanol for 1 min, rinsed twice in sterile water and plated onto half strength potato dextrose agar (½PDA) in 90 mm. petri dishes. The plates were incubated at 25 °C for 5–7 d before the most occurring fungi were subcultured onto potato dextrose agar (PDA). Pure cultures of the fungi were incubated at the same conditions for 7–14 d before identification (Beales, 2012).

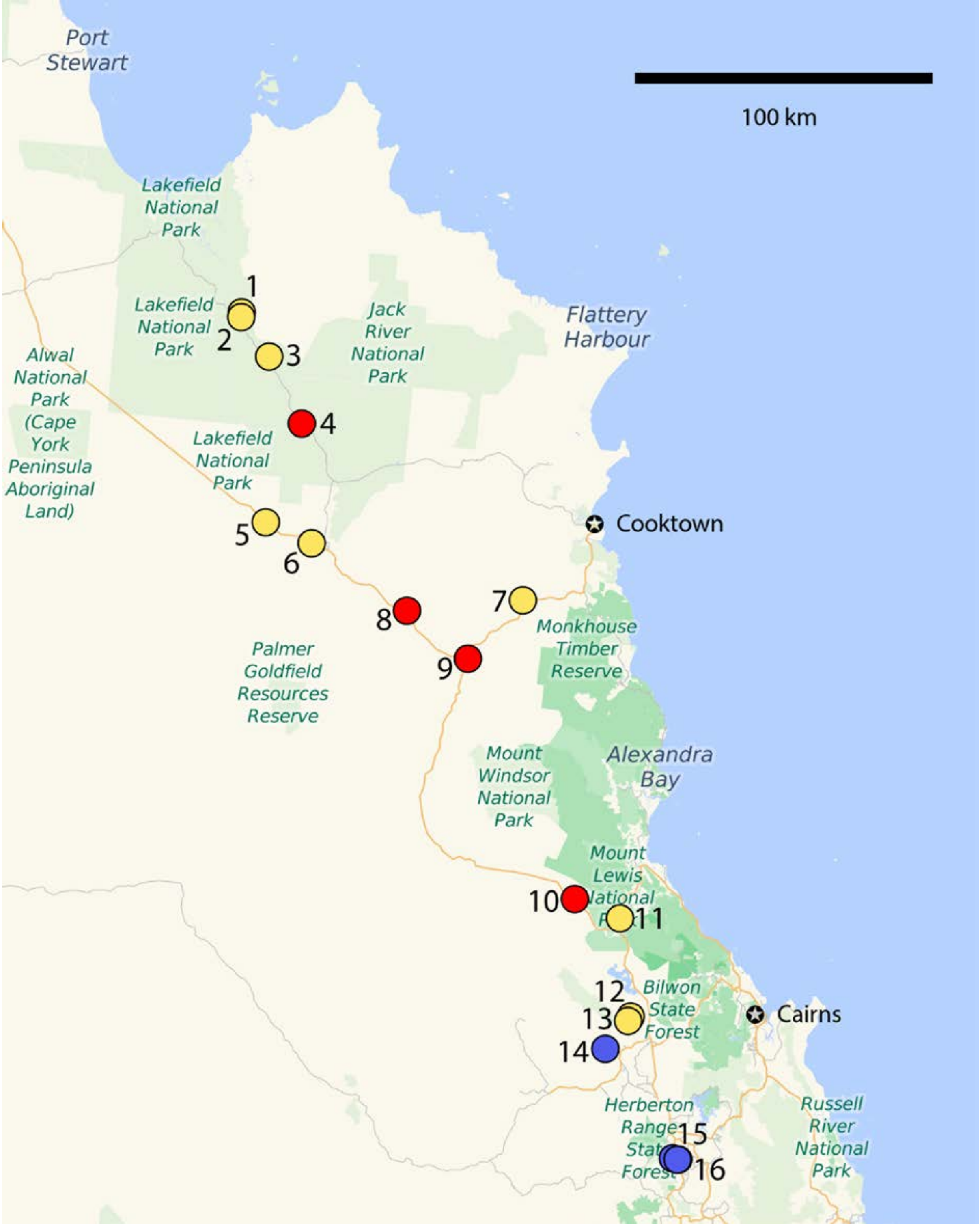

Figure 4.1 Locations of specimens collecting in northern Queensland during 2014-2015, where red, yellow and blue circles represent *Oryza australiensis*, undescribed *Oryza* AA genome taxa and *Oryza sativa*, respectively.

4.2.2 Fungal identification

4.2.2.1 Morphological examination

Fungal isolates were obtained from the lesions following surface sterilization with 70% ethanol, plating onto half strength potato dextrose agar (½PDA) and incubating at 24 °C for 5 d. Morphological characters including colony appearance on ½PDA and conidial shape were observed. Fungal conidia were mounted on glass slides in 100 % lactic acid and gently heated to boiling before microscopic examination. Observations were made with a Leica DFC 500, images were captured with camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

4.2.2.2 Molecular examination

Fungal DNA extraction and PCR amplification

Fungal DNA was extracted from fresh mycelium following the method in chapter 2. The primer pairs ITS1/ITS4 (White et al., 1990); RPB-Ac/RPB-Cr (Castlebury et al., 2004; Matheny et al., 2002), EF1983F/2218R (Schoch et al., 2009) and gpd1/gpd2 (Berbee et al., 1999) were used to amplify ITS, *RPB1*, *TEF1* and *GPDH* genes, respectively. PCR amplifications were done in 20 µl final reaction volume with 10 µl of high fidelity Phusion DNA Polymerase (Biolabs), forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl and distill water 7 µl. Thermal cycling conditions for the ITS, *TEF1* and *GPDH* loci were as provided by Deng et al., (2015). For the *RPB1* locus, thermal cycling parameters were: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10s, 59 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min. PCR products were sent to Macrogen, South Korea for direct sequencing using the amplification primers.

Plant DNA extraction and PCR amplification

DNA was extracted from dried plant tissue with a Power Plant® DNA Isolation Kit (MO BIO Laboratories, Inc.) with minor modification to manufacturer's protocol. An additional 0.02 g of glass beads (0.5 mm diameter) and proteinase K enzyme to a final concentration of 0.8 µg/ µL were added to the supplied extraction buffer then incubated at 65°C at least 4 hours before mechanical lysis. The primers pairs matK-F1/R1, psbZ-F1/tRNAGly-R1, InterG-F1/R1, rpoC2-F1/R1, atpA-F1/ rps14-R1 and rpl14-F1/rpl16-R1 were used to amplified *matK*, *psbZ*, *InterG*, *rpoC2*, *rps14* and *rpl16* respectively. PCR amplification was performed in 20 µl reactionwith 10 µl of high fidelity Phusion® DNA Polymerase (New England Biolabs), forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl and distill water 7 µl,using the manufacturer-specified cycling and conditions. Thermal cycling conditions were as follows: 98 ºC for 30 s, followed by 30 cycles of 98 ºC for 10 s, 61–65 ºC (Table 1) for 30 s and 72 ºC for 1 min, with a final extension of 72 ºC for 10 min. PCR products were sent to Macrogen, South Korea, for direct sequencing using the amplification primers. Primers used in this study and PCR conditions are showed in Table 4.1.

Table 4.1 PCR primers used for *Oryza* DNA barcoding

Primer name	**Sequence**	**Position**[a]	**Anneal. temp.**	**Frag.length (bp)**
matK-F1	TAATTAAGAGGATTCACCAG	1669-1688	61.0	1628
matK-R1	ATGCAACACCCTGTTCTGAC	3277-3296		
psbZ-F1	TTGTATTTGCTTCTCCTGATG	12012-12032	63.5	1447
tRNAGly-R1	AAGGATCTATGGATGAAGATACA	c13436-13458		
InterG-F1	CACTGCATCCACTTAATTTC	17012-17031	65.0	614
petN-R1	GGCTGCTTTAATGGTAGTCT	c17606-17625		
rpoC2-F1	GTTCGCCATTCCATTACAGT	26459-26478	64.0	1011
rpoC2-R1	TCATACGCGACTGCAGGT	c27452-27469		
atpA-F1	CTAGCAAGACATTCACCGA	35644-35662	63.5	773
rps14-R1	CGGAAGACCTAGAGCTAACTATC	c36394-36416		
rpl14-F1	CGTCTTCGCATTTGAACT	77410-77427	63.5	589
rpl16-R1	GAGCAATGACACGATATGC	c77980-77998		

[a] Position on the *Oryza sativa* cv. Nipponbare chloroplast genome, GenBank accession GU592207.1.

Sequence alignments and phylogenetic analyses

Sequences were edited and assembled using Sequencher v. 5.1 for Windows (Gene Codes Corp., Ann Arbor, USA). The assembled consensus sequences were aligned with MAFFT using default setting (www.ebi.ac.uk/Tools/msa/mafft) and manually adjusted where necessary (Katoh and Standley, 2013). Individual loci were aligned separately, and then the alignments concatenated for the phylogenetic analyses. Combined ITS and *RPB1* sequence datasets were used to identify the *Pyricularia* species while ITS, *GPDH* and *TEF1* sequence datasets to identify *Bipolaris* and *Curvularia* species (Manamgoda et al., 2014; Deng et al., 2015). Sequences and outgroup used in this study are shown in Table 4.2. Maximum-likelihood phylogenetic analyses of DNA sequence data were done with RAxML v7.2.6 (Stamatakis, 2006). For the analyses, the concatenated alignments were analysed as partitioned datasets, using the GTRGAMMA model of evolution with rapid bootstrapping (command –f a) using a random starting tree and 1,000 replicates. Bayesian phylogenetic inference was performed with MrBayes v3.1.2 to conduct a Markov Chain Monte Carlo (MCMC) (Ronquistet al., 2012).

Table 4.2 *Oryza* specimens used in this study and associated GenBank numbers

Species	**BRIP Acc. No.**	**Location**	**GenBank accession no.**					
			MatK	**rpoC2**	**InterG**	**rps14**	**rpl16**	**psbZ**
Oryza australiensis (EE)	61669	Mary Creek, Mulligan Hwy, Qld	KU923970	KU923886	KU923942	KU923833	KU923913	KU923861
	61670	Mary Creek, Mulligan Hwy, Qld	KU923971	KU923887	KU923943	KU923834	KU923914	KU923862
	61679	12 Mile Lagoon Rd,Laura, Qld	KU923979	KU923894	KU923951	KU923842	KU923922	KU923869
	61674	Laura River Crossing, Qld	KU923975	KU923890	KU923947	KU923838	KU923918	KU923865
	61675	Laura River Crossing, Qld	KU923976	KU923891	KU923948	KU923839	KU923919	KU923866
	61677	Laura River Crossing, Qld	KU923977	KU923892	KU923949	KU923840	KU923920	KU923867
	62888	Lakeland Downs, Qld	KU923997	KU923911	KU923968	KU923859	KU923940	KU923885
Oryza spp. (AA)	61667	Abattoir Swamp, Qld	KU923969	-	KU923941	KU923832	KU923912	KU923860
	61684	7.5 km NW of Laura, Qld	KU923984	KU923899	KU923956	KU923847	KU923927	KU923874
	61686	Clancy’s Lagoon, Mareeba, Qld	KU923985	KU923900	KU923957	KU923848	KU923928	KU923875
	62876	Abattoir Swamp, Qld	KU923991	KU923906	KU923963	KU923854	KU923934	KU923881
	62877	Abattoir Swamp, Qld	KU923992	KU923907	KU923964	KU923855	KU923935	-
	62879	Abattoir Swamp, Qld	KU923993	KU923908	KU923965	KU923856	KU923936	KU923882

	62880	Lakeland Downs, Qld	KU923994	KU923909	KU923966	KU923857	KU923937	KU923883
	61673	Rinyirru National Park, Qld	KU923974	KU923889	KU923946	KU923837	KU923917	KU923864
	61681	Sandy Creek, Laura, Qld	KU923981	KU923896	KU923953	KU923844	KU923924	KU923871
	61682	Red Lily Lagoon, Rinyirru, Qld	KU923982	KU923897	KU923954	KU923845	KU923925	KU923872
	61683	Red Lily Lagoon, Rinyirru, Qld	KU923983	KU923898	KU923955	KU923846	KU923926	KU923873
	61687	Pandanus Lagoon, Mareeba, Qld	KU923986	KU923901	KU923958	KU923849	KU923929	KU923876
	62881	Lakeland Downs, Qld	KU923995	KU923910	KU923967	KU923858	KU923938	KU923884
	61671	Rinyirru National Park, Qld	KU923972	-	KU923944	KU923835	KU923915	KU923863
	61672	Rinyirru National Park, Qld	KU923973	KU923888	KU923945	KU923836	KU923916	-
	61678	White Lily Lagoon, Rinyirru, Qld	KU923978	-	KU923950	KU923841	KU923921	KU923868
	61680	25 km NW of Laura, Qld	KU923980	KU923895	KU923952	KU923843	KU923923	KU923870
O. sativa var. Doongara	61688	Arriga, Qld	KU923987	KU923902	KU923959	KU923850	KU923930	KU923877
	61689	East Barron Rd, Atherton, Qld	KU923988	KU923903	KU923960	KU923851	KU923931	KU923878
	61690	East Barron Rd, Atherton, Qld	KU923989	KU923904	KU923961	KU923852	KU923932	KU923879
	61691	Mareeba, Qld	KU923990	KU923905	KU923962	KU923853	KU923933	KU923880

BRIP: Plant pathology herbarium, Department of Agriculture, Fisheries and Forestry, Queensland, Australia

Table 4.3 Fungi, hosts, isolates and GenBank accession numbers used in the phylogenetic analyses (ex-type cultures are in bold).

Species	Host	Locality	Accession no.	GenBank accession no.			
				ITS	**RPB1**	**GPDH**	**TEF**
Alternaria alternata	Unknown	India	**EGS 34.0160**	AF071346	-	AF081400	-
Barretomyces calatheae	*Calathea longifolia*	Brazil	**CBMAI 1060**	GU294490	-	-	-
Bipolaris chloridis	*Chloris gayana*	Australia	**CBS 242.77**	JN192372	-	JN600961	-
B. clavata	*Dactyloctenium radulan*	Australia	BRIP 12530	KJ415524	-	KJ415422	KJ415471
B. microlaenae	*Microlaena stipoides*	Australia	**BRIP 15613**	JN601032	-	JN600974	JN601017
B. oryzae	*Oryza sativa*	Thailand	**MFLUCC 10-0715**	JX256416	-	JX276430	JX266585
	Oryza sp. Taxon A	Australia	BRIP 61667	KU552185	-	KU552175	KU552150
	O. australiensis	Australia	BRIP 61669b	KU552186	-	KU552169	KU552144
	Oryza sp. Taxon B	Australia	BRIP 61673b	KU552187	-	KU552170	KU552145
	O. australiensis	Australia	BRIP 61674	KU552188	-	-	-
	O. australiensis	Australia	BRIP 61675	KU552189	-	KU552171	KU552146
	O. australiensis	Australia	BRIP 61677	KU552190	-	-	-
	Oryza sp. Taxon B	Australia	BRIP 61678	KU552191	-	-	-
	O. australiensis	Australia	BRIP 61679a	KU552192	-	KU552172	KU552147

	Oryza sp. Taxon B	Australia	BRIP 61681	KU552193	-	KU552173	KU55214
	Oryza sp. Taxon B	Australia	BRIP 61682	KU552194	-	-	-
	Oryza sp. Taxon A	Australia	BRIP 61684b	KU552195	-	KU552177	KU552152
	grass	Australia	BRIP 61685b	KU552196	-	KU552178	KU552153
	Oryza sp. Taxon A	Australia	BRIP 61686a	KU552197	-	KU552179	KU552154
	Oryza sp. Taxon A	Australia	BRIP 62876	KU552198	-	KU552176	KU552149
B. panici-miliacei	*Panicum miliaceum*	Japan	**CBS 199.29**	KJ909773	-	KM042896	KM093788
B.peregianensis	*Cynodon dactylon*	Australia	**BRIP 12790**	JN601034	-	JN600977	JN601022
Curvularia alcornii	*Zea mays*	Thailand	**MFLUCC 10-0703**	JX256420	-	JX276433	JX266589
	Oryza sp. Taxon B	Australia	BRIP 61672a	KU552202	-	KU552164	KU552157
C. aeria	Air	Brazil	**CBS 294.61**	NR130689	-	-	-
	Oryza sativa	Australia	BRIP 61232b	KU552200	-	KU552162	KU552155
C. asianensis	*Panicum* sp.	Thailand	**MFLUCC 10-0711**	JX256424	-	JX276436	JX266593
	Oryza sativa	Australia	BRIP 61689	KU552206	-	-	KU552160
C. australiensis	*Oryza sativa*	Australia	**IMI 53994**	KC424595		KC747744	KC503939

C. clavata	Unknown	Canada	DAOM 148084	AF071336		AF081391	
	Oryza sp. Taxon B	Australia	BRIP 61672b	KU552203	-	KU552165	KU552158
	Oryza sp. Taxon B	Australia	BRIP 61680	KU552205	-	KU552167	KU552159
C. coicis	*Coix lacryma*	Japan	**CBS 192.29**	AF081447	-	AF081410	JN601006
C. ellisii	Air	Pakistan	**CBS 193.62**	JN192375	-	JN600963	JN601007
C. graminicola	unknown	Australia	**BRIP 23186**	JN192376	-	JN600964	JN601008
C. heteropogonis	*Heteropogon contortus*	Australia	**CBS 284.91**	JN192379	-	JN600969	JN601013
C. hominis	Cornea	USA	**UTHSC09-464**	HG779011	-	HG779106	-
C. lunata	human lung biopsy	USA	**CBS 730.96**	JX256429	-	JX276441	JX266596
	Oryza sp. Taxon A	Australia	BRIP 62880a	KU552207			KU552161
C. muehlenbeckiae	*Muehlenbeckia* sp.	India	**CBS 144.63**	HG779002	-	HG779108	-
	Oryza sp. Taxon B	Australia	BRIP 61671	KU552201	-	KU552163	KU552156
C. ovariicola	*Eragrostis interrupta*	Australia	**CBS 470.90**	JN192384	-	JN600976	JN601020
C. ravenelii	*Sporobolus fertilis*	Australia	**BRIP 13165**	JN192386	-	JN600978	JN601024
C. tripogonis	unknown	Australia	**BRIP 12375**	JN192388	-	JN600980	JN601025
C. tsudae	*Chloris gayana*	Japan	**ATCC 44764**	KC424596	-	KC747745	KC503940
C. tuberculata	*Zea mays*	India	**CBS 146.63**	JX256433	-	JX276445	JX266599

Curvularia sp.1	*Oryza australiensis*	Australia	BRIP 61674	KU552204	-	KU552166	-
Pyricularia ctenantheicola	*Ctenanthe oppenheimiana*	Greece	**GR0002**	KM484879	KM485099	-	-
P. grisea	*Digitaria* sp.	USA	M82	JX134670	JX134724	-	-
	Digitaria sp.	USA	M83	JX134671	JX134725	-	-
P. oryzae		USA	70-15 = ATCC MYA-4617	Genome	Genome	-	-
	Oryza sp. Taxon B	Australia	BRIP 61678	KU552208	KU552180	-	-
	Oryza sp. Taxon B	Australia	BRIP 61680 a	KU552209	KU552183	-	-
	Oryza sativa	Australia	BRIP 61688 c	KU552210	KU552181	-	-
	Oryza sativa	Australia	BRIP 61689 b	KU552211	KU552182	-	-
	Oryza sativa	Australia	BRIP 61690 a	KU552212	KU552184	-	-
	Oryza sp. Taxon A	Australia	BRIP 62879	KU552213	-	-	-
P. penniseticola	*Pennisetum typhoides*	**Mali**	**ML0031**	KM484929	KM485148	-	-
P. pennisetigena	*Pennisetum* sp.	**Mali**	**ML0036**	KM484935	KM485153	-	-

[a] Abbreviations are: ATCC - American Type Culture Collection, Manassas, Virginia; BRIP, Plant Pathology Herbarium, Brisbane, Queensland, Australia; CBMAI - Coleção Brasileira de Microrganismos de Ambiente e Industria of the UNICAMP, Brazil; CBS - Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, the Netherlands; DAOM - Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada; MFLUCC - Mae Fae Luang University Culture Collection, Chiang Rai, Thailand; Genome: Broad Institute, USA.

4.3 Results

Fungal identification

Fungi were isolated from 31 specimens with symptoms of foliar diseases, of which 27 were from wild rice and four were from rice crops on the Atherton Tablelands. Morphological characters such as colony appearance on PDA and conidial shape were used to triage isolates prior to further molecular examination. Almost half of the fungal isolates were *Nigrospora* species, which are common saprobes or endophytes on grasses (Naik, 2009). *Bipolaris* was the next most frequently isolated genus from the wild rices, and phylogenetic analyses using concatenated ITS-TEF1-GPDH alignments identified all as *Bipolaris oryzae* (Fig. 4.2), the cause of rice brown spot disease throughout the world (Barnwal et al., 2013). Brown spot disease symptoms and associated *Bipolaris oryzae* isolates were present on the wild rice populations at Abattoir Swamp and Clancy's Lagoon but were not observed on rice crops of cv. Doongara grown within a radius of 50–100 km. The third most frequently isolated genus of fungi was *Curvularia* and large species diversity was observed given the relatively small number of specimens (Fig. 4.3). *Curvularia alcornii*, *Curvularia lunata*, *Curvularia clavata* and *Curvularia muehlenbeckiae* were each isolated once from wild *Oryza* AA genome-type plants. *Curvularia aeria* was isolated from a cultivated rice specimen from the Burdekin region, which had suspected brown leaf spot disease and was collected independently of the surveys and submitted as a diagnostic specimen. *Curvularia asianensis* was also isolated from a cultivated rice specimen collected during the survey of the Atherton Tablelands in 2014. Finally, an undescribed *Curvularia* species was isolated from an *Oryza australiensis* plant that was growing near the Laura River crossing on the Peninsula Developmental Road.

Typical blast disease symptoms were rarely observed on the wild rice populations that were surveyed. During May 2014, *Pyricularia oryzae* was found twice (BRIP 61678 and BRIP 61680) on individual *Oryza* AA genome-type plants, one growing at White Lily Lagoon within Rinyirru National Park and the second at an isolated pond north of Laura, which was the JPN2 site of Sotowa et al. (2013). In March 2015, *Pyricularia oryzae* was found at a single location, on an *Oryza* AA genome-type plant (BRIP 62879) growing on the fringe of a dam by the Mulligan Highway between Lakeland Downs and Cooktown (Fig 4.4).

Host identification

The *Oryza* species that are indigenous to Queensland are poorly described and there is no morphological key. Consequently, a DNA barcoding strategy for host identification was devised. An *in silico* analysis was first done using reference chloroplast genome sequences on GenBank to determine the discriminatory power of each locus that was selected for sequencing. MatK, which is one of two recommended DNA barcodes for plants (Hollingsworth et al., 2009), was only useful for differentiating *Oryza australiensis* from the AA genome-type taxa. By contrast, rpl14–rpl16 discriminated each reference chloroplast genome, as well as *Oryza sativa*.

During the surveys, diseased specimens of *Oryza australiensis* and wild *Oryza* AA genome-type plants were collected (Table 4.1). Of the six regions of the chloroplast genome that were sampled by PCR, sequences from the atpA–rps14, psbZ–tRNAGly and rpl14–rpl16 amplicons were most phylogenetically informative and used for classification (Fig. 4.5). All polymorphisms within these amplicons mapped to intergenic regions in the chloroplast genome, except for single non-synonymous substitutions in the rpl16 gene in a small number of *Oryza australiensis* and AA genome-type plants. Among the newly collected plant specimens, sequences from the matK, rpoC2 and interG–petN amplicons were invariate for each *Oryza* genome group, except for a single specimen of *Oryza australiensis* (BRIP 61679), which had a 19 nucleotide deletion in a non-coding region of the interG–petN amplicon relative to other specimens of this species.

Significant chloroplast haplotype diversity was observed for both *Oryza australiensis* and the AA genome-type plants (Fig. 4.5). GenBank accession KF428978.1 from Abattoir Swamp near Julatten was considered the reference sequence for *Oryza* sp. Taxon A (Brozynska et al., 2014) and this chloroplast haplotype was also collected from Clancy’s Lagoon in Mareeba Wetlands Reserve and from an unnamed dam on the Mulligan Highway between Lakeland Downs and Cooktown (Fig. 4.1). During 2014, the wild rice population at the JPN2 site of Sotowa et al. (2013) was surveyed, and the specimen from this site (BRIP 61680) was considered representative of *Oryza* sp. Taxon B. No other specimen had an identical sequence to BRIP 61680, although several from Rinyirru National Park were closely related. Given the lack of information on what constitutes a distinct species the different chloroplast haplotypes were simply labelled using letters of the alphabet.

