SYSTEMATICS AND ENZYMATIC ACTIVITY OF FOLIAR ASCOMYCETES (*FUNGI*) ON *LEPIDOZAMIA PEROFFSKYANA* AND *MACROZAMIA LUCIDA* IN EASTERN AUSTRALIA

A Thesis submitted by

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ABSTRACT

The kingdom *Fungi* is deeply entangled in the evolutionary history and ecology of life on Earth. The diversity of fungi is poorly known with only 150,000 species described from at least 4 million species by most estimates. The leaf surfaces of plants shelter an extensive diversity of fungi, including filamentous, single-celled, and dimorphic forms. Many extant species may become extinct before their discovery and preservation, due to habitat destruction and a changing climate. Many plants are further under threat in their natural habitats from illegal removal because of their horticultural value. Cycads are one of these horticulturally valuable plants. Cycads are the earliest seed-bearing plants. The fungi found on cycad leaves are often subject to extreme conditions of temperature, humidity, and ultraviolet radiation, which makes them interesting candidates for bioprospecting.

This thesis reports the foliar fungi found in association with the leaves of two endemic Australian cycads, *Lepidozamia peroffskyana* and *Macrozamia lucida*. *Samsoniella* sp. and *Penicillium* sp. were isolated from *L. peroffskyana* and represent novel species. *Periconia cyperacearum* was isolated from *L. peroffskyana* and represents a new host record. A novel species of *Acrocalymma* and an unidentified fungus were isolated from *M. lucida*. Several *Cladosporium* species were isolated from *L. peroffskyana* and *M. lucida*. The production of proteases, amylases, cellulases, and mannanases by these fungi was qualitatively investigated using skim milk, starch, cellulose, and galactomannan, as substrates, respectively. *Acrocalymma* sp. (BRIP 71369a) produced amylase. *Cladosporium* spp. (BRIP 71372a, BRIP 71364a and BRIP 71173c), and *Samsoniella* sp. (BRIP 71359b) produced protease and amylase.

CERTIFICATION OF THESIS

This is entirely the work of Lachlan Bartrop except where otherwise acknowledged. This work is original and has not been previously submitted for any other award, except where acknowledged.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDICES

ABBREVIATIONS

Abbreviation	Full term
ACT	Actin
aLRT	Approximate likelihood ratio test
BLAST	Basic local alignment search tool
Bp	Base pairs
BRIP	Queensland Plant Pathology Herbarium
BTUB	β-tubulin
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
DNA	Deoxyribonucleic acid
GTR	General time-reversible
h	Hour
ITS	Internal transcribed spacer
LSU	Large sub-unit
Ma	Million years before present
MEA	Malt extract agar
ML	Maximum likelihood
NGS	Next generation sequencing
nrRNA	Non-ribosomal transcribed RNA
OA	Oatmeal agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PUR	Polyurethane
QLD	Queensland
rDNA	Nuclear ribosomal DNA
RNA	Ribonucleic acid
RPB2	RNA Polymerase II
SNA	Synthetic nutrient-poor agar
SSU	Small sub-unit
TEF1	Translation-elongation factor 1α
UF	Ultrafast
Unident.	Unidentified
UV	Ultraviolet
v.	Version
wk	Week

1. CHAPTER 1: LITERATURE REVIEW

1.1 Fungal diversity

The kingdom *Fungi* is estimated to contain between 2.2 to 3.8 million species, of which about 8% have been formally named and described (Hawksworth & Lücking, 2017). Scientific discovery and the naming of new taxa facilitates unambiguous communication in research studies in phylogeny, conservation, ecology, and biotechnology (Cheek et al., 2020). The cryopreservation of fungal cultures as type specimens underpins nomenclature (Turland, 2019).

Until about 20 years ago, the classification of fungi was based mostly on morphology (Schoch et al., 2012). The weakness of this approach was that taxa that shared similar morphological traits through convergent evolution may not be phylogenetically related (Taylor et al., 2000). The ability of mycologists to discover and differentiate cryptic (morphologically similar) species requires a molecular (DNA) approach.

The classification of all fungi is now mostly based on inferences drawn from phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA, which was designated the official barcode locus for most fungi (Schoch et al., 2012). Phylogenetic species recognition is routinely used to classify taxa (species) on the basis that changes in the DNA sequences are conserved (Taylor et al., 2000). Molecular phylogenetic methods have resulted in rapid classification and re-classification of fungi, as reflected in (i) the tripling of fungal phyla in the last 20 years (from 4 to 12), and (ii) the rate of increase in number of published fungal names over decades (Hawksworth & Lücking, 2017; James et al., 2020). This phenomenon has been catalysed by improved affordability, efficiency, and availability of molecular DNA methods (Xu, 2016).

Metagenomics is the analysis of the collective genomes of microorganisms in their natural environment independent of specimens (cultures) (Nilsson et al., 2019). Metagenomics has revealed previously unknown branches of the tree of life (Kalsoom Khan et al., 2020; Tedersoo et al., 2014), as well as provided evidence of cryptic fungi in many environments (Baeza et al., 2017; Léveillé-Bourret et al., 2021; Runnel et al., 2021). The fungi that are only known to science from metagenomic studies have been coined the “dark taxa” (Kalsoom Khan et al., 2020). The fungal “dark taxa” cannot be

accommodated within the current framework of fungal nomenclature, as there are no physical specimens or cultures that can serve as types and link new taxa to names (Ryberg & Nilsson, 2018). Metagenomics have shown that an enormous diversity of fungi await discovery in understudied habitats (Blackwell, 2011).

Phylogenetic analysis looks for statistically well-supported monophyletic clades, which are considered as representative of taxa (Taylor et al., 2000). Living cultures of fungi permanently preserved in a living, albeit metabolically inactive, state can serve as type specimens. Type specimens are required to support the valid publication of new names, according to the *International Code of Nomenclature for algae, fungi, and plants* (Turland, 2019). The current rate of classification of fungi is slow, relative to the estimated number of species that await discovery and naming (Hibbett, 2016). The collection and preservation of new and beneficial fungi has never been more urgent as habitat destruction and a changing climate threaten to make many taxa extinct before their detection. The discovery of these fungi is a critical step toward their conservation (Cheek et al., 2020). Subsequently, it may be possible to understand the roles that these organisms play in ecosystems as well as their beneficial and exploitable characteristics (Antonelli et al., 2020; Blackwell, 2011).

1.2 Foliar microfungi

The fungal kingdom is well known for species that form edible or toxic fruiting bodies, e.g., mushrooms (Agaricales). Yet a far larger diversity of microfungi exist in most habitats. Microfungi are particularly understudied in extreme niches, e.g., in the guts of mammals and insects, in soil and in association with plants, and in marine environments (Duo Saito et al., 2018; Liggenstoffer et al., 2010; Peay et al., 2016; Richards et al., 2012; Teixeira et al., 2017; Višňovská et al., 2020).

All plants accommodate a rich biological diversity of foliar fungi as endophytes (within leaf tissues) and epiphytes (on leaf tissues) (Aslam et al., 2017; Heitman, 2011; Unterseher, 2011; Vorholt, 2012, Figure 1.1). The surface of plants as a habitat for microorganisms is referred to as the phylloplane. The global leaf area (total phylloplane) has been estimated at 5.5×10^6 km^2 and is about twice as large as the Earth's land surface area (Vorholt, 2012).

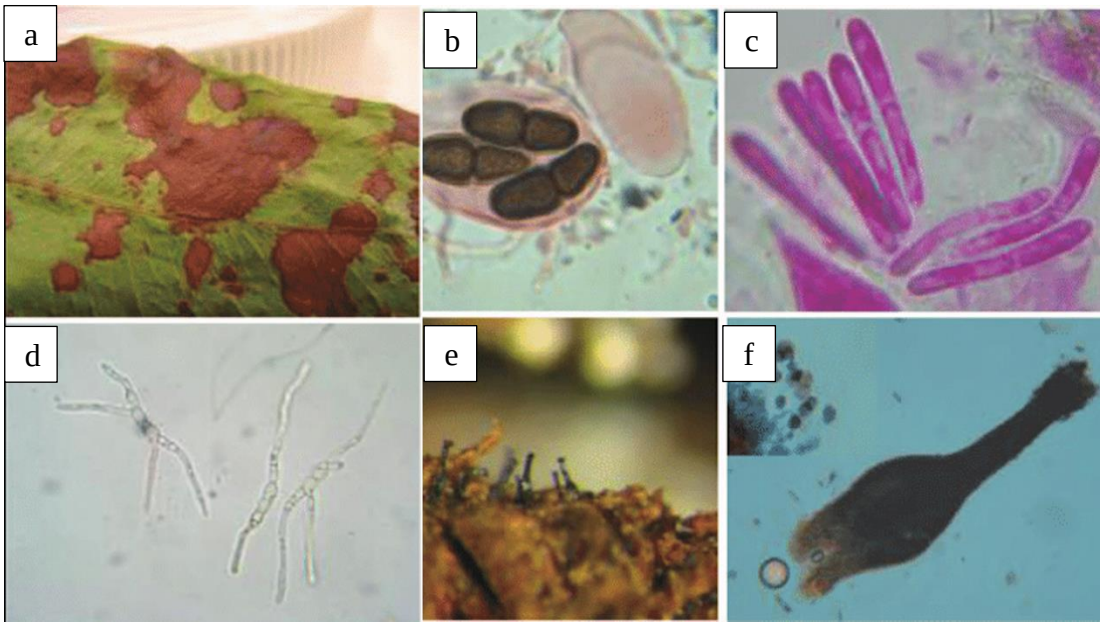

Figure 1.1. Foliar fungi from Australian *Eucalyptus* sp. (Carnegie, 2007). (a) Red-brown leaf spots associated with *Mycosphaerella marksii*. (b) Ascospore germination of *Mycosphaerella nubilosa*. (c) Ascospores of *Lembosina corymbiae*. (d) Ascospores of *Mycosphaerella 'eucalypti'*. (e, f) Ascocarps of *Corymbia*. sp. *'pleomorpha'*

The saprophytic, pathogenic, and commensal relationships that fungi exhibit in the phylloplane are complex. Phylloplane fungi can be either beneficial, e.g., increase stress tolerance (Redman et al., 2002), or detrimental (Berger et al., 2007).

1.2.1 Foliar epiphytes, endophytes, and pathogens

Epiphytic microfungi are abundant in the phylloplane, where they inhabit the surface of leaf tissues in filamentous, non-filamentous (single-celled yeasts) or dimorphic forms. The leaf surface provides a microclimate with essential nutrients and water (Limtong & Nasanit, 2017). Nutrients come in the form of either plant exudates (simple sugars, amino acids, and sugar alcohols) leached from leaf tissues (Carver & Gurr, 2006) or from external deposits of organic (i.e., insect frass, spores, pollen) and inorganic (e.g., nitrogen and phosphorous) nutrients that supplement the energy resource (Bannister et al., 2016; Dong et al., 2021).

Endophytic microfungi inhabit interior spaces of healthy leaf tissues for part, or all of their life cycle (Rodriguez et al., 2009). Endophytes are underestimated as ecosystem drivers and stabilisers (Strobel, 2018). Gamboa et al. (2002) found that ~16 fungal endophytes per 20 mm^2 could occupy leaf material in plants. These cryptic fungi do not typically induce disease and have been found in mutualistic and commensal

relationships within specialised host specific interactions or in chance associations (as a consequence of horizontal transmission) (Rodriguez et al., 2009).

Microfungi inhabiting the phylloplane as epiphytes and endophytes may also adopt a pathogenic lifestyle. They live biotrophically (requiring living tissue to grow and reproduce) using specialised feeding structures called haustoria, or necrotrophically, by emitting enzymatic cocktails of toxins to destroy host tissue that has undergone senescence (Berger et al., 2007; Shao et al., 2021; Thomma et al., 2001). Opportunistic infection directly into the host cell plasma membrane is aided by the emittance of effector molecules (virulence factors) (J. D. G. Jones & Dangl, 2006).

Subtle changes in the expression of transcription factors influence multiple metabolic functions of the fungal pathogen (S. de Vries et al., 2020). If these changes prove successful, they will be selected and contribute to the success and evolution of the species (Morris & Moury, 2019). Genetic expression also enables a complex symbiotic continuum of fungal lifestyles (Carroll, 1988; Saikkonen et al., 1998), e.g., endophytes can become (i) pathogens under certain conditions (Schulz & Boyle, 2005); and (ii) saprobes, after senescence of the host leaf and plant death (Hyde et al., 2007; Promputtha et al., 2007; D. Zhou & Hyde, 2001).

Effects of plant-pathogen interactions include (i) the additional energy cost to the host to assimilate defence responses; (ii) manipulation of host carbohydrate metabolism; and (iii) the inhibition of photosynthesis at necrotic foliar sites (Jain et al., 2019). Biotrophic fungal pathogens include basidiomycetous smut and rust fungi and ascomycetous powdery mildews, that cause a variety of cankers, leaf spots and leaf blights, sometimes resulting in reduced yields and death of the host (Ellis et al., 2007). Economical loss is caused by plant pathogens on many cultivated crops and ornamental plants (Fones et al., 2020; Kakoti et al., 2020).

1.3 Fungal biodiscovery

Fungi have benefited society on a global scale, i.e., by the synthesis of biofuels; in bread and alcohol production; and in the bioremediation of contaminated environments (Tang et al., 2006, Grondin et al., 2015, Buzzini et al., 2017; Jourbet & Doty 2018, Willis, 2018). The well-known baker's yeast, *Saccharomyces cerevisiae*, was the first eukaryote to have its full genome sequenced (Kurtzman et al., 2015)

which led to a greater understanding of human biology, as many of the genes that regulate cellular, metabolic, and molecular processes are similar in humans and fungi (Willis, 2018).

Fungal cultures are critical as a resource for the discovery of novel secondary metabolites (bioprospecting) that have uses in medicine, agriculture, food processing, biotechnology, and nutraceuticals (Antonelli et al., 2020). For example, fungal secondary metabolites can serve as antibiotics i.e., penicillin (Gaynes, 2017), immuno-suppressants, i.e., cyclosporine (Borel, 2002), and anticancer compounds, i.e., taxol (Hao et al., 2013).

1.3.1 Foliar microfungi biodiscovery

The phylloplane is an extreme environment (Barge et al., 2019). It is microbially dense which creates competition, has high ultraviolet (UV) exposure, and periodic nutrient and water regimes guided by seasonal changes (Arnold, 2007). Fungi in this environment have adapted to the harsh conditions in a variety of unusual ways, consequently making them the targets of biodiscovery (Hyde et al., 2019; Rai et al., 2021; Rigobelo & Baron, 2021). Epiphytes have been reported playing a role as the hosts extended immune system via synthesis of secondary metabolites used in the host's function (André et al., 2017). For example, an unidentified *Cladosporium* interfered with the lifecycles of rice pathogens (Chaibub et al., 2020). Fungal endophytes produce exploitable compounds such as phyto-hormones, organic acids, and enzymes in exchange for a protective habitat and a nutrient source (Waqas et al., 2015).

1.3.2 Enzymes from microfungi

Fungi secrete enzymatic cocktails to externally digest food sources and absorb nutrients (Pirozynski et al., 1988). Accordingly, 60% of industrial used enzymes are of fungal origin (Østergaard & Olsen, 2010). Only five genera, *Aspergillus, Humicola, Penicillium, Rhizopus* and *Trichoderma* account for 75% of all enzymes produced (Østergaard & Olsen 2010).

Foliar fungi produce hydrolytic and oxidative enzymes to penetrate and colonise their plant hosts, obtain nutrients, and enhance resistance against disease causing pathogens

(Crippa et al., 2019; Mishra et al., 2019). Phylloplane fungi have been targeted for their fermentation capabilities. Suryanarayanan et al. (2012) found endophytes such as *Phoma* spp. and *Pestalotiopsis* spp. produce a range of enzymes, across phylogenetically distant plants. *Pestalotiopsis microspora* can break down polyurethane plastic (PUR) by using a serine protease (Russell et al., 2011), and *Aspergillus niger* can degrade sugar cane biomass by producing glycohydrolases (Robl et al., 2013). Several xylanases produced by *Trichoderma* spp., and *Thermomyces* spp. have been commercialised in the pulp and paper industries (Østergaard & Olsen 2010).

Novel and natural sources of enzyme-producing phylloplane fungi are highly sought after as fungi are adaptable to variable environmental conditions (Tiquia-Arashiro & Grube, 2019). For example, endophytes isolated from plants in the Baima Snow Mountain Nature Reserve, China, possess enzymatic capabilities at low temperatures, i.e., for use as organic solvents (Cavicchioli et al., 2002; H.-Y. Li et al., 2012). Fungal enzymes have also been used to catalyse reactions and reduce toxic by-products, without needing extreme environmental conditions (Chapla et al., 2012). Epiphytic fungi may produce industrially exploitive enzymes given they have direct contact with the external environment, unlike endophytes, that produce enzymes only in the presence of certain host substrates (Arguelles et al., 2016; Arnold, 2007).

1.3.3 Amylase

Amylases are one of the most exploited enzymes in a range of industries (i.e., food, textiles, chemical) used to convert starch into various sugar solutions (R. Gupta et al., 2003). There are three main types, α-amylase (1,4 α-glucan glucanohydrolase), β-amylase (1,4 α-glucan maltohydrolase), and glucoamylase (1,4 α-glucan glucohydrolase) (Mouyna et al., 2013; Q. Zhang et al., 2017). Lim and Oslan (2021) detail the mechanism of α-amylases, which consists of catalysing the endo-hydrolysis of α-1,4-D-glycosidic bonds in starch into glucose, maltose, and dextrin, without losing the α-anomeric configuration in the products. *Aspergillus* spp. and *Rhizopus* spp. are the largest fungal producers of amylases in industry (Corrêa et al., 2014; Souza & Magalhães, 2010).

1.3.4 Protease

Proteases are enzymes which catalyse the hydrolysation of peptide bonds in proteins and polypeptides (de Souza et al., 2015). They are used in food processing, detergents, pharmaceuticals, and bioremediation and comprise over 60% of the enzyme market (Neetu et al., 2014). Their mechanism of action involves attacking interior peptide bonds, i.e., pepsin and papain, or by the removal of the terminal amino acids from the polypeptide (aminopeptidases). Phylloplane fungi shown to produce proteases are *Pencillium* sp. *Phoma tropica*, *Pestalotiopsis* sp. *Alternari* sp, *Fusarium* sp., *Talaromyces flavus*, *Xylaria* sp., *Cladosporium cladiosporioides*, *Acrimonium terrícola*, *Colletotrichum* sp., *Drechslera hawaiiensis*, *Curvularia vermiformis* and *Aspergillus* sp (Corrêa et al., 2014).

1.3.5 Cellulase

Cellulases are a class of enzymes that breakdown lignocellulosic material (Panchapakesan & Shankar, 2016). In nature, lignocellulose makes up the combination of lignin (a polymer) with cellulose and hemicellulose (carbohydrate polymers), which creates a rigid solid for structure formation in plants and trees (Fengel & Wegener, 2003). Cellulases are exploited in biofuel production for the degradation of lignocellulosic material to bioethanol (Costa et al., 2021; Sukumaran et al., 2021) and have use in bioremediation, e.g., to degrade waste hydrocarbons, (Al-Zaban et al., 2021). The cellulases involved in lignocellulose degradation are exo- and endo-glucanases, β-glucosidases, exo- and endo-xylanases and β-xylosidases (Jayasekara & Ratnayake, 2019). Additional catalysis is needed by oxidative enzymes, specifically, laccase, manganese peroxidase, lignin peroxidase, hemicellulases and oxidoreductases (Corrêa et al., 2014; Strakowska et al., 2014). Phylloplane fungi produce cellulase to depolymerise leaf tissue and degrade plant cell walls (El-Said et al., 2014; Thomma et al., 2001).

1.3.6 Mannanase

Hemicellulose is the second most abundant plant polysaccharide in nature (El Khadem, 2003). It is composed of three residues with different backbones, namely, xylan, xyloglucan, and galactomannan (Aulitto et al., 2019; van den Brink & de Vries, 2011). Galactomannans are the dominant hemicellulose fraction of gymnosperms and consist of a backbone of β-1,4-linked d-mannose residues, which can be substituted by d-galactose residues via an α-1,6-linkage (Aspinall, 1980; R. P. de Vries & Visser, 2001). Consequently, several enzymes called mannanases are required for complete hydrolysis of galactomannan. In industry, mannanases are used to produce sugar from plant biomass for biofuel (Bien-Cuong et al., 2009; López-Mondéjar et al., 2016). They also have use in detergents (Kirk et al., 2002), pre-bleaching of softwood pulps (Golestani, 2020) and in food processing (Dawood & Ma, 2020). Phylloplane fungi produce mannanase (R. P. de Vries & Visser, 2001), e.g., *A. awamori* hydrolysed konjak and locust bean gum, by producing β-Mannanase (Kurakake & Komaki, 2001).

1.4 Cycads (Cycadales)

Cycads (Cycadales) are gymnosperms that originated about 300 million years ago (Ma) in the mid-Permian (Gao & Thomas, 1989), and peaked in distribution and diversity in the Jurassic-Cretaceous (Jones, 2002). Extant cycad species re-diversified around 12 Ma (Mankga et al., 2020; Nagalingum et al., 2011). Cycads are entomophilous plants that developed a palm-like habit with stout trunks and large evergreen pinnate leaves (Jones, 2002). Cycads are gymnosperms that produce naked seeds, i.e., the ovules are not enclosed in an ovary but lie exposed on leaflike structures (Norstog & Nicholls, 2019). Cycads are unlike other gymnosperms, and similar to ferns, in that they produce flagellated free-swimming sperm (Renzaglia et al., 2002).

Pollen is transferred from male plants to the ovules of female plants, which are both formed on cones on separate male and female plants (dioecious). The cones comprise spirally arranged sporophylls (Jones, 2002). All genera, except *Cycas*, reproduce from a determinate female cone that generate (when pollinated) colourful seeds with an outer layer called a sarcotesta, which encourages dispersal by foraging birds and mammals (Zheng et al., 2017). Pollination is by host-specific insect pollinators for most genera (Pascoa, 2013; Terry, 2001). The prolonged period of cycad pollination is aided by a multilayered sporoderm that protects the pollen, a convergent feature of

anemophilous (wind-pollinated) germplasm in other gametophytes (Nadarajan et al., 2018).

1.4.1 Cycad classification

The cycads are classified in the Cycadales, a monophyletic order that contains three families, Cycadaceae, Stangeriaceae and Zamiaceae (Calonje et al., 2013). The cycads comprise roughly 348 species in 10 genera, namely *Bowenia* (2), *Ceratozamia* (30), *Cycas* (114), *Dioon* (15), *Encephalartos* (65), *Lepidozamia* (2), *Macrozamia* (41), *Microcycas* (1), *Stangeria* (1) and *Zamia* (77) (Calonje et al., 2019; Osborne et al., 2012). Cycads are classified by phenotypic characters that included vegetative and reproductive structure, morphological traits (pollen and leaflet anatomy), chemical composition, isozyme profiles, and geographical location (Walters et al., 2004).

Molecular data has provided complete phylogenies for many cycads (Calonje et al., 2019; Chaw et al., 2005; Hill et al., 2003). The ITS has been successful at classifying cycads at the rank of genus or species (Xiao & Möller, 2015). All phylogenies show *Cycas* as a sister group to the remaining Cycadales. *Encephalartos*, *Lepidozamia* and *Macrozamia*, form a monophyletic group (Sangin et al., 2008), and *Microcycas* and *Zamia* form a separate clade (Calonje et al., 2019).

With Next Generation Sequencing (NGS), entire genomes have become available for phylogenetic analyses of cycads (Jiang et al., 2016). Jiang et al. (2016) found *Cycas* first diverged in the Cycadales, followed by *Dioon* which agrees with Salas et al. (2013). *Stangeria* is polyphyletic and sister to *Microcycas* and *Zamia* (Lei et al., 2018; Figure 1.2).

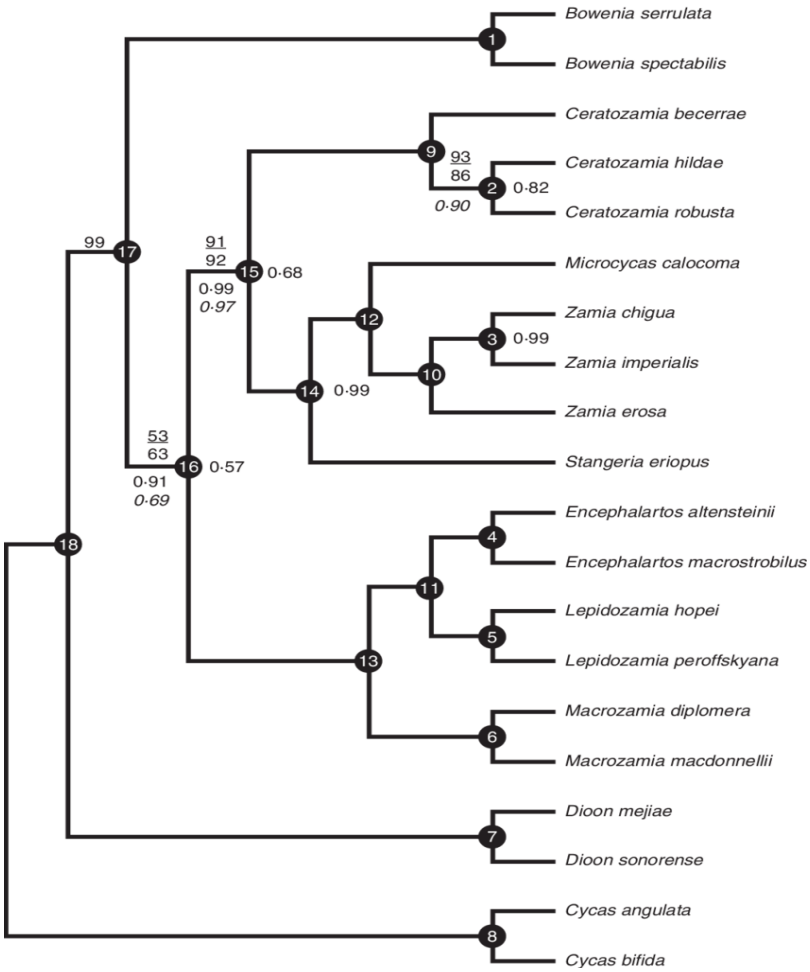

Figure 1.2. Phylogeny of Cycadales (Salas et al. 2013)

1.4.2 Cycad distribution

The global distribution of cycads is clustered and restricted to the tropical and subtropical areas of the globe, largely between 27°S and 18°N latitude (Fragnière et al., 2015; Rull, 2020), with an almost symmetrical distribution around the equator (Figure 1.3). Cycads prefer high summer rainfall and inhabit relatively low elevations, with only 4 species found above 2000 m (Jones, 2002).

Cycas is the most geographically distributed and phylogenetically diverse genus (Osborne et al., 2012; Zheng et al., 2017). Fossil evidence points to Asia as its origin (Xiao & Möller, 2015). From there, *Cycas* extended southward to Australia, eastern Africa, and the Pacific Islands (Xiao & Möller, 2015). Walters et al. (2004) and Mangka et al. (2020) reported a thick spongy layer of the seeds (the sarcotesta),

compared to a fleshy layer in other genera, which allows for a longer survival time in salt water and may have contributed to their spread.

Encephalartos (Zamiaceae) are indigenous to Africa, where they are commonly referred to as bread palms due to their edible pith (www.kew.org/plants/). *Encephalartos* has 65 species (Encyclopaedia Britannica, 2017) and their recent domestic global trade as horticultural plants has endangered many species in their native habitats (Donaldson, 2003). South Africa is a diversity hub for *Encephalartos*, with 37 species, although 70% are threatened with extinction and listed in CITES Appendix 1 (Williamson et al., 2017). Additionally, *Stangeria eriopus* (monotypic) is found on the African continent. *Ceratozamia* (Zamiceae) has 30 species and is mostly found in Mexico, with several listed as most endangered in CITES Appendix 1 (https://cites.org/eng/app/appendices.php).

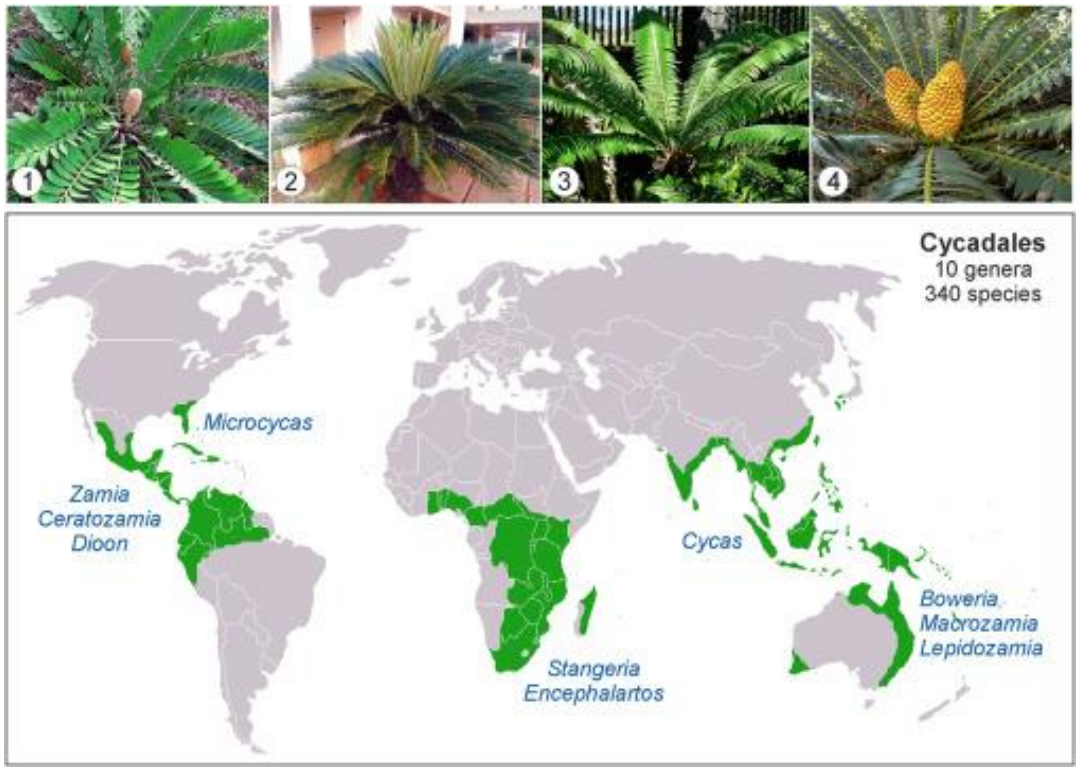

Figure 1.3. World cycad distribution and examples of most renowned genera. (1) *Zamia*, (2) *Cycas*, (3) *Dioon*, and (4) *Encephalartos* (Rull, 2020)

Australia has four endemic genera of cycads, *Macrozamia*, *Lepidozamia*, *Bowenia* and *Cycas* (Pacsoa, 2013a). *Macrozamia* spp. occur in small clusters of vast density in forest understories (Laidlaw & Forster, 2012). Hall and Walter (2013) found that 97% of *Macrozamia miquelli* seed was dispersed less than one metre from the parent plant. Additionally, Snow and Walter (2007) reported 97% of *Macrozamia lucida* seeds remained within 0.25 m of the maternal female. Both studies concluded that historical megafauna were largely responsible for seed dispersal. This is likely analogous for *Lepidozamia peroffskyana* and *Lepidozamia hopei*. These species are endemic to eastern rainforest regions of Australia and *L. peroffskyana* is restricted to a lower latitude than the latter (Atlas of Living Australia, 2021a). *Lepidozamia* have some of the heaviest seeds of cycads (up to 18g), thus fauna are the likely means of dispersal (Dickie & Pritchard, 2002).

1.5 Diversity of foliar microfungi on cycads

Cycads represent understudied and interesting candidates for fungal phylloplane investigations, in part due to (i) their ancient lineage (Jiang et al., 2016); (ii) known presence of many microbial symbionts within cycad roots and seeds (Zheng & Gong, 2019); and (iii) their relative rarity and threatened habitats (Jones, 2002).

A review of the literature for molecularly verifiable microfungi isolated from the phylloplane of cycads (Cycadales) from around the world, found all species were from the subphylum Pezizomycota (Ascomycota), predominantly the Dothideomycetes and the Sordariomycetes (Appendix A). In total, 32 cycad fungi have been reported in the literature, across 12 orders (Figure 1.4). The two dothideomyceteous orders, Pleosporales and Capnodiales, had eight and five species, respectively. *Cycas* spp. have been the most studied cycad for foliar fungi, with 11 studies mostly in Asia. *Encephalartos* spp. were also well researched with nine studies from Africa (Appendix A).

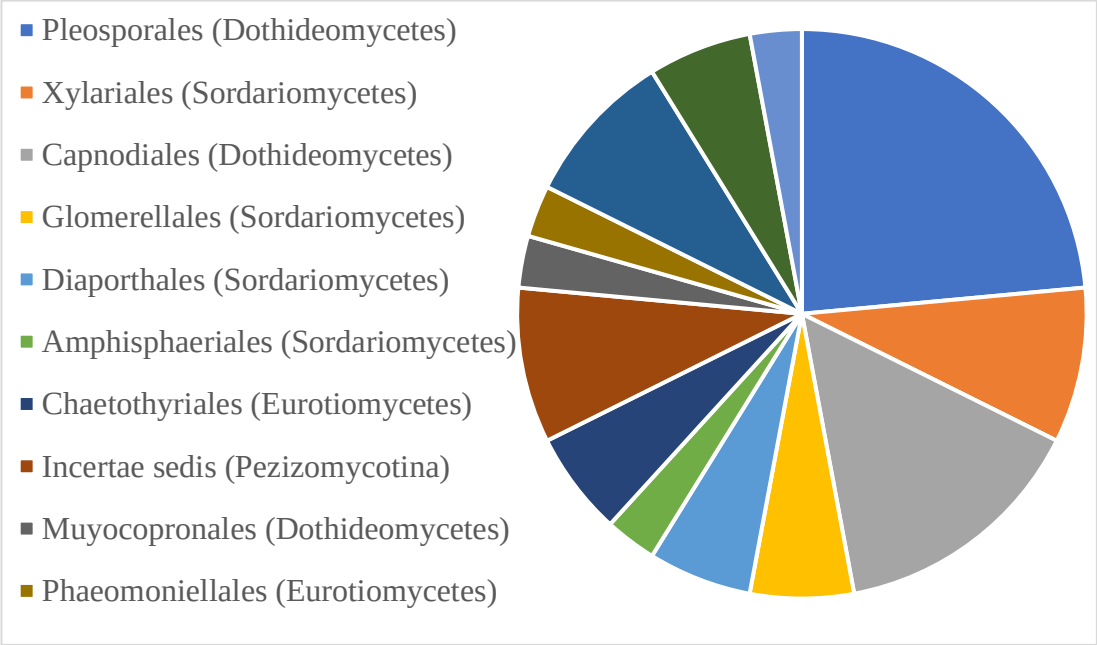

Figure 1.4. Taxonomic orders of verified foliar fungi found on cycads

A search of *Index Fungorum* (2021) for records of foliar fungi on cycads, which included those that had not been verified by molecular data, revealed 186 species, in 89 genera of 14 orders. Unfortunately, unverifiable taxa may have been misidentified or misclassified. The relationships between the most reported genera of microfungi, their continental location and their cycad host are shown in Figure 1.5.

Cladosporium spp. were often reported on the leaves of *Cycas* spp. in Asia, which was expected as these are ubiquitous airborne fungi (Andersen et al., 2009; do Nascimento et al., 2019). Other dominant genera include *Ascochyta* on *Cycas* spp. in Asia; *Fusarium* spp. on a range of cycads globally; and *Phoma* spp. and *Phyllosticta* spp. on *Cycas* spp. (Figure 1.5). *Cycas* is the most explored genus of cycad for foliar fungi investigations (Figure 1.5).

Muyocopron zamiae appears to have an association with *Zamia* spp., isolated twice in the USA (Hernández-Restrepo et al., 2019). *Mycoleptodiscus indicus* is closely phylogenetically related and has also been isolated from *Zamia* spp. (EI-Gholl & Alfieri, 1991; Sutton, 1973; Tang, 2002). *Mycoleptodiscus* spp. appears to have an association with *Zamia* spp. (Figure 1.5).

Two confirmed pathogenic fungi have been reported from cycads, namely *Boeremia exigua* var. *exigua*, causing necrotic lesions on leaves of *Cycas circinalis* in India

(Banerjee & Panja, 2020) and *Colletotrichum siamense*, causing brown leaf spots on *Cycas debaoensis* in China (Han et al., 2021). Both species have been isolated as pathogens on other hosts (Gorny et al., 2015; Y. Zhang et al., 2020). *Colletotrichum cycadis* has also been reported from leaf spots of *Cycas revoluta* in Australia (Crous et al., 2020a). *Phoma herbarum* and *Phomopsis cycadis* have been reported as pathogens on *C. revoluta* in Pakistan (Nayab & Akhtar, 2016) and China (Xiaoxia et al., 2014), respectively, although these identifications are not verifiable. *Xenocylindrosporium kirstenboschense*. Was isolated from *Encephalartos friderici-guilielmi* and although the authors reported that the fungus appeared pathogenic, this was not confirmed with Koch's postulates (Crous et al., 2009a).