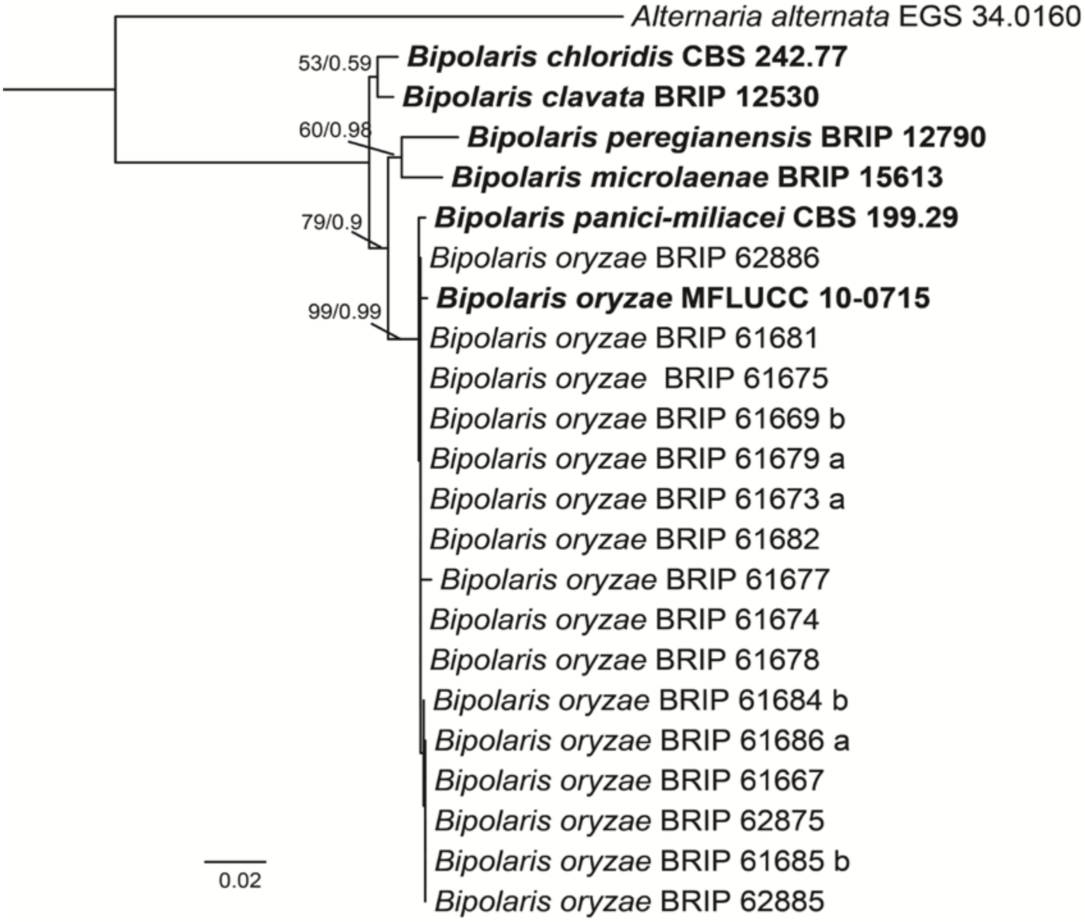

Figure 4.2 Phylogenetic relationships of *Bipolaris* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3- phosphate dehydrogenase (GPDH) genes. Bootstrap values (>70%) from maximum likelihood and posterior probabilities (>0.95) from Bayesian inference are shown on the nodes respectively and ex-type cultures are written in bold. *Alternaria alternata* was included as an outgroup

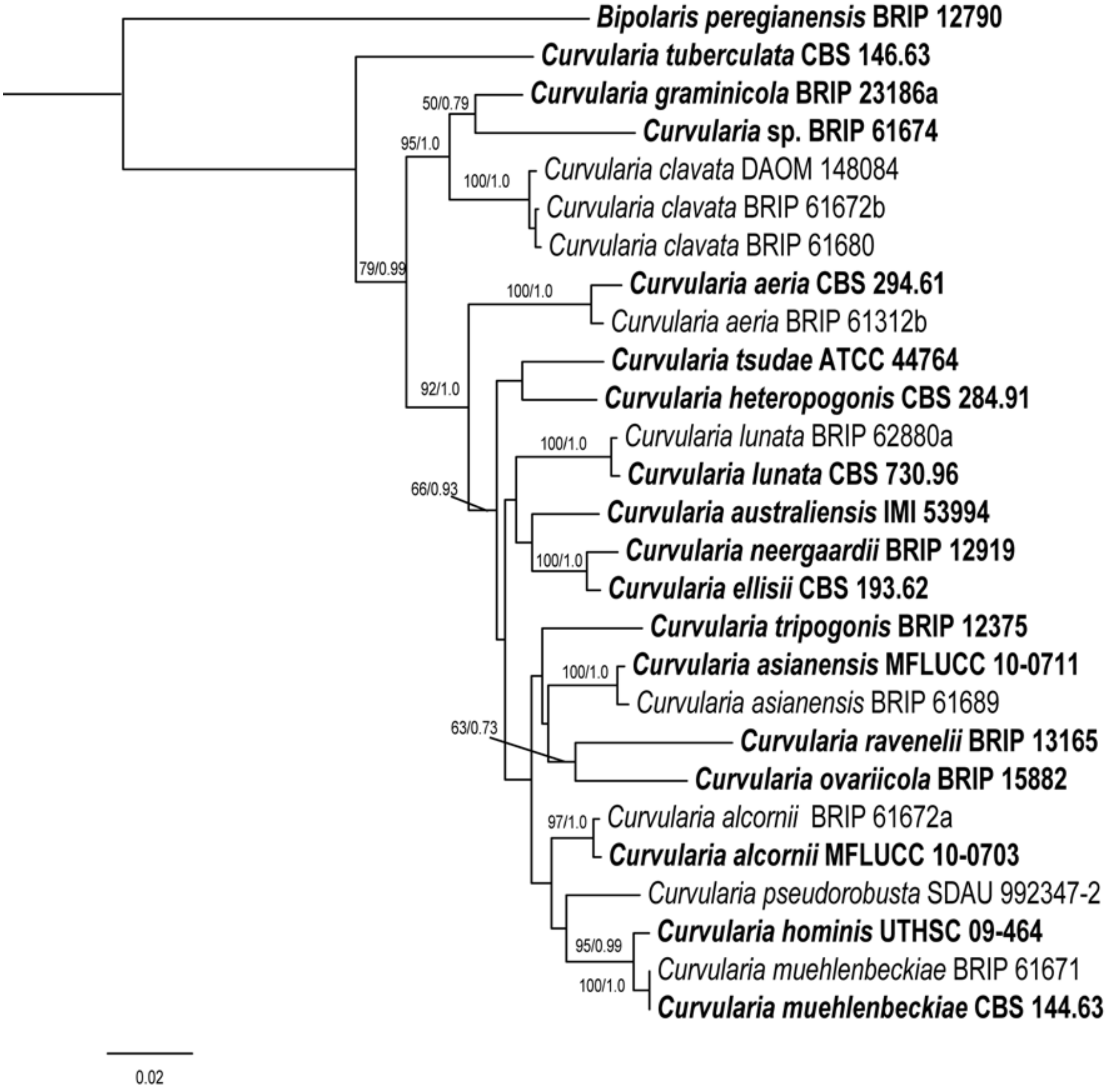

Figure 4.3 Phylogeny of *Curvularia* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes. Bootstrap values (>70%) from maximum likelihood and posterior probabilities (>0.95) from Bayesian inference are shown on nodes respectively.ex-type cultures are written in bold. *Bipolaris peregianensis* was included as an outgroup

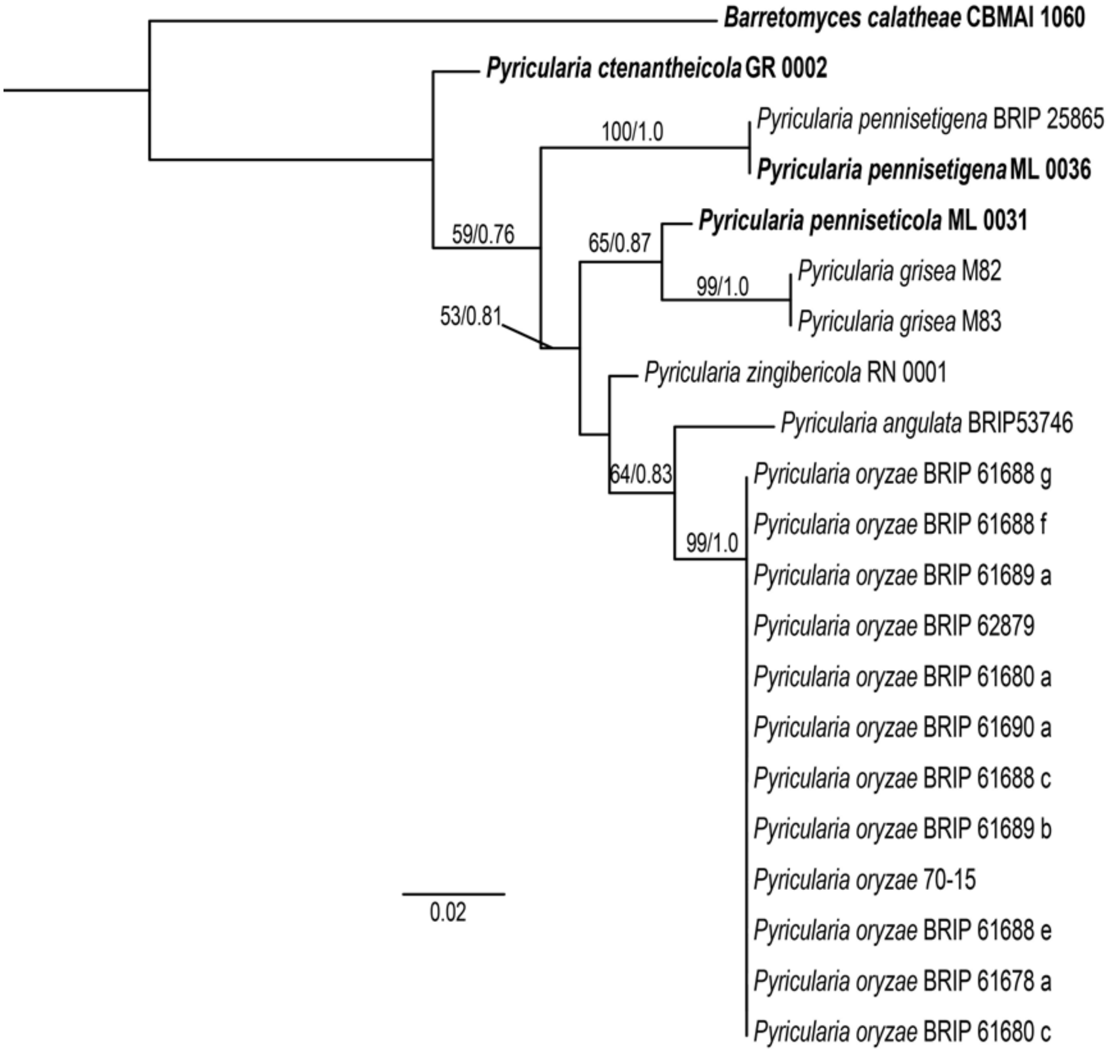

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Figure 4.4 Phylogenetic relationships of *Pyricularia* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS) and the large subunit of RNA polymerase II. Bootstrap values (>70%) from maximum likelihood and posterior probabilities (>0.95) from Bayesian inference are shown on the nodes respectively.and ex-type cultures are written in bold. *Barretomyces calatheae* was included as an outgroup.

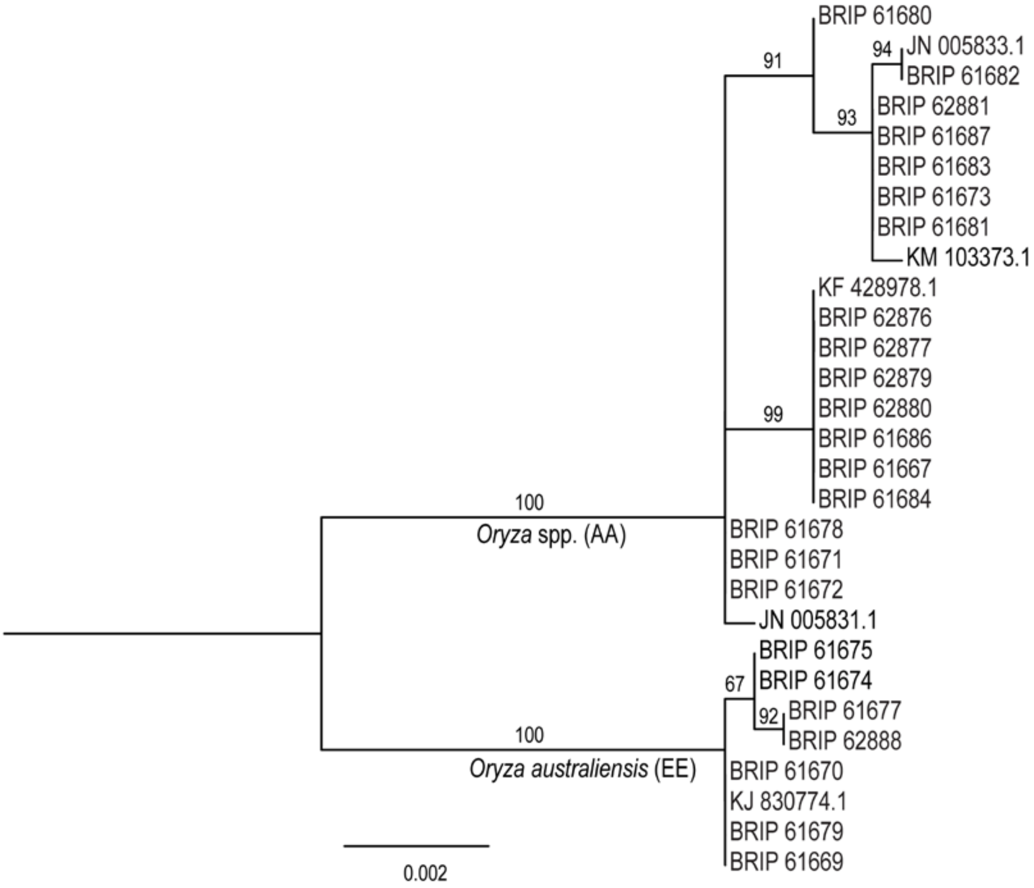

Figure 4.5 Phylogenetic relationships of the wild rices, inferred using DNA sequence data from the atpA–rps14, rpl14–rpl16 and psbZ–tRNAGly PCR amplicons. The analysis was done using the maximum likelihood (ML) method, as implemented in RaxML ver 7.2.6. Bootstrap values (>70 %) from 1,000 replicates in an ML search are shown above nodes. Details of plant specimens used in the analysis are provided in Table 4.2.

4.4 Discussion

During the surveys, a range of fungi were found to be associated with foliar diseases of wild rices species, including *Pyricularia oryzae*, the most serious disease of cultivated rice worldwide (Ou, 1985). Whether the wild rices are potentialsource of inoculum for rice blast epidemics in Queensland remains unanswered. *Oryza* AA genome type species (*Oryza rufipogon* and *Oryza meridionalis*) have been recorded as far south as the Burdekin Region (Atlas of Living Australia), which has been identified as one of the most promising new areas for rice production (Marshall, 2015). However, *Pyricularia oryzae* has also been recorded in Queensland from *Brachiaria mutica*, *Hordeum vulgare*, *Pennisetum clandestinum*, *Phalaris canariensis*, *Rhynchelytrum repens* and *Setaria italica* (Chapter 2) which are all introduced grasses. Some of these alternative grass hosts are more widely distributed, as weeds or through cultivation, than the wild rices in the agricultural regions of northern Australia.

Pyricularia oryzae was isolated, albeit rarely, from two undescribed AA genome-type taxa (*Oryza* sp. Taxon A and B), but not from *Oryza australiensis*. The locations where *Pyricularia oryzae* was discovered were very remote (*c*. 300 km distance) from the nearest major cropping region, the Atherton Tableland. The discovery of *Pyricularia oryzae* on wild rice species represents the second record of this fungus on an indigenous Australian host plant, which introduces the possibility that the fungus is native to Australia. If this hypothesis is correct, it could be expected that local isolates of *Pyricularia oryzae* would form discrete genetic populations when compared with isolates from South-East Asia using DNA markers such as microsatellites (Maciel et al., 2014) and AFLPs (Onaga et al., 2015) or by whole genome resequencing (Mach, 2009). Furthermore, the distribution of avirulence (*Avr*) genes among Australian isolates of *Pyricularia oryzae* would likely be different to that of fungal isolates from South-East Asia, as resistance (*R*) genes have not been deployed in Australian rice cultivars and the wild rices may contain novel *R* genes that are not present in cultivated rice.

The most common plant pathogenic fungus on wild rices was *Bipolaris oryzae*, which is the cause of brown spot disease. Brown spot is predisposed by physiological stresses such as soil infertility or drought but nevertheless causes average yield losses of around 10% in lowland rice crops of South and South-East Asia (Ou, 1985). *Bipolaris oryzae* was infamously associated with the Great Bengal Famine (India and Bangladesh) of 1942 (Barnwal et al. 2013). It is also likely that *Bipolaris oryzae* is an indigenous Australian pathogen of wild rices. *Bipolaris oryzae* was not isolated from rice crops on the Atherton Tablelands, but this may reflect the good growing conditions in these crops. However, *Bipolaris oryzae* remains a threat to rice production in northern Australia, especially as this region is drought-prone and the soils are often ancient and nutrient-poor (Australian Soil Resource Information System). Major genes for brown spot disease resistance have yet to be identified in *Oryza sativa*, although loci conferring partial resistance are known (Katara et al., 2010; Sato et al., 2015).

The final group of well-recognized rice pathogens that was isolated from both wild and cultivated rices in northern Australia was *Curvularia* spp., specifically, *Curvularia aeria, Curvularia alcornii, Curvularia asianensis, Curvularia clavata, Curvularia lunata* and *Curvularia muehlenbeckiae*. None of these species are regarded as major pathogens of *Oryza sativa*, except *Curvularia lunata*, which is one of the commonest causes of rice kernel discolouration (Ou, 1985), and has also been observed to cause leaf spotting, leaf blight and spikelet rot (Huang et al., 2011; Liu et al., 2013; Majeed et al., 2015). *Curvularia aeria* (syn. *Curvularia lunata* var. *aeria*), *Curvularia asianensis* and *Curvularia clavata* have also been isolated from rice grain (Nakada et al., 1994; Wu, 1994; Hilda Nenínger et al., 2003; Manamgoda et al., 2012) and an isolate of *Curvularia aeria* from the Philippines on *Echinochloa* sp. was a weak leaf-spotting pathogen of rice under experimental conditions (Zhang et al. 1996). To our knowledge, *Curvularia alcornii* and *Curvularia muehlenbeckiae* have not been previously reported on *Oryza* spp. anywhere in the world (Manamgoda et al., 2012; 2015), and neither have been previously reported from Australia on any plant species (Hyde and Alcorn, 1993).

This study has provided baseline data on the diversity of leaf spot pathogens on species of wild and cultivated rice in Queensland. The accurate diagnosis of fungal pathogens is fundamental to the development of disease control strategies, as without this information it is not possible to properly determine host or geographic ranges, nor extrapolate from previous studies. If the pathogens that were identified have co-evolved with the species of wild rice, it could be expected that some plant populations may have novel resistance genes, and these may be able to be exploited for rice breeding programs.

Acknowledgement

The authors would like to acknowledge the support of the Australian Government's Cooperative Research Centres Program (project PBCRC 62082). We also thank Steve Rogers and Mike Hedditch from SunRice for useful discussions, helping to organize visits to the rice farms on the Atherton Tablelands and for providing rice seed.

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Chapter 5 Pathogenicity of Australian isolates of *Pyricularia oryzae*

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Abstract

Pathogenicity tests of Australian isolates of *Pyricularia oryzae* were conducted to investigate pathotype diversity. Four Australian isolates of *Pyricularia oryzae,* two from domesticated rice (*Oryza sativa*) and two isolates from wild rice (*Oryza* spp. AA genome), were inoculated onto nine commercial rice cultivars, namely Doongara, Langi, Kyeema, Illabong, Sherpa, Reiziq, Koshi, Opus and Quest. The results demonstrated that all rice cultivars were susceptible to infection, with cvs Quest and Sherpa the most and least susceptible, respectively. Australian isolates of *Pyricularia oryzae*; two from domesticated rice, two from wild rice and one isolate from barley (*Hordeum vulgare*), were inoculated on four important cereal crops namely, barley cv. Malebo, domesticated rice cv. Reiziq, wheat (*Triticum aestivum*) cv. Gregory and oats (*Avena sativa*) cv. Taipan. All four isolates of *Pyricularia oryzae* from rice were able to infect rice and barley but not wheat. Additionally, one isolate from wild rice, BRIP62879, infected oat. The symptom of blast disease was found on rice and barley when inoculated with *Pyricularia oryzae* from barley. Isolates of *Pyricularia oryzae* from *Setaria italica,* and *Pennisetum clandestinum* were inoculated onto their original hosts, domesticated rice cv. Reiziq, barley cv. Malebo and wheat cv. Gregory. The results demonstrated that both infected their original host as well as barley, but did not infect rice or wheat. The causal organism was reisolated from blast lesions and identified with morphological characters to complete Koch's postulation.

5.1 Introduction

Pyricularia oryzae causes blast-like symptoms on many grasses, and is one of the most widely disseminated and destructive plant pathogens (Urashima and Silva, 2011). Specificity between plant genera and the fungal pathotypes is under the control of gene-for-gene interactions (Murakami et al., 2000; Takabayashi et al., 2002; Tosa et al., 2006; Tosa and Chuma, 2011). *Pyricularia oryzae* contains several pathotypes defined by differences in host ranges, particularly some key indicator species (Tosa and Chuma, 2014). Representative pathotypes are the (i) *Oryza* pathotype, which is pathogenic on *Oryza* spp. including domesticated rice, (ii) *Setaria* pathotype, which is pathogenic on *Setaria* spp. including foxtail millet (*Setaria italica*), (iii) *Panicum* pathotype, which is pathogenic on *Panicum* spp.

including common millet (*Panicum miliaceum*), (iv) *Eleusine* pathotype, which is pathogenic on *Eleusine* spp. including finger millet (*Eleusine coracana*) (Kato et al., 2000), (v) *Triticum* pathotype, which is pathogenic on *Triticum* spp. including common wheat (*Triticum aestivum*) (Urashima et al., 1993) and (vi) *Avena* pathotype, which is pathogenic on *Avena* spp. including oats (*Avena sativa*) (Oh et al., 2002).

Pathotypes of *Pyricularia oryzae* are sometimes not clearly delineated. For example, in the Philippines, several rice varieties were susceptible to isolates from *Rottboellia exaltata, Echinochloa colonum* and *Leersia hexandra.* Furthermore, the grasses *Brachiaria distachya, Echinochloa colonum, Leptochloa chinensis, Rottboellia exaltata* and *Leersia hexandra* were susceptible to some isolates of *Pyricularia oryzae* originating from rice (Mackill and Bonman, 1986). In Brazil, the *Triticum* pathotype was found to infect barley (Anjos and Charchar, 2000; Urashima et al., 2004) and *Setaria geniculata* but not rice (Urashima and Kato, 1998; Maciel et al., 2013). Recently, Castroagudín et al. (2016) showed that *Pyricularia oryzae* pathotype *Triticum* and *Pyricularia graminis-tritici* are pathogenic on barley, oats, signal grass (*Urochloa brizantha*) and wheat but not rice, whereas *Pyricularia oryza*e pathotype *Oyrza* was pathogenic on barley, oats, rice and wheat but not signal grass. The possibility of cross-infection is an important consideration from the perspective of disease scouting and management. Many common weedy grasses in rice fields could potentially act as alternative hosts and provide green bridges between rice crops (Castroagudín et al., 2016).