Fusarium oxysporum and *Fusarium proliferatum* were reported as pathogens on *Lepidozamia peroffskyana* and *C. revoluta*, respectively (Mirhosseini et al., 2017; Nkosi, 2020). The identity of these two *Fusarium* species was based on translation-elongation factor 1α (TEF1) (*F. oxysporum*) and ITS *(F. proliferatum*), which is insufficient for full taxonomic resolution (Lombard et al., 2019). *Fusarium* spp. have been isolated from the phylloplane of several cycads (Table 1.1), although identification was not confirmed with molecular data. *Fusarium* spp. are economically significant pathogens on cereals crops such as wheat (Ghimire et al., 2020).

Table 1.1. *Fusarium* spp. reported from the leaves of cycads

Species	Host	Country	Reference
Fusarium oxysporum	*Dioon spinulosum; Encephalartos princeps; Encephalartos transvenosus*	New Zealand; India; South Africa	Braithwaite et al. (2006); Hassan et al. (2019); Nesamari et al. (2017)
F. phyllophilum	*D. spinulosum*	New Zealand	Braithewaite et al. (2006)
F. proliferatum	*Cycas revoluta*	Iran	Mirhosseini et al. (2017)
Fusarium sp.	*Zamia* spp.*; E. transvenosus*	USA; Dominican Republic; South Africa	Forgacs (1971); Nesamari et al. (2016)
F. equiseti	*Cycas panzhihuanesis*	China	Zheng and Gong (2019)

Phyllosticta capitalensis was reported as an endophyte of *Encephalartos ferox*, *Encephalartos latifrons*, and *Zamia integrifolia* (Baayen et al., 2002). *Phyllosticta* spp. have also been isolated from the phylloplane of numerous other cycad hosts, without supporting molecular data aside from *Phyllosticta encephalarticola* (Table 1.2).

Table 1.2. *Phyllosticta* spp. reported from the leaves of cycads

Species	Host	Country	Reference
Phyllosticta capitalensis	*Encephalartos ferox; Encephalartos latifrons; Zamia integrifolia*	South Africa; USA	Baayen et al. (2002)
P. cycadina	*Cycas revoluta*	India	Rao and Baheker (1964); Tandon and Bilgrami (1957)
P. cycadis	*Cycas circinalis; C. revoluta*	Pakistan	Ahmad (1948)
P. encephalarticola	*Encephalartos* sp.	South Africa	Crous et al. (2019a)
Phyllosticta sp.	*Cycas panzhihuaensis*	China	Yi et al. (2013)
P. stangeriae	*Stangeria paradoxa*	-	Zimmerman (1909)

Four species of *Cladosporium* have been reported from the phylloplane of cycads, *Cladosporium cycadicola, Cladosporium apicale, Cladosporium cycadis* and *Cladosporium cycadacearum*. Of these, only *C. cycadicola* has supporting molecular data (Crous et al., 2014a). *Cladosporium apicale* has been isolated from Sri Lanka on *Cycas circinalis* and in India on *Cycas revoluta* (Bensch et al., 2012). *Cladosporium cycadacearum* on *C. revoluta* in India has similar morphology to *C. apicale* and may be a synonym (Bensch et al., 2012; S. Kumar et al., 2007).

Circinotrichum cycadis has been reported from *Cycas* sp. (Appendix A). *Circinotrichum* is polyphyletic and belongs to the Xyriales (Sordariomycetes) (Crous et al., 2015; D.-W. Li et al., 2017). *Gyrothrix encephalarti* was recently found on *Encephalartos* sp. leaves by Crous et al. (2020) and has similar morphology to *Circinotrichum* (Seifert & Gams, 2011). These two genera are closely related phylogenetically and have an association with cycads (Cunningham, 1974).

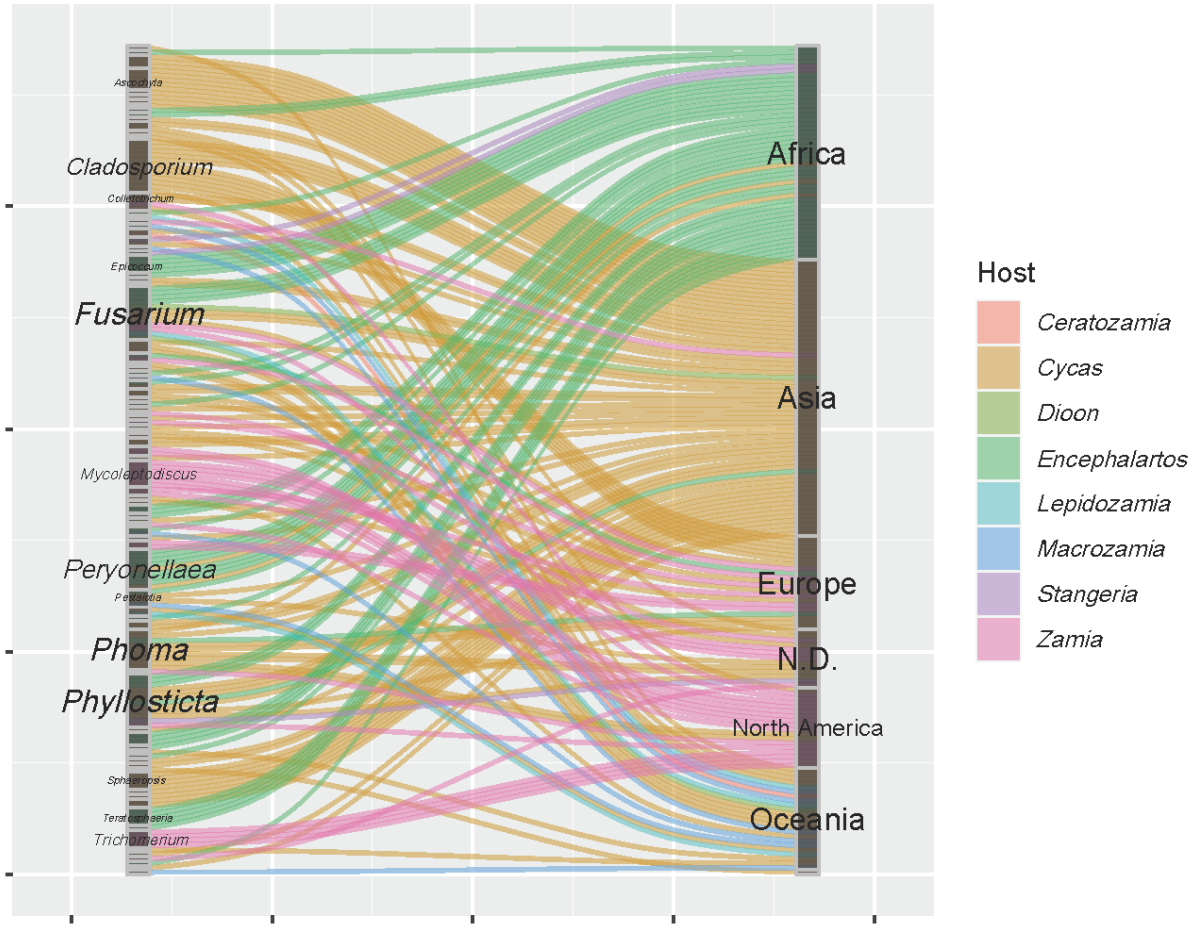

Figure 1.5. Alluvial plot (Brunson, 2020) depicting relationship between unverified reports of microfungi, cycad host, and the continent isolated. Created in R Studio v. 1.4.1717 (Rstudio Team, 2020). Fungal genera are labelled if the genus has been reported three or more times

Only ascomycetes have been isolated from the phylloplane of cycads, which aligns with other foliar fungi investigations (Reynolds & Gilbert, 2005; Salazar-Cerezo et al., 2018; S.-L. Zhou et al., 2015). This may reflect bias in the isolation method that favours the removal of ascomycetous conidia from the leaf surface by scraping or washing. These culture-based approaches tend to favour rapidly growing fungi and exclude biotrophic species (Cordier et al., 2012).

1.6 Summary

Culture independent analysis techniques such as DNA barcoding and NGS reveal the hidden diversity of fungi and their phylogenetic relationships (Aslam et al., 2017; Nilsson et al., 2016; Ryberg & Nilsson, 2018). Many of these fungi are yet to be discovered due to (i) their cryptic life history in niche ecosystems; (ii) many being obligate biotrophs and unculturable; and (iii) the trickle of microfungi studies that name new taxa, relative to those predicted to exist (Hawksworth & Lücking, 2017).

Cycads are an ancient lineage of gymnosperms and possibly the first seed plants that were insect pollinated (Jones, 2002). Many of the remaining 200 extant species of cycads have diminished in their natural habitats and now face extinction (Donaldson, 2003). The unique microfungi that inhabit the cycad phylloplane face similar extinction, most before their discovery. These fungi have certainly had a long ecological and evolutionary association with their hosts (Hongsanan, 2016).

The collection and naming of microfungi that inhabit the phylloplane of cycads is necessary for a full understanding of the fungi that occupy this planet. Studies that have studied the microfungi on the cycad phylloplane around the world hint at a vast and undiscovered diversity. The diversity of foliar microfungi associated with cycads in Australia is poorly known and has received little attention (Appendix A).

Phylloplane fungi rely on extracellular enzymes to colonise their plant hosts, obtain nourishment, and outcompete other microorganisms (Arguelles et al., 2016; Mishra et al., 2019). While endophytes have been investigated for enzymatic activity (Corrêa et al., 2014; Mishra et al., 2019), the study of epiphytic fungal enzymes is less common. The microfungi on cycads are candidates for novel enzymes. Abass (2005) showed that *Fusarium solani*, responsible for decline in *Cycas revoluta* plants, produces proteases, cellulases, lipases, amylases, and phenol oxidases. Widening the arsenal of

fungi that produce industrial enzymes is paramount to the sustainable advancement of biotechnology.

1.7 Thesis overview

This thesis proposes to investigate the diversity of foliar microfungi isolated from the phylloplane of *Lepidozamia peroffskyana* and *Macrozamia lucida*, which are two cycads endemic to eastern Australia. Microfungi were isolated from leaves of *L. peroffskyana* and *M. lucida* and cryopreserved as living cultures in the Queensland Plant Pathology Herbarium (BRIP), Ecosciences Precinct, Dutton Park. An investigation of a reference isolate in BRIP collected from *Zamia hamannii* in northern Queensland, was also studied.

This research also investigates the ability of fungal isolates from the phylloplane of *L. peroffskyana* and *M. lucida* to produce protease, amylase, cellulase, and mannanase during solid-state fermentation of skim milk, starch, cellulose, and galactomannan, respectively.

1.7.1 Host plants

Lepidozamia peroffskyana (Zamiaceae) is endemic to the east coast of Australia extending from Gympie, Queensland, south to Taree, New South Wales (Atlas of Living Australia, 2021a). It can reach 7 m in height and has a columnar trunk approximately 35 cm in diameter. It has dark green, glossy fronds which are produced in flushes of 50 – 60 (max) that can grow up to 3 m in length. Cones do not have a peduncle and reach up to 60 cm in length and 25 cm in diameter (Pacsoa, 2013b).

Macrozamia lucida (Zamiaceae) is endemic to south-east Queensland in coastal, wet sclerophyll forest (Atlas of Living Australia, 2021b). It has thin, glossy leaves, non-pungent leaflets with white callous bases (clearly differentiating it from *L. peroffskyana*), that protrude from a stem of 8 – 20 cm diameter (Pacsoa, 2013c). Leaves are 0.2 – 1.5 cm wide and 70 – 120 cm long and the pollen combs are fusiform, 12 – 15 cm long and 3.5 – 4 cm in diameter (Pacsoa, 2013c).

1.8 Research questions

This study is driven by the following research questions:

1. Are novel or rare fungal species present on the phylloplane of two native Australian cycads, *L. peroffskyana* and *M. lucida,* in south-eastern Queensland?
2. Are cycad phylloplane fungi a potential source of proteases, amylases, cellulases, and mannanases?

The research objectives of this study to:

1. Sample, isolate, preserve, identify, and classify fungi isolated from the phylloplane of two native Australian cycads, *L. peroffskyana* and *M. lucida*, in south-eastern Queensland;
2. Investigate whether any novel species of fungi exist among isolates using molecular phylogenetic approaches in accordance with the rules of the *International Code of Nomenclature for algae, fungi, and plants* (https://www.iapt-taxon.org); and
3. Assay a selection of these fungi for their ability to produce protease, amylase, cellulase, and mannanase.

2. CHAPTER 2: IDENTIFICATION OF FOLIAR FUNGI ISOLATED FROM *LEPIDOZAMIA PEROFFSKYANA* AND *MACROZAMIA LUCIDA* IN EASTERN AUSTRALIA

2.1 Introduction

Cycads are living relics of once abundant and diverse groups of the earliest seed-bearing plants (Nadarajan et al., 2018) which make them attractive for evolutionary studies (Bogler & Francisco-Ortega, 2004; Davis & Schaefer, 2011). The diversification of the most specious genera, *Encephalartos*, *Macrozamia*, *Zamia* and *Cycas* either occurred in the Palaeogene (66–23 Ma) or the Neogene (23–2.6 Ma) (Condamine et al., 2015). Molecular dating showed that *Lepidozamia* and *Macrozamia* evolved from a common ancestor at 33.9 and 16.1 Ma, respectively (Condamine et al., 2015). Anthropogenic destruction of the equatorial habitat of cycads has made them one of the most endangered of plant groups based on IUCN Red List assessments (Marler & Marler, 2015).

Microfungi require conservation priority due to their high diversity and the threats from habitat destruction and climate change (Moore et al., 2001; Heilmann-Clausen et al., 2015). Less than 0.025 % of the estimated global fungal diversity have an assessed conservation status (IUCN, 2021). The scientific community, governments and NGOs have realized the significance of fungi, which has prompted the launch of a global initiative to make fungi one of the priorities within conservation and agricultural policy frameworks, e.g., the Fauna, Flora, and Fungal Proposal (Kuhar et al., 2018).

There have been limited studies of endemic foliar microfungi that inhabit the phylloplane of cycads (Cycadales). The literature shows that only 32 fungal species associated with cycads have been isolated and their identities verified by molecular data (Appendix A). Within Australia, four species on *Macrozamia*, four on *Cycas* and one on *Ceratozamia* have been reported. All of these fungal species belonged to the most diverse ascomycetous subphylum, the Pezizomycota (Ascomycota).

In this study, the diversity of fungi associated with leaves of two cycads, *Lepidozamia peroffskyana* and *Macrozamia lucida*, was determined using molecular and morphological methods. A discussion of these results in the context of fungal

taxonomy and ecology is given. The discovery of foliar microfungi inhabiting ancient plants is a critical step toward their conservation (Cheek et al., 2020).

2.2 Materials and methods

2.2.1 Fungal isolates

Leaves of *L. peroffskyana* and *M. lucida* (Figure 2.1) were collected from sub-tropical rainforest at Mount Glorious, 40 km north-west of Brisbane, Queensland, in May and June of 2020. Leaves of *L. peroffskyana* had irregular patches of darken mycelium growing on the upper side (Figure 2.2a, c). On the corresponding lower leaf surface, the leaf tissue was chlorotic or necrotic (Figure 2.2b). Leaves of *M. lucida* had small brown necrotic leaf spots (Figure 2.2d).

The climate in the region is humid sub-tropical with hot, wet summers, and warm, dry winters (Bureau of Meteorology, 2021). Climate data collected from the Mount Glorious weather station (27.33 °S, 152.77 °E), shows February 2019 had the highest mean rainfall (249.6 mm) and September 2019 the lowest (55.9 mm) since 1941. The annual average rainfall of the area since 1941 is 1631 mm. Annual temperature has averaged 20.9 °C since 1941, with December and July being the hottest and coolest months, respectively (Bureau of Meteorology, 2021).

Mature leaves of *L. peroffskyana* and *M. lucida* that showed symptoms of disease or had visible fungal material on their surface (Figure 2.2) were transferred into paper bags. Diseased leaves were targeted to increase the likelihood of fungal isolation Fungal conidia were removed by scraping the leaf surface area of the lesion with a sterile scalpel blade and placing in sterile water on petri dishes containing potato dextrose agar (PDA; Oxford, Hampshire, England). Plates were serially diluted to obtain single colonies that were sub-cultured onto PDA plates. Colonies were grown on PDA, synthetic nutrient-poor agar (SNA), malt extract agar (MEA), and oatmeal agar (OA) (Gams et al., 1998) and incubated under continuous near-UV light at 25 °C to promote sporulation. Pure cultures of isolated fungi were deposited in the Queensland Plant Pathology Herbarium (BRIP), Ecosciences Precinct, Dutton Park and permanently preserved at -80 °C under glycerol (Table 2.1). A reference culture (BRIP 66260a) previously isolated in 2017 from leaves of *Zamia hamannii* growing in the Cairns Botanical Gardens was also examined.

2.2.2 Morphology

Images of selected isolates were made after 2 – 4 wk of growth on PDA at 25 °C. Slide preparations from colonies sporulating on PDA were mounted in lactic acid. Sections through conidiomata were made by hand. Cells mounted on glass slides in 100% lactic acid were measured using a Leica DM5500B compound microscope. Images of cells were captured under Nomarski interference with a Leica DFC500 camera.

Figure 2.1. Cycads at Mount Glorious. (a, b) *L. peroffskyana* and (c) *M. lucida*

2.2.3 DNA sequences

Polymerase Chain Reaction (PCR) amplicons of the internal transcribed spacer (ITS), β-tubulin (BTUB) and RNA Polymerase II (RPB2) from genomic DNA from selected isolates were provided by the BRIP, Ecosciences Precinct, Dutton Park (see Appendix B for methods) and DNA was sequenced from the purified PCR amplicons by Macrogen (Seoul, Korea) using Sanger sequencing.

2.2.4 Phylogenetic analyses

The DNA sequences were analysed, and a consensus of the forward and reverse sequences was computed using Unipro UGENE v. 34.0 (Okonechnikov et al., 2012). A BLAST search against the National Centre for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/) against type specimens was used to initially identify isolates generated in this study. Sequences obtained in this study have not been registered in GenBank. Reference sequences used in the phylogenetic analyses were downloaded from the NCBI GenBank nucleotide database (Appendix

C). Sequences were aligned using UGENE v. 34.0 (Okonechnikov et al., 2012). Alignments were manually checked and improved where necessary using MEGA v. 7 (Kumar et al., 2016) and were concatenated (for BRIP 71434a) using the same software.

The ITS, BTUB and RPB2 sequences were aligned in MAFFT (Katoh et al., 2009) (available: http://www.ebi.ac.uk/Tools/msa/mafft/), and analysed via the phylogenetic criteria, maximum likelihood (ML). Maximum likelihood was applied as a search criterion in IQ-TREE v. 1.6.12 (Nguyen et al., 2015). The nucleotide substitution model used was general time-reversible (GTR, command -m GTR+F). The IQ-TREE analyses were run with an Ultrafast (UF) Bootstrap analysis (command -bb) with 1000 ML bootstrap replicates and an approximate likelihood ratio test (aLRT) search (command -alrt) with 10000 replicates. Phylograms were visualized with FigTree v1.4.4 program (available: http://tree.bio.ed.ac.uk/software/figtree/) and edited in Microsoft PowerPoint v. 16.42 (2020) and Adobe Acrobat Pro® v. 2017.008.30051 (2017, Adobe®, San Jose, CA). Minimum spanning networks were generated using POPART v. 1.7 (Leigh & Bryant, 2015).