In this study, the pathogenicity of isolates of *Pyricularia oryzae* from domesticated rice, wild rice, barley and some weedy grasses is described. The study set out to answer the following questions: (i) can isolates from wild rice infect domesticated rice; (ii) do Australian isolates infect other cereal crops, particularly wheat; (iii) is there any diversity in host range?

5.2 Materials and methods

5.2.1 Host preparation

Seeds of barley (*Hordeum vulgare* cv. Malebo), wheat (*Triticum astivum* cv. Gregory), oat (*Avena sativa* cv. Taipan), kikuyu grass (*Pennisetum clandestinum*), foxtail millet *(Setaria italica* cv. Golden German) and nine rice cultivars (Doongara, Langi, Kyeema, Illabong, Sherpa, Reiziq, Koshi, Opus and Quest) were soaked in water for 24 h, pre-germinated in moistened filter paper for 48 h and then sown in 7.5 cm diam. pots containing a commercial potting mix (Searles Premium) at a rate of five seeds per pot with three replicates per variety. Seedlings were grown in a glasshouse at 25 ºC and 8 h of daylight for approximately 21 d (until the 3-4 leave stage) prior to inoculation.

5.2.2 Inoculum preparation and infection assay

Seven isolates of *Pyricularia oryzae* were grown on ½PDA for 14 d. Conidia were harvested by washing plates with 10 ml of distilled water and scraping the mycelium with an inoculation loop. The mycelium was then filtered from the slurry by using an open-weaved synthetic cloth. The concentration of spore suspensionwas measured with a haemocytometer and standardized to 1 x 10^5 conidia/ml. Tween 20 (0.01% v/v) was added to improve the effectiveness of spraying. Plants in each experiment were inoculated separately by spraying spore suspension 20 ml per pot using a Preval hand sprayer, and then placed in a moist chamber in the dark for 48 h before transfer back to a glasshouse. Disease symptoms were evaluated 5 d after inoculation.

5.2.3 Disease evaluation

The symptoms were evaluated based on the size of lesions and scored as follows:

0 = no evidence of infection;

1 = brown specks smaller than 0.5 mm in diameter, no sporulation, uniform or scattered brown specks;

2 = brown specks about 0.5-1 mm in diameter, no sporulation, small lesions with distinct tan centres surrounded by a darker brown margin approximately 1 mm in diameter, no sporulation;

3 = roundish to elliptical lesion about 1-3 mm in diameter with a grey centre surrounded by a brown margin, lesions capable of sporulation, small eyespot lesions less than one and a half times the interval between thin veins or less than 1.5 mm in diameter and surrounded by a dark brown margin;

4 = typical spindle-shaped blast lesion capable of sporulation, 3 mm or longer with necrotic grey centres and water-soaked brown margins little or no coalescence of lesions,

intermediate sized eyespot lesions less than twice the interval between thin veins or less than 2 mm in diameter.

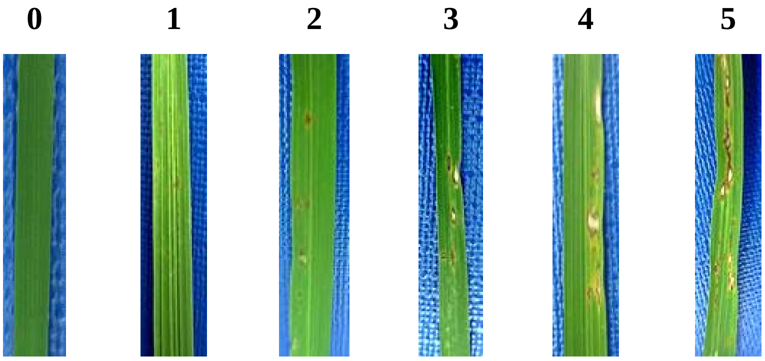
5 = lesions as in 4 large eyespot lesions more than twice the interval between thin veins or more than 2 mm in diameter. (Hayashi and Fukuta, 2009).

Disease severity (DS) was calculated using the formula below, and the level of significance tested using Duncan's Multiple Range Test, as implemented in GenStat 16th edition (VSN International Ltd, UK).

$$DS = \frac{\Sigma\, n \times v}{N \times V} \times 100$$

n = number of leaves infected by blast
v = value score of each category attack
N = number of leaves observed
V = value of the highest score

Disease scale used in this study (Hayashi et al., 2009)



5.2.3 Pathogenicity experiments

Fungal isolates and plant species that were challenged in the pathogenicity experiments are shown in Table 5.1. Host reactions were conducted5 d after inoculation. Each infection experiment was tested individually and repeated for two times. When symptoms of blast were observed, the causal fungus was reisolated and identified to complete Koch's postulates.

Table 5.1 Fungal isolates and challenge plant species used in pathogenicity studies

Exp.	Fungal isolates		Target hosts
	BRIP	**Host**	
1	58447	*Oryza sativa* cv. Quest	*Oryza sativa* cv. Doongara
	61689	*Oryza sativa* cv. Doongara	*Oryza sativa* cv. Langi
	61678	*Oryza* spp. AA genome	*Oryza sativa* cv. Kyeema
	62879	*Oryza* spp. AA genome	*Oryza sativa* cv. Illabong
			Oryza sativa cv. Sherpa
			Oryza sativa cv. Reiziq
			Oryza sativa cv. Koshi
			Oryza sativa cv. Opus
			Oryza sativa cv. Quest
2	58447	*Oryza sativa* cv. Quest	*Hordeum vulgare* cv. Malebo
	61689	*Oryza sativa* cv. Doongara	*Avena sativa* cv. Taipan
	61678	*Oryza* spp. AA genome	*Triticum aestivum* cv. Gregory
	62879	*Oryza* spp. AA genome	*Oryza sativa* cv. Reiziq
	39772	*Hordeum vulgare* cv. Malebo	
3	14879	*Pennisetum cladestinum*	*Pennisetum cladestinum*
	62336	*Setaria italica*	*Setaria italica*
			Hordeum vulgare cv. Malebo
			Triticum aestivum cv. Gregory
			Oryza sativa cv. Reiziq

5.3 Results

In the first experiment, all four isolates of *Pyricularia oryzae* caused blast disease to varying degrees on seedlings of all nine domesticated rice cultivars (Table 5.2). There was significant variation in the aggressiveness of the fungal isolates. No statistically significant difference in disease severity was observed across the cultivars when isolate BRIP61678 was used. The cultivar Quest was most severely diseased when challenged with fungal isolates from domesticated rice (BRIP 58447 and 61689). Quest showed the most severity with statistically significant difference when inoculated with isolate BRIP58447, while isolate BRIP61689 also showed the highest severity and significantly different from Langi, Kyeena, Sherpa and Koshi. The cultivar Langi was most susceptible to infection using BRIP 62879 from wild rice as the challenge isolate, whereas the cultivar Opus was the least susceptible to infection.

Table 5.2 Disease severity (%) after inoculation of rice (*Oryza sativa*) with *Pyricularia oryzae*

Rice variety	**Percent Disease Severity (5 DAI)**			
	BRIP 61678 (wild rice)	**BRIP 62879 (wild rice)**	**BRIP58447 (rice)**	**BRIP 61689 (rice)**
Doongara	5.66 a	26.85 cd	20.29 d	7.45 ab
Langi	1.68 a	35.55 d	10.28 abc	1.67 a
Kyeema	3.70 a	19.55 abc	5.48 ab	0.56 a
Illabong	2.22 a	15.26 abc	15.03 bcd	3.80 ab
Sherpa	0.90 a	10.79 ab	4.75 a	2.10 a
Reiziq	5.56 a	21.93 abc	19.96 d	8.50 ab
Koshi	0.93 a	24.34 bcd	17.76 cd	1.65 a
Opus	4.32 a	9.53 a	8.11 abc	3.82 ab
Quest	5.19 a	16.05 abc	40.40 e	15.06 b

This analysis was performed by GenStat 16th edition to test the significance. Each column were analysed separately. Values are means of three replicates. Values with the same letter are not statistically different based on Duncan’s Multiple Range Test (DMRT) at $P<0.05$.

The second experiment tested the pathogenicity of *Pyricularia oryzae* from cultivated and wild rice as well as barley on four cereal crops (Table 5.3). Both rice and barley were able to infect by all isolates, but not wheat or oats, bar one exception: one wild rice isolate (BRIP 62879) caused very low levels of disease on oats which is significantly different from rice and barley and isolate from barley (BRIP39772) showed blast symptom on rice with low degree of severity and significantly from their original host.

Table 5.3 Pathogenicity of *Pyricularia oryzae* from rice and barley on cereal crops

Host	**Percent Disease Severity (5 DAI)**				
	BRIP 61678 (wild rice)	**BRIP 62879 (wild rice)**	**BRIP 58447 (rice)**	**BRIP 61689 (rice)**	**BRIP 39772 (barley)**
Oryza sativa cv Reiziq (rice)	2.6 a	16.0 a	15.3 a	5.0 a	0.4 b
Hordeum vulgare (barley)	2.7 a	17.0 a	4.6 a	3.2 a	15.1 a
Avena sativa (oats)	0 b	1.15 b	0 b	0 b	0 b
Triticum aestivum (wheat)	0 b	0 b	0 b	0 b	0 b

This analysis was performed by GenStat 16th edition to test the significance. Each column were analysed separately. Values are means of three replicates. Values with the same letter are not statistically different based on Duncan's Multiple Range Test (DMRT) at $P<0.05$.

In the third experiment, the pathogenicity of isolates of *Pyricularia oryzae* from weedy grasses was tested. Fungal isolates from *Setaria italica* and *Pennisetum clandestinum* were inoculated onto their original hosts, rice, barley and wheat. The isolates caused blast disease on their original hosts and barley, but did not cross-infect rice and wheat (Table 5.4).

Table 5.4 Pathogenicity of *Pyricularia oryzae* from weeds on cereal crops

Host	**Percent Disease Severity (5 DAI)**	
	BRIP 14879 **(*Pennisetum cladestinum*)**	**BRIP 62336** **(*Setaria italica*)**
Pennisetum cladestinum	12.5 a	NT
Setaria italica	NT	5.8 a
Hordeum vulgare (barley)	1.5 b	1.7 b
Oryza sativa (rice)	0 c	0 c
Triticum aestivum (wheat)	0 c	0 c

This analysis was performed by GenStat 16th edition to test the significance. Each column were analysed separately. Values are means of three replicates. Values with the same letter are not statistically different based on Duncan's Multiple Range Test (DMRT) at $P<0.05$.

5.4 Discussion

Pyricularia oryzae is a species complex which includes several pathotypes (Kato et al., 2000; Tosa et al., 2004; Yamagashira et al., 2008). The possibility of cross-infection is an important consideration from the perspective of disease scouting and management. In thisstudy, we investigated the pathogenicity and cross-infectivity of several isolates of *Pyricularia oryzae* on domesticated rice and other grasses by artificial inoculation methods. The results of the first experiment showed that nine cultivated rice varieties were susceptible to *Pyricularia oryzae* pathotype *Oryza*, although wild rice plants were not included in the experiment as seed from these plants was not available as they have not yet been successfully grown in the glasshouse because of seed dormancy (Takahashi, 1984). The first experiment and the second one were set up and conducted in different period of time, which could be a reason of why the disease severity in second experiment showed less severe than the first experiment.

Most of the cultivated rice cultivars in Australia are the japonica-type, which is perfectly suited to the dry temperate micro-climate of Australian rice growing (www.sunrice.com.au). Eight out of nine rice cultivarsin this study are commonly grown in Australia and can be considered to represent the genetic diversity of Australian commercial rice production (www.sunrice.com.au). There is evidence that *Pyricularia oryzae* is a threat to rice production in northern Queensland, as seen with the epidemic of blast disease on rice cv. Doongara at Mareeba in 2014, which led to the crop not being harvested (Chapter 4). The results of this experiment showed that the rice cultivars Quest, Doongara and Langi are likely to be more susceptible to rice blast disease epidemics. Why these cultivars have not succumbed to blast disease in southern Australia is not known, but may reflect an absence of the pathogen in this region or perhaps environmental conditions which are not amenable to epidemic development, despite the fact that modelling suggests otherwise (Lanoiselet et al., 2002).

The ability of Australian isolates of *Pyricularia oryzae* to infect both rice and barley is not surprising. The occurrence of barley blast has been documented in many parts of the world in Brazil (Anjos and Charchar, 2000), Japan (Kawai et al., 1979) and Thailand (Sato et al., 2001). In some countries, such as northern Thailand and Japan, blast disease is highly important because the barley fields are located near rice fields, produced in proximity during the same growing season and can be infected by the same pathogen (Sato et al., 2001).Even though, in Australia, Pyricularia blast on barley and oats is less important than rice because the main production areas of barley are located in the southern part of Western Australia, Victoria and New South Wales, where climate conditions are not favourable for *Pyricularia oryzae*, this study presented the possibility of blast disease on both crops.

Isolates of *Pyricularia oryzae* from *Pennisetum cladestinum* and *Setaria italica* were able to infect their original host as well as barley but did not infect rice and wheat. This provides evidence that there are probably both *Pennisetum* and *Setaria* pathotypes of *Pyricularia oryzae* in Australia. Neither of these two pathotypes poses a threat to rice production in northern Australia. These results further indicate that barley is a susceptible crop that can be infected by *Pyriculaira oryzae*. However, this experiment was tested a single isolate of each pathotype, more isolates may require for further study.

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Chapter 6 Conclusions and General Discussion

6.1 Overview

The *Magnaporthales* (*Sordariomycetes*, *Ascomycota*) comprises three families, *Magnaporthaceae*, *Pyriculariaceae* and *Ophioceraceae* (Klaubauf et al., 2014). This order contains 28 genera and over 200 species, which includes many plant pathogens of significance in agricultural (Kirk et al., 2008; Zhang et al., 2016b). In Australia, the best known fungus in this order is *Gaeumannomyces graminis* var. *tritici*, which causes the highly destructive disease known as take-all of wheat (Macnish and Speijers, 1982; Wilhelm et al., 1988). Another important plant pathogen is *Pyricularia oryzae*, the cause of blast disease on rice and other cereals (Stahl, 1955; You et al., 2012).

In Australia, the first record of *Pyricularia oryzae* was in 1955 on rice in the Northern Territory (Stahl, 1955). Recently, the fungus was reported on rice in Western Australia (You et al., 2012). *Pyricularia oryzae* is one of the most devastating and widespread species on grasses around the world. Although fungicides can be used for disease control, these chemicals generate additional production costs as well as potential contamination of food products and the environment. The most practical and economical approach to control *Pyricularia oryzae* is to use disease resistant plant varieties, and breeding for blast resistance is a major objective in rice improvement programs (Divya et al., 2014). However, the high genetic variability in populations of *Pyricularia oryzae* has resulted in host resistance breaking down within a few years after the introduction of the varieties (Taheri and Irannejad, 2014).

This study presents a species level taxonomic examination of some Australian plant pathogenic fungi in the order *Magnaporthales*, particularly in the families *Pyriculariaceae* and *Magnaporthaceae*. In these families, morphology alone was inadequate for species identification, although it has some value as a primary character at a generic level. DNA based phylogenetic analyses of nuclear ribosomal DNA within the Internal Transcribed Spacer (ITS) and Large Subunit region (LSU), as well as six protein coding genes, were used together with morphological data to identify and classify species of *Pyriculariaceae* in Australia (Chapter 2), as well as two novel species of *Maganporthaceae* (*Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis*) which used sequences of ITS, LSU, RPB1 and TEF1 for idnetification (Chapter 3). This phylogenetic study resulted in the proposed transfer of *Pyricularia rabaulensis* to *Barretomyces* (Chapter 2) and the establishment of a new genus (*Wongia*) to accommodate *Magnaporthe garrettii* and *Magnaporthe griffinii* (Chapter 3).

The species of wild rice found in Australian (*Oryza* spp.) represent virtually untapped gene pools for the improvement of domesticated rice (Henry et al., 2010). However, concern has been expressed that these wild rice species may represent threats to tropical cereal crops (rice, maize and sorghum) and the sugarcane industry, both as reservoirs of indigenous pests and pathogens, as well as infection pathways for exotic pests and pathogens that occur in countries to the north of Australia (Petrovic et al., 2013). In this study, two surveys of leaf spot diseases on wild rice, with particular focus on blast disease, were completed during 2014 and 2015 (Chapter 4). *Pyricularia oryzae*, the rice blast pathogen, was found on individual *Oryza* AA genome-type plants, while *Bipolaris oryzae*, the cause of brown spot disease, and a large diversity of *Curvularia* species that can cause seed discoloration, were recovered on both *Oryza* AA genome-type and *Oryza australiensis* (Chapter 4). Subsequently, the pathogenicity of the *Pyricularia oryzae* isolates was tested on nine commercial rice cultivars as well as barley, oats, wheat and two introduced weed species (Chapter 5).

6.2 Key findings and contributions

This study provides an indepth understanding of the identification, classification, epidemiology and pathogenicity of some Australian plant pathogens in the *Magnaporthales*, with focus on *Pyricularia oryzae*, the cause of rice blast disease. The key findings of this study were

- Taxonomic resolution of several species of fungi in the order *Magnaporthales* using multigene analysis;
- The presence of *Pyricularia oryzae* on species of wild rice in remote regions of northern Queensland;
- The pathogenicity and cross-infectivity of *Pyricularia oryzae* from cultivated rice, wild rice and grasses;

The findings are discussed in more detail as follows.

Taxonomy and species identification of *Magnaporthales* isolated from Australia (Chapter 2 and Chapter 3)

This study identified two novel species of root pathogenic fungi in the *Magnaporthaceae,* namely, *Budhanggurabania cynodonticola* and *Magnaporthiopsis agrostidis*, using a combination of morphological and molecular characters (Wong et al., 2015a; Wong et al., 2015b). *Budhanggurabania cynodonticola* shared several morphological characteristics with *Pseudophialophora*, while *Magnaporthiopsis agrostidis* was similar to *Magnaporthiopsis incrustans* in culture on PDA, but was highly divergent based on molecular analysis. The DNA sequences of four gene regions, namely ITS, LSU, *RPB1* and *TEF1*, for these two new species were deposited in GenBank.

In addition, the holotypes of two root-infecting fungi from Australia, *Magnaporthe garrettii* and *Magnaporthe griffinii*, were studied to resolve the classification of these fungi. These two species were classified in *Magnaporthe* based on morphological characters (Wong et al., 2012). To date, the genus *Magnaporthe* was proposed for suppression by Zhang *et al*. (2016), in essence because *Magnaporthe* is congeneric with *Nakataea* (Hara 1939), as the types of both genera, *Magnaporthe salvinii* (basionym *Leptosphaeria salvinii*) and *Nakataea sigmoidea* (basionym *Helminthosporium sigmoideum*), refer to the same species (Krause and Webster, 1972; Luo and Zhang, 2013). Subsequently, most of members in this genus have been transferred to other genera, *Magnaporthiopsis*, *Nakataea* and *Pyricularia*, using molecular analysis (Luo and Zhang, 2013). In this study, the multigene analyses confirmed that *Magnaporthe garrettii* and *Magnaporthe griffinii* were sister species of uncertain family (*Papulocaceae*) in the *Diaportheomycetidae* (Maharachchikumbura et al., 2015) and the genus *Wongia* was proposed to accommodate these two species, as *Wongia garrettii* and *Wongia griffinii*, respectively.

The genus *Pyricularia* contains over 80 species that cause blast disease on plants, especially on grasses and other monocot species (Zhang et al., 2016b). The results from this study confirmed that three species of *Pyricularia* are found in Australia, namely *Pyricularia oryzae, Pyricularia angulata* and *Pyricularia pennisetigina*. *Pyricularia oryzae* was discovered from nine grass species (*Brachiaria mutica, Hordeum vulgare, Oryza rufipogon, Oryza sativa, Pennisetum clandestinum*, *Phalaris canariensis*, *Melinis repens*, *Setaria italica* and

Stenotaphrum secundatum). *Pyricularia pennisetigena* on *Cenchrus ciliaris* is reported for the first time in Australia. It has previously been reported on *Cenchrus echinatus* and *Echinochloa colonum* in Brazil, *Cenchrus ciliaris* in Japan, *Pennisetum* sp. in Mali and *Pennisetum glaucum* in USA (Klaubauf et al., 2014). *Pyricularia angulata* is a leaf spot pathogen on banana that has been reported from Australia (Male et al., 2011) and Korea (Kim et al., 1987). *Pyricularia angulata* and *Pyricularia pennisetigina* are morphological similar to *Pyricularia oryzae*, but differ in host pathogenicity and DNA sequence (Kato et al., 2000; Klaubauf et al., 2014).

Another species, *Pyricularia rabaulensis,* has been reported on *Alpinia caerulea* (*Zingiberaceae*) in Australia (KE EMU database). Although the holotype of this species was destroyed in the 1995 Kobe earthquake, the neotype is provisionally designated in this study. *Pyricularia rabaulensis* is known on *Musa* x *paradisiaca* (*Musaceae*) in Papua New Guinea (Matsushima, 1971), on submerged leaf in Brazil (Barbosa and Gusmão, 2011) and on *Alpinia caerulea* in Australia, where it is endemic to sub-tropical and tropical regions of the east coast. This species needs to be transferred to *Barretomyces*, based on morphological and molecular evidence.

Foliar diseases on wild rice (Chapter 4)

This study has provided baseline information about the diversity of leaf spot fungal pathogens on wild and cultivated rices in northern Queensland. Fungal isolates from seven genera, namely, *Bipolaris, Colletotrichum*, *Curvularia*, *Nigrospora*, *Pestalotiopsis, Phoma* and *Pyricularia*, were collected during the surveys in 2014 and 2015. Isolates of *Pyricularia*, *Bipolaris* and *Curvularia* were identified to species level by a combination of multigene analyses of ITS, *RPB1*, *TEF1* and *GPDH* sequences, together with morphology. *Pyricularia oryzae*, the cause of rice blast disease, was identified from wild rice (*Oryza* spp. AA genome type) and cultivated rice (*Oryza sativa*) but not from *Oryza australiensis*. The rice brown spot pathogen, *Bipolaris oryzae*, was also found on species of wild rice in northern Queensland, on both *Oryza* spp. AA genome type and *Oryza australiensis*, but not from cultivated rice. It is likely that these two fungi are indigenous to Australia. The last group of well-recognized rice pathogens was *Curvularia* spp., specifically, *Curvularia aeria*, *Curvularia alcornii*, *Curvularia asianensis, Curvularia clavata, Curvularia lunata* and *Curvularia muehlenbeckiae* (Khemmuk et al., 2016). None of these species are regarded as serious pathogens of rice, except *Curvularia lunata*, which is one of the more frequent causes of rice kernel discolouration (Ou, 1985).

Pathogenicity of ***Pyricularia oryzae*** (Chapter 5)

The pathogenicity and cross-infectivity of Australian isolates of *Pyricularia oryzae* were investigated by artificial inoculation on several hosts, including domesticated rice, wheat, barley, oats and two introduced weedy grass species. Nine rice varieties were shown to be susceptible to infection by isolates of *Pyricularia oryzae* from both cultivated and wild rice. These results indicated that rice production in Australia is potentially at threat from natural reservoirs of *Pyricularia oryzae* in wild rice populations. The ability of isolates of *Pyricularia oryzae* from rice and barley to cross-infect indicates that *Pyricularia oryzae* has a various host in Australia, as *Oryza* and *Hordeum* are distantly related grasses (Kawai et al., 1979). This finding is supported by the work of others, who found that barley is compatible with *Pyricularia oryzae* from rice (Kato et al., 2000; Castroagudín et al., 2016).

Isolates of *Pyricularia oryzae* from *Pennisetum cladestinum* and *Setaria italica* were also able to infect barley as well as their original hosts but interestingly, not rice and wheat, demonstrating that they represent a different pathotype. This further shows that barley is very susceptible to *Pyricularia oryzae*, including both *Oryza* pathotypes and isolates from other grasses (Kato et al., 2000; Sato et al., 2001).

6.3 Recommendations for future research

Differential system for classifying blast race

The relationship between the avirulence genes of the pathogen and the resistance genes of rice cultivars is explained by the gene-for-gene theory (Flor, 1971). On the basis of this theory, a set of nine differentials varieties each with known resistance genes was proposed in Japan (Yamada et al., 1976). However, this differential set has limited value as it, (i) does not comprise isogenic lines, (ii) may have other unknown resistance genes present in cultivars, and (iii) fails to describe adequately the diversity of pathotypes in the tropics (Ou, 1980). Considering this, a decade later, the International Rice Research Institute (IRRI) developed a set of five near isogenic lines (NILs) of cultivar CO39 for characterizing populations of *Pyricularia oryzae* (Mackill and Bonman, 1992). Kobayashi et al. (2007) developed the first set of monogenic lines (MLs) as international differential varieties, which targeted a large number of resistance genes while harboring a single one in each genetic background. A collaborative project between IRRI and the Japan International Research Center for Agricultural Sciences (JIRCAS) has further developed three universal differential variety sets of near isogenic lines based on a Japonica type (variety Lijiangxintuanheigu), an Indica type (variety CO39) and a universal susceptible line US-2 (Fukuta et al., 2009). These differential variety sets are useful tools for the identification of the rice blast races, genes for blast resistance, as well as for monitoring changes in rice blast populations (Khush and Jena, 2009; Kawasaki-Tanaka et al., 2016).

In the order for the rice industry to expand in northern Australia, it will be necessary to breed for rice blast resistance using locally-adapted varieties. To assist the plant breeders, it will be necessary to screen a large panel of *Pyricularia oryzae* isolates from Australia against a differential variety set to identify resistance genes, which can then be deployed in the breeding program.

Consideration of wheat blast

Wheat blast is a serious disease caused by the phylogenetically distinct species, *Pyricularia oryzae* pathotype *triticum* and *Pyricularia graminis-tritici* (Castroagudín et al., 2016). There is no evidence of cross infection between isolates of *Pyricularia* from wheat and rice (Castroagudín et al., 2016). Wheat blast was first reported in 1985 in Brazil (Igarashi et al., 1986) then in 2011 at a research plot in Kentucky, USA (Pratt, 2012). In February 2016, wheat blast was first reported in Asia, where it was responsible for loss of over 15,000 hectares of cultivated wheat in Bangladesh (Callaway, 2016). Although a serious epidemic has not yet been reported outside South America, there is an increasing concern that wheat blast could become a threat to wheat production worldwide. Molecular studies showed that the wheat blast pathogen in Bangladesh was related to the Brazilian isolates rather than the one from North America. The source of wheat blast pathogen found in Bangladesh is not known, although it is possible that it was carried in seed from South America. Another possibility is a host shift from grasses to wheat, as happened in Kentucky, USA when the ryegrass strain of *Pyricularia oryzae* moved onto wheat (Callaway, 2016). Although wheat blast has never been reported in Australia, there are over ten grass species reported as hosts of *Pyricularia oryzae*. The possibility of a dynamic host shift across grass genera cannot be dismissed, and it is important that wheat is included in host range studies to alert authorities of the emergence of a new pathotype.

Genomic studies of *Pyricularia oryzae*

Genomic information is the key to understanding the molecular events that lead to fungal pathogenicity as well as developing plant disease control strategies (Zhang et al., 2016a). Whole genome studies of fungal pathogens can be used to better understand many aspects of biology including climate adaptation and host range shift, as well as to monitor the breakdown of plant disease resistance or the emergence of fungicide resistance (Chiapello et al., 2015). To date, 53 isolates of *Pyricularia oryzae* have been sequenced and published (http://www.ncbi.nlm.nih.gov), which is a reflection of the importance of this species, however there are no genomes sequenced from Australian isolates. Different isolates have been shown to possess similar genome sizes and gene numbers (Dean et al., 2005; Xue et al., 2012; Chen et al., 2013; Chiapello et al., 2015; Dong et al., 2015). These multiple genome sequences provide information about many specific genes that are responsible for the infection process as well as the mechanisms of genetic variation in pathogen-host interactions. If the hypothesis that *Pyricularia oryzae* is indigenous to Australia is correct, it could be expected that there is extensive variation among field isolates.

Mating types *Pyricularia oryzae*

Sexual reproduction can enhance and influence the genotypic variability of *Pyricularia oryzae* populations and recombinant progenies may provide a potential mechanism for increasing genetic diversity of *Pyricularia oryzae* resulting in an ability to infect new host cultivars (Chen et al., 2003). The sexuality of *Pyricularia oryzae* is controlled by a single locus, *MAT1* (Yoder et al., 1986), and comprises of two distinct mating types (*MAT1-1* and

MAT1-2). However, the ability to produce a fertile stage (perithecia) is apparently controlled by multiple genes that segregate independently of mating type and pathogenicity on different hosts (Kolmer and Ellingboe, 1988). The distribution of mating types has been used to characterize the genetic structure and dynamics of heterothallic populations of *Pyricularia oryzae* (Zeng et al., 2009). Both mating types were found in almost equal distribution in populations of *Pyricularia oryzae* in western Africa (Ivory Coast, Mali and Burkina Faso) and the Himalayan foot hills (South China, Laos, North Thailand and western Nepal), while only *MAT1-1* was found in Europe (France, Italy and Spain) (Li et al., 2016). The distribution of mating types in Australian populations of *Pyricularia oryzae* is not known and warrants further study.

Rice blast resistance genes and population diversity

Rice cultivation is expanding in northern Australia, where the climate is ideal for the development of rice blast disease (Lanoiselet et al., 2002). The results from Chapter 4 and Chapter 5 showed that most of the common rice cultivarsin Australia are susceptible to rice blast disease and there is a potential for epidemics. Breeding for durable rice blast resistance employs strategies that use molecular markers to determine progeny which carried resistance genes and a lineage exclusion strategy to target resistance gene combinations that are most likely to provide an effective barrier to *Pyricularia oryzae* (Zeigler et al., 1995).

The use of resistant varieties with multiple genes is one of the most economically and environmentally efficient ways to protect crop losses from rice blast disease (Zeigler et al., 1995; Skamnioti and Gurr, 2009). More than 73 blast resistance genes have been identified (Ballini et al., 2008). However, single-locus resistance may be short-lived, often lasting for only 2–4 years (Bonman et al., 1992). This is particularly evident in large acreages of genetically uniform rice cultivars, where adaptive selection pressures cause isolates of *Pyriculaia oryzae* to race-shift towards those isolates that escape host recognition. The resistance genes from species of wild rice may be important resources for stacking new blast resistance genes (Jia et al., 2016). These wild rice populations may represent an important and underutilized genetic resource for the improvement of cultivated rice cultivars.

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Appendices

Appendix 1: The diversity of *Pyricularia* on Australia

P 5.4.7

Scientific Theme: 5 Phylogenetics, Evolution and Systematics
Session: 5.4 Genomes, genes and morphology: Making sense of the Ascomycota

Account ID IMC0496
Abstract ID ABS0251

The diversity of *Pyricularia* in Australia

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The genus *Pyricularia* contains species that cause blast diseases in many grasses (Poaceae) around the world, including *P. oryzae* on rice (*Oryza sativa*), and *P. grisea* on wheat, maize and barley. Severe rice blast epidemics have recently occurred in several locations across northern Australia and it is unclear whether these epidemics have arisen as a consequence of an incursion of *P. oryzae* from south-east Asia or instead, have been caused by an endemic *Pyricularia*. Northern Australia is a centre of diversity for wild rice (*Oryza* spp.). To address this question, twenty-five isolates of *Pyricularia* from various native and introduced grasses in Australia were studied using morphological and molecular phylogenetic methods. Phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA and the large subunit of RNA polymerase II gene (*RPB1*) indicated that *Pyricularia* species in Australia comprise several cryptic taxa. Of significance was that none of the Australian specimens were phylogenetically close to the type specimens of *P. grisea* and *P. oryzae*. Specimens of *Pyricularia* from wild and domesticated rice in Australia appear to represent at least two novel taxa, and these taxa have probably had very long associations with wild rice.

Keywords: Phylogeny, taxonomy

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Appendix 2: The co-evolution of wild rice and its pathogens, especially *Pyricularia* spp.

(Science Exchange 2014, 6-9 May 2014, Sunshine Coast, Brisbane, Australia)

Title: The co-evolution of wild rice and its pathogens, especially *Pyricularia* spp**.**

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PBCRC project number:
62082

Project summary:
Pyricularia spp. is one of the most devastating pathogen to staple crop such as rice, wheat and millet worldwide. In Australia, ***Pyricularia oryzae*** has been recorded in the Northern Territory since 1955 but not in rice growing area. Currently, this pathogen was found in rice fields in WA and QLD in non-epidemic level. In the future, it is likely that Australian's rice industry will expand in a large scale that means the epidemic potential of rice diseases will be increased.

The relevance of this project to Australian plant biosecurity is that there is evidence that strains of rice (***Pyricularia oryzae***) in Australia differ from those known from overseas. This means that Australian rice production is still at risk from exotic strains of rice blast. Understanding the genetic and pathogenic variability in rice blast in Australia will facilitate the management of and control of this disease (rated recently as one of the world's top ten pathogens of importance) in Australia. This project will improve and fill the knowledge of ***Pyricularia*** spp. which found in Australia and this advantage will be utilized to rice breeding program to develop resistant rice varieties.

About the author:
Wanporn Khemmuk enrolled as a PhD student at the University of Queensland in April 2013. She studies on ***Pyricularia*** spp. which found in Australia including variation and pathogenicity.

Wanporn worked as an associate researcher in Royal Project Foundation Thailand for 1 year (2004-2005) and the agriculture export company for 2 years (2005-2007). Currently, she works with Rice Department, Ministry of Agriculture and Cooperatives,Thailand since 2007 as a plant pathologist.

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Appendix 3: *Budhanggurabania cynodonticola*

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Budhanggurabania P. Wong, Khemmuk & R.G. Shivas, *gen. nov.*

Etymology. Name derived from the combination of *budhang* and *guraban*, meaning black and fungus, respectively, in the traditional language of Wiradjuri people of Australia, on whose country the fungus was first discovered.

Classification – *Magnaporthaceae, Magnaporthales, Sordariomycetes.*

Mycelium hyaline, becoming dark grey to black with age; hyphae septate, branched, smooth. *Conidiophores* hyaline, single or branched. *Conidiogenous cells* hyaline, phialidic, straight to slightly curved, narrowed at the base. *Conidia* hyaline, aggregated in slimy heads, cylindrical or slightly curved, apex rounded, base acute, aseptate, hyaline, smooth. *Ascomata* on infected stolons, rhizomes and roots, ostiolate perithecia, ampulliform, includes a short apical neck, periphysate, dark brown to black, superficial, solitary. *Asci* obovoid to saccate, 8-spored, ascus wall deliquescent at maturity within the ascomata releasing the ascospores that extrude from the ostiole. *Ascospores* multi-seriate, ellipsoidal with rounded ends, 3 dark brown septa, slightly constricted at each of the septa; central cells brown, with 4–6 oblique striations in lateral view; apical cells subhyaline to pale brown, smooth.

Type species. Budhanggurabania cynodonticola.

MycoBank MB811696.

Budhanggurabania cynodonticola P. Wong, Khemmuk & R.G. Shivas, *sp. nov.*

Etymology. Named after the host genus, *Cynodon*, from which it was isolated.

Mycelium hyaline becoming dark grey to black with age; hyphae septate, branched, smooth, 1–3 µm wide. *Conidiophores* hyaline, single or branched. *Conidiogenous cells* hyaline, phialidic, straight to slightly curved, 5–29 × 1.5–3 µm, narrowed at the base. *Conidia* hyaline, aggregated in slimy heads, cylindrical or slightly curved, 5.5–9(–12) × 1.5–2 µm, apex rounded, base acute, aseptate, hyaline, smooth. *Ascomata* occasionally formed on agar, present on infected stolons, rhizomes and roots, ostiolate perithecia, ampulliform, 300–400 µm high × 200–350 µm diam, includes a short apical neck 75–100 µm high × 70–80 µm wide, periphysate, dark brown to black, superficial, solitary. *Asci* obovoid to saccate, 50–75 × 25–35 µm, 8-spored, ascus wall deliquescent at maturity within the ascomata releasing the ascospores that extrude from the ostiole. *Ascospores* multi-seriate, ellipsoidal with rounded ends, 25–38 × 10–15 µm, with 3 dark brown septa, slightly constricted at each of the septa, middle septum 3.5–4.5 µm wide, apical and basal septa 1–2.5 µm wide; central cells brown, with 4–6 oblique striations in lateral view; apical cells 3–6 µm long, subhyaline to pale brown, smooth.

Culture characteristics — On quarter-strength potato dextrose agar (QPDA), colonies reaching 3.5–4 cm diam after 1 wk at 25 °C in the dark, mostly adpressed with sparse to moderately abundant gray aerial mycelium, becoming darker with age; reverse dark grey to black, paler at the margin. On PDA, colonies grow more slowly, reaching 3 cm diam after 1 wk.

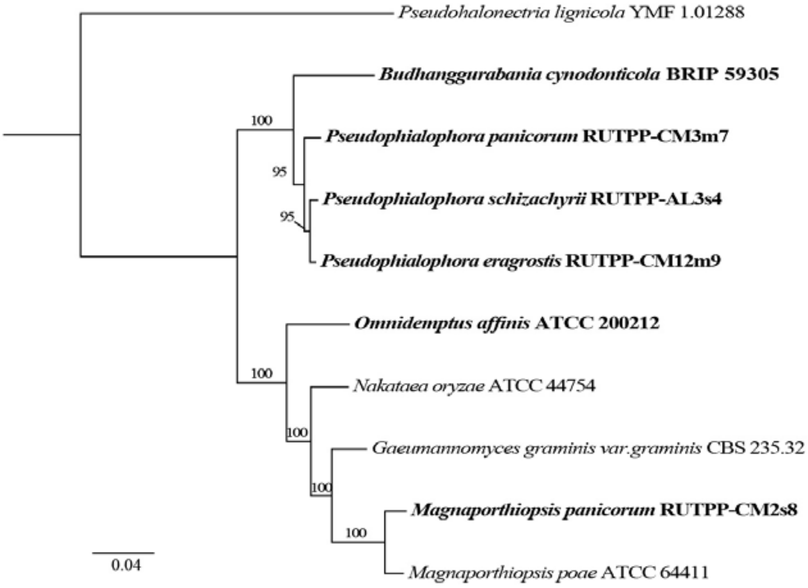
Typus. AUSTRALIA, New South Wales, Deniliquin, Deniliquin Golf Club, from rotted roots of *Cynodon dactylon*, 1 Mar. 2012, *P.T.W. Wong* (holotype BRIP 59305, includes ex-type culture, ITS sequence GenBank KP162134, LSU sequence GenBank KP162140, SSU sequence GenBank KP162130, TEF1 sequence GenBank KP162138, *mcm7* sequence GenBank KP162131 and RPB1 sequence GenBank KP162143, MycoBank MB811697).

Additional material examined — AUSTRALIA, *loc. id*., from rotted roots of *C. dactylon*, 1 Mar. 2012, *P.T.W. Wong* (BRIP 59306, BRIP 59307); Northern Territory, Darwin, Darwin Bowling Club, from rotted roots and stolons of *C. dactylon*, Feb. 2013, *P.T.W. Wong*, PW13051 (BRIP 61692); Queensland, Townsville, Townsville Golf Club, from rotted roots and stolons of *C. dactylon*, Oct. 2014, *P.T.W. Wong*, PW14042 (BRIP 61818).

Notes — Phylogenetic analysis places *Budhanggurabania* in the *Magnaporthaceae* close to other genera with similar phialidic conidial states, including *Buergenerula*, *Gaeumannomyces*, *Magnaporthiopsis* and *Pseudophialophora* (Klaubauf et al. 2014, Luo et al. 2014). *Budhanggurabania* differs from these genera by producing distinctive 3-septate ascospores, with the larger central cells dark brown and striate; and the smaller end cells pale brown and smooth.

Budhanggurabania cynodonticola was isolated from diseased roots and stolons of *Cynodon dactylon* from bowling greens and golf course fairways and greens with a serious patch disease in New South Wales, Queensland and the Northern Territory. Pathogenicity tests in the glasshouse have shown that *B. cynodonticola* is highly pathogenic to *C. dactylon*, causing extensive root and stolon rotting. Ascomata of *B. cynodonticola* are occasionally formed in culture and often occur on diseased roots, stolons and rhizomes of *C. dactylon* when incubated in humid chambers at 20–30 °C in diffuse daylight for several weeks. Ascospores from these ascomata germinate at 25 °C on QPDA + 100 mg of novobiocin/L of medium, to form colonies that produce the asexual state within a few days. The conidia also germinate readily on QPDA or PDA.

Colour illustrations. The patch disease on a fairway at Deniliquin Golf Club, Deniliquin, New South Wales; conidiophore and conidia from ex-holotype culture; ascomata, asci with 8 ascospores and ascospores from infected roots of *Cynodon dactylon*. Scale bars (from left to right) = 10 µm, 100 µm, 10 µm, 10 µm.



Maximum-likelihood tree obtained from the combined DNA sequence data from six loci (SSU, ITS, LSU, *mcm7*, RPB1, TEF1) using RAxML v7.2.6 with GTRGAMMA as a model of evolution. The scale bar indicates the expected changes per site. Ex-type species are in **bold** face. ATCC = American Type Culture Collection, USA; BRIP = Queensland Plant Pathology Herbarium, Australia; CBS = Centraalbureau voor Schimmelcultures, The Netherlands; RUTPP = Rutgers Mycological Herbarium, USA; YMF = Yunnan Microbiological Fermentation Culture Collection Center, China.

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Appendix 4: *Magnaporthiopsis agrostidis*

Fungal Planet 2015

Magnaporthiopsis agrostidis P. Wong, Khemmuk & R.G. Shivas, *sp. nov.*

Etymology: Named after the host genus, *Agrostis,* from which the fungus was isolated.

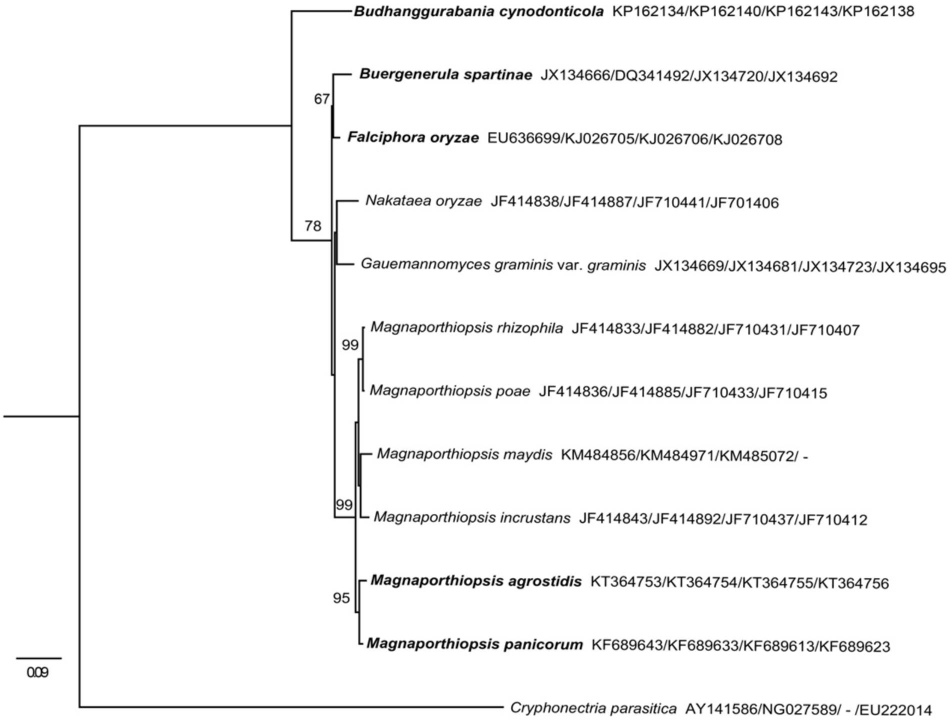
Classification — *Magnaporthaceae*, *Magnaporthales*, *Sordariomycetes*.

Mycelium hyaline, becoming dark grey to dark brown with age; hyphae septate, branched, smooth, 1–4 µm wide, forming mycelial strands and fans at the margins. *Conidiophores* brown, single and terminal or penicillate and integrated. *Conidiogenous cells* brown or slightly pigmented, phialidic, cylindrical to lageniform, 5–20 x 1.5–3 µm, tapering to a conspicuous flared collarette c. 3 µm high x 1.5 µm wide. *Conidia* hyaline, aseptate, smooth, filiform, rounded at the apex and narrowed towards the base, curved to lunate, 4–6 x 1 µm, aggregated in slimy heads. Ascomata have not been observed in culture.

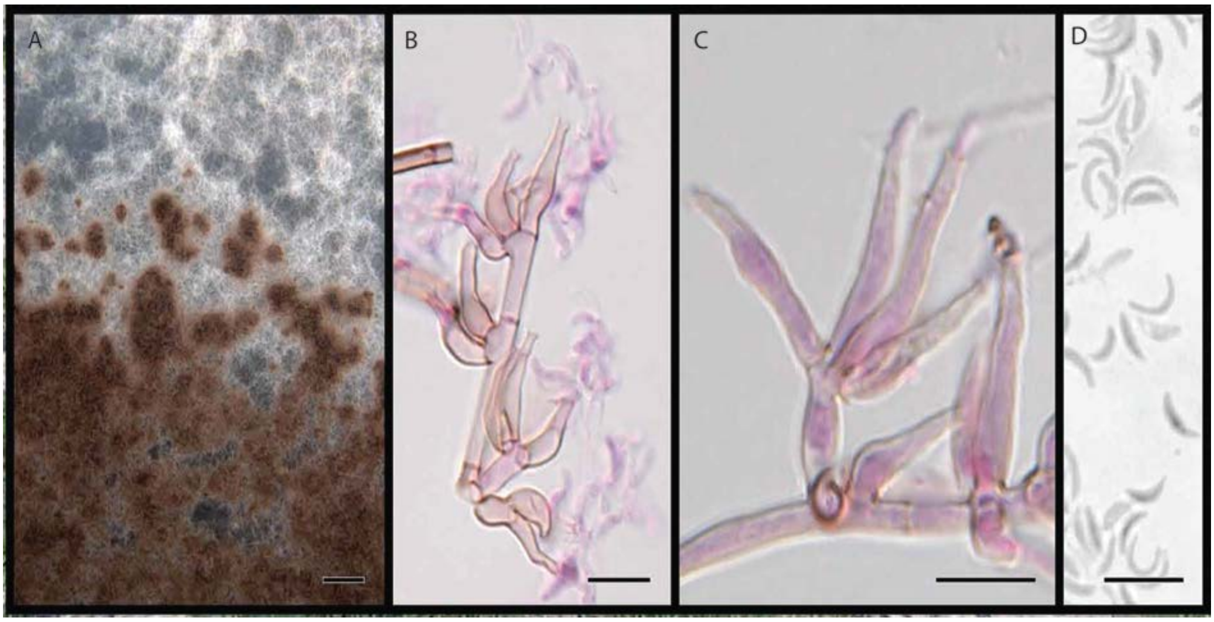
Culture characteristics — On PDA, colonies reaching 7.5 cm diam. after 1 wk at 25°C in the dark; moderately abundant grey aerial mycelium, becoming olivaceous brown with age and forming dark grey to dark brown crust-like mycelial aggregations on the agar surface in older cultures (> 4 wk); reverse dark grey to olivaceous brown, paler at the margin. The crust-like mycelial aggregations were formed more commonly on quarter-strength PDA amended with novobiocin (100 mg/L).

Typus. AUSTRALIA, New South Wales, Little Bay, New South Wales Golf Club, from rotted roots of *Agrostis stolonifera*, May 2013, *P.T.W. Wong PW13010* (holotype BRIP 59300, includes ex-type culture, ITS sequence GenBank KT364753, LSU sequence GenBank KT364754, RPB1 sequence GenBank KT364755, TEF1 sequence GenBank KT 364756, MycoBank MB 814222).

Notes — Phylogenetic analysis placed *Magnaporthiopsis agrostidis* in a monophyletic clade that includes the type species of the genus, *M. poae* (*Magnaporthaceae*). It is the sixth known species of *Magnaporthiopsis* (Luo & Zhang 2013, Luo et al. 2014). All species of *Magnaporthiopsis* are root-inhabiting fungi or root-infecting pathogens of grasses. *M. agrostidis* produces harpophora-like conidia (Gams 2000), which differ from other *Magnaporthiopsis* species by being smaller, filiform, and more curved. They have not been shown to germinate on PDA and may have a role as spermatia in sexual reproduction (Wong & Walker 1975). *Magnaporthiopsis agrostidis* produces superficial crust-like mycelial aggregations that become dark grey to dark brown in older cultures, similar to those of *M. incrustans* (Landschoot & Jackson 1989). *Magnaporthiopsis agrostidis* was isolated from diseased roots of *Agrostis stolonifera* from a golf green with symptoms of a patch disease. It has been shown to be pathogenic to *A. stolonifera* in glasshouse pathogenicity tests.