2.3 Results

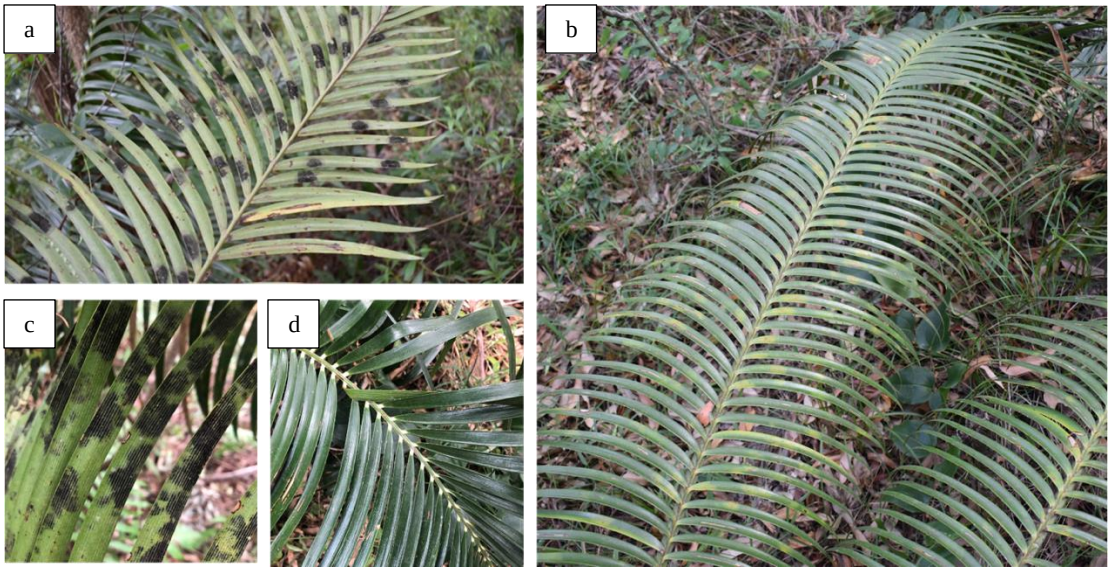

Figure 2.2. Leaf symptoms of host plants. (a, b, c) *L. peroffskyana* leaf symptoms. (d) Small brown necrotic regions on leaf spots on *M. lucida*

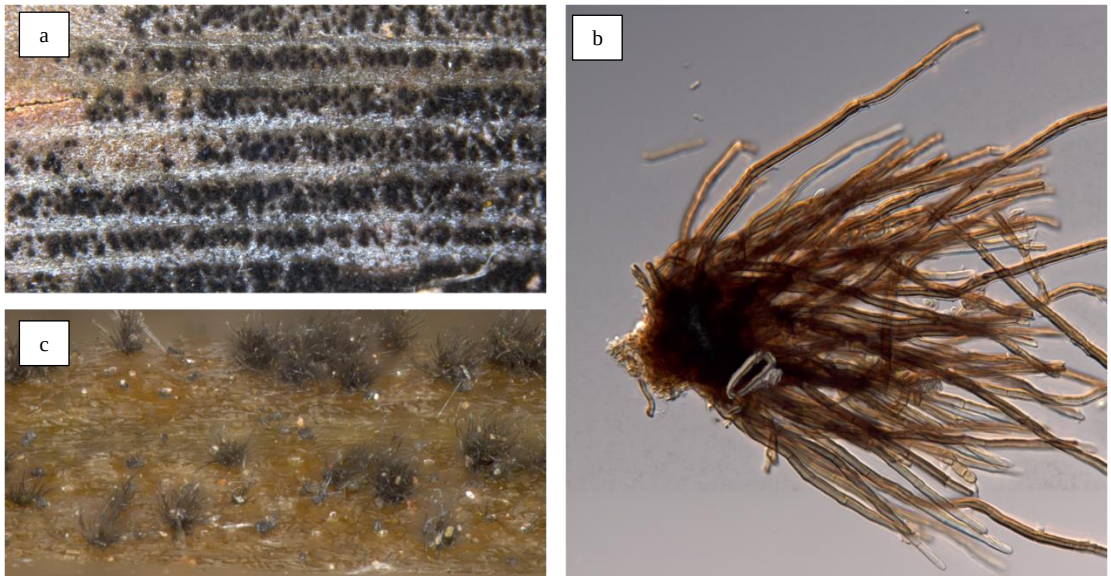

Figure 2.3. Black leaf spot on *L. peroffskyana*. (a, c) Conidiogenous cells on host substrate. (b) Fascicle of conidiophores

2.3.1 Morphology

Fourteen fungal cultures were isolated from the leaves of *L. peroffskyana* (6 isolates) and *M. lucida* (8 isolates) (Table 2.1). All isolates were associated with leaf spots (Figure 2.2; Figure 2.3). The colony characteristics were imaged, and morphology of isolates was determined (Figure 2.4-2.6).

Table 2.1. Fungal isolates examined in this study

Species	Culture*	Host	Collection location	Collection date
Acrocalymma sp.	BRIP 71369a	*Macrozamia lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71173c	*Lepidozamia peroffskyana*	Site 2	12/05/2020
Cladosporium sp.	BRIP 71173d	*L. peroffskyana*	Site 2	12/05/2020
Cladosporium sp.	BRIP 71173e	*L. peroffskyana*	Site 2	12/05/2020
Cladosporium sp.	BRIP 71367a	*M. lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71372a	*M. lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71368a	*M. lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71370a	*M. lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71363a	*M. lucida*	Site 1	17/06/2020
Cladosporium sp.	BRIP 71364a	*M. lucida*	Site 1	17/06/2020
Muyocopron zamiae	BRIP 66260a	*Zamia hamannii*	Site 3	19/09/2017
Penicillium sp.	BRIP 71434a	*L. peroffskyana*	Site 1	12/05/2020
Periconia cyperacearum	BRIP 71173a	*L. peroffskyana*	Site 1	12/05/2020
Samsoniella sp.	BRIP 71359b	*L. peroffskyana*	Site 4	17/06/2020
Unidentified sp.	BRIP 71373a	*M. lucida*	Site 1	17/06/2020

Site 1: Mount Glorious, QLD, Australia, 27°17’13”S 152°45’22”
Site 2: Mount Glorious, QLD, Australia, 27°17’2”S 152°45’27”E
Site 3: Cairns Botanical Gardens, Cairns, QLD, Australia, 16° 54' 1”S 145° 44' 57”E
Site 4: Mount Glorious, QLD, Australia, 27°17’13”S 152°45’20”E
*BRIP = Queensland Plant Pathology Herbarium, Dutton Park, Brisbane

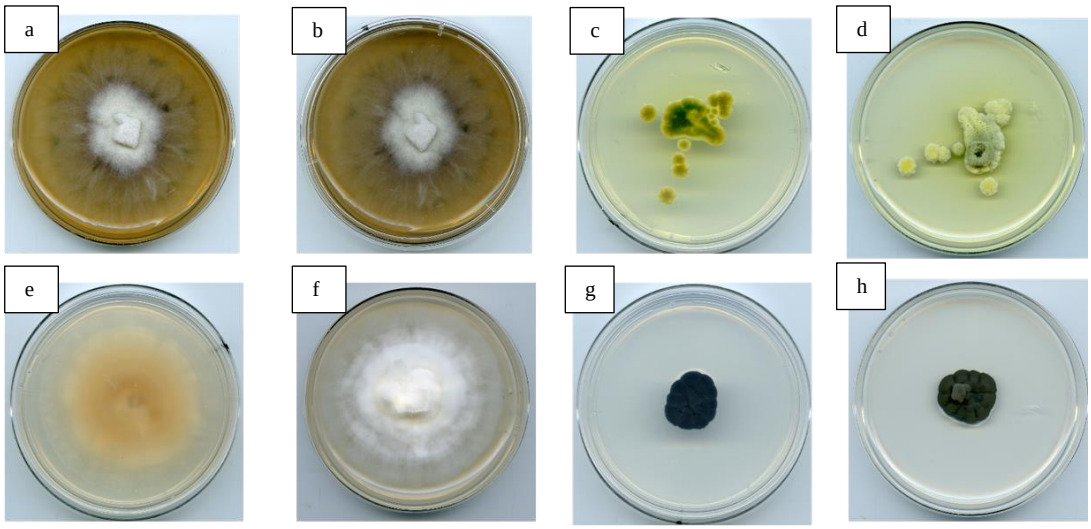

Figure 2.4. Colonies on PDA after 4 wk at 25 °C. (a, b) BRIP 71369a front and back. (c, d) BRIP 71434a front and back. (e, f) BRIP 71359b front and back. (g, h) BRIP 71373a front and back

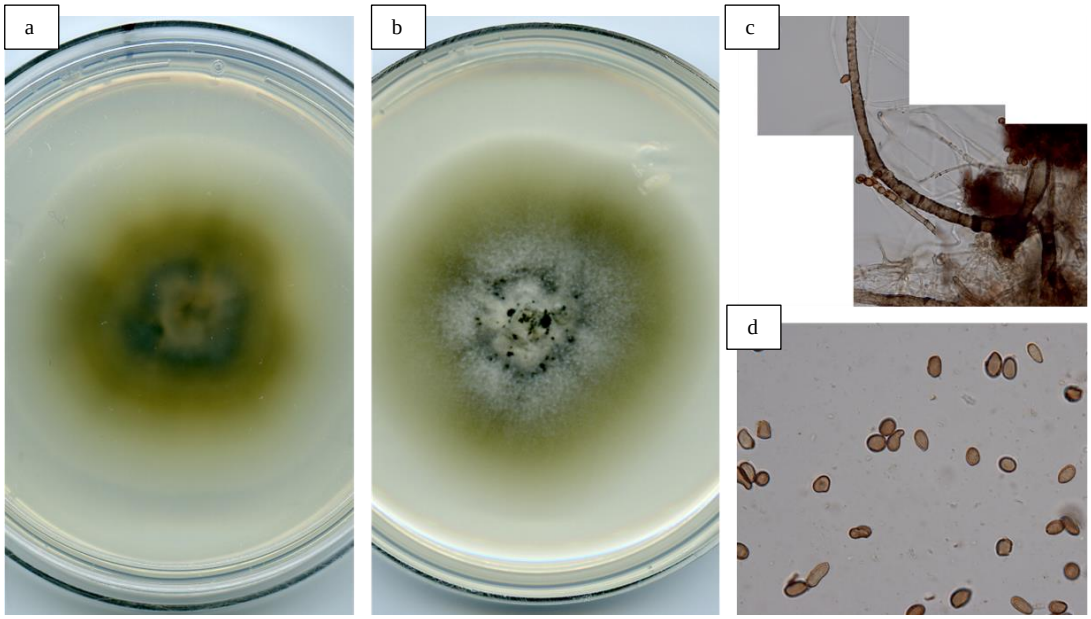

Figure 2.5. BRIP 71173a. (a, b) Colonies after 4 wk at 25 °C front and back. (c) Conidiogenous cells and conidia. (d) Conidia

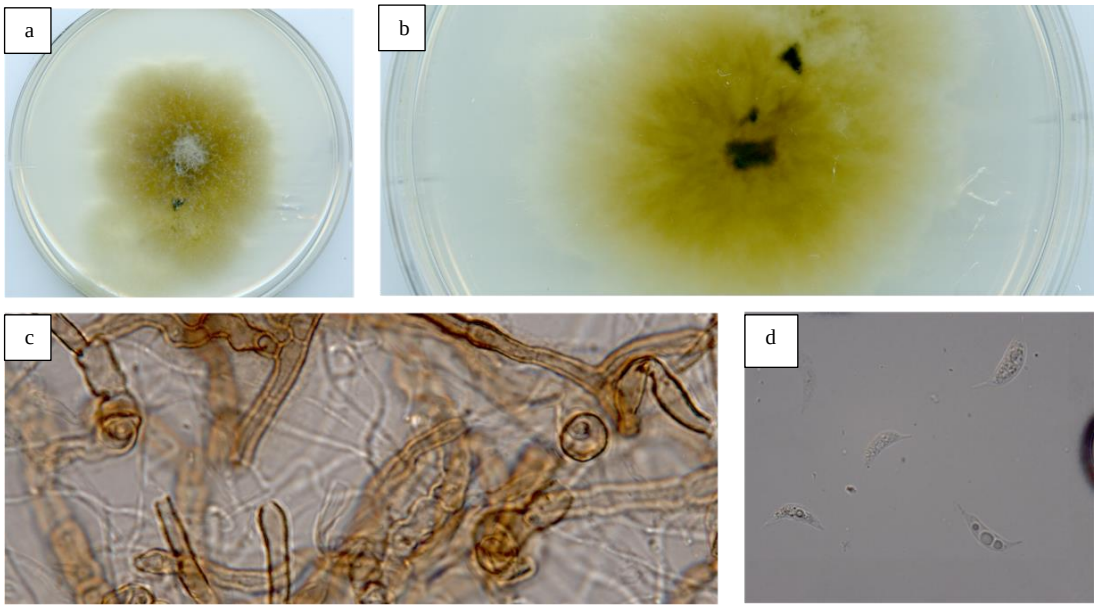

Figure 2.6. BRIP 66260a. (a, b) Colonies after 4 wk at 25 °C upper and lower. (c) Developing conidiogenous cells. (d) Conidia

2.3.2 Phylogeny

Analysis of the ITS region identified the isolates as Ascomycota comprising Dothideomycetes (11), Eurotiomycetes (2) and Sordariomycetes (1). One previously unidentified reference isolate (BRIP 66260a) from *Zamia hamannii* was shown to belong to the Dothideomycetes (Ascomycota).

Phylogenies were made for isolates obtained in this study. Phylogenetic trees are presented based on multiple sequences alignments of the ITS region. Additional loci were used to classify BRIP 71434a (BTUB and RPB2). The morphology of two isolates, BRIP 71173a and BRIP 66260a, was determined. Novel species will be formally described according to the rules of the *International Code of Nomenclature for algae, fungi, and plants* (https://www.iapt-taxon.org), at a later date.

2.3.3 Taxonomy

***Acrocalymma* sp. (BRIP 71369a)**

Classification — *Acrocalymmaceae, Pleosporales, Dothideomycetes.*

All species of *Acrocalymma* are morphologically similar and can only be reliably separated by molecular phylogenetic analysis. Based on a mega-BLAST search of related sequences from type material, the ITS sequence of BRIP 71369a is closest related to *Acrocalymma pterocarpi* (GenBank NR_163327.1; Identities = 469/475

(99%), Gaps = 4/475 (0%)), and *A. fici* (GenBank NR_137953.1; Identities = 486/514 (95%), Gaps = 1/514 (0%)). Nucleotide sequences of the ITS, LSU and SSU regions were obtained from GenBank (Appendix C) to determine the evolutionary relationships between the strain *Acrocalymma* sp. (BRIP 71369a) and other strains.

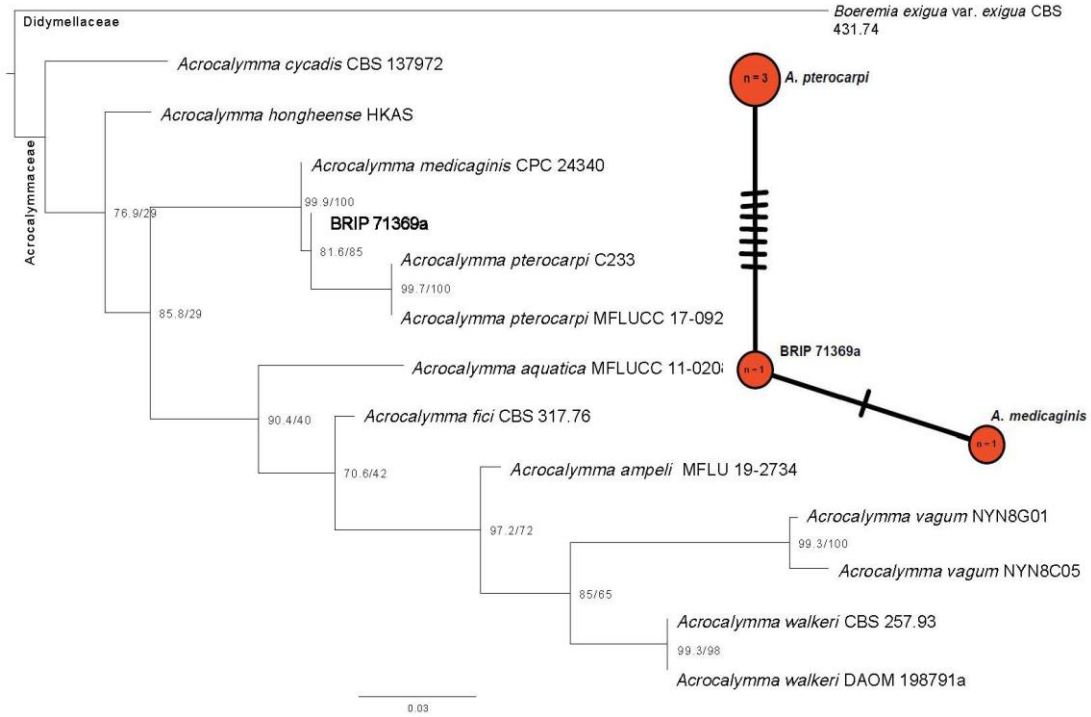

Figure 2.7. Phylogenetic tree of BRIP 71369a. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. Minimum spanning network of *Acrocalymma* ITS sequences generated using POPART v. 1.7 (Leigh & Bryant, 2015); hashes indicate number of parsimony informative characters between taxa. The outgroup is *Boeremia exigua* var. *exigua* CBS 431.74 (Didymellaceae)

Acrocalymma is a monotypic genus in its own lineage of Pleosporales and is sister to other genera in Morosphaeriaceae (H. Zhang et al., 2012). *Acrocalymma* contains eleven species (*Index Fungorum*, 2021). *Acrocalymma* sp. (BRIP 71369a) is recognised as a novel species based on a phylogenetic species hypothesis (Figure 2.7). *Acrocalymma* sp. (BRIP 71369a) is the second associated with cycads, along with *A. cycadis*, collected from leaf litter of *Cycas calcicola* (Crous et al., 2014a). *Acrocalymma medicaginis* is a known pathogen that reddens roots and causes crown rot on lucerne (*Medicago sativa*) in Queensland (Alcorn & Irwin, 1987). *Acrocalymma walkeri* is also known from lucerne in Australia (Trakunyingcharoen et al., 2014). *Acrocalymma* spp. have been isolated from soil in Korea (Das et al., 2020), a decaying

Pterocarpus indicus seed pod and submerged wood in Thailand (Jayasiri et al., 2019; H. Zhang et al., 2012), from *Ficus* sp. in India (Trakunyingcharoen et al., 2014) and Taiwan (Tennakoon et al., 2021), from twigs of *Pittosporum* and dried leaves of *Quercus gluaca* in China (Mortimer et al., 2021), and from *Amaranthusm* sp., *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita* and *Vitis viniferain* in Spain and the USA (Trakunyingcharoen et al., 2014).

***Samsoniella sp.* (BRIP 71359b)**

Classification— *Cordycipitaceae, Hypocreales, Sordariomycetes* .

All species of *Samsoniella* are morphologically similar and can only be reliably separated by molecular phylogenetic analysis. Based on a mega-BLAST search of related sequences from type material, the ITS sequence of BRIP 71359b is closest related to *Samsoniella hepiali* (GenBank NR_160318.1; Identities = 576/579 (99%), Gaps = 1/579 (0%)), and the type species, *S. inthanonensis* (GenBank NR_164420.1; Identities = 535/539 (99%), Gaps = 3/539 (1%)). Nucleotide sequences of the ITS regions were obtained from GenBank (Appendix C) to determine the evolutionary relationships between *Samsoniella* sp. (BRIP 71359b) and other strains.

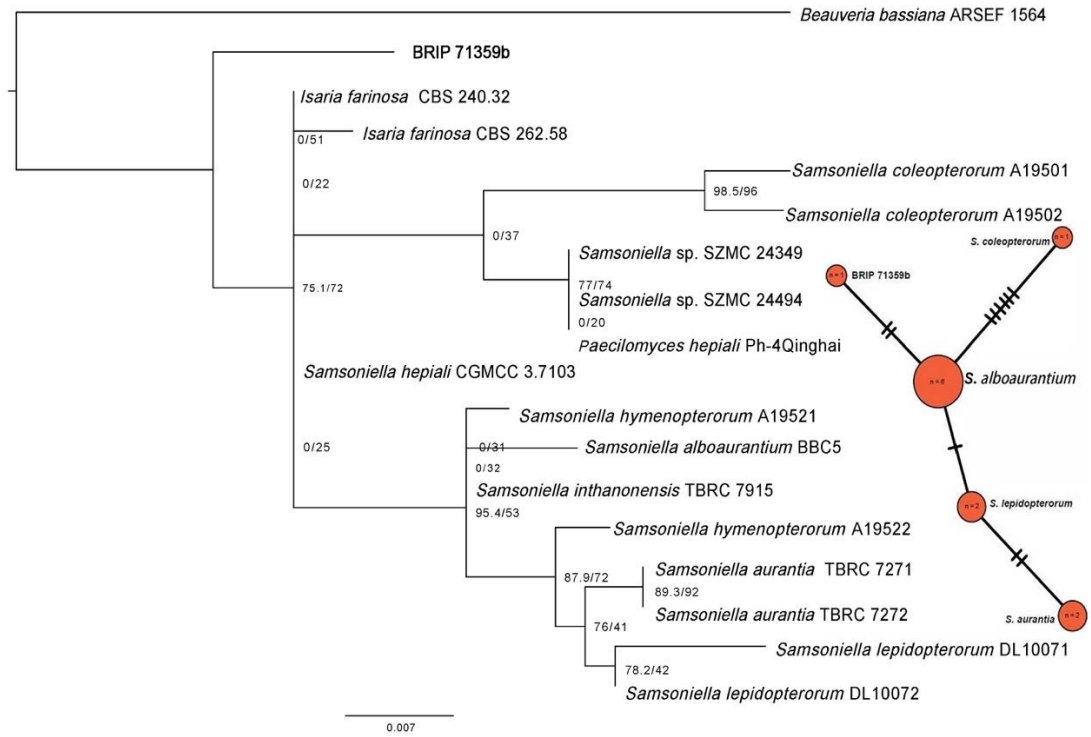

Figure 2.8. Phylogenetic tree of BRIP 71359b. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000

UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. Minimum spanning network of all available *Samsoniella* ITS sequences generated using POPART v. 1.7 (Leigh & Bryant, 2015); hashes indicate number of parsimony informative characters between taxa. The outgroup is *Beauveria bassiana* ARSEF 1564 (Cordycipitaceae)

Based on the phylogenetic analysis, *Samsoniella* sp. (BRIP 71359b) represents a taxonomic novelty, making it a first record for Australia and the only species known from a cycad (Figure 2.8). Mongkolsamrit et al. (2018) established *Samsoniella* based on a combination of molecular and morphological evidence. Since then, more taxa have been assigned to the genus (Chen et al., 2020; Kovač et al., 2020; Y.-B. Wang et al., 2020). Morphological data showed *Samsoniella* is distinguished by way of oval to fusiform conidia, and bright red-orange stromata. *Samsoniella* is in the Cordycipitaceae (Kepler et al., 2017), which are a diverse group of entomopathogenic fungi. The type, *S. inthanonensis,* was collected from Doi Inthanon National Park in Thailand, from lepidopteran larvae (Mongkolsamrit et al., 2018). The phylogenetic analysis showed *Isaria farinosa* (CBS 240.32), *I. farinosa* (CBS 262.58) and *Paecilomyces hepiali* (Ph-4Qinghai) represent species of *Samsoniella* (Figure 2.8), in agreement with other studies (Mongkolsamrit et al., 2018; Wang et al., 2020). *Index Fungorum* (2021) currently lists 16 species of *Samsoniella* that have come from insect hosts (Table 2.2).

Table 2.2. Verified reports of *Samsoniella*

Species	Substrate	Country	Reference
Samsoniella aurantia	Larvae (Lepidoptera)	Thailand	Mongkolsamrit et al. (2018)
S. alboaurantium	Soil and insect pupa	Great Britain	Mongkolsamrit et al. (2018)
S. alpina	Larvae of *Hepialus baimaensis*	China	Y.-B. Wang et al. (2020)
S. antleroides	Larvae (Noctuidae)	China	Y.-B. Wang et al. (2020)
S. cardinalis	Pupae (Limacodidae)	China	Y.-B. Wang et al. (2020)
S. coleopterorum	Snout beetle (Curculionidae)	China	Chen et al. (2020)
S. cristata	Pupae (Saturniidae)	China	Y.-B. Wang et al. (2020)

S. hepiali	Larvae and pupae (Lepidoptera)	China; Vietnam	Y.-B. Wang et al. (2020)
S. hymenopterorum	Bee (Vespidae)	China	Chen et al. (2020)
S. inthanonensis	Larvae (Lepidoptera)	Thailand	Mongkolsamrit et al. (2018)
S. kunmingensis	Pupae (Lepidoptera)	China	Y.-B. Wang et al. (2020)
S. lanmaoa	Pupae (Lepidoptera)	China	Y.-B. Wang et al. (2020)
S. lepidopterorum	Pupae (Lepidoptera)	China	Chen et al. (2020)
S. ramosa	Pupae (Limacodidae)	China	Y.-B. Wang et al. (2020)
S. tortricidae	Pupae of Tortricidae (Lepidoptera)	China	Y.-B. Wang et al. (2020)
S. yunnanensis	Pupae (Limacodidae), *Cordyceps* sp. and *Cordyceps cicadae*	China	Y.-B. Wang et al. (2020)

***Muyocopron zamiae* Hern.-Restr. & Crous (BRIP 66260a)**

Classification — *Muyocopronaceae, Muyocopronales, Dothideomycetes.*

Colonies on PDA after 14 d at 25 ˚C olivaceous, flattened, 3 cm diam, margin entire, reverse slate blue (2.6a, b). *Conidiomata* sporodochium-like, dark brown, superficial, scattered, irregular and confluent, conidiomata 20–60 µm. *Conidiogenous cells* integrated, narrowly ellipsoidal to irregular, terminal, 9–15 × 2–5 µm, pale brown, sympodial, poly-blastic (Figure 2.6c). *Conidiogenous loci* terminal, *c.* 1 µm wide, terminal, conspicuous, darkened-refractive. *Conidia* in simple or branched short chains, ellipsoidal to oval, 5–9 × 3–4 µm, subhyaline to pale olivaceous brown, thin-walled, with minute darkened-refractive hila (Figure 2.6d).

All species of *Muyocopron* are morphologically similar and can only be reliably separated by molecular phylogenetic analysis. Based on a mega-BLAST search of ITS sequences, BRIP 66260a was closely related to *Muyocopron zamiae* (NR_172555.1; Identities = 613/617 (99%), Gaps = 1/617 (0%)). This was confirmed using nucleotide sequences of the ITS regions obtained from GenBank (Appendix C) to determine the evolutionary relationships between BRIP 66260a and related strains. Based on a phylogenetic species hypothesis, BRIP 66260a was conspecific with *M. zamiae* (Figure 2.9).

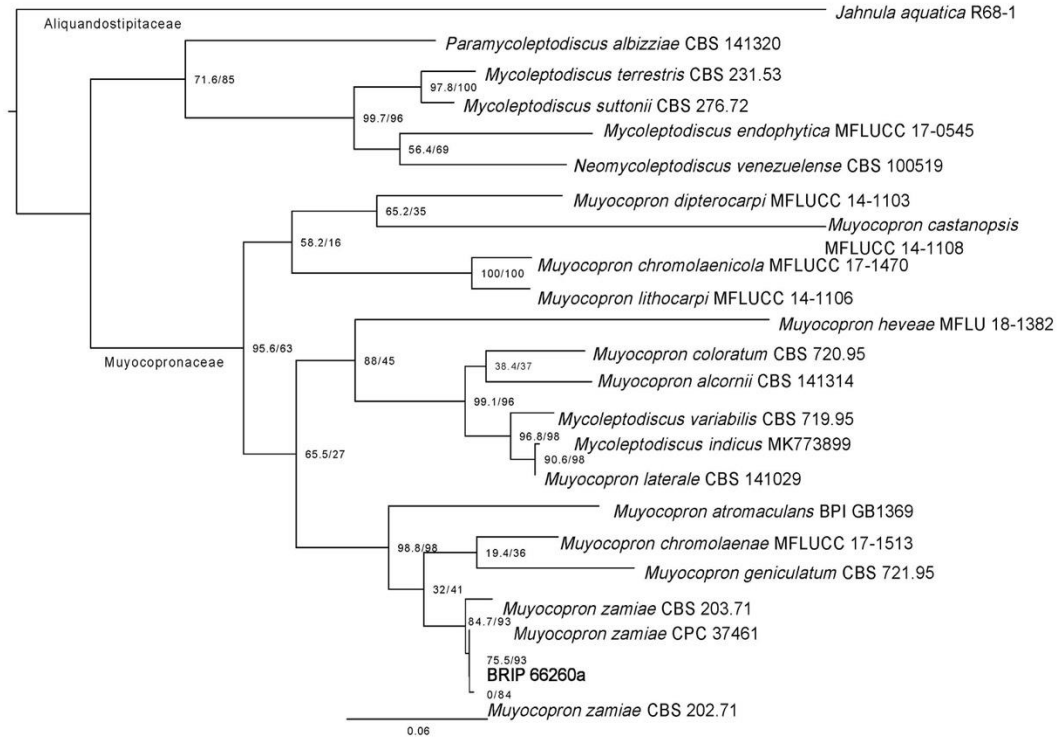

Figure 2.9. Phylogenetic tree of BRIP 66260a. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. The outgroup is *Jahnula aquatica* R68-1 (Aliquandostipitaceae)

Muyocopron spp. are widespread foliar saprobes that have been isolated from a diversity of plants (Hyde et al., 2013; Mapook et al., 2016; Wu et al., 2011). *Muyocopron* comprises 56 accepted species (*Index Fungorum*, 2021). The type species of this genus is *M. corrientinum,* which was isolated from *Oncidium* sp. (Spegazzini, 1881).

Hernandez-Restrepo et al. (2019) showed species of *Muyocopron* and *Mycoleptodiscus* are closely related, and along with *Arxiella, Leptodiscella, Neochlearomyces* and *Paramycoleptodiscus* form the Muyocopronales. *Muyocopron* typically occur as small black spots of ascomata on the host surface (Mapook et al., 2016). *Muyocopron alcornii* was described from Australia on *Epidendrum* sp. (Hernández-Restrepo et al., 2019). Hernandez-Restrepo et al. (2019) reported *M. zamiae* as the cause of leaf spots on *Zamia fischeri* and *Z. integrifolia* in the USA. Earlier morphology-based reports of *M. indicus* on *Zamia* spp. may refer to *M. zamiae* (EI-Gholl & Alfieri, 1991; Sutton, 1973). *Muyocopron zamiae* (BRIP 66260a) on *Zamia hamanii,* represents a new host record for this fungus and the third species from

Zamia.

Penicillium **sp. (BRIP 71434a)**

Classification — *Aspergillaceae, Eurotiales, Eurotiomycetes.*

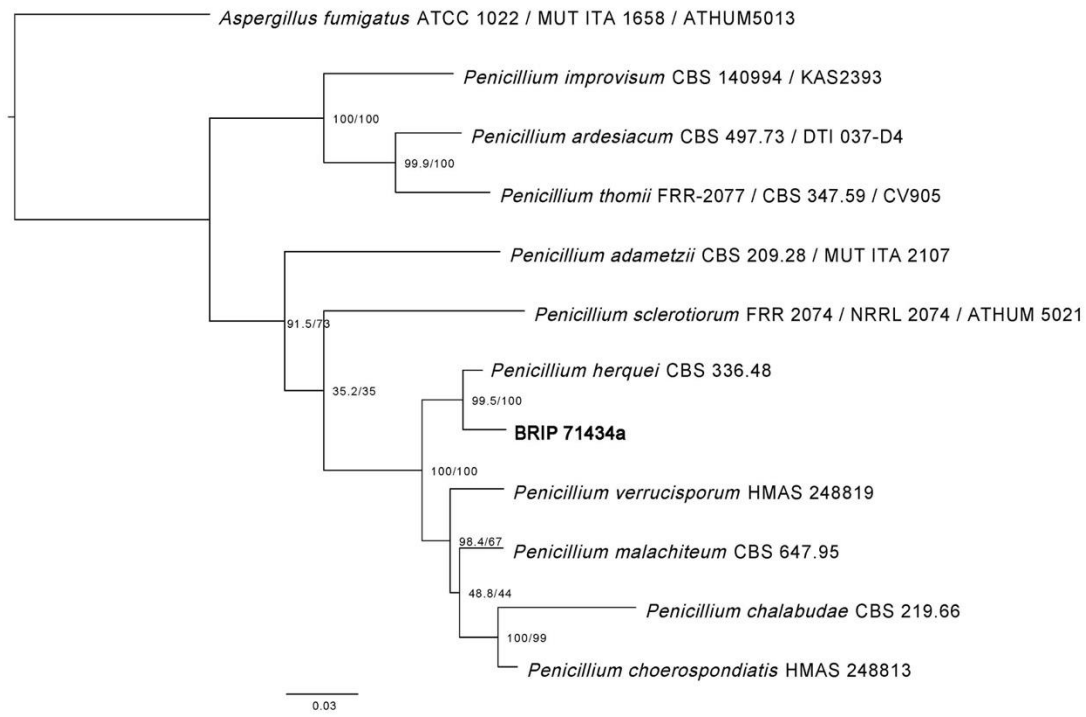

Figure 2.10. Phylogenetic tree of BRIP 71434a. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. The outgroup is *Aspergillus fumigatus* ATCC-1022 / MUTITA1658 / ATHUM013 (Aspergillaceae)

Species of *Penicillium* are morphologically similar and can only be reliably separated by molecular phylogenetic analysis. Based on a mega-BLAST search of related sequences from type material, the ITS sequence of BRIP 71434a has the closest match with *Penicillium herquei* (GenBank: NR_103659.1; Identities = 549/550 (99%), Gaps = 0/550 (0%)), and *P. malachiteum* (GenBank NR_120271.1; Identities = 552/556 (99%), Gaps = 3/556 (0%)). The BTUB sequence of BRIP 71434a has the closest match with *P. herquei* (GenBank: JN625970.1; Identities = 448/446 (96%), Gaps = 2/466 (0%)), and *P. verrucisporum* (GenBank KX885049.1; Identities = 414/448 (92%), Gaps = 3/448 (0%)). The RPB2 sequence of BRIP 71434a has the closest match with *P. herquei* (GenBank: JN121494.1; Identities = 828/837 (99%)), Gaps = 0/837 (0%)). Nucleotide sequences of the ITS, BTUB and RPB2 regions of selected isolates

were obtained from GenBank (Appendix C) to determine the evolutionary relationships between *Penicillium* sp. (BRIP 71434a) and related strains.

Penicillium has had over 1000 epithets, although many have since been transferred to other genera, which leaves 354 accepted species (Visagie et al., 2014). *Penicillium* spp. belong to the Aspergillaceae with two subgenera, *Aspergilloides* and *Penicillium* (Wang et al., 2017). Houbraken and Samson (2011) further subdivided *Penicillium* into 25 clades. Older valid names that do not have an associated culture cannot be verified by molecular data (Visagie et al., 2014). These older names are effectively lost.

Penicillium is one of the most common fungal genera occurring in a range of environments, including food, air, soil, and plants. Eight species produce antibiotic activity against gram-positive bacteria (Houbraken et al., 2011, 2016). *Penicillium echinulatum* and *P. oxalicum* produce β-glucosidase in the biofuel industry (Schneider et al., 2016; G. Yao et al., 2016). Other species produce a range of mycotoxins and other bioactive molecules (Frisvad et al., 2004). Some species have a pivotal role in food fermentation (Kalai et al., 2017). In nature, *Penicillium* spp. are important nutrient recyclers and have been isolated from soil (Choi et al., 2021), on cordyceps fruiting bodies (Guo et al., 2020), and as saprophytes and endophytes (Kim et al., 2008; Ouhibi et al., 2018; Peterson et al., 2005) that may promote plant growth (Khan et al., 2008). Taxonomic novelties are often isolated, and the genus currently comprises over 400 species (Choi et al., 2021; L.-J. Liang et al., 2021; Ouhibi et al., 2018; Park et al., 2015, p. 2; X.-C. Wang et al., 2017; You et al., 2014).

Penicillium sp. (BRIP 71434a) may represent a taxonomic novelty, and based on the ITS, BTUB and RPB2 genes, is closely related to *P. herquei* (Figure 2.10). *Penicillium herquei* is known from a leaf of *Agauria pyrifolia* (Pitt, 1979), and has been investigated for its secondary metabolite production, enzymatic activity, pigmentation, and antiviral compounds (Feng et al., 2019; Guo et al., 2020; Luo et al., 2021; Nishikori et al., 2016; Stodola et al., 1951). *Penicillium* sp. (BRIP 71434a) is the first verifiable *Penicillium* reported from a cycad.