Caption for tree. Multilocus phylogenetic tree inferred from Maximum-likelihood using RAxML ver.7.2.6 with GTRGAMMA as a model of evolution. Bootstrap support values are indicated at the nodes. The alignment consisted of four partial loci, namely ITS, LSU, RPB1 and TEF1 (represented by respective GenBank accession numbers in the tree). The scale bar indicates the expected changes per site. Ex-type species are in **bold**.



Colour illustrations. New South Wales Golf Club, Little Bay, New South Wales, Australia; crust-like mycelial aggregations; conidiophores; and conidia from ex-holotype culture.— Scale bars = 1 mm (left), 10 µm (others).

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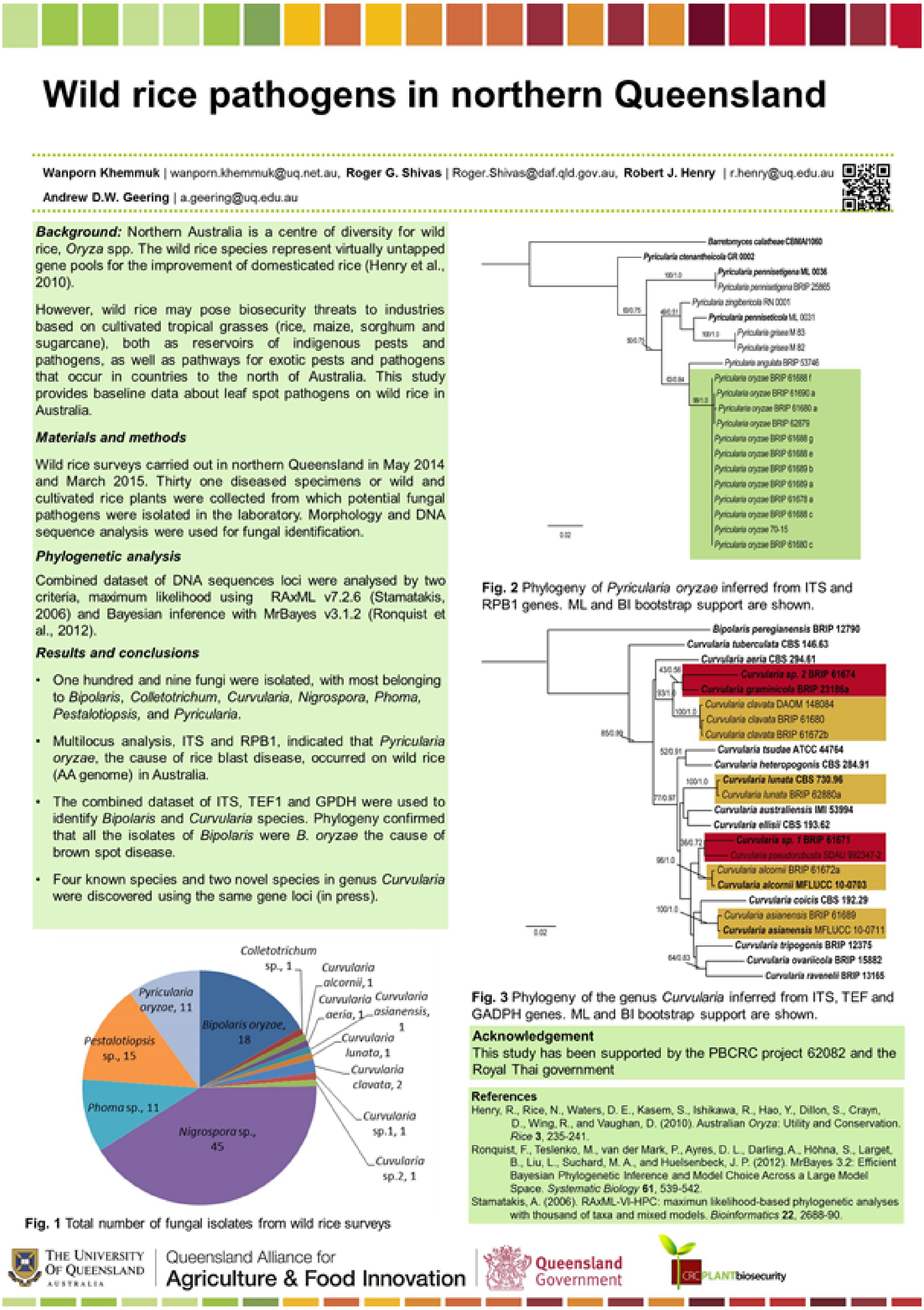
Appendix 5: Wild rice species in northern Queensland



Wild rice pathogens in northern Queensland

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Background: Northern Australia is a centre of diversity for wild rice, *Oryza* spp. The wild rice species represent virtually untapped gene pools for the improvement of domesticated rice (Henry et al., 2010).

However, wild rice may pose biosecurity threats to industries based on cultivated tropical grasses (rice, maize, sorghum and sugarcane), both as reservoirs of indigenous pests and pathogens, as well as pathways for exotic pests and pathogens that occur in countries to the north of Australia. This study provides baseline data about leaf spot pathogens on wild rice in Australia.

Materials and methods

Wild rice surveys carried out in northern Queensland in May 2014 and March 2015. Thirty one diseased specimens or wild and cultivated rice plants were collected from which potential fungal pathogens were isolated in the laboratory. Morphology and DNA sequence analysis were used for fungal identification.

Phylogenetic analysis

Combined dataset of DNA sequences loci were analysed by two criteria, maximum likelihood using RAxML v7.2.6 (Stamatakis, 2006) and Bayesian inference with MrBayes v3.1.2 (Ronquist et al., 2012).

Results and conclusions

- One hundred and nine fungi were isolated, with most belonging to *Bipolaris*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Phoma*, *Pestalotiopsis*, and *Pyricularia*.
- Multilocus analysis, ITS and RPB1, indicated that *Pyricularia oryzae*, the cause of rice blast disease, occurred on wild rice (AA genome) in Australia.
- The combined dataset of ITS, TEF1 and GPDH were used to identify *Bipolaris* and *Curvularia* species. Phylogeny confirmed that all the isolates of *Bipolaris* were *B. oryzae* the cause of brown spot disease.
- Four known species and two novel species in genus *Curvularia* were discovered using the same gene loci (in press).

Fig. 1 Total number of fungal isolates from wild rice surveys

Fig. 2 Phylogeny of *Pyricularia oryzae* inferred from ITS and RPB1 genes. ML and BI bootstrap support are shown.

Fig. 3 Phylogeny of the genus *Curvularia* inferred from ITS, TEF and GADPH genes. ML and BI bootstrap support are shown.

Acknowledgement
This study has been supported by the PBCRC project 62082 and the Royal Thai government

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Appendix 6: Fungi associated with foliar diseases of wild and cultivated rice (*Oryza* spp.) in northern Queensland

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Short title: Fungi associated with foliar diseases of rice in northern Queensland

Abstract

During surveys of wild and cultivated rice in northern Queensland in 2014 and 2015, 92 fungal isolates were obtained from plants that were afflicted by foliar diseases, including the rice blast pathogen, *Pyricularia oryzae*, and the brown spot pathogen, *Bipolaris oryzae*. Seven species of *Curvularia* were found, viz. *Curvularia aeria*, *C. alcornii*, *C. asianensis*, *C. clavata*, *C. lunata*, *C. muehlenbeckiae* and an undescribed species. To remove uncertainty about the identity of the host plants from which the fungi were isolated, a DNA barcoding strategy was developed using regions of the chloroplast genome. Pathogenicity tests using wild rice isolates of *P. oryzae* indicated that many local rice varieties are susceptible to infection.

Additional Keywords

Wild rice; Rice blast disease; Brown spot disease

Introduction

The genus *Oryza* comprises 22–24 species, which are pantropical in distribution and divided into nine diploid (AA to JJ) and three tetraploid (BBCC, CCDD and HHJJ) genome types (Ge et al. 1999; Henry et al. 2010; Nayar, 2014; Vaughan et al. 2008). Rice (*O. sativa*) is an example of an AA genome-type species, and some of its closest relatives are endemic to Australia, including one annual species, *O. meridionalis* (Ng et al. 1981), and possibly two undescribed perennial species, variously referred to as the 'r-type' and 'm-type' (Sotowa et al., 2013) or *Oryza* sp. Taxon A and Taxon B, respectively (Brozynska et al., 2014). The perennial taxa are distinguishable by anther length, although other floral characteristics such as awn length and the number of spikelets per panicle may also be diagnostic (Sotowa et al., 2013). The only other common wild rice species in Australia is *O. australiensis*, which is the single representative of the EE genome (Henry et al. 2010). *Oryza officinalis*, which is primarily distributed from India to New Guinea, is recorded twice from Australia, specifically at Moa Island in the Torres Strait as well as from eastern Arnhem Land (Henry et al. 2010).

The Australian wild rices represent untapped gene pools for the improvement of domesticated rice (Brozynska et al. 2015; Henry et al. 2010). However, concern has also been expressed that they may represent biosecurity threats to tropical cereal crops (rice, maize and sorghum) and the sugarcane industry, both as reservoirs of indigenous pests and pathogens, as well as infection pathways for exotic pests and pathogens that occur in countries to the north of Australia. For example, *Fusarium sacchari*, which is a common endophyte or weak pathogen of *O. australiensis* in remote parts of the Northern

Territory, is capable of causing crown rots of maize and sorghum, and pre-emergence ‘damping off’ of cultivated rice in an experimental situation (Petrovic et al. 2013).

There are renewed efforts to grow rice in Queensland and northern Western Australia, in anticipation of future water shortages in the Riverina region (south-western NSW), the present hub of the industry (Cockfield et al. 2012). This relocation of the industry to the north raises the prospect of crossover of pathogens between wild and cultivated rice species. Among the foliar fungal pathogens that have been reported from cultivated rice in northern Australia, *Pyricularia oryzae*, the cause of rice blast disease, is of greatest concern to the industry (You et al. 2012). *Pyricularia oryzae* was first recorded in Australia in 1955 on the rice varieties Caloro, Kentjana and Radin-China, about 100 km south of Darwin in the Northern Territory, and then again in 1963–64 in the same general vicinity on both cultivated and wild rice (as *O. sativa* var. *fatua*) (Heaton 1964). It has also been reported from Western Australia (You et al. 2012) and Queensland (unpublished records). Fortunately, *P. oryzae* has never been recorded in the Riverina Region, despite the fact that CLIMEX and DYMEX modelling indicate that it could establish there and cause epidemics over a significant proportion of years (Lanoiselet et al. 2002).

The diversity of foliar pathogens of wild rice in northern Australia has not been catalogued in a comprehensive manner. In the largest survey that has been done in Australia, unidentified *Bipolaris* and *Curvularia* spp. (as *Helminthosporium*) were frequently detected on wild rice but not *Pyricularia* spp. (Cother et al. 2009). In this paper, we report the fungi associated with foliar diseases of wild and cultivated rice in northern Queensland. Furthermore, we have tested the hypothesis that *P. oryzae* isolates from wild rice can cross-infect cultivated rice. Other leaf spotting pathogens have been identified to species level using multi-locus analyses. In order to remove uncertainty about the identity of the host plants from which the fungi were isolated, we have used DNA sequences from the rice chloroplast genome to barcode the wild rice specimens.

Materials and Methods

Plant materials and fungal isolations

Leaf spots of wild rices were collected from north Queensland during 12−14 May 2014 and 27−29 March 2015. Thirteen sites were surveyed from Atherton Tableland in the south to Rinyirru National Park in the north (Fig. 1). Sites were chosen based on known and easily accessible locations for wild rices as determined by recent surveys in northern Queensland (Brozynska et al. 2014; Sotowa et al., 2013). Samples of leaves with disease symptoms, together with whole plants for host identification, were collected. For comparative purposes, three cultivated rice crops (var. Doongara) growing on the Atherton Tablelands were also surveyed on 15 May 2014. Fungal isolates were obtained from the lesions following surface sterilization with 70 % ethanol, plating onto half strength potato dextrose agar (½PDA) and incubating at 24 °C for 5 d (Beales, 2012).

Plant DNA extractions and PCR amplifications

DNA was extracted from dried plant tissue using a Power Plant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) as per the manufacturer’s protocol with the following minor modifications. Glass beads (0.02 g of 0.5 mm diam.) and proteinase K (0.8 μg/μl) were added to the supplied extraction buffer. The tubes were incubated at 65 °C overnight before mechanical lysis.

For plant DNA barcoding purposes, six regions of the chloroplast genome were selected for sequencing: maturase K (matK); intergenic region–cytochrome b6/f complex subunit VIII (interG–petN); DNA-directed RNA polymerase subunit β (rpoC2); ATP synthase subunit α–30S ribosomal protein S14 (atpA–rps14); 50S ribosomal protein L14–50S ribosomal protein L16 (rpl14–rpl16); photosystem II reaction center protein Z–tRNA-Gly (psbZ–tRNAGly). MatK gene sequences were amplified using the MatKF1/R1 primer pair of Ge et al. (1999). To design new PCR primers, relevant DNA sequences from *O. sativa* cv. Nipponbare and the Australian wild rice species were aligned with

MUSCLE, PCR primers on either side of variable regions selected by eye, and primer parameters measured using the OligoEvaluator software tool (http://www.oligoevaluator.com). Primer sequences are listed in Table 1.

All PCR amplifications were done in a 20 µL reaction volume using high fidelity Phusion DNA Polymerase (Biolabs) as per the manufacturer's instructions. Thermal cycling conditions were as follows: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 61–65 °C (Table 1) for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 10 min. PCR products were sent to Macrogen, South Korea, for direct sequencing using the amplification primers.

Fungal DNA extractions and PCR amplifications

Fungal DNA was extracted from fresh mycelium following the method of Lee and Taylor (1990). The primer pairs ITS1/ITS4 (White et al. 1990); RPB-Ac/RPB-Cr (Castlebury et al. 2004; Matheny et al. 2002); EF1983F/2218R (Schoch et al. 2009) and gpd1/gpd2 (Berbee et al. 1999) were used to amplify the rDNA internal transcribed spacer (ITS), the large subunit of RNA polymerase II (RPB1), translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes, respectively. PCR amplifications were done with high fidelity Phusion DNA Polymerase (Biolabs) in a 20 µL final reaction volume. Thermal cycling conditions for the ITS, TEF1 and GPDH loci were as provided by Deng et al. (2015). For the RPB1 locus, thermal cycling parameters were: 98 °C for 30 s, followed by 30 cycles of 98 °C for 10s, 59 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min. PCR products were sent to Macrogen, South Korea, for direct sequencing using the amplification primers.

Sequence alignments and phylogenetic analyses

Sequences were edited and assembled using Sequencher v. 5.1 for Windows (Gene Codes Corp., Ann Arbor, USA). The assembled consensus sequences were aligned with MAFFT using default setting (www.ebi.ac.uk/Tools/msa/mafft) and manually adjusted where necessary (Katoh and Standley, 2013). For both the plant and fungal DNA sequences, individual loci were aligned separately, and then the alignments concatenated for the phylogenetic analyses. Combined ITS and RPB1 sequence datasets were used to identify the *Pyricularia* species and ITS, GPDH and TEF1 sequence datasets to identify *Bipolaris* and *Curvularia* species.

Phylogenetic analyses using the maximum likelihood method were done using RAxML v7.2.6 (Stamatakis, 2006). For the analyses, the concatenated alignments were run as partitioned datasets, the GTRGAMMA model of evolution was specified and rapid bootstrapping (command –f a) using a random starting tree and 1,000 replicates used to assess the degree of support for the clades. MrBayes was used to conduct a Markov Chain Monte Carlo (MCMC) search with Bayesian inference (Ronquist and Huelsenbeck 2003). Four runs, each consisting of four chains, were implemented for 10 million generations. The cold chain was heated at a temperature of 0.25. Substitution model parameters were sampled every 1000 generations and trees were saved every 1000 generations. Convergence of the Bayesian analysis was confirmed using AWTY (Nylander et al. 2008) (available at: ceb.csit.fsu.edu/awty/).

Pathogenicity of *Pyricularia oryzae* isolates on commercial rice varieties

Rice varieties Doongara, Langi, Kyeema, Illabong, Sherpa, Reiziq, Koshi, Opus and Quest propagated from seed (Mike Hedditch, SunRice) were inoculated with *P. oryzae* isolates BRIP 61678 and BRIP 62879. To aid germination, the seed was soaked in water for 24 hr, incubated in square Petri dishes for 48 hr and then sown in 7.5 cm diam. pots containing a commercial potting mix (Searles Premium) at a rate of five seeds per pot and three pots per variety. Inoculum was prepared from 14 d old cultures by adding 10 ml of sterile distilled water to each plate, scraping with an inoculating loop and then filtering out the mycelium from the slurry using an open-weaved synthetic cloth. The concentration of conidia, as measured using a haemacytometer, was adjusted to 10^4 conidia per ml and a drop of Tween

20 added. At 21 d post-sowing (3–4 leaf seedling stage), the rice plants were inoculated by spraying the spore suspension using a Preval hand sprayer, and then placed in a moist chamber in the dark for 48 hr before transferring back to a glasshouse.

Disease symptoms were evaluated in 5 d after inoculation using a 0 to 5 progressive scale as follows: 0 = no lesions; 1 = uniform or scattered brown specks; 2 = small lesions with distinct tan centers surrounded by a darker brown margin approximately 1 mm in diam.; 3 = small eyespot lesions less than one and a half times the interval between thin veins or less than 1.5 mm. in diam. surrounded by dark brown; 4 = intermediate size eyespot lesions less than twice the interval between thin veins or less than 2 mm. in diam.; 5 = large eyespot lesions more than twice the interval between thin veins or more than 2 mm in diam. (Anonymous, 2002). Disease severity (DS) was calculated using the formula below, and the significance of differences tested using Duncan's Multiple Range Test, as implemented in GenStat 16th edition (VSN International Ltd, UK).

$$DS = \frac{\Sigma\, n \times v}{N \times V} \times 100$$

n = number of leaves infected by blast

N = number of leaves observed

v = value score of each category attack

V = value of the highest score

Results

Identification of *Oryza* spp. in northern Queensland

The *Oryza* species that are indigenous to Queensland are poorly described and there is no morphological key. Consequently, a DNA barcoding strategy for host identification was devised. An *in silico* analysis was first done using reference chloroplast genome sequences on GenBank to determine the discriminatory power of each locus that was selected for sequencing. MatK, which is one of two recommended DNA barcodes for plants (Hollingsworth et al. 2009), was only useful for differentiating *O. australiensis* from the AA genome-type taxa. By contrast, rpl14–rpl16 discriminated each reference chloroplast genome, as well as *O. sativa*.

During the surveys, diseased specimens of *Oyza australiensis* and wild *Oryza* AA genome-type plants were collected (Table 2). Of the six regions of the chloroplast genome that were sampled by PCR, sequences from the atpA–rps14, psbZ–tRNAGly and rpl14–rpl16 amplicons were most phylogenetically informative and used for classification (Fig. 2). All polymorphisms within these amplicons mapped to intergenic regions in the chloroplast genome, except for single non-synonymous substitutions in the rpl16 gene in a small number of *O. australiensis* and AA genome-type plants (Supplementary Tables 1 and 2). Among the newly collected plant specimens, sequences from the matK, rpoC2 and interG–petN amplicons were invariate for each *Oryza* genome group, except for a single specimen of *O. australiensis* (BRIP 61679), which had a 19 nucleotide deletion in a non-coding region of the interG–petN amplicon relative to other specimens of this species.

Significant chloroplast haplotype diversity was observed for both *O. australiensis* and the AA genome-type plants (Fig. 2). GenBank accession KF428978.1 from Abattoir Swamp near Julatten was considered the reference sequence for *Oryza* sp. Taxon A (Brozynska et al., 2014) and this chloroplast haplotype was also collected from Clancy's Lagoon in Mareeba Wetlands Reserve and from an unnamed dam on the Mulligan Highway between Lakeland Downs and Cooktown (Fig. 1). During 2014, the wild rice population at the JPN2 site of Sotowa et al. (2013) was surveyed (site 5 in Fig. 1), and the specimen from this site (BRIP 61680) was considered representative of *Oryza* sp. Taxon B. No other specimen had an identical sequence to BRIP 61680, although several from Rinyirru National

Park were closely related (Supplementary Fig. 1). Given the lack of information on what constitutes a distinct species, the different chloroplast haplotypes were simply labelled using letters of the alphabet.

Identification of fungi

Fungi were isolated from 31 specimens with symptoms of foliar diseases, of which 27 were from wild rice and four were from rice crops on the Atherton Tablelands. Morphological characters such as colony appearance on PDA and conidial shape were used to triage isolates prior to further molecular examination. Almost half of the fungal isolates were *Nigrospora* species. *Bipolaris* was the next most frequently isolated genus from the wild rices (Table 3), and phylogenetic analyses using concatenated ITS-TEF1-GPDH alignments identified all as *B. oryzae* (Fig. 2), the cause of rice brown spot disease throughout the world (Barnwal et al. 2013). *Oryza australiensis* and wild *Oryza* AA genome-type plants were all identified as hosts of *B. oryzae*. Brown spot disease symptoms and associated *B. oryzae* isolates were present on the wild rice populations at Abattoir Swamp and Clancy's Lagoon (Tables 2 and 3), but were not observed on rice cv. Doongara crops grown within a radius of 50–100 km.

The third most frequently isolated genus of fungi was *Curvularia* and large species diversity was observed given the relatively small number of specimens (Table 3 and Fig. 3). *Curvularia alcornii*, *C. lunata*, *C. clavata* and *C. muehlenbeckiae* were each isolated once from wild *Oryza* AA genome-type plants. *Curvularia aeria* was isolated from a cultivated rice specimen from the Burdekin region, which had suspected brown leaf spot disease and was collected independently of the surveys and submitted as a diagnostic specimen. *Curvularia asianensis* was also isolated from a cultivated rice specimen collected during the survey of the Atherton Tablelands in 2014. Finally, an undescribed *Curvularia* species was isolated from an *O. australiensis* plant that was growing near the Laura River crossing on the Peninsula Developmental Road.

Typical blast disease symptoms were rarely observed on the wild rice populations that were surveyed. During May 2014, *P. oryzae* was found twice (BRIP 61678 and BRIP 61680) on individual *Oryza* AA genome-type plants, one growing at White Lily Lagoon within Rinyirru National Park and the second at an isolated pond north of Laura, which was the JPN2 site of Sotowa et al. (2013) (Table 3). In March 2015, *P. oryzae* was found at a single location, on an *Oryza* AA genome-type plant (BRIP 62879) growing on the fringe of a dam by the Mulligan Highway between Lakeland Downs and Cooktown (Table 3).

Serious rice blast disease epidemics occurred in cultivated rice (all variety Doongara) at three locations on the Atherton Tablelands that were visited in 2014 (Fig. 4). At Arriga, west of Mareeba, the grain harvest did not proceed because the yield effect from disease was so severe (S. Rogers, pers. comm.). *Pyricularia oryzae* (BRIP 61688, BRIP 61689 and BRIP 61690) was isolated from diseased plants in this crop. *Pyricularia oryzae* was also isolated from plants showing typical blast symptoms in two other crops. All *P. oryzae* isolates from the wild and cultivated rices had identical ITS and RPB1 sequences (Fig. 5).

Susceptibility of rice to *Pyricularia oryzae*

Representative isolates of *P. oryzae* from the wild *Oryza* AA genome-type plants (BRIP 62879 and BRIP 61678), were used to inoculate a set of nine commercial rice varieties and all varieties were susceptible to infection and developed typical rice blast symptoms (Table 4). There was a noticeable difference in the aggressiveness of the two fungal isolates, with isolate BRIP 62879 consistently causing more severe disease symptoms across all rice varieties. When challenged with isolate BRIP 62879, variety Opus had the lowest average disease severity score, while Doongara, which was the variety grown on the Atherton Tablelands during 2014, was among the most susceptible to infection.

Discussion

During the surveys, a range of fungi were found associated with foliar diseases of wild rices, including *Pyricularia oryzae*, the most serious disease of cultivated rice worldwide (Ou, 1985). The results of the pathogenicity tests provide evidence that isolates of *P. oryzae* from wild rice can infect nine different cultivated rice varieties. Whether the wild rices are an important source of inoculum for rice blast epidemics in Queensland remains an open question. *Oryza* AA genome type species (*O. rufipogon* and *O. meridionalis*) have been recorded as far south as the Burdekin Region (Atlas of Living Australia), which has been identified as one of the most promising new areas for rice production (Marshall, 2015). However, *P. oryzae* has also been recorded in Queensland from *Brachiaria mutica*, *Hordeum vulgare*, *Pennisetum clandestinum*, *Phalaris canariensis*, *Rhynchelytrum repens* and *Setaria italica* (http://collections.daff.qld.gov.au/web/), which are all introduced grasses. Some of these alternative grass hosts are more widely distributed, as weeds or through cultivation, than the wild rices in the agricultural regions of northern Australia.

Pyricularia oryzae was isolated, albeit rarely, from three undescribed AA genome-type plants, but not from *O. australiensis*. The locations where *P. oryzae* was discovered were very remote (*c*. 300 km distance) from the nearest major cropping region, the Atherton Tableland. The discovery of *P. oryzae* on wild rices in both Queensland and the Northern Territory (Heaton, 1964) introduces the possibility that the fungus is also native to Australia. If this hypothesis is correct, it could be expected that local isolates of *P. oryzae* would form discrete genetic populations when compared with isolates from South-East Asia using DNA markers such as microsatellites (Maciel et al. 2014) and AFLPs (Onaga et al. 2015) or by whole genome resequencing (Mach, 2009). Furthermore, the distribution of avirulence (*Avr*) genes among Australian isolates of *P. oryzae* would likely be different to that of fungal isolates from South-East Asia, as resistance (*R*) genes have not been deployed in Australian rice cultivars and the wild rices may contain novel *R* genes that are not present in cultivated rice.

The most common plant pathogenic fungus isolated from the wild rices was *Bipolaris oryzae*, which is the cause of rice brown spot disease. Brown spot is predisposed by physiological stresses such as soil infertility or drought but nevertheless causes average yield losses of around 10 % in lowland rice crops of South and South-East Asia and was associated with the Great Bengal Famine (India and Bangladesh) of 1942 (Barnwal et al. 2013). As with *P. oryzae*, it is likely that *B. oryzae* is an indigenous pathogen of the wild rices. *Bipolaris oryzae* was not isolated from rice crops on the Atherton Tablelands, but this may reflect the good growing conditions in these crops. However, *B. oryzae* remains a threat to rice production in northern Australia, especially as this region is drought-prone and the soils are often nutrient deficient (Australian Soil Resource Information System). Major genes for brown spot disease resistance have yet to be identified in *O. sativa*, although quantitative trait loci conferring partial resistance are known (Katara et al. 2010; Sato et al. 2015).

The final group of well-recognized rice pathogens that was isolated from both wild and cultivated rices in northern Australia was *Curvularia* spp., specifically, *C. aeria, C. alcornii, C. asianensis, C. clavata*, *C. lunata* and *C. muehlenbeckiae*. None are regarded as serious pathogens of *O. sativa*, except *C. lunata*, which is one of the commonest causes of rice kernel discolouration (Ou, 1985), and has also been observed to cause leaf spotting/blight and spikelet rot (Huang et al. 2011; Liu et al. 2013; Majeed et al. 2015). *Curvularia aeria* (syn. C. *lunata* var. *aeria*), *C. asianensis* and *C. clavata* have also been isolated from rice grain (Hilda Nenínger et al. 2003; Manamgoda et al. 2012; Nakada et al. 1994; Wu, 1994). An isolate of *C. aeria* from the Philippines on *Echinochloa* sp. was a weak leaf-spotting pathogen of rice under experimental conditions (Zhang et al. 1996). To our knowledge, *C. alcornii* and *C. muehlenbeckiae* have not been previously reported on *Oryza* spp. anywhere in the world (Manamgoda et al. 2012, 2015), and neither have been previously reported from Australia on any plant species (Australasian Plant Disease Database; Hyde and Alcorn, 1993).

This study has provided baseline data on the diversity of leaf spot pathogens on wild and cultivated rices in Queensland. The accurate diagnosis of fungal pathogens is fundamental to the development of disease control strategies, as without this information it is not possible to properly determine host or geographic ranges, nor extrapolate from previous studies. There was also significant diversity between wild rice populations, and this diversity needs to be taken into account in host range and disease resistance studies. If the pathogens that were identified have co-evolved with the wild rices, it could be expected that some plant populations may have novel resistance genes, and these may be able to be incorporated in rice breeding programs. The ability of Australian isolates of *Bipolaris oryzae* and *Curvularia* spp. from wild rices in Australia, to infect cultivated rice remains to be studied.

Acknowledgements

We thank Steve Rogers and Mike Hedditch from SunRice for useful discussions, helping to organize visits to the rice farms on the Atherton Tablelands and for providing rice seed. The authors would like to acknowledge the support of the Australian Government's Cooperative Research Centres Program.

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Table 1 PCR primers used for DNA barcoding of *Oryza* spp.

Primer name	Sequence	Position[a]	Anneal. temp.	Frag.length (bp)
matK-F1	TAATTAAGAGGATTCACCAG	1669-1688	61.0	1628
matK-R1	ATGCAACACCCTGTTCTGAC	3277-3296		
psbZ-F1	TTGTATTTGCTTCTCCTGATG	12012-12032	63.5	1447
tRNAGly-R1	AAGGATCTATGGATGAAGATACA	c13436-13458		
InterG-F1	CACTGCATCCACTTAATTTC	17012-17031	65.0	614
petN-R1	GGCTGCTTTAATGGTAGTCT	c17606-17625		
rpoC2-F1	GTTCGCCATTCCATTACAGT	26459-26478	64.0	1011
rpoC2-R1	TCATACGCGACTGCAGGT	c27452-27469		
atpA-F1	CTAGCAAGACATTCACCGA	35644-35662	63.5	773
rps14-R1	CGGAAGACCTAGAGCTAACTATC	c36394-36416		
rpl14-F1	CGTCTTCGCATTTGAACT	77410-77427	63.5	589
rpl16-R1	GAGCAATGACACGATATGC	c77980-77998		

[a] Position on the *Oryza sativa* cv. Nipponbare chloroplast genome, GenBank accession GU592207.1.

Table 2 *Oryza* specimens used in this study and associated GenBank numbers

Species	BRIP Acc.	Location[1]	GenBank accession no.					
			MatK	rpoC2	InterG	atpA-rps14	rpl14-rpl16	psbZ-tRNAGly
O. australiensis	61669	Mary Creek (10)	KU923970	KU923886	KU923942	KU923833	KU923913	KU923861
	61670	Mary Creek (10)	KU923971	KU923887	KU923943	KU923834	KU923914	KU923862
	61679	12 Mile Lagoon Rd (4)	KU923979	KU923894	KU923951	KU923842	KU923922	KU923869
	61674	Laura River Crossing (8)	KU923975	KU923890	KU923947	KU923838	KU923918	KU923865
	61675	Laura River Crossing (8)	KU923976	KU923891	KU923948	KU923839	KU923919	KU923866
	61677	Laura River Crossing (8)	KU923977	KU923892	KU923949	KU923840	KU923920	KU923867
	62888	Lakeland Downs substation (9)	KU923997	KU923911	KU923968	KU923859	KU923940	KU923885
Oryza AA genome	61667	Abattoir Swamp (11)	KU923969	-	KU923941	KU923832	KU923912	KU923860
	61684	Clancy’s Lagoon (12)	KU923984	KU923899	KU923956	KU923847	KU923927	KU923874
	61686	Clancy’s Lagoon (12)	KU923985	KU923900	KU923957	KU923848	KU923928	KU923875
	62876	Abattoir Swamp (11)	KU923991	KU923906	KU923963	KU923854	KU923934	KU923881
	62877	Abattoir Swamp (11)	KU923992	KU923907	KU923964	KU923855	KU923935	-
	62879	Abattoir Swamp (11)	KU923993	KU923908	KU923965	KU923856	KU923936	KU923882
	62880	Unnamed dam, Mulligan	KU923994	KU923909	KU923966	KU923857	KU923937	KU923883

		Hwy (7)						
	61673	Unnamed road, Rinyirru (3)	KU923974	KU923889	KU923946	KU923837	KU923917	KU923864
	61681	Sandy Creek (6)	KU923981	KU923896	KU923953	KU923844	KU923924	KU923871
	61682	Red Lily Lagoon (1)	KU923982	KU923897	KU923954	KU923845	KU923925	KU923872
	61683	Red Lily Lagoon (1)	KU923983	KU923898	KU923955	KU923846	KU923926	KU923873
	61687	Pandanus Lagoon (13)	KU923986	KU923901	KU923958	KU923849	KU923929	KU923876
	62881	Unnamed dam, Mulligan Hwy (7)	KU923995	KU923910	KU923967	KU923858	KU923938	KU923884
	61671	Rinyirru National Park	KU923972	-	KU923944	KU923835	KU923915	KU923863
	61672	Rinyirru National Park	KU923973	KU923888	KU923945	KU923836	KU923916	-
	61678	White Lily Lagoon (2)	KU923978	-	KU923950	KU923841	KU923921	KU923868
	61680	25 km NW of Laura (5)	KU923980	KU923895	KU923952	KU923843	KU923923	KU923870
O. sativa var. Doongara	61688	Arriga (14)	KU923987	KU923902	KU923959	KU923850	KU923930	KU923877
	61689	East Barron Rd, Wongabel (16)	KU923988	KU923903	KU923960	KU923851	KU923931	KU923878
	61690	East Barron Rd, Wongabel (16)	KU923989	KU923904	KU923961	KU923852	KU923932	KU923879
	61691	Wongabel Rd, Wongabel (15)	KU923990	KU923905	KU923962	KU923853	KU923933	KU923880

[1] Numbers in parentheses refer to the sites shown in Fig. 1.

Table 3 Fungi, hosts, isolates and GenBank accession numbers used in the phylogenetic analyses (ex-type cultures are in bold).

Species	**Host**	**Country**	**Accession no.**	**GenBank accession no.**			
				ITS	**RPB1**	**GPDH**	**TEF**
Alternaria alternata	Unknown	India	**EGS 34.0160**	AF071346	-	AF081400	-
Barretomyces calatheae	*Calathea longifolia*	Brazil	**CBMAI 1060**	GU294490	-	-	-
Bipolaris chloridis	*Chloris gayana*	Australia	**CBS 242.77**	JN192372	-	JN600961	-
B. clavata	*Dactyloctenium radulan*	Australia	BRIP 12530	KJ415524	-	KJ415422	KJ415471
B. microlaenae	*Microlaena stipoides*	Australia	**BRIP 15613**	JN601032	-	JN600974	JN601017
B. oryzae	*Oryza sativa*	Thailand	**MFLUCC 10-0715**	JX256416	-	JX276430	JX266585
	Oryza AA genome hp A	Australia	BRIP 61667	KU552185	-	KU552175	KU552150
	O. australiensis hp B	Australia	BRIP 61669b	KU552186	-	KU552169	KU552144
	Oryza AA genome hp E	Australia	BRIP 61673b	KU552187	-	KU552170	KU552145
	O. australiensis hp D	Australia	BRIP 61674	KU552188	-	-	-
	O. australiensis hp D	Australia	BRIP 61675	KU552189	-	KU552171	KU552146
	O. australiensis hp E	Australia	BRIP 61677	KU552190	-	-	-
	Oryza AA genome hp G	Australia	BRIP 61678	KU552191	-	-	-
	O. australiensis hp F	Australia	BRIP 61679a	KU552192	-	KU552172	KU552147
	Oryza AA genome hp E	Australia	BRIP 61681	KU552193	-	KU552173	KU55214

	Oryza AA genome hp C	Australia	BRIP 61682	KU552194	-	-	-
	Oryza AA genome hp A	Australia	BRIP 61684b	KU552195	-	KU552177	KU552152
	grass	Australia	BRIP 61685b	KU552196	-	KU552178	KU552153
	Oryza AA genome hp A	Australia	BRIP 61686a	KU552197	-	KU552179	KU552154
	Oryza AA genome hp A	Australia	BRIP 62876	KU552198	-	KU552176	KU552149
B. panici-miliacei	*Panicum miliaceum*	Japan	**CBS 199.29**	KJ909773	-	KM042896	KM093788
B.peregianensis	*Cynodon dactylon*	Australia	**BRIP 12790**	JN601034	-	JN600977	JN601022
Curvularia alcornii	*Zea mays*	Thailand	**MFLUCC 10-0703**	JX256420	-	JX276433	JX266589
	Oryza AA genome hp G	Australia	BRIP 61672a	KU552202	-	KU552164	KU552157
C. aeria	Air	Brazil	**CBS 294.61**	NR130689	-	-	-
	Oryza sativa	Australia	BRIP 61232b	KU552200	-	KU552162	KU552155
C. asianensis	*Panicum* sp.	Thailand	**MFLUCC 10-0711**	JX256424	-	JX276436	JX266593
	Oryza sativa	Australia	BRIP 61689	KU552206	-	-	KU552160
C. australiensis	*Oryza sativa*	Australia	**IMI 53994**	KC424595		KC747744	KC503939
C. clavata	Unknown	Canada	DAOM 148084	AF071336		AF081391	

	Oryza AA genome hp G	Australia	BRIP 61672b	KU552203	-	KU552165	KU552158
	Oryza AA genome hp B	Australia	BRIP 61680	KU552205	-	KU552167	KU552159
C. coicis	*Coix lacryma*	Japan	**CBS 192.29**	AF081447	-	AF081410	JN601006
C. ellisii	Air	Pakistan	**CBS 193.62**	JN192375	-	JN600963	JN601007
C. graminicola	unknown	Australia	**BRIP 23186**	JN192376	-	JN600964	JN601008
C. heteropogonis	*Heteropogon contortus*	Australia	**CBS 284.91**	JN192379	-	JN600969	JN601013
C. hominis	human cornea	USA	**UTHSC09-464**	HG779011	-	HG779106	
C. lunata	human lung biopsy	USA	**CBS 730.96**	JX256429	-	JX276441	JX266596
	Oryza AA genome hp A	Australia	BRIP 62880a	KU552207			KU552161
C. muehlenbeckiae	*Muehlenbeckia* sp.	India	**CBS 144.63**	HG779002	-	HG779108	-
	Oryza AA genome hp G	Australia	BRIP 61671	KU552201	-	KU552163	KU552156
C. ovariicola	*Eragrostis interrupta*	Australia	**CBS 470.90**	JN192384	-	JN600976	JN601020
C. ravenelii	*Sporobolus fertilis*	Australia	**BRIP 13165**	JN192386	-	JN600978	JN601024
C. tripogonis	unknown	Australia	**BRIP 12375**	JN192388	-	JN600980	JN601025
C. tsudae	*Chloris gayana*	Japan	**ATCC 44764**	KC424596	-	KC747745	KC503940
C. tuberculata	*Zea mays*	India	**CBS 146.63**	JX256433	-	JX276445	JX266599
Curvularia sp.1	*Oryza australiensis* hp D	Australia	BRIP 61674	KU552204	-	KU552166	-
Pyricularia ctenantheicola	*Ctenanthe oppenheimiana*	Greece	**GR0002**	KM484879	KM485099	-	-
P. grisea	*Digitaria* sp.	USA	M82	JX134670	JX134724	-	-

	Digitaria sp.	USA	M83	JX134671	JX134725	-	-
P. oryzae		USA	70-15 = ATCC MYA-4617	Genome	Genome	-	-
	Oryza AA genome hp G	Australia	BRIP 61678	KU552208	KU552180	-	-
	Oryza AA genome hp B	Australia	BRIP 61680 a	KU552209	KU552183	-	-
	Oryza sativa	Australia	BRIP 61688 c	KU552210	KU552181	-	-
	Oryza sativa	Australia	BRIP 61689 b	KU552211	KU552182	-	-
	Oryza sativa	Australia	BRIP 61690 a	KU552212	KU552184	-	-
	Oryza AA genome hp A	Australia	BRIP 62879	KU552213	-	-	-
P. penniseticola	*Pennisetum typhoides*	**Mali**	**ML0031**	KM484929	KM485148	-	-
P. pennisetigena	*Pennisetum* sp.	**Mali**	**ML0036**	KM484935	KM485153	-	-

[a] Abbreviations are: ATCC - American Type Culture Collection, Manassas, Virginia; BRIP, Plant Pathology Herbarium, Brisbane, Queensland, Australia; CBMAI - Coleção Brasileira de Microrganismos de Ambiente e Industria of the UNICAMP, Brazil; CBS - Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, the Netherlands; DAOM - Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada; MFLUCC - Mae Fae Luang University Culture Collection, Chiang Rai, Thailand; Genome: Broad Institute, USA.

Table 4 Disease severity (%) after inoculation of cultivated rice (*Oryza sativa*) with *Pyricularia oryzae* isolates from the wild rices

Rice variety	PDS[a]	
	BRIP 61678	BRIP 62879
Doongara	6 a	27 cd
Langi	2 a	36 d
Kyeema	4 a	20 abc
Illabong	3 a	15 abc
Sherpa	1 a	11 ab
Reiziq	6 a	22 abc
Koshi	1 a	25 bcd
Opus	5 a	10 a
Quest	8 a	16 abc

[a] Values are the means of three replicates and those with the same letter are not statistically different based on Duncan's Multiple Range Test (DMRT) at $P<0.05$.

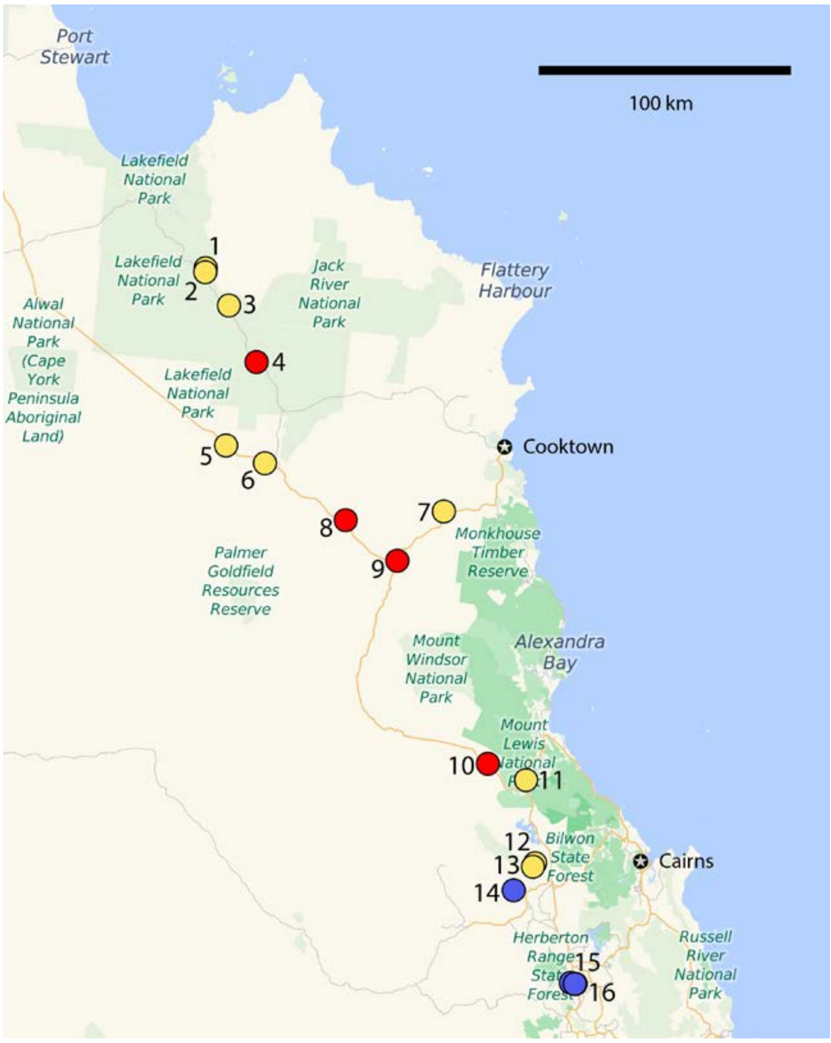

Fig. 1 Survey sites in north Queensland, where red, yellow and blue circles represent *Oryza australiensis*, undescribed *Oryza* AA genome taxa and *Oryza sativa*, respectively. The sites, as numbered, are: (1) Red Lily Lagoon, Rinyirru (-14.8494, 144.1678); (2) White Lily Lagoon, Rinyirru (-14.8611, 144.1664); (3) unnamed road, Rinyirru (-14.9794, 144.2528); (4) 12 Mile Lagoon Road, New Laura (-15.1772, 144.3522); (5) unnamed pond, Peninsula Developmental Road, 25 km north of Laura (-15.4694, 144.2422); (6) unnamed lagoon near Sandy Creek, Peninsula Developmental Road (-15.5308, 144.3831); (7) unnamed dam, Mulligan Highway (-15.699359, 145.02981); (8) near Laura River Crossing, Peninsula Developmental Road (-15.7303, 144.6747); (9) Lakeland Downs substation (-15.872457, 144.860644); (10) Mary Creek Crossing, Mulligan Highway (-16.5792, 145.1889); (11) Abattoir Swamp (-16.8539, 145.4475); (12) Clancy's Lagoon, Mareeba Wetlands Reserve; (13) Pandanus Lagoon, Mareeba Wetlands Reserve (-16.9269, 145.3603); (14) Arriga (-17.0183, 145.2814); (15) Wongabel Road, Wongabel (-17.3386, 145.4886); (15) Wongabel Road, Wongabel (-17.3386, 145.4886); (16) East Barron Road, Wongabel (-17.3422, 145.505).

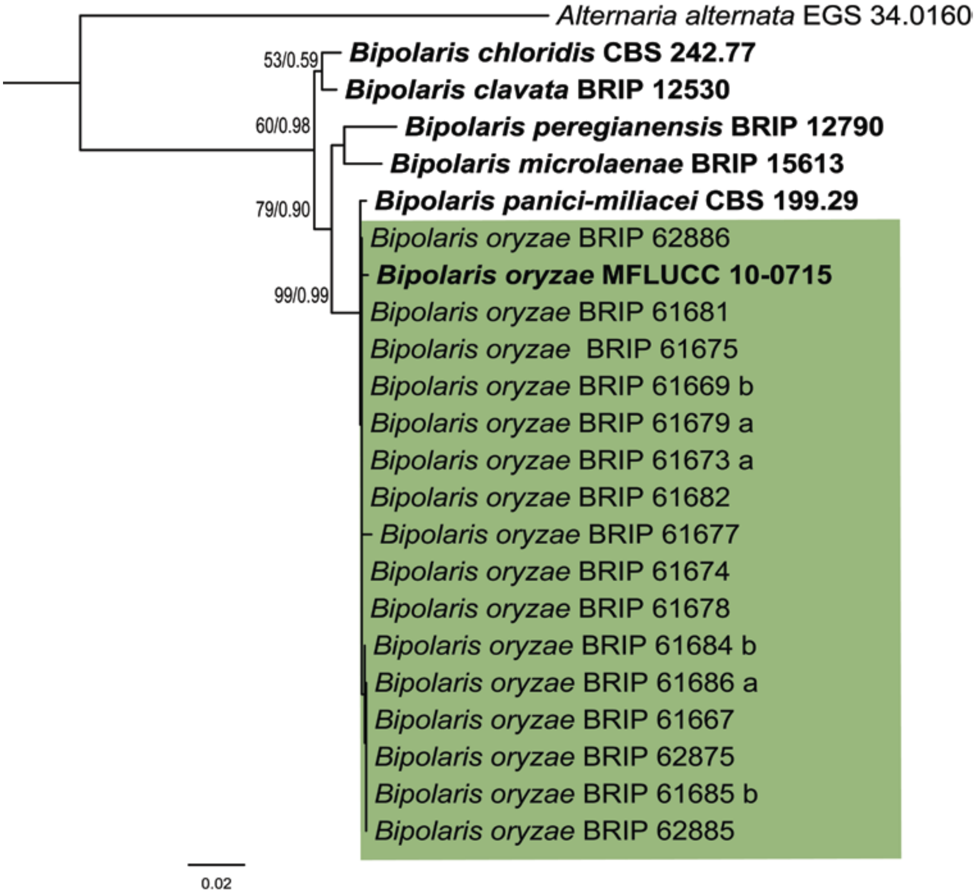

Fig. 2 Phylogenetic relationships of *Bipolaris* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes. Analysis done using the maximum likelihood (ML) method, as implemented in RaxML. Bootstrap values (>70 %) from 1,000 replicates in an ML search shown above nodes; posterior probabilities (>0.95) from Bayesian inference shown below nodes. Details of fungal isolates used in the analysis are provided in Table 3, and ex-type cultures are written in bold. *Alternaria alternata* was included as an outgroup.