***Periconia cyperacearum* Crous (BRIP 71173a)**

Classification — *Periconiaceae, Pleosporales, Dothideomycetes.*

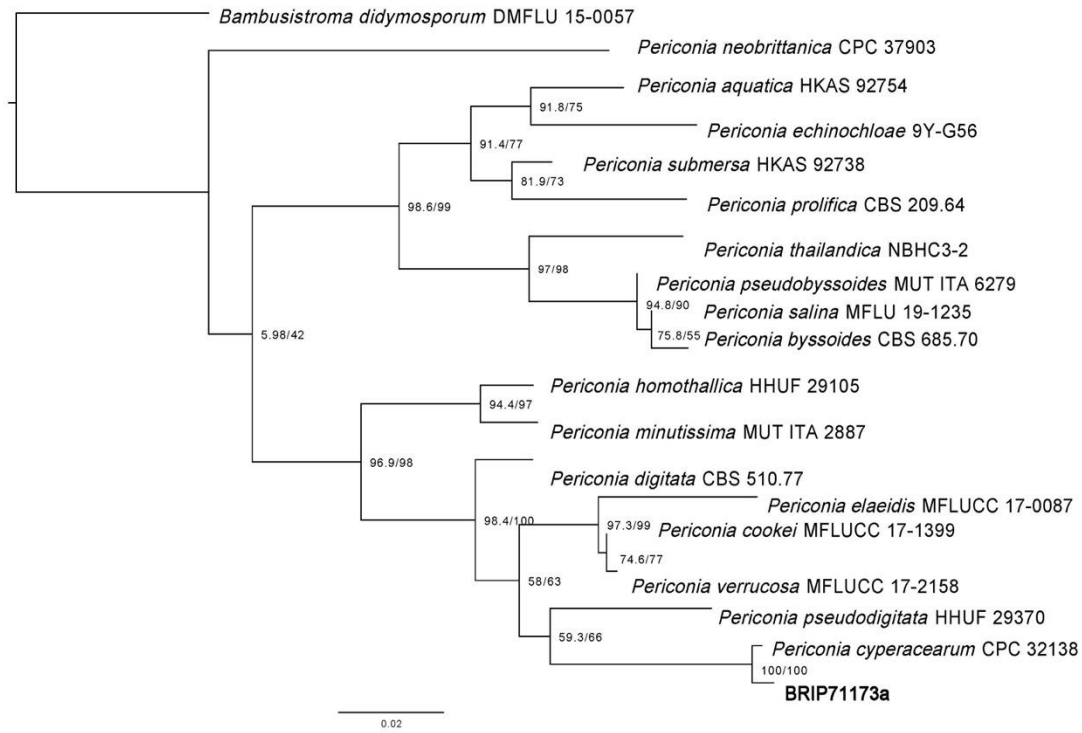

Figure 2.11. Phylogenetic tree of BRIP 71173a. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. The outgroup is *Bambusistroma didymosporum* MFLU 15-0057 (Periconiaceae)

Colonies on PDA after 4 wk at 25 °C reaching 35 mm diam, isabelline becoming darker at centre, with scant aerial mycelium, margin even; reverse olivaceous (Figure 2.5a, b). *Conidiophores* solitary, erect, walls roughened and medium brown at base, becoming paler and subhyaline towards apex, septate; basal cells swollen, 15–20 µm diam, bearing a cluster of dry conidia. *Conidiogenous* cells phialidic, 8 x 4 µm, hyaline to pale brown, cylindrical to ampulliform, tapered at apex (Figure 2.5c). Conidia aseptate, ellipsoid to obovoid or slightly irregular, reddish brown, verruculose, 6–11 × 3–5 µm (Figure 2.5d).

All species of *Periconia* are morphologically similar and can only be reliably separated by molecular phylogenetic analysis. Based on a mega-BLAST search of related sequences from type material, the ITS sequence of BRIP 71173a differs from *Periconia cyperacearum* (GenBank NR_160375.1; Identities = 547/550 (99%), Gaps = 0/550 0%)), and from *P. verrucosa* (GenBank NR_171873.1; Identities = 480/511 (94%), Gaps = 2/511 (0%)). Nucleotide sequences of the ITS region were obtained

from GenBank (Appendix C) to determine the evolutionary relationships between BRIP 71173a and related strains.

The phylogenetic analysis inferred that isolate BRIP 71173a is *P. cyperacearum* (Figure 2.11). This is the first report of *P. cyperacearum* from *L. peroffskyana* and the first time this species has been isolated in association with cycads. *Periconia cyperacearum* is only known from leaves of Cyperaceae in New South Wales, Australia (Crous et al., 2018). *Periconia* is known by the type *Periconia lichenoides,* from stems of various plants in Germany (Tode, 1801). However, this specimen was later lost, which left only Tode's drawing for taxonomic work. *Index Fungorum* (2021) lists 206 species of *Periconia*. However, *Periconia* is paraphyletic and in need of revision (Tanaka et al., 2015). Previously it was treated as a member of the Massarinaceae (Zhang et al., 2012) although Tanaka et al. (2015) recently placed them in the Periconiaceae.

Unident. sp. (BRIP 71373a)

Classification — *Incertae sedis, Chaetothyriales, Eurotiomycetes*.

Based on a mega-BLAST search of type sequences of the ITS region, the closest matches for BRIP 71373a was shared between *Ceramothyrium melastoma* (GenBank NR_111822.1; Identities = 552/643, Gaps = 31/643 (4%)) and *Trichomerium deniquelatum* (GenBank NR_132965.1; Identities = 557/649; Gaps = 46/649 7%)), both with 86% similarity. *Ceramothyrium melastoma* was isolated by Crous et al. (2012) in North Sumatra, Lake Toba, on leaves of *Melastoma* sp. (Melastomataceae). *Ceramothyrium melastoma* clusters in a basal lineage to the Chaetothyriales, rendering *Ceramothyrium* paraphyletic (Figure 2.12) which agrees with (Crous et al., 2012). *Trichomerium* also belongs to Chaetothyriales, previously placed in Capnodiaceae and Chaetothyriaceae (Chomnunti et al., 2012). Nucleotide sequences of the ITS regions were obtained from GenBank (Appendix C) to determine the evolutionary relationships between BRIP 71373a and related strains. Phylogenetic analysis showed that BRIP 71373a is likely a taxonomic novelty and sister to both *Ceramothyrium* and *Trichomerium* (Figure 2.12).

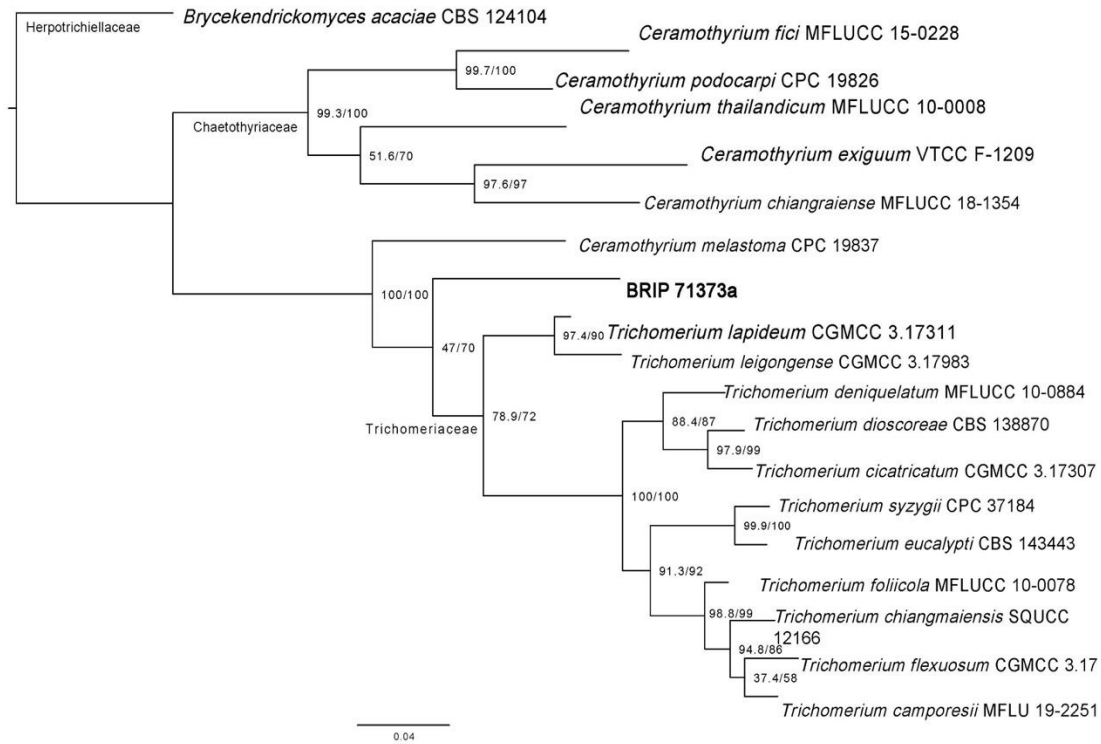

Figure 2.12. Phylogenetic tree of BRIP 71373a. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF Bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT and using GTR as the model of evolution. The aLRT and UF Bootstrap values are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. The outgroup is *Brycekendrickomyces acaciae* CBS 124104 (Herpotrichiellaceae)

Cladosporium spp. (BRIP 71173c, BRIP 71173d, BRIP 71173e, BRIP 71370a, BRIP 71368a, BRIP 71367a, BRIP 71364a, BRIP 71363a and BRIP 71372a)

Classification — *Cladosporiaceae*, *Capnodiales*, *Dothideomycetes.*

A total of nine isolates belonging to *Cladosporium* were identified from molecular sequencing (Table 2.1). The ITS sequences were searched against the NCBI database using BLAST (Table 2.3). A multiple sequence alignment of the ITS region of the isolated *Cladosporium* isolates was performed with selected similar ITS sequences from type material downloaded from GenBank (Appendix C).

Cladosporium currently has 859 epithets in *Index Fungorum* (2021), however many have been since transferred to other taxa. *Cladosporium* spp. are characterised by conspicuous scars and conidia in acropetal chains (Bensch et al., 2012; David, 1997). *Cladosporium* spp. are ubiquitous fungi, often isolated as endophytes and saprobes (Bensch et al., 2010; El-Morsy, 2000; Iturrieta-González et al., 2021).

Using multi locus sequence typing, *Cladosporium* has been split into three complexes, *Cladosporium cladosporioides* (Bensch et al., 2010), *Cladosporium herbarum* (Schubert et al., 2007) and *Cladosporium sphaerospermum* (Zalar et al., 2007). The phylogenetic analysis showed that *Cladosporium* isolates BRIP 71173c, BRIP 71173d, BRIP 71173e, BRIP 71370a, BRIP 71368a, BRIP 71367a, BRIP 71364a, BRIP 71363a and BRIP 71372a belonged to the *C. cladosporioides* complex (Figure 2.13). *Cladosporium cycadicola* has been reported from the phylloplane of *Cycas media* in Australia, which is the only other verified *Cladosporium* from a cycad (Crous et al., 2014a). It is worth noting that the morphology of *C. apicale*, isolated from leaves of *Cycas circinalis* in Sri Lanka (Bensch et al., 2012), resembles that of the fungus on the black leaf spot on *L. peroffskyana* (Figure 2.3). *Cycas circinalis* was synonymised with *C. cycadaceurum* from *Cycas revoluta,* based on morphology (S. Kumar et al., 2007).

Table 2.3. Identification of *Cladosporium* spp. isolates obtained in this study determined by BLAST

Closest species by ITS sequences match in NCBI nucleotide database

Culture	GenBank ITS highest similarity	Accession number	Similarity (%)	Sequence length (bp)
BRIP 71367a	*Cladosporium austroafricanum*	NR_152288.1	100%	539
BRIP 71173c	*C. chasmanthicola*	NR_152307.1	100%	484
BRIP 71173d	*C. pini-ponderosae*	NR_119730.1	99%	533
BRIP 71173e	*C. pseudochalastosporoides*	NR_152296.1	99%	521
BRIP 71372a	*C. pini-ponderosae*	NR_119730.1	99%	558
BRIP 71368a	*C. colombiae*	NR_119729.1	99%	570
BRIP 71370a	*C. austroafricanum*	NR_152288.1	100%	556
BRIP 71363a	*C. pini-ponderosae*	NR_119730.1	100%	549
BRIP 71364a	*C. pini-ponderosae*	NR_119730.1	99%	565

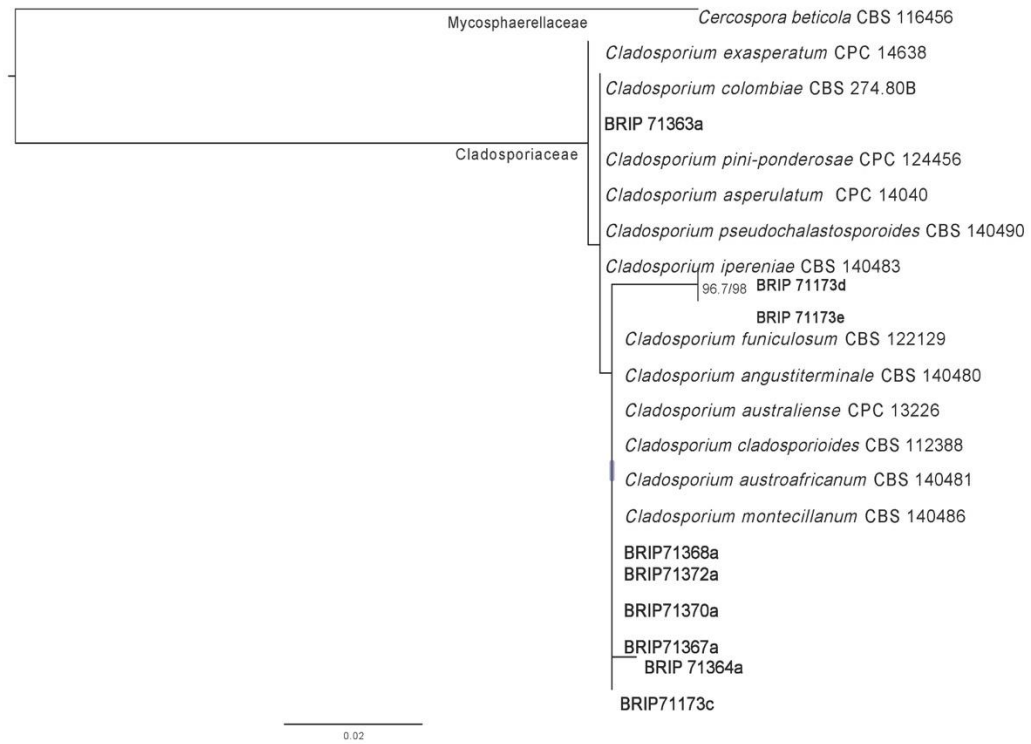

Figure 2.13. Phylogenetic tree of *Cladosporium* isolates. Phylogenetic tree created from a maximum likelihood search using IQ-TREE v. 1.6.12 (Nguyen et al., 2015) with 1000 UF bootstraps (Hoang et al., 2018), 10000 replicates of an aLRT, and using GTR as the model of evolution. The aLRT and UF values (>90) are indicated at nodes. The scale bar represents the expected number of nucleotide substitutions per site. The outgroup is *Cercospora beticola* CBS 116456 (Mycosphaerellaceae)

2.4 Discussion

This study found fourteen foliar fungi associated with leaf spots on *L. peroffskyana* and *M. lucida* (Table 2.1). *Periconia cyperacearum* (BRIP 71173a) on *L. peroffkskyana* represents a new host record. *Acrocalymma* sp. (BRIP 71369a) on *M. lucida,* as well as *Samsoniella* sp. (BRIP 71359b) and *Penicillium* sp. (BRIP 71434a) from *L. peroffskyana,* all represent novel species that will be described at a future date. *Muyocopron zamiae* (BRIP 66260a) from *Z. hamannii* represents a new host record.

Several *Cladosporium* sp. were identified from leaf spots of *L. peroffksyana* (3) and *M. lucida* (6) based on ITS sequences. Isolates of *Cladosporium*, dominated the phylloplane investigation (9 out of 14), providing evidence for *Cladosporium* as established foliar fungi, which aligns with other studies (Bensch et al., 2012; Marin-Felix et al., 2017; Schubert et al., 2009; Torres et al., 2017). *Cladosporium* produce a wide spectrum of secondary metabolites that likely facilitate their adaptation to diverse ecological habitats (Răut et al., 2021). Four isolates had 100% similarity with sequences on GenBank and the remaining five had 99% (Table 2.3). The ITS as a barcode is only useful for identification to the genus rank. Additional genes, i.e., BTUB and Actin (ACT), are needed to identify these taxa using a phylogenetic species hypothesis (Bensch et al., 2015).

An unidentified species (BRIP 71373a) was obtained from *M. lucida.* This isolate had 86% similarity in ITS with *C. melastoma* (GenBank NR_111822.1) and *Trichomerium deniquelatum* (GenBank NR_132965.1). The phylogenetic analysis inferred that BRIP 71373a represents a novel *Ceramothyrium* sp. or *Trichomerium* sp., however additional molecular and morphological data are required to resolve this taxon to the species rank.

The fungi that inhabit the leaves of two cycads, *L. peroffskyana* and *M. lucida,* are diverse across 5 orders (Capnodiales, Chaetothyriales, Pleosporales, Eurotiales and Hypocreales), predominantly Dothideomycetes (11 out of 14 isolates), and all in the most diverse subphylum, Pezizomycota (Ascomycota). This large diversity aligns with other studies in the literature (Appendix A). All microfungi were isolated as epiphytes, though it is likely that some can transition to an endophytic lifestyle, as other species in the same genera are known endophytes (Jin et al., 2018; Nakashima et al., 2020;

Toghueo & Boyom, 2020; Verma et al., 2011) *Samsoniella* are entomopathogenic fungi (Table 2.2) and the identification of *Samsoniella* sp. (BRIP 71359b) is the first known from a leaf surface. All fungi were isolated from leaf spots and chlorotic or necrotic leaf tissue of *L. peroffskyana* and *M. lucida*, though Koch's Postulates are needed for future studies to determine if any isolates were the cause of leaf symptoms (Duhe, 2011).

The study represents the first investigation of foliar fungi that are associated with leaf spots of *L. peroffskyana* and *M. lucida*. All identifications have been confirmed with molecular sequence data. These isolates have been deposited in BRIP and taxonomic novelties will be preserved for further research and made available for the wider scientific community.

2.5 Summary

Phylloplane microfungi of *L. peroffskyana* and *M. lucida* have rarely been investigated. In this chapter, a collection of foliar microfungi were isolated from leaf spots of *L. peroffskyana* and *M. lucida* and identified via DNA sequencing. A reference isolate from *Z. hamannii* was also investigated. All isolates were members of the Ascomycota, which generally dominate phylloplane investigations (Crous et al., 2012; Dong et al., 2021; Pecoraro et al., 2021; Reynolds & Gilbert, 2005). Novel species of *Acrocalymma, Penicillium* and *Samsoniella* were found. Further, a new host record of *M. zamiae* and *P. cyperacearum*. *Cladosporium* spp. were often isolated from the leaves of cycads. An undescribed biodiversity of Australian phylloplane fungi has been discovered and it shows that more await discovery in understudied habitats. These microfungi are now preserved and available for future study.