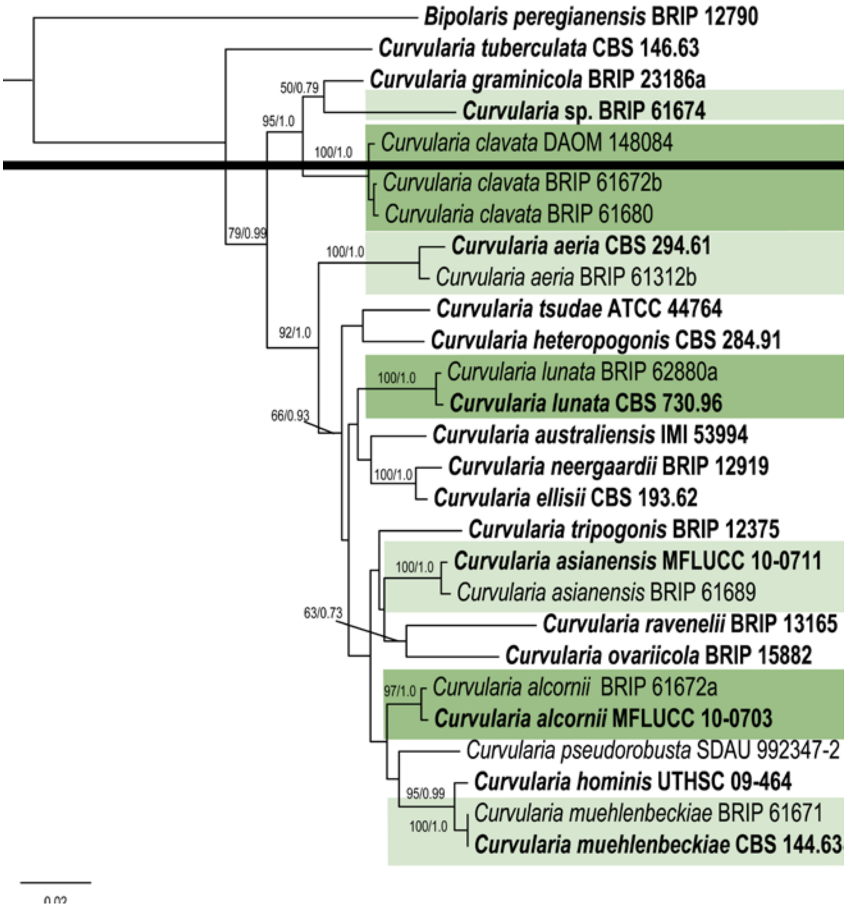

Fig. 3 Phylogenetic relationships of *Curvularia* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF1) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes. The analysis was done using the maximum likelihood (ML) method, as implemented in RaxML. Bootstrap values (>70 %) from 1,000 replicates in an ML search shown above nodes; posterior probabilities (>0.95) from Bayesian inference shown below nodes. Details of fungal isolates used in the analysis are provided in Table 3, and ex-type cultures are written in bold. *Bipolaris peregianensis* was included as an outgroup.

Fig. 4 Rice blast disease symptoms in a rice variety Doongara crop at Atherton, Queensland, in March 2014.

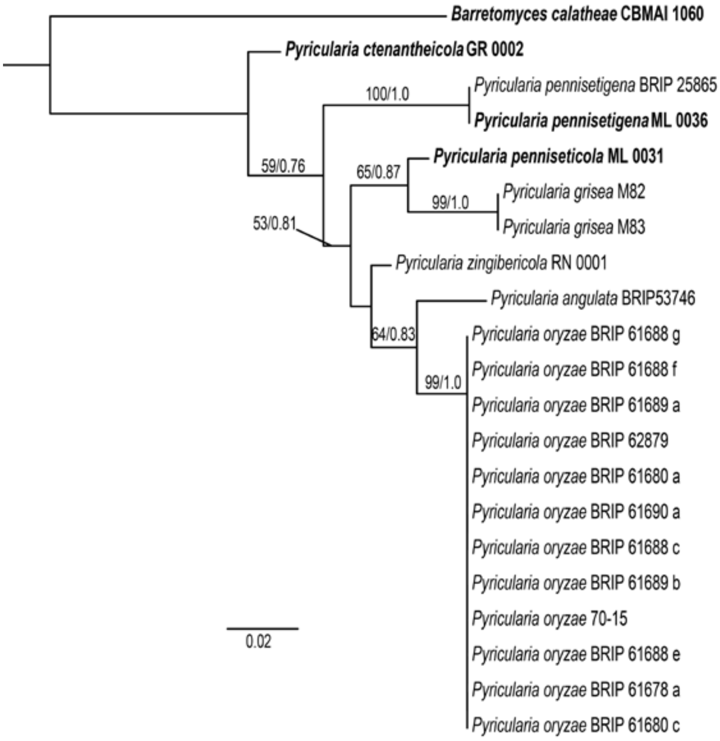

Fig. 5 Phylogenetic relationships of *Pyricularia* species, inferred using DNA sequence data from the rDNA internal transcribed spacer (ITS) and the large subunit of RNA polymerase II. The analysis was done using the maximum likelihood (ML) method, as implemented in RaxML. Bootstrap values (>70 %) from 1,000 replicates in an ML search shown above nodes; posterior probabilities (>0.95) from Bayesian inference shown below nodes. Details of fungal isolates used in the analysis are provided in Table 3, and ex-type cultures are written in bold. *Barretomyces calatheae* was included as an outgroup.

Appendix 7: ***Wongia*** **gen. nov. (*Papulosaceae, Sordariomycetes*), new generic name for two root-infecting fungi from Australia**

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Abstract: The classification of two root-infecting fungi, *Magnaporthe garrettii* and *M. griffinii*, was examined by phylogenetic analysis of multiple gene sequences. This analysis demonstrated that *M. garrettii* and *M. griffinii* were sister species that formed a well-supported separate clade in *Papulosaceae* (*Diaporthomycetidae*, *Sordariomycetes*), which clusters outside of the *Magnaporthales*. *Wongia* gen. nov, is established to accommodate these two species, which are not closely related to other species classified in *Magnaporthe* nor to other genera, including *Nakataea*, *Magnaporthiopsis* and *Pyricularia*, which all now contain other species once classified in *Magnaporthe*.

Key words: *Ascomycota*, *Cynodon*, *Diaporthomycetidae*, multigene analysis, one fungus-one name, molecular phylogenetics, root pathogens

Article info: Received: 5 July 2016; Accepted: 7 October 2016; Published: 11 October 2016.

INTRODUCTION

The taxonomic and nomenclatural problems that surround generic names in the *Magnaporthales* (*Sordariomycetes*, *Ascomycota*), together with recommendations for the suppression and protection of some of these names, were explained by the *Pyricularia/Magnaporthe* Working Group established under the auspices of the International Commission on the Taxonomy of Fungi (ICTF; Zhang *et al*. 2016). One of these generic names, *Magnaporthe,* was proposed for suppression by Zhang *et al*. (2016) because *Magnaporthe* is congeneric with *Nakataea* (Hara 1939) as the types of both genera, *Magnaporthe salvinii* (syn. *Leptosphaeria salvinii*) and *Nakataea sigmoidea* (syn. *Helminthosporium sigmoideum*) are conspecific(Krause & Webster 1972, Luo & Zhang 2013).

Magnaporthe was morphologically characterised by having dark perithecia with long necks immersed in host tissue, unitunicate asci, and 4-celled fusiform hyaline to pale brown ascospores (Krause & Webster 1972). Subsequently, seven species were assigned to *Magnaporthe* based on morphology, namely, *M. salvinii* (Krause & Webster 1972), *M. grisea* (Barr 1977), *M. rhizophila* (Scott & Deacon 1983), *M. poae* (Landschoot & Jackson 1989), *M. oryzae* (Couch & Kohn 2002), and *M. garrettii* and *M. griffinii* (Wong et al. 2012). Most of these species belong to other genera, specifically *Magnaporthiopsis*, *Nakataea*, and *Pyricularia* (Luo & Zhang 2013). The two exceptions are the Australian ectotrophic species, *M. garrettii* and *M. griffinii*, which infect roots of some turf grasses (Wong *et al.* 2012). One of these species, *M. griffinii*, was found by Klaubauf et al. (2014) to be distant from *Sordariomycetes* based on ITS sequences (GenBank JQ390311, JQ390312).

This study aims to resolve the classification of *M. garrettii* and *M. griffinii* using molecular sequence data from the type specimens. Four loci from the nuclear genome namely, ITS) and the large subunit (LSU) of rDNA, translation elongation factor 1-alpha (TEF1), and the largest subunit of RNA polymerase II (RPB1) were selected for analysis.

MATERIALS AND METHODS

Fungal cultures and DNA extraction

Dried specimens which were the holotypes of *Magnaporthe garrettii* (DAR 76937) and *M. griffinii* (DAR 80512) were borrowed from the Plant Pathology Herbarium, New South Wales Agriculture (DAR). Dried perithecia were excised with a needle and soaked in extraction buffer overnight at 65 ºC before extraction of DNA with an UltraClean® Microbial DNA Isolation Kit (MoBIO Laboratories) as per the manufacturer's instructions. An additional culture of *M. griffinii* (BRIP 60377) was grown on PDA for 6 wk before enough mycelium was produced for DNA extraction.

PCR amplification

The primer pairs ITS1/ITS4 (White *et al.* 1990), RPB-Ac/RPB-Cr (Castlebury *et al*. 2004, Matheny *et al*. 2002), LR5/LROR and EF1983F/2218R (Schoch *et al*. 2009) were used to amplify ITS, RPB1, LSU, and TEF1 sequences, respectively. PCR amplifications were conducted in a 20 µl reaction volume containing 1 µl of 5-10 ng DNA, 10 µl of high fidelity Phusion DNA Polymerase (New England Biolabs), 1 µl of primers (10 µM) and 7 µl of sterile water with the thermal cycling program as follows: 98 ºC for 30s, 30 cycles of 98 ºC for 10 s, 58–62 ºC for 30 s and 72 ºC for 1 min, and a final extension of 72 ºC for 10 min. PCR products were sent to Macrogen (Korea) for direct sequencing using the amplification primers.

Phylogenetic analysis

All sequences were assembled with Sequencher v. 5.1 (Gene Codes Corp., Ann Arbor, MI). Alignments were generated for individual loci using MAFFT v. 6.611 (Katoh & Toh 2008), and then the alignments concatenated for the phylogenetic analyses. DNA sequences were deposited in GenBank with the accession numbers listed in Table 1 and the final curated alignment deposited in TreeBASE under accession no, ID 19968. Phylogenetic trees were reconstructed with two phylogenetic criteria, Maximum likelihood (ML) and Bayesian Inference (BI). ML was carried out with RAxML v. 7.2.6 using GTRGAMMA as the model of evolution (Stamatakis 2006), choosing the rapid bootstrap analysis (command –f a) with a random starting tree and 1000 maximum likelihood bootstrap replications. BI was done with MrBayes v. 3.1.2 (Ronquist *et al*. 2012), utilizing four parallel MCMC chains, which were allowed to run for 10 million generations, with sampling every 1000 generations and saving trees every 5 000 generations. The cold chain was heated at a temperature of 0.25. All phylogenetic trees were visualized using FigTree (Morariu *et al.* 2009).

RESULTS

Molecular phylogeny

The phylogenetic trees recovered from the ML and BI analyses had identical topologies and were well-supported by bootstrap and posterior probabilities (Fig. 1). The analyses comprised 36 taxa belonging to eight orders and two families in the subclass *Diaporthomycetidae* (*Sordariomycetes*). *Camarops ustulinoides* (*Boniliales*, *Sordariomycetes*) was used as the outgroup (Table 1). The phylogenetic analysis revealed *Magnaporthe garrettii* (DAR 76937) and *M. griffinii* (DAR 80512) as sister species that formed a distinct well-supported (100/1.0) monophyletic clade in *Papulosaceae* that sat outside *Magnaporthales*. The analysis provided moderate support (67/0.93) for placement of *M. garrettii* and *M. griffinii* in *Papulosaceae*, which has not yet been assigned to any order of *Diaporthomycetidae*. Based on this analysis, a new generic name is established here to accommodate *M. garrettii* and *M. griffinii*.

TAXONOMY

Wongia Khemmuk, Geering & R.G. Shivas, **gen. nov.**

MycoBank MB817529

Etymology: Named after the eminent Australian mycologist and plant pathologist, Percy T.W. Wong (University of Sydney), who first studied and classified these fungi.

Diagnosis: Differs from all other genera in the subclass *Diaporthomycetidae* in having non-amyloid apical rings in the asci with 3-septate ascospores that have dark brown middle cells and pale brown to subhyaline shorter distal cells.

Type species: *Wongia garrettii* (P. Wong & M.L. Dickinson) Khemmuk *et al.* 2016

Classification: *Ascomycota*, *Sordariomycetes*, *Diaporthomycetidae.*

Description: *Mycelium* comprised of brown, straight or flexuous hyphae, with simple hyphopodia. *Ascomata* perithecial, superficial and immersed, mostly solitary or sometimes aggregated in small groups, globose, black, ostiolate, with a long or short neck, perithecial wall composed of textura epidermoidea, external cell much darker. *Paraphyses* thin-walled, hyaline, filiform, septate.

Asci unitunicate in structure, cylindrical, mostly straight, short stalked, tapered towards a rounded apex, with a light refractive, non-amyloid apical ring, 8-spored. *Ascospores* uniseriate, cylindrical to fusiform, straight or slightly curved with rounded ends, 3-septate, middle cells dark brown and distal cells pale brown to subhyaline and shorter.

Wongia garrettii (P.T.W. Wong & M.L. Dickinson) Khemmuk, Geering & R.G. Shivas, **comb. nov.**

(Fig. 2A–B)

MycoBank MB817530

Basionym: *Magnaporthe garrettii* P.T.W. Wong & M.L. Dickinson, *Australian Plant Pathology* **41**: 326 (2012).

Type: Australia: *South Australia*: Adelaide, Colonel Light Gardens Bowling Club, on *Cynodon dactylon*, 30 Oct. 2004, *M.L. Dickinson* (DAR 76937 – holotype).

Description and illustration: Wong *et al.* (2012)

Wongia griffinii (P.T.W. Wong & A.M. Stirling) Khemmuk, Geering & R.G. Shivas, **comb. nov.**

(Fig. 2C–D)

MycoBank MB817531

Basionym: *Magnaporthe griffinii* P.T.W. Wong & A.M. Stirling, *Australian Plant Pathology* **41**: 327 (2012).

Type: Australia: *Queensland*: Coolum, Hyatt Coolum Golf Club, on *Cynodon dactylon* × *transvaalensis*, 13 Mar. 2008, *M. Whatman* (DAR 80512 – holotype).

Description and illustration: Wong *et al*. (2012)

Other specimens examined: Australia: *New South Wales*: Cobbitty, on *Cynodon dactylon*, 19 Apr. 2013, *G. Beehag*, (BRIP 60378). *Queensland*: Brisbane, on on *Cynodon dactylon* × *transvaalensis*, Jan. 2000, *A.M. Stirling* (BRIP 60377).

DISCUSSION

Magnaporthe is a synonym of *Nakataea* as their respective type species, *Magnaporthe salvinii* and *Nakataea sigmoidea*, refer to the same species (Krause & Webster 1972, Luo & Zhang 2013, Klaubauf *et al*. 2014, Zhang *et al*. 2016). This led us to re-examine two Australian species, *M. garrettii* and *M. griffinii*, pathogenic on roots of couch (*Cynodon dactylon*) and hybrid couch (*C. dactylon* × *transvaalensis*) (Wong *et al.* 2012). We established *Wongia* here to accommodate these two species, based on molecular and morphological analysis.

Multigene analyses placed *W. garrettii* and *W. griffinii* in *Papulosaceae* (*Diaporthomycetidae*, *Sordariomycetes*; Maharachchikumbura *et al.* 2015) with moderate bootstrap support (Fig. 1). The *Papulosaceae* has not yet been placed in an order within *Sordariomycetes* (Winka & Erikson 2000). *Wongia* is the fourth genus to be placed in *Papulosaceae*, along with *Brunneosporella* (Ranghoo & Hyde 2001), *Fluminicola* (Wong *et al.* 1999). and *Papulosa* (Kohlmeyer & Volkmann-Kohlmeyer 1993). Most members in this family are found on submerged wood in freshwater habitats and grow slowly in culture on potato dextrose agar (Ranghoo & Hyde 2001). *Wongia garrettii* and *W. griffinii* are morphologically different from other genera of *Papulosaceae* in having non-amyloid apical rings in the asci using Melzer's reagent, while others have amyloid apical rings (Winka and Eriksson 2000). The long perithecial necks of *W. garrettii* differentiate it from *W. griffinii* (Wong *et al.* 2012), which also has larger ascospores (24–35 x 6–9 µm) than *W. garrettii* (19–25 x 5–7 µm) (Wong *et al*. 2012). Asexual morphs have not been found in either *W. garrettii* or *W. griffinii* in nature nor in cultures grown on artificial media under laboratory conditions (Wong *et al*. 2012).

ACKNOWLEDGEMENTS

We acknowledge support of the Australian Government's Cooperative Research Centres Program (project no. PBCRC62082), and the Plant Pathology Herbarium, New South Wales Agriculture (DAR), for lending us the type specimens of the two species for sequencing.

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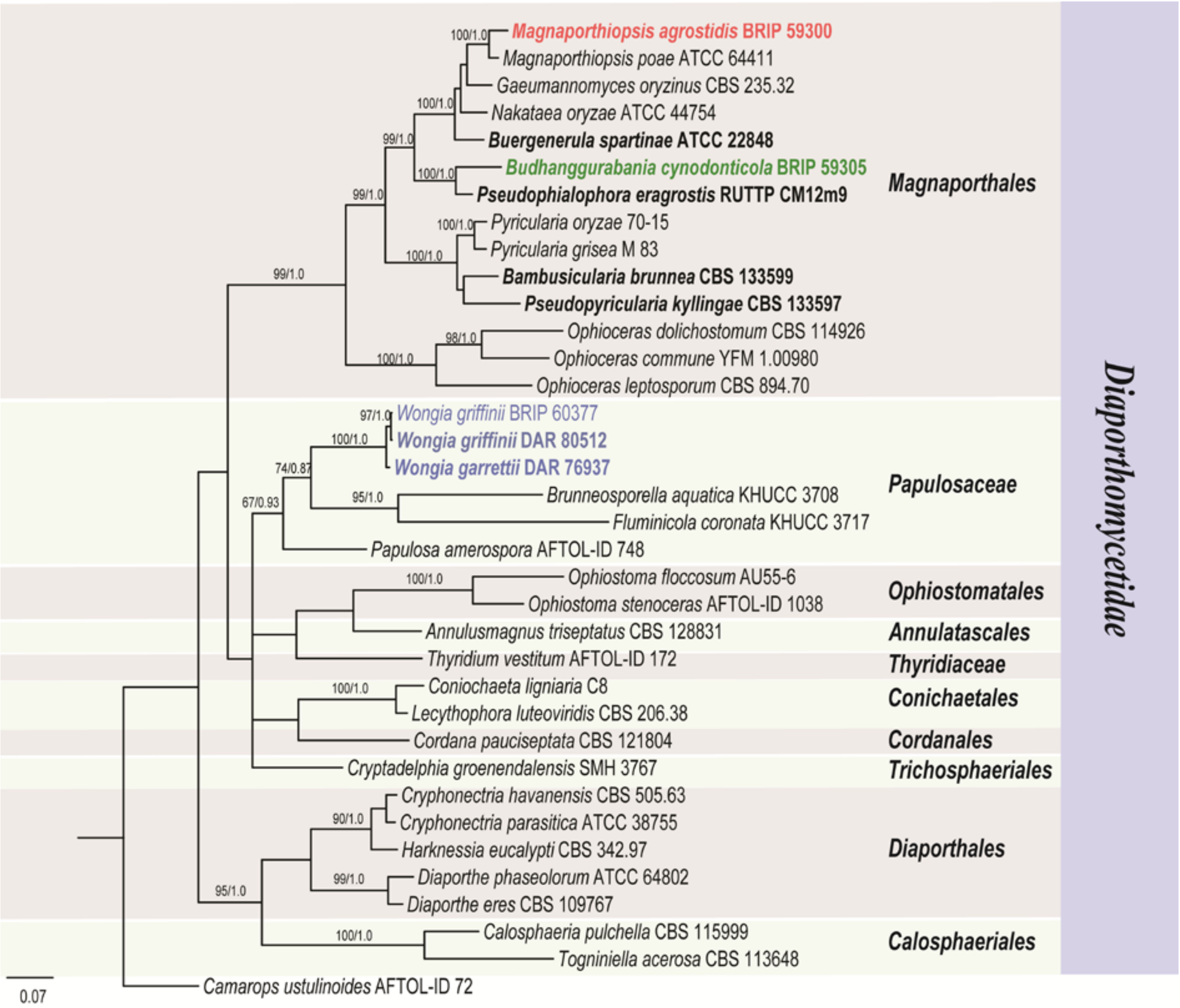

Fig.1 Phylogenetic tree obtained from a maximum likelihood analysis of the combined ITS/LSU/RPB1/TEF1 alignment. The bootstraps support values from 1 000 replicates and posterior probabilities obtained in Bayesian analysis are indicated at the nodes. The scale bar indicates the expected changes per site. Ex-type cultures of species are indicated in bold.

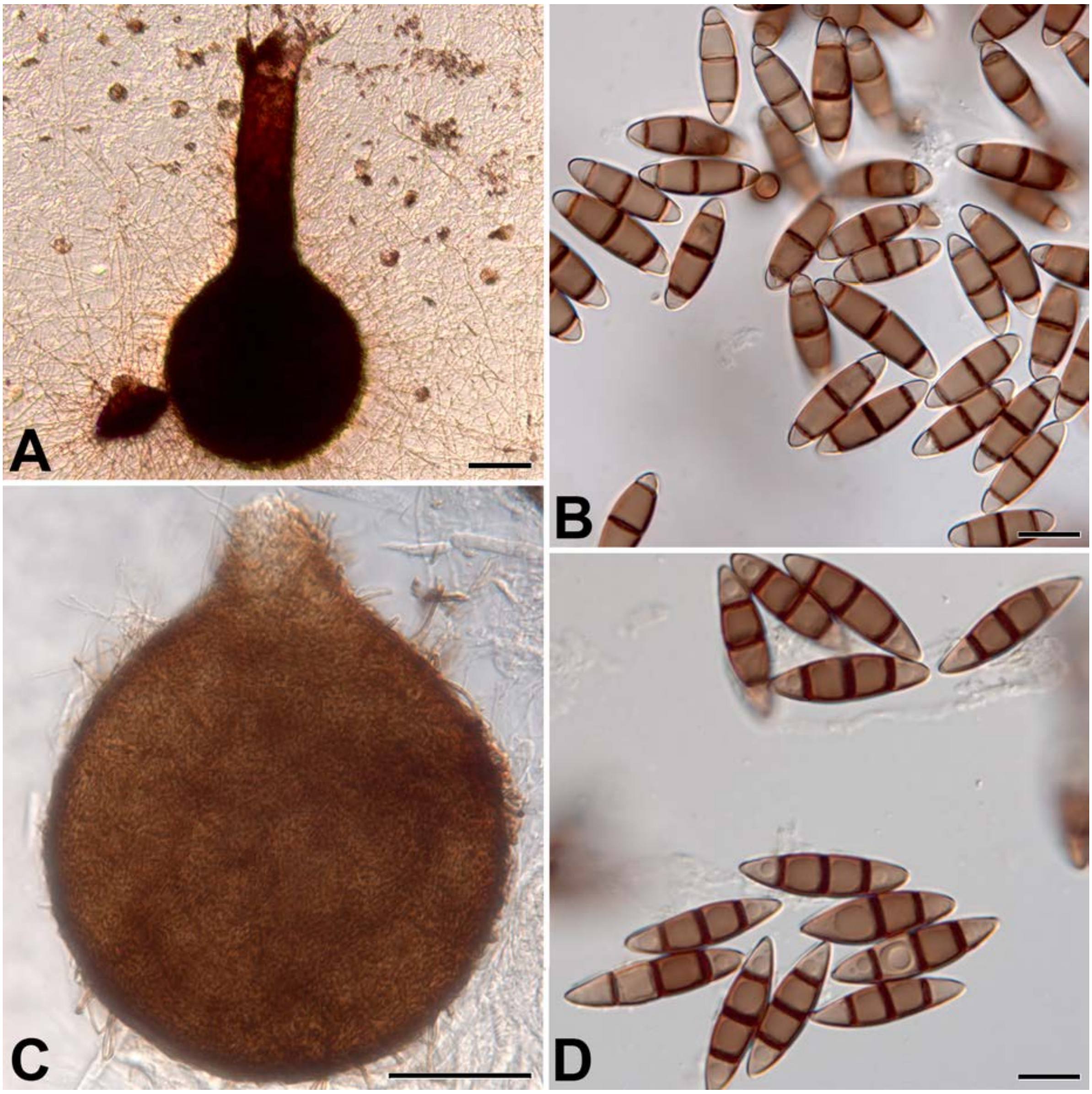

Fig. 2 Morphological features of *Wongia* species. A–B. *W. garrettii* (DAR 76937 – holotype). A. Perithecium. B. Ascospores. C–D. *W. griffinii* (BRIP 60378). C. Perithecium. D. Ascospores. Bars: A, D = 100 µm; B, D = 10 µm.