Phylloplane microfungi have potential as sources of novel enzymes. Selected isolates were studied for their ability to produce protease, amylase, cellulase, and mannanase (Chapter 3).

3. CHAPTER 3: ENZYMATIC ACTIVITY OF FOLIAR FUNGI OF *LEPIDOZAMIA PEROFFSKYANA* AND *MACROZAMIA LUCIDA*

3.1 Introduction

Enzymes have a universal range of application, including in textiles, food processing, bioremediation, biofuel production, biosensing and pharmaceuticals (Adegboye et al., 2021; R. Gupta et al., 2003; L. Kumar & Bharadvaja, 2019; Robinson, 2015). Fungi are well recognised as sources of natural and novel enzymes (Hoffmeister & Keller, 2007; Kango et al., 2019), particularly phylloplane fungi (S. Gupta & Chaturvedi, 2015; Peay et al., 2016; Santos et al., 2019). Their pathogenic, endophytic, and epiphytic lifestyles require the use of extracellular enzymes for host colonisation, nourishment, and competition (Corrêa et al., 2014; Ohm et al., 2012). Compared to endophytic fungal enzymes, epiphytic fungi have rarely been explored. The phylloplane represents a hostile environment with constant UV exposure, high competition, and varying nutrient and water availability guided by seasonal changes (Gomes et al., 2018). The fungi inhabiting the cycad phylloplane are likely candidates to possess unexplored enzymatic capabilities.

A selection of the foliar microfungi isolated from cycads in Chapter 2, were studied for their ability to produce proteases, amylases, cellulases, and mannanases. This study was a qualitative investigation of the solid-state fermentative capabilities of microfungi. The results are discussed within the context of fungal ecology and potential biotechnology.

3.2 Materials and methods

A selection of microfungi reported in Chapter 2 (BRIP 66260a, BRIP 71173a, BRIP 71359b, BRIP 71369a, BRIP 71173c, BRIP 71364a and BRIP 71372a) were assayed for their ability to produce protease, amylase, cellulase, and mannanase. Two isolates, *Aspergillus awamori* (FRR-3550) and *Aspergillus oryzae* (FRR-3863) were purchased from the CSIRO Culture Collection Catalogue (https://fungi.csiro.au/) and used as positive controls.

3.2.1 Protease

Fungal strains were assayed for proteolytic activity using amended methods of Karimi et al. (2019). Nutrient medium consisting of 20 g agar (dry weight, ChemSupply Australia, Port Adelaide, SA, Australia; AL027) and 20 ml skim long life milk (Farmdale, Aldi, Essen, Germany), was diluted in 1 L sterilised water in a reagent bottle and then autoclaved under 1 atm pressure at 130 °C for 98 minutes. The culture medium was then transferred to petri dishes and allowed to cool. Fungal colonies were streaked onto petri dishes and stored in an incubator at 25 °C for 120 h. Images were captured of the plates using a Nikon D5600 and edited in Microsoft PowerPoint v. 16.42 (2020). The presence of protease was determined by a clear zone surrounding the colony, indicating hydrolysis of casein. *Aspergillus oryzae* (FRR-3863) was used as a positive control (Gea et al., 1996).

3.2.2 Amylase

Nutrient medium consisting of 20 g agar (dry weight, Sigma-Aldrich, St Louis, MO; 05040) and 20 g starch (dry weight, ChemSupply Australia, Port Adelaide, SA, Australia; AL027) was diluted in 1 L sterilised water in a reagent bottle and autoclaved under 1 atm pressure at 130 °C for 98 minutes. The medium was then transferred to petri dishes and allowed to cool. Fungal colonies were streaked onto petri dishes and stored in an incubator at 25 °C for 120 h. A dilution of 1:100 ml of iodine (Fisher Scientific, Waltham, MA) in sterilised water was used to flush the colonies. Images were captured of the plates using a Nikon D5600 and edited in Microsoft PowerPoint v. 16.42 (2020). The presence of clear white patches after approximately 30 seconds indicated the presence of amylolytic activity (Hankin & Anagnostakis, 1975). *Aspergillus awamori* (FRR-3550) and *Aspergillus oryzae* (FRR-3863) were used as positive controls (Anindyawati et al., 1998; Sivaramakrishnan et al., 2007). *Aspergillus oryzae* (FRR-3863) was screened for amylolytic activity on a different day to the remaining isolates and has been included as an additional positive control

3.2.3 Cellulase

Nutrient medium consisting of 20 g agar (dry weight, ChemSupply Australia, Port Adelaide, SA, Australia; AL027) and 20 g alpha-cellulose (dry weight, Sigma-Aldrich, St Louis, MO; C8002) was diluted in 1 L sterilised water in a reagent bottle and autoclaved under 1 atm pressure at 130 °C for 98 minutes. The culture medium was transferred to petri dishes and allowed to cool. Fungal colonies were streaked onto petri dishes and stored in an incubator at 25 °C for 120 h. Images were captured of the plates using a Nikon D5600 and edited in Microsoft PowerPoint v. 16.42 (2020). The presence of cellulase was determined by the growth of colonies on the substrate.

3.2.4 Mannanase

Nutrient medium consisting of 20 g agar (dry weight, ChemSupply Australia, Port Adelaide, SA, Australia; AL027) and 20 g galactomannan (dry weight, Sigma-Aldrich, St Louis, MO; G4129) was diluted in 1 L sterilised water in a reagent bottle and autoclaved under 1 atm pressure at 130 °C for 98 minutes. The nutrient medium was transferred to petri dishes and allowed to cool. Fungal colonies were streaked onto the petri dishes and stored in an incubator at 25 °C for 120 h. Images were captured of the plates using a Nikon D5600 and edited in Microsoft PowerPoint v. 16.42 (2020). The presence of mannanase was determined by the growth of colonies on the substrate. *Aspergillus oryzae* (FRR-3863) was used as a positive control (Sakai et al., 2017).

3.3 Results

All seven isolates and positive controls were assayed for enzymatic activity as described. An additional step for plates containing starch consisted of flooding with iodine solution. Plates were inspected for colony growth, which was reported as positive or negative (Table 3.1). Images of colony growth on substrates are shown below (Figure 3.1 - 3.4).

Table 3.1: Results of enzymatic assays. (+) Positive result. (-) Negative result. (n/a) Not applicable

	Enzymatic assays			
	Protease	Amylase	Cellulase	Mannanase
Acrocalymma sp. (BRIP 71369a)	+	+	-	+
Aspergillus awamori (FRR-3550)	n/a	+	n/a	n/a
Aspergillus oryzae (FRR-3863)	+	+	n/a	+
Cladosporium sp. (BRIP 71372a)	+	+	+	+
Cladosporium sp. (BRIP 71173c)	+	+	+	+
Cladosporium sp. (BRIP 71364a)	+	+	+	+
Muyocopron zamiae (BRIP 66260a)	-	-	-	-
Periconia cyperacearum (BRIP 71173a)	-	-	-	-
Samsoniella sp. (BRIP 71359b)	+	+	+	+

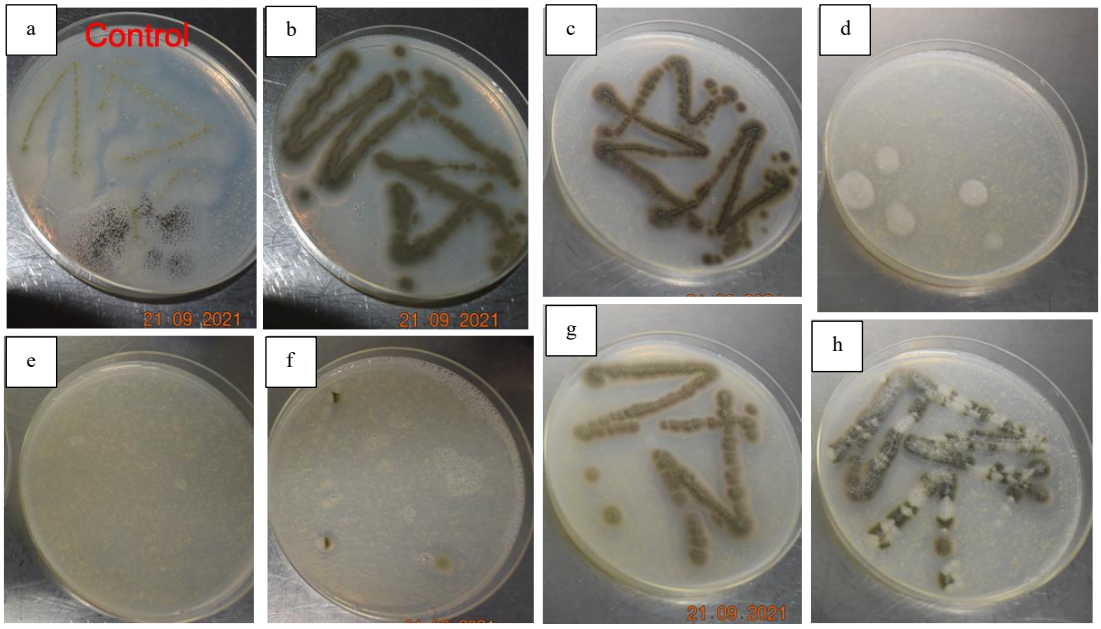

Figure 3.1. Application of isolates to skim milk agar. Clear patches around colonies indicate areas of protease production. (a) *Aspergillus oryzae* (FRR-3863), control. (b) *Cladosporium* sp. (BRIP 71364a). (c) *Cladosporium* sp. (BRIP 71372a). (d) *Acrocalymma* sp. (BRIP 71369a). (e) *Muyocopron zamiae* (BRIP 66260a). (f) *Periconia cyperacearum* (BRIP 71173a). (g) *Cladosporium* sp. (BRIP 71173c). (h) *Samsoniella* sp. (BRIP 71359b)

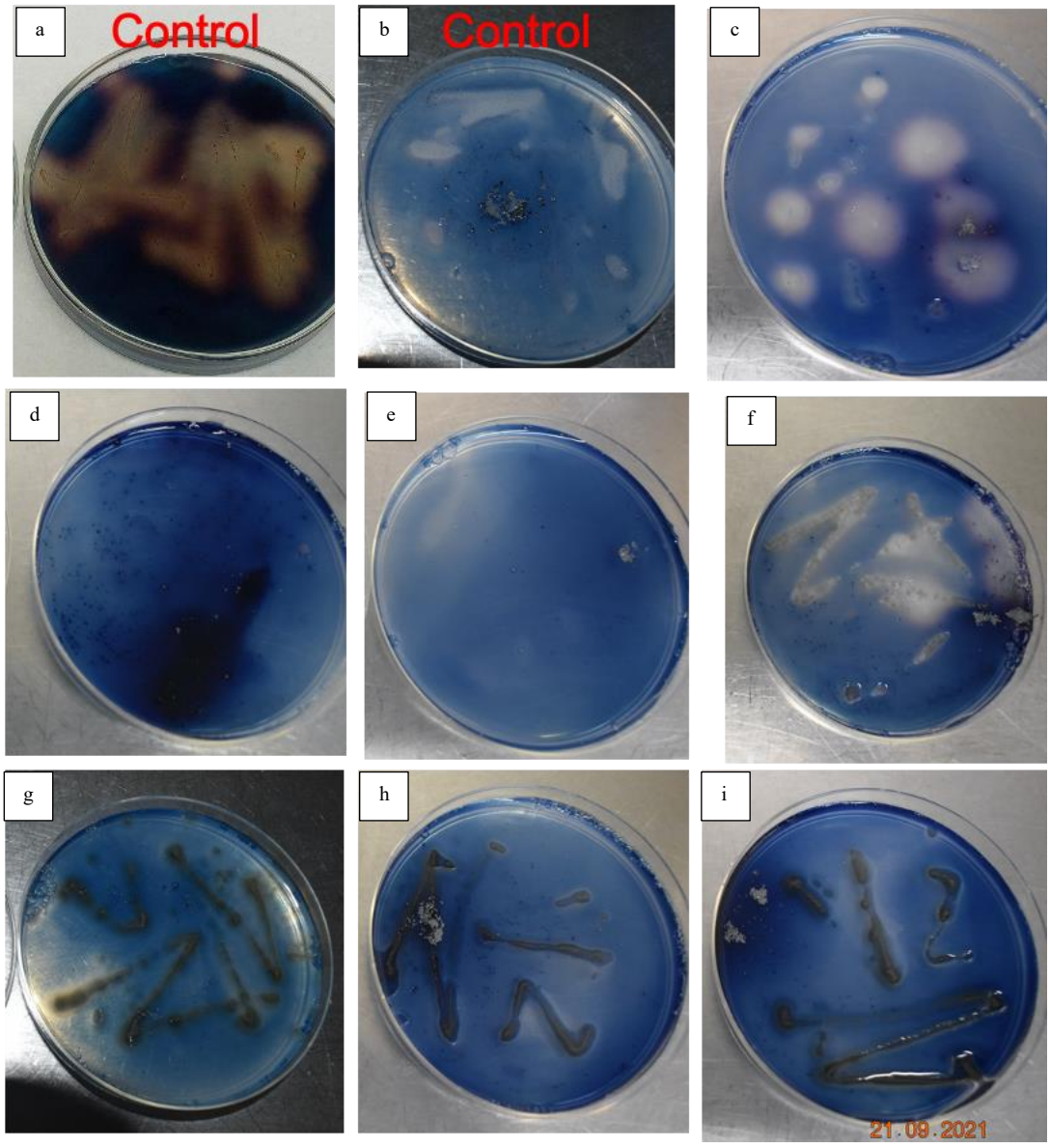

Figure 3.2. Application of isolates to starch agar. White patches around colonies indicate areas of amylase production. (a) *Aspergillus oryzae* (FRR-3863), control. (b) *Aspergillus awamori* (FRR-3550), control. (c) *Acrocalymma* sp. (BRIP 71369a). (d) *Periconia cyperacearum* (BRIP 71173a). (e) *Muyocopron zamiae* (BRIP 66260a). (f) *Samsoniella* sp. (BRIP 71359b). (g) *Cladosporium* sp. (BRIP 71364a). (h) *Cladosporium* sp. (BRIP 71173c). (i) *Cladosporium* sp. (BRIP 71372a)

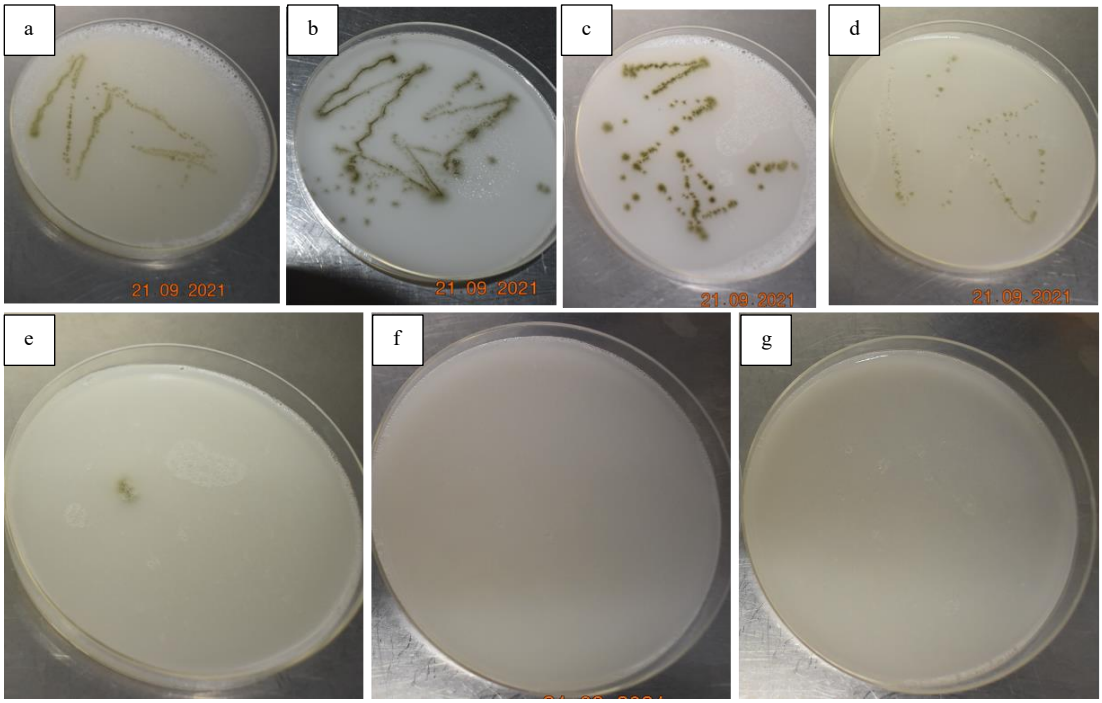

Figure 3.3. Application of isolates to cellulose agar. (a) *Cladosporium* sp. (BRIP 71364a). (b) *Cladosporium* sp. (BRIP 71173c). (c) *Cladosporium* sp. (BRIP 71372a). (d) *Samsoniella* sp. (BRIP 71359b). (e) *Muyocopron zamiae* (BRIP 66260a). (f) *Periconia cyperacearum* (BRIP 71173a). (g) *Acrocalymma* sp. (BRIP 71369a)

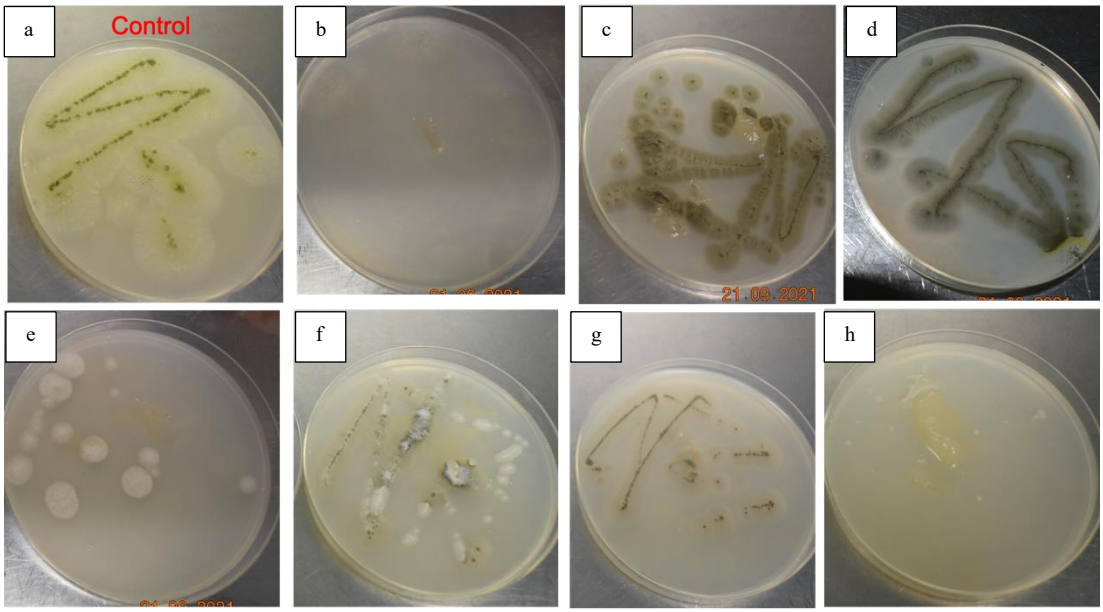

Figure 3.4. Application of isolates to galactomannan agar. (a) *Aspergillus oryzae* (FRR-3863), control. (b) *Muyocopron zamiae* (BRIP 66260a). (c) *Cladosporium* sp. (BRIP 71372a). (d) *Cladosporium* sp. (BRIP 71364a). (e) *Acrocalymma* sp. (BRIP 71369a). (f) *Samsoniella* sp. (BRIP 71359b). (g) *Cladosporium* sp. (BRIP 71173c). (h) *Periconia cyperacearum* (BRIP 71173a)

3.4 Discussion

The microfungi isolated from *L. peroffskyana* and *M. lucida* exhibited novel (first-detection) enzymatic activity. *Samsoniella* sp. (BRIP 71359b), *Acrocalymma* sp. (BRIP 71369a) and the *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a) all produced protease. *Acrocalymma* sp. (BRIP 71369a) produced amylase. Amylase production was also observed in *Samsoniella* sp. (BRIP 71359b) and the *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a) with comparison to the positive controls, *A. oryzae* (FRR-3863) and *A. awamori* (FRR-3550). Growth of colonies on cellulose agar was observed in the *Cladosporium* spp. isolates (BRIP 71173c, BRIP 71372a and BRIP 71364a) and *Samsoniella* sp. (BRIP 71359b). Growth of colonies on the galactomannan agar was observed in the *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a), *Samsoniella* sp. (BRIP 71359b), and *Acrocalymma* sp. (BRIP 71369a).