Table 1. Collection details and GenBank accession numbers of isolates included in this study.

				GenBank accession no.[2]			
Species	**Voucher**[1]	**Substrate**	**Locality**	**ITS**	**LSU**	**RPB1**	**TEF1**
Annulusmagnus triseptatus	CBS 128831	decayed wood	France		GQ996540		
Bambusicularia brunnea	CBS 133599^{T}	Sasa sp.	Japan	KM484830	KM484948	KM485043	
Barretomyces calatheae	CBMAI 1060^{T}	Calathea longifolia	Brazil	GU294490			
Brunneosporella aquatica	HKUCC 3708	submerged wood	Hong Kong		AF132326		
Budhanggurabania cynodonticola	BRIP 59305^{T}	Cynodon dactylon	Australia	KP162134	KP162140	KP162143	KP162138
Buergenerula spartinae	ATCC 22848^{T}	Spartina alterniflora	-	JX134666	DQ341492	JX134720	JX134692
Calosphaeria pulchella	CBS 115999	Prunus avium	France		AY761075		
Camarops ustulinoides	AFTOL-ID 72	-	-		DQ470941	DQ471121	DQ471050
Coniochaeta ligniaria	NRRL 30616	soil	-		AY198388		
Cordana pauciseptata	CBS 121804	-	Spain		HE672160		
Cryphonectria havanensis	CBS 505.63	Eucalyptus saligna	Russia		AF408339		
C. parasitica	ATCC 38755	Castanea dentata	USA	Genome[3]	Genome[a]	Genome[3]	Genome[3]
Diaporthe eres	CBS 109767	Acer campestre	Austria		AF408350		
Diaporthe phaseolorum	ATCC 64802	-	-		AY346279		
Fluminicola coronata	HKUCC 3717	-	Hong Kong		AF132332		
Gaeumannomyces oryzinus	CBS 235.32	Oryza sativa	USA	JX134669	JX134681	JX134723	JX134695
Harknessia eucalypti	CBS 342.97	Eucalyptus regnans	Australia		AF408363		
Lecythophora luteoviridis	CBS 206.38	-	Switzerland		FR691987		
Magnaporthiopsis agrostidis	BRIP 59300^{T}	Agrostis stolonifera	Australia	KT364753	KT364754	KT364755	KT689623
M. poae	ATCC 64411	Triticum aestivum	USA	JF414836	JF414885	JF710433	JF710415
Nakataea oryzae	ATCC 44754	Oryza sativa	Japan	JF414838	JF414887	JF710441	JF701406
Neurospora crassa	MUCL 19026	-	-		AF286411		
Ophioceras leptosporum	CBS 894.70	dead stemt	UK	JX134678	JX134690	JX134732	JX134704
O. dolichostomum	CBS 114926	rotten wood	China	JX134677	JX134689	JX134731	JX134703
O. commune	YMF1.00980	rotten wood	China	JX134675	JX134687	JX134729	JX134701

Ophiostoma floccosum	AU55-6 in G	Pinus sp.	Canada		AF234836		
O. stenoceras	AFTOL-ID 1038	-	-		DQ836904		
Papulosa amerospora	AFTOL-ID 748	-	-		DQ470950	DQ471143	DQ471069
Pseudophialophora eragrostis	RUTTP-CM12m9[T]	Eragrostis sp.	USA	KF689648	KF689638	KF689618	KF689628
Pseudopyricularia kyllingae	CBS 133597[T]	Kyllinga brevifolia	Japan	KM484876	KM484992	KM485096	
Pyricularia grisea	M 83	Digitaria sp.	USA	JX134671	JX134683	JX134725	JX134697
P. oryzae	70-15	-	USA	Genome[4]	Genome[4]	Genome[4]	Genome[4]
Togniniella acerosa	CBS 113648	decayed wood	New Zealand		AY761076		
Wongia garrettii	DAR 76937[T]	Cynodon dactylon	Australia	**KU850474**		**KU850469**	**KU850467**
W. griffinii	DAR 80512[T]	Cynodon dactylon × transvaalensis	Australia	**KU850473**	**KU850471**		
W. griffinii	BRIP 60377	Cynodon dactylon × transvaalensis	Australia	**KU850472**	**KU850470**	**KU850468**	**KU850466**

1AFTOL: Assembling the Fungal Tree of Life; ATCC: American Type Culture Collection, Manassas, VA; BRIP: Plant Pathology Herbarium, Department of Agriculture and Forestry, Queensland, Australia; CBMAI: Coleção Brasileira de Microrganismos para Ambiente e Indústria, TOWN ???, Brazil ; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; DAR: Plant Pathology Herbarium, Orange Agriculture Institute, NSW, Australia; F: Field Museum Mycology Herbarium, Chicago, IL; G: Culture Collection of the Wood Science Department, University of British Columbia, Vancouver, BC, Canada; HKUCC: Hong Kong University Culture Collection; MUCL: Mycothèque de l´Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL: American Research Service (ARS) culture collection, Beltsville, MD; RUTPP = Rutgers Mycological Herbarium, TOWN? NJ; YMF: Yunnan Microbiological Fermentation Culture Collection Center, TOWN?, Yunnan, China.

2 GenBank accession numbers of sequences newly generated in this study are in bold.

3 Joint Genome Institute, Walnut Creek, CA.

4 Broad Institute, Cambridge, MAA.

T Type specimen or ex-type culture.

Appendix 8 Total herbarium specimens of *Pyricularia* accessioned in the Plant Pathology Herbarium (BRIP), Department of Agriculture and Forestry (April, 2016)

Accession no.		Scientific name	Hosts	Locations	State	Country
BRIP	25184 a	*Magnaporthe grisea*	*Pennisetum clandestinum*	Kenilworth	QLD	Australia
BRIP	20142 c	*Magnaporthe grisea*	*Megathyrsus maximus*	Tuncul Park	QLD	Australia
BRIP	5626 a	*Magnaporthe grisea*	*Phyllostachys bambusoides*	Maroochy Horticultural Research Station, Nambour	QLD	Australia
BRIP	20141 a	*Magnaporthe grisea*	*Megathyrsus maximus* var. *pubiglumis*	Tuncul Park	QLD	Australia
BRIP	25247 a	*Magnaporthe grisea*	*Digitaria didactyla*	Maclean	QLD	Australia
BRIP	5623 a	*Magnaporthe grisea*	*Panicum coloratum*	Beerwah	QLD	Australia
BRIP	23180 a	*Magnaporthe grisea*	*Curcuma australasica*	Darnley Island	QLD	Australia
BRIP	20146 b	*Magnaporthe grisea*	*Megathyrsus maximus*	Tuncul Park	QLD	Australia
BRIP	7659 a	*Magnaporthe grisea*	*Brachiaria decumbens*	Centre for Wet Tropics Agriculture, South Johnstone	QLD	Australia
BRIP	25225 a	*Magnaporthe grisea*	*Digitaria didactyla*	Maclean	QLD	Australia
BRIP	5629 a	*Magnaporthe grisea*	*Setaria italica*	Toowoomba	QLD	Australia
BRIP	7580 a	*Magnaporthe grisea*	*Eleusine indica*	Horticultural Research Station, Kamerunga	QLD	Australia
BRIP	7577 a	*Magnaporthe grisea*	*Melinis minutiflora*	Mission Beach	QLD	Australia
BRIP	7684 a	*Magnaporthe grisea*	*Digitaria decumbens*	South Johnstone Research Station, South Johnstone	QLD	Australia

BRIP	24838 a	*Magnaporthe grisea*	*Stenotaphrum secundatum*	Clifton Park Turf Supplies, Maclean	QLD	Australia
BRIP	5634 a	*Magnaporthe grisea*	*Zea mays*	Walkamin Research Station, Walkamin	QLD	Australia
BRIP	5631 a	*Magnaporthe grisea*	*Setaria italica*	Mount Tyson	QLD	Australia
BRIP	16645 a	*Magnaporthe grisea*	*Chloris gayana*	Tuchekoi	QLD	Australia
BRIP	5632 a	*Magnaporthe grisea*	*Setaria* sp.	CSIRO Pasture Research Station, Samford	QLD	Australia
BRIP	16609 a	*Magnaporthe grisea*	*Oryza meridionalis*	Arriga	QLD	Australia
BRIP	22943 a	*Magnaporthe grisea*	*Boesenbergia* sp.	Mossman	QLD	Australia
BRIP	7660 a	*Magnaporthe grisea*	*Zea mays*	Kairi Research Station, Kairi	QLD	Australia
BRIP	5620 a	*Magnaporthe grisea*	*Eleusine indica*	Eumundi	QLD	Australia
BRIP	44103 a	*Magnaporthe grisea*	*Cenchrus ciliaris*	Gympie	QLD	Australia
BRIP	5630 a	*Magnaporthe grisea*	*Setaria italica*	Felton	QLD	Australia
BRIP	44102 a	*Magnaporthe grisea*	*Cenchrus ciliaris*	Gympie	QLD	Australia
BRIP	16629 a	*Magnaporthe grisea*	*Megathyrsus maximus* var. *pubiglumis*	Kairi Research Station, Kairi	QLD	Australia
BRIP	25413 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Kairi Research Station, Kairi	QLD	Australia
BRIP	51194 a	*Magnaporthe grisea*	*Oryza sativa*	Nevin		East Timor
BRIP	39549 a	*Magnaporthe grisea*	*Zingiber officinale*	-	Pohnpei	Federated States of Micronesia
BRIP	17762 a	*Magnaporthe grisea*	*Oryza sativa*			Guyana
BRIP	50449 a	*Magnaporthe grisea*	*Oryza sativa*	Timika Jaya		Indonesia
BRIP	50450 a	*Magnaporthe grisea*	*Oryza sativa*	Timika Jaya		Indonesia
BRIP	17818 a	*Magnaporthe grisea*	*Oryza sativa*			Unknown

BRIP	5612 a	*Magnaporthe grisea*	*Brachiaria mutica*	Woombye	QLD	Australia
BRIP	16630 a	*Magnaporthe grisea*	*Megathyrsus maximus*	Kairi Research Station, Kairi	QLD	Australia
BRIP	5624 a	*Magnaporthe grisea*	*Megathyrsus maximus* var. *pubiglumis*	Beerwah	QLD	Australia
BRIP	40249 a	*Magnaporthe grisea*	*Setaria* sp.	Malanda	QLD	Australia
BRIP	25843 a	*Magnaporthe grisea*	*Cenchrus ciliaris*	Karumba	QLD	Australia
BRIP	5621 a	*Magnaporthe grisea*	*Eleusine indica*	Woombye	QLD	Australia
BRIP	5627 a	*Magnaporthe grisea*	*Setaria anceps*	Samford	QLD	Australia
BRIP	19271 a	*Magnaporthe grisea*	*Echinochloa* sp.	Kowanyama	QLD	Australia
BRIP	5617 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Indooroopilly	QLD	Australia
BRIP	13240 a	*Magnaporthe grisea*	*Eleusine indica*	Kenmore	QLD	Australia
BRIP	16407 a	*Magnaporthe grisea*	*Cenchrus ciliaris*	south of Rolleston	QLD	Australia
BRIP	5633 a	*Magnaporthe grisea*	*Stenotaphrum secundatum*	South Stradbroke Island	QLD	Australia
BRIP	16604 a	*Magnaporthe grisea*	*Echinochloa colona*	Tuchekoi	QLD	Australia
BRIP	5619 a	*Magnaporthe grisea*	*Eleusine indica*	Indooroopilly	QLD	Australia
BRIP	44104 a	*Magnaporthe grisea*	*Cenchrus ciliaris*	Gympie	QLD	Australia
BRIP	25249 a	*Magnaporthe grisea*	*Lolium multiflorum*	Malanda	QLD	Australia
BRIP	5625 a	*Magnaporthe grisea*	*Pennisetum natalense*	CSIRO Pasture Research Station, Samford	QLD	Australia
BRIP	5616 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Christmas Creek	QLD	Australia
BRIP	40195 a	*Magnaporthe grisea*	*Lolium* sp.	Clifton	QLD	Australia
BRIP	29398 a	*Magnaporthe grisea*	*Stenotaphrum secundatum*	Redlands Research Station, Cleveland	QLD	Australia
BRIP	19435 a	*Magnaporthe grisea*	*Megathyrsus maximus*	Mabuiag Island	QLD	Australia
BRIP	5614 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Kingaroy	QLD	Australia

BRIP	39215 a	*Magnaporthe grisea*	*Pennisetum clandestinum*	Redlands Research Station, Delancey Street, Cleveland	QLD	Australia
BRIP	5628 a	*Magnaporthe grisea*	*Setaria anceps*	Lawes	QLD	Australia
BRIP	5622 a	*Magnaporthe grisea*	*Eragrostis poaeoides*	Christmas Creek	QLD	Australia
BRIP	5613 a	*Magnaporthe grisea*	*Digitaria adscendens*	Nambour	QLD	Australia
BRIP	5618 a	*Magnaporthe grisea*	*Eleusine indica*	Redland Bay	QLD	Australia
BRIP	5637 a	*Magnaporthe grisea*	*Panicum coloratum*	Beerwah	QLD	Australia
BRIP	5615 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Eumundi	QLD	Australia
BRIP	15562 a	*Magnaporthe grisea*	*Stenotaphrum secundatum*	Burnt Pine	Norfolk Island	Australia
BRIP	13272 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Duncombe Bay	Norfolk Island	Australia
BRIP	14717 a	*Magnaporthe grisea*	*Digitaria ciliaris*	Baulkham Hills	NSW	Australia
BRIP	14679 a	*Magnaporthe grisea*	*Setaria italica*	In crop, Edgeroi	NSW	Australia
BRIP	11821 a	*Magnaporthe grisea*	*Stenotaphrum secundatum*	Ashfield	NSW	Australia
BRIP	27779 a	*Magnaporthe grisea*	*Digitaria* sp.	Duranbah	NSW	Australia
BRIP	51341 a	*Magnaporthe grisea*	*Pennisetum pedicellatum*	Amadjibalk	NT	Australia
BRIP	51133 a	*Magnaporthe grisea*	*Pennisetum* sp.	Virginia	NT	Australia
BRIP	12582 a	*Magnaporthe grisea*	*Eleusine indica*	Christmas Island	WA	Australia
BRIP	51385 a	*Magnaporthe grisea*	*Cenchrus brownii*	Browse Island	WA	Australia
BRIP	39932 a	*Magnaporthe grisea*	*Cenchrus setiger*	Broome	WA	Australia
BRIP	25591 a	*Magnaporthe oryzae*	*Oryza australiensis*	Broome	WA	Australia
BRIP	39772 a	*Magnaporthe oryzae*	*Hordeum vulgare*	Goodger	QLD	Australia
BRIP	15815 a	*Magnaporthe oryzae*	*Brachiaria mutica*	Julatten	QLD	Australia
BRIP	5636 a	*Magnaporthe oryzae*	*Oryza sativa*	Burdekin	QLD	Australia
BRIP	15748 a	*Magnaporthe oryzae*	*Oryza rufipogon*	Brandon	QLD	Australia
BRIP	15526 a	*Pyricularia angulata*	*Musa* 'Cavendish'	Tully	QLD	Australia
BRIP	59458 a	*Pyricularia angulata*	*Musa* 'Williams'	Mareeba	QLD	Australia

BRIP	53746 a	*Pyricularia angulata*	*Musa* 'Cavendish'	Tully	QLD	Australia
BRIP	53869 a	*Pyricularia angulata*	*Musa* 'Cavendish'	Tully	QLD	Australia
BRIP	5610 a	*Pyricularia caricis*	Carex riparia			France
BRIP	60387 a	*Pyricularia grisea*	*Oryza sativa*	Paddys Green	QLD	Australia
BRIP	61688 a	*Pyricularia oryzae*	*Oryza sativa*	Cairns	QLD	Australia
BRIP	61690 a	*Pyricularia oryzae*	*Oryza sativa*	Cairns	QLD	Australia
BRIP	61691 a	*Pyricularia oryzae*	*Oryza sativa*	Cairns	QLD	Australia
BRIP	61680 a	*Pyricularia oryzae*	*Oryza rufipogon*	Cairns	QLD	Australia
BRIP	62336 a	*Pyricularia oryzae*	*Setaria italica*	Warwick	QLD	Australia
BRIP	61678 a	*Pyricularia oryzae*	*Oryza rufipogon*	Cairns	QLD	Australia
BRIP	61689 a	*Pyricularia oryzae*	*Oryza sativa*	Cairns	QLD	Australia
BRIP	15855 a	*Pyricularia oryzae*	*Pennisetum clandestinum*	Evelyn	QLD	Australia
BRIP	5638 a	*Pyricularia penniseti*	*Pennisetum clandestinum*	Jimboomba	QLD	Australia
BRIP	14879 a	*Pyricularia* sp. aff. *penniseti*	*Pennisetum clandestinum*	North Queensland	QLD	Australia
BRIP	15666 a	*Pyricularia* sp. aff. *penniseti*	*Pennisetum clandestinum*	Maleny	QLD	Australia
BRIP	24847 a	*Pyricularia rabaulensis*	*Alpinia arctiflora*	Mount Lewis, near Julatten	QLD	Australia
BRIP	24373 c	*Pyricularia* sp. aff. rabaulensis	*Hornstedtia scottiana*	Mossman Gorge	QLD	Australia
BRIP	22632 b	*Pyricularia rabaulensis*	*Alpinia caerulea*	Maiala National Park	QLD	Australia
BRIP	23325 a	*Pyricularia rabaulensis*	*Alpinia caerulea*	Mount Tamborine	QLD	Australia
BRIP	23858 a	*Pyricularia rabaulensis*	*Alpinia caerulea*	Levers Plateau	NSW	Australia

BRIP	23904 a	*Pyricularia rabaulensis*	*Alpinia caerulea*	Indooroopilly	QLD	Australia
BRIP	23928 a	*Pyricularia rabaulensis*	*Alpinia caerulea*	Indooroopilly	QLD	Australia
BRIP	17407 a	*Pyricularia* sp.	*Pennisetum clandestinum*	Redland Bay	QLD	Australia
BRIP	16085 a	*Pyricularia* sp.	*Orthothylax glaberrimus*	Ipswich	QLD	Australia
BRIP	16313 a	*Pyricularia* sp.	*Ottochloa gracillima*	Highvale	QLD	Australia
BRIP	16208 a	*Pyricularia* sp.	*Digitaria eriantha*	'Roxborough', Miles	QLD	Australia
BRIP	7582 a	*Pyricularia* sp.	*Pennisetum clandestinum*	Kairi Research Station, Kairi	QLD	Australia
BRIP	5641 a	*Pyricularia* sp.	*Musa* sp.	Rockhampton	QLD	Australia
BRIP	7583 a	*Pyricularia* sp.	*Pennisetum clandestinum*	Kairi Research Station, Kairi	QLD	Australia
BRIP	14253 a	*Pyricularia* sp.	*Pennisetum clandestinum*	Mount Tamborine	QLD	Australia
BRIP	17558 a	*Pyricularia* sp.	*Cenchrus ciliaris*	Pan Da Research Station, near Samoeng	Chiang Mai	Thailand
BRIP	44845 a	*Pyricularia* sp.	*Zingiber* sp.	Afunarani	Malo	Vanuatu
BRIP	15824 b	*Pyricularia* sp.	*Eleusine indica*	Rex range, near Julatten	QLD	Australia
BRIP	23123 a	*Pyricularia* sp.	*Cenchrus ciliaris*	Home Gorge, near Musselbrook Mining Camp, Musselbrook Reserve	QLD	Australia
BRIP	19582 d	*Pyricularia* sp.	*Carex gaudichaudiana*	West Gap Creek, Cunninghams Gap	QLD	Australia
BRIP	27675 a	*Pyricularia* sp.	*Stenotaphrum secundatum*	Redlands Horticultural Research Station, Cleveland, Brisbane	QLD	Australia
BRIP	44101 a	*Pyricularia* sp.	*Pennisetum typhoides*	Gympie	QLD	Australia
BRIP	5639 a	*Pyricularia* sp.	*Digitaria adscendens*	Kenmore, Brisbane	QLD	Australia

BRIP	20663 a	*Pyricularia* sp.	*Panicum effusum*	Lyra	QLD	Australia
BRIP	8835 a	*Pyricularia* sp.	*Brachiaria mutica*	Rochedale, Brisbane	QLD	Australia
BRIP	5640 a	*Pyricularia* sp.	*Eleusine indica*	Calavos	QLD	Australia
BRIP	12835 a	*Pyricularia* sp.	*Cenchrus ciliaris*	Narayen, near Mundubbera	QLD	Australia
BRIP	12782 a	*Pyricularia* sp.	*Stenotaphrum secundatum*	Kalbar	QLD	Australia
BRIP	19699 a	*Pyricularia* sp.	*Pennisetum polystachyon*	Chonburi	Chonburi	Thailand
BRIP	61933 b	*Pyricularia* sp.	*Musa paradisiaca*	Bumbora Reserve	Norfolk Island	Australia
BRIP	59659 a	*Pyricularia* sp.	*Musa*	Two Chimneys Road	Norfolk Island	Australia
BRIP	51720 a	*Pyricularia* sp.	*Oryza sativa*	Noelbaki	West Timor	Indonesia
BRIP	53096 a	*Pyricularia* sp.	*Panicum* sp.	Irvingdale	QLD	Australia
BRIP	14654 a	*Pyricularia* sp.	*Cenchrus ciliaris*	Katherine	NT	Australia
BRIP	51443 a	*Pyricularia* sp.	*Pennisetum pedicellatum*	Deleye	NT	Australia
BRIP	14656 a	*Pyricularia* sp.	*Pennisetum pedicellatum* subsp. pedicellatum	Darwin	NT	Australia
BRIP	61930 b	*Pyricularia* sp.	*Stenotaphrum secundatum*	Emily Bay	Norfolk Island	Australia
BRIP	60350 a	*Pyricularia* sp.	*Digitaria ciliaris*	Stockyard Road	Norfolk Island	Australia
BRIP	59379 a	*Pyricularia* sp.	*Cenchrus purpurascens*	Rooty Hill Road	Norfolk Island	Australia
BRIP	61254 a	*Pyricularia* sp.	*Cenchrus purpurascens*	Mission Road	Norfolk Island	Australia
BRIP	61597 a	*Pyricularia* sp.	*Cenchrus pedicellatus*	Bickerton Island	NT	Australia
BRIP	61526 a	*Pyricularia* sp.	*Pennisetum* sp.	Doi Mae Salong	Chiang Rai	Thailand

BRIP	53870 a	*Pyricularia* sp.	*Phalaris canariensis*	'Mirradong', Ryeford via Clifton	QLD	Australia
BRIP	25865 a	*Pyricularia* sp.	*Cenchrus ciliaris*	NW of Banana	QLD	Australia
BRIP	64068 a	*Pyricularia* sp.	*Cenchrus* sp.	Wagait Beach	NT	Australia
BRIP	15828 a	*Pyricularia* sp.	*Rhynchelytrum repens*	Rex range, near Julatten	QLD	Australia
BRIP	60367 a	*Pyricularia* Sacc.	*Digitaria sanguinalis*	Springwood	NSW	Australia
BRIP	60370 a	*Pyricularia* Sacc.	*Bouteloua* Lag.	Springwood	NSW	Australia
BRIP	60368 a	*Pyricularia* Sacc.	*Digitaria sanguinalis*	Cobbity	NSW	Australia
BRIP	60369 a	*Pyricularia* Sacc.	*Bouteloua* Lag.	Springwood	NSW	Australia