Cellulase and mannanase production was determined by growth of colonies on cellulose and galactomannan, respectively. A second screening step staining with Congo red, Grams iodine or 1% hexadecyltrimethyl ammonium bromide (Coronado-Ruiz et al., 2018; Florencio et al., 2012; Kasana et al., 2008; Soni et al., 2017; Y. Wang & Easteal, 1999) would have indicated if the isolates metabolised cellulose or galactomannan as a carbon source. When employing this method, a clear hydrolysed boundary is produced which allows for clear comparison of cellulase and mannanase production. A positive control for cellulase would have also supported the results obtained. The growth of colonies on galactomannan and cellulose indicated enzyme production.

The production of protease was determined by the presence of a clear zone of hydrolysis surrounding the fungal colonies. Small light-coloured spots on petri dishes indicated the presence of bacterial contamination which has prevented clear qualitative examination. The control, *A. oryzae* (FRR-3863), returned a positive result (Figure 3.1a). Amylolytic activity was observed in both positive controls by the presence of clear white patches around colonies, that indicated areas of hydrolysed starch (Figure 3.2a, b).

Cladosporium spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a), showed proteolytic activity (Figure 3.1b, c, g). *Cladosporium* spp. are known producers of protease (Acosta et al., 2017; Chaibub et al., 2020; Karimi Jashni et al., 2020; Patil et al., 2015). Karimi Jashni et al (2020) investigated protease expression of the plant pathogen, *Cladosporium fulvum*. Transcriptome data showed only 14 out of the 59 predicted proteases are expressed during *in vitro* and *in planta* growth, indicating tightly regulated expression. Minor amounts of protease genes were expressed during infection of tomato, owing to the stealth pathogenicity of the fungus (Karimi Jashni et al., 2020). Although the pathogenicity of the *Cladosporium* spp. was not confirmed in this study, the presence of proteases might aid in biotrophic growth and pathogenesis on the cycad host.

Cladosporium spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a) produced amylase when compared to the positive controls (Figure 3.2g, h, i). This aligns with other studies (Abe et al., 2015; Patil et al., 2015). *Cladosporium* have been shown to produce amylase, for e.g., in the sponge-associated fungi *Cladosporium tenuissimum* (Sibero et al., 2019), and in *Cladosporium cladospoiroides* with carrot peel as a starch source (Mushimiyimana, 2019), indicating intraspecific genetic variation. Studies have investigated *C. cladosporioides* as producers of a range of enzymes including cellulase and protease (Acosta et al., 2017; Thulluri et al., 2015), which is closely phylogenetically related to the screened isolates. The *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a), produced colonies on the cellulose and galactomannan agar. *Cladosporium* have been shown to produce mannanase in other studies (R. Ma et al., 2018; Soni et al., 2017), however a qualitative investigation for cellulase production by Patil et al. (2015), returned negative results. *Cladosporium* are commonly found in a range of environments and thus possess an enzymatic arsenal (Bensch et al., 2010; 2012).

Samsoniella sp. (BRIP 71359b) exhibited proteolytic activity (Figure 3.1h). Entomopathogenic fungi are renowned producers of proteases, for e.g., *Beauveria bassiana* (Bidochka & Khachatourians, 1987) *Lecanicillium psalliotae* (Yang et al., 2005) and *Isaria fumosorosea* (Xu et al., 2017). Díaz-Godínez et al. (2016) proposed that the mode of infection for entomopathogenic fungi is achieved both mechanically and enzymatically. Proteases are likely secreted to degrade insect cuticles (Cortez-Madrigal et al., 2014; Nunes et al., 2010; Tiago et al., 2002). The growth of

Samsoniella sp. (BRIP 71359a) on cellulose (Figure 3.3d) and galactomannan agar (Figure 3.4f) has not been observed elsewhere. Enzymatic activity of *Samsoniella* sp. (BRIP 71359b) is possibly attributed to the complex lifestyle of entomopathogenic fungi. For example, *Samsoniella* sp. was found to secrete enzymes only during penetration of a nematode egg to preserve the nutrients inside for later growth (Y.-J. Liang et al., 2020). This suggests that *Samsoniella* possess more enzymatic potential under favoured environmental conditions.

Periconia cyperacearum (BRIP 71173a) and *Muyocopron zamiae* (BRIP 66260a) returned negative results to all enzymatic assays (Table 3.1). These species might be more fastidious, potentially in specific relationships with their cycad hosts. Knapp et al. (2018) investigated the functional heterogeneity of comparative genomics between *Cadophora* sp. and *Periconia macrospinosa*, which are common root endophytes. Genome sequencing revealed the presence of proteases and hypothesised that these genes are likely upregulated during colonisation of hosts. *Periconia* sp. have been shown to successfully produce amylase, cellulase, and tyrosinases (Mandyam et al., 2010; Mandyam & Jumpponen, 2005). No other studies investigating the enzymatic potential of *Muyocopron* sp. were found in the literature.

Acrocalymma sp. (BRIP 71369a) showed strong amylase activity (Figure 3.2c), however little protease activity (Figure 3.1d) and no growth on cellulose (Figure 3.3g). This is interesting because no amylase (and cellulase) activity was observed by *Acrocalymma vagum* in a recent study (Susilowati et al., 2020). Patil et al. (2015) suggested that endophytic fungi produce amylase to degrade starch as it becomes available when the plant senesces. *Acrocalymma* have also been investigated for their ability to produce chitinases, glucanases, and polyphenol oxidases (Silveira et al., 2020). Growth of *Acrocalymma* sp. (BRIP 71369a) on starch was comparable to the positive controls, which indicates amylase production and has potential for biotechnological exploitation.

Phylloplane fungi rely on an external excretion of enzymes to digest organic substrates (i.e., leaves, insect frass), for competition and colonisation (Chaibub et al., 2020; Gilbert, 2002; Promputtha et al., 2007, 2010). A range of enzymatic activity was observed in the screen isolates. This implies that despite originating from the same

habitat, these fungi evolved along different evolutionary trajectories and likely possess considerable functional differences within their epiphytic lifestyles.

3.5 Summary

This chapter explored the enzymatic capabilities of six fungi that were isolated from the phylloplane of *L. peroffskyana* and *M. lucida*, and one other fungus, isolated from *Z. hamannii. Cladosporium* spp. (BRIP 71173c, BRIP 71372a, BRIP 71364a), *Acrocalymma* sp. (BRIP 71369a) and *Samsoniella* sp. (BRIP 71359b) could produce protease. *Acrocalymma* sp. (BRIP 71369a) exhibited good amylase activity. *Periconia cyperacearum* (BRIP 71173a) and *M. zamiae* (BRIP 66260a) returned negative results to all enzymatic tests performed. The enzymatic activity of fungi under solid-state fermentation is likely affected by genetic expression and environmental factors. This study has shown the enzymatic potential of foliar fungi isolated from the cycad phylloplane which may have biotechnological significance.

4. CHAPTER 4: DISCUSSION AND CONCLUSION

Fungi are deeply entangled in the evolutionary history and ecology of life on Earth (Peay et al., 2016). Microfungi (yeasts and filamentous forms) comprise the largest proportion of *Fungi* and exert direct and indirect influence on all life on this planet, whether in soil (Eastwood et al., 2011) or associated with plants (Owen & Hundley, 2004), and invertebrates (Lo et al., 2013). Many epiphytic fungi await discovery given that only 8% of the entire fungal kingdom has been discovered (Hawksworth & Lücking, 2017). A hyphal lifestyle allows for rapid response to changes in the substrate and enables redistribution of scarce resources (Boddy, 1999).

Cycads are an ancient gymnosperm (Jones, 2002), and two-thirds of species are endangered due to habitat destruction and illegal removal from the environment (Donaldson, 2003). The microfungi that inhabit the phylloplane of cycads are poorly known. This project investigated the diversity of microfungi associated with the leaves of two cycads, *L. peroffskyana* and *M. lucida*, which are endemic to eastern Australia. The fungi in the cycad phylloplane rely on enzymes to digest nutrient sources and survive. The ability of these fungi to produce protease, amylase, cellulase, and mannanase, was examined on different organic substrates.

Ascomycetes from 5 orders (Capnodiales, Chaetothyriales, Pleosporales, Eurotiales and Hypocreales) were isolated from *L. peroffkskyana* and *M. lucida*. Dothideomycetous species (11 out of 14 isolates) were most abundant. A reference culture (BRIP 66260a) isolated in 2017 from *Zamia hamannii* in the Cairns Botanical Gardens was also identified as a Dothideomycetous species. Dothideomycetes are common phylloplane fungi and their association with plants has been dated to the lower cretaceous (125-113 Ma), by *Bleximothyrium ostiolatum*, an extinct fly speck fungus (Renard et al., 2021).

A novel species of *Acrocalymma* (BRIP 71369a) from *M. lucida* produced amylase comparable to the positive controls. This is the first member of this genus to degrade starch. *Samsoniella* sp. (BRIP 71359b) and *Penicillium* sp. (BRIP 71434a) represent novel species from *L. peroffskyana*. *Samsoniella* sp. (BRIP 71359b) produced protease and amylase and was able to grow on cellulose and galactomannan agar. An unidentified fungus (BRIP 71373a) represents a novel species from *M. lucida*,

belonging to *Ceramothyrium* or *Trichomerium.* These taxa reflect the hidden diversity of fungi on cycad leaves.

Nine *Cladosporium* isolates from *L. peroffskyana* and *M. lucida* could not be identified to the species level. ITS was not reliable for species resolution in this genus. Three *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a) were screened for enzymatic activity. These isolates produced protease, amylase and grew on cellulose agar. The *Cladosporium* isolates successfully colonised galactomannan agar in addition to *Acrocalymma* sp. (BRIP 71369a). Phylloplane fungi produce cellulase and mannanase for colonisation, competition, and nourishment (Arguelles et al., 2016; R. Ma et al., 2018; Y. Ma et al., 2015; Ohm et al., 2012). Although growth of fungal colonies on cellulose and galactomannan was observed, the production of cellulase and mannanase is inconclusive given the agar may have been the preferred nutrient source.

Muyocopron zamiae (BRIP 66260a) from *Z. hamannii* represents a new host record. The isolate represents the third *Muyocopron* species from *Zamia* spp. and is evidence for a specific *Muyocopron*-cycad association. *Periconia cyperacearum* (BRIP 71173a) represents a new host record from *L. peroffskyana*. *Periconia cyperacearum* is also known from Cyperaceae in Australia (Crous et al., 2018). *Muyocopron zamiae* (BRIP 66260a) and *P. cyperacearum* (BRIP 71173a) did not produce enzymes in any of the assays. This infers a fastidious utilisation of nutrients from their cycad hosts (*Z. hamannii* and *L. peroffskyana*, respectively). *Periconia* and *Muyocopron* are known to produce other bioactive metabolites, e.g., production of piperine by *Periconia* sp. (Verma et al., 2011), and azaphilones by the endophytic, *Muyocopron laterale* (Nakashima et al., 2020).

Only fungi with an epiphytic lifestyle were isolated in this study. Epiphytes and endophytes have been found to differ in composition, despite living in close proximity (Dong et al., 2021; Kharwar et al., 2010; Santamaría & Bayman, 2005; H. Yao et al., 2019). Difference in the composition of fungal communities is also influenced by the techniques applied, i.e., culture-dependent, and culture-independent (NGS). For example, Dissanayake et al. (2018) found only a 53% similarity between fungal taxa from grapevine using both detection methods. Shinohara et al. (2021) highlighted

biases, i.e., *Penicillium* were frequently cultured and *Toxicocladosporium*, *Verrucocladosporium*, and *Sterigmatomyces* were unculturable.

In this study, known endophytes, *Periconia*, *Muyocopron*, *Acrocalymma* and *Cladosporium*, were detected (Bensch et al., 2012; He et al., 2019; Hernández-Restrepo et al., 2019; Pereira et al., 2019). An isolate of *Samsoniella* was unexpected as this is a known entomopathogen (Y.-B. Wang et al., 2020). Enzyme production of assayed taxa is likely influenced by genetic regulation dependent on their nutritional modes (i.e., Alazi & Ram, 2018; Jones et al., 2019). Transcription factors are known to regulate expression of enzyme activity in filamentous fungi, e.g., genes coding for plant-cell wall degradation are induced in the presence of certain molecules and are suppressed under conditions where these enzymes aren't required (Aro et al., 2005). Endophytes and epiphytes may become saprobes following senescence of the host substrate by using leaf degrading enzymes (Promputtha et al., 2010). *Samsoniella aurantia* and *S. inthanonensis* have been isolated from pupae within leaf litter (Mongkolsamrit et al., 2018). It is possible that *Samsoniella* possess an arsenal of plant cell-wall degrading enzymes, e.g., *Samsoniella* sp. (BRIP 71359b) colonised agar containing cellulose and galactomannan. In this study, the use of different nutrient agar or other molecules may have activated genetic pathways for enzyme production (Alazi & Ram, 2018).

The enzymatic activity of isolates in this study have potential for biotechnological use, i.e., protease production by *Cladosporium* spp. (BRIP 71173c, BRIP 71372a and BRIP 71364a) and *Samsoniella* sp. (BRIP 71359b); amylase production by *Acrocalymma* sp. (BRIP 71369a). *Cladosporium* and *Samsoniella* are known producers of protease (Acosta et al., 2017; Karimi Jashni et al., 2020; Y.-J. Liang et al., 2020). This is the first study that demonstrates amylase production by *Acrocalymma* sp.

This study sampled, isolated, preserved, and identified fungi from the phylloplane of two native Australian cycads, *L. peroffskyana* and *M. lucida*, at Mount Glorious, QLD. A large diversity of foliar fungi was found. All isolates were members of the Ascomycota. Novel species of *Acrocalymma*, *Penicillium*, and *Samsoniella* were found which will be named later, according to the rules of the *International Code of Nomenclature for algae, fungi, and plants* (https://www.iapt-taxon.org). Additionally, new host records of *P. cyperacearum* on *L. peroffskyana and M. zamiae* on *Z.*

hamannii, were discovered. *Cladosporium* spp. were ubiquitous fungi in the cycad phylloplane. The collection of these fungal taxa and future naming of taxonomic novelties will facilitate their preservation and conservation. All these isolates are now stored in BRIP for future study.

This study assayed isolates for their ability to produce protease, amylase, cellulase, and mannanase. The *Cladosporium* spp., produced protease and amylase and growth was observed on agar containing cellulose and galactomannan. *Acrocalymma* sp. (BRIP 71369a) produced amylase and growth was observed on agar containing galactomannan. *Samsoniella* sp. (BRIP 71359b) produced protease and amylase and growth was observed on agar containing cellulose and galactomannan. This research has shown the enzymatic potential of foliar cycad fungi for future study and highlighted the hidden diversity of fungi on cycad leaves.

4.1 Future prospects

The diversity of foliar fungi isolated from the phylloplane of *L. peroffskyana* and *M. lucida* and their enzymatic potential warrants further investigation. For a more complete picture of the foliar fungal diversity of *L. peroffksyana* and *M. lucida*, a culture-independent investigation using NGS, would enable comparison of fungal taxonomic composition and identify cryptic taxa that are not culturable (Bálint et al., 2015; Kembel & Mueller, 2014). Many unculturable fungi are endophytes and pathogens (H. Yao et al., 2019; Zheng & Gong, 2019), including Basidiomycetes. Culturable Basidiomycetes are typically slower growing than Ascomycetes (Arnold et al., 2007; Martin et al., 2015).

The application of Koch's postulates (Cohen, 2017) is needed to confirm the cause of the distinct necrotic leaf spots on the upper leaf surfaces of *L. peroffskyana* (personal observation). Although *Lepidozamia* are not endangered, habitat destruction threatens their isolated habitats, and further study of their fungal diseases would promote their conservation.

For most isolates, the molecular barcode of the ITS region was successful in resolution to the species rank. *Cladosporium* is a heterogeneous genus of fungi, and one of the largest with over 772 names (Dugan et al., 2004). Phylogenetic analysis using ITS alone does not provide resolution of *Cladosporium* species (Braun et al., 2003).

Additional genes, i.e., ACT and TEF1, are needed for molecular delimitation at the species rank. The unidentified isolate (BRIP 71373a) has phylogenetic support as a novel *Ceramothyrium* or *Trichomerium*. Sun et al. (2020) successfully identified novel taxa of the Chaetothyriales using combined analyses of five loci. As such, further investigation should involve sequencing ACT and TEF1 loci to identify the *Cladosporium* isolates and the unidentified isolate (BRIP 71373a) to the species rank.

A quantitative measurement of the amylase production by *Acrocalymma* sp. (BRIP 71369a) would allow for comparison with industrial production, from *Aspergillus* spp. and *Pencillium* spp. (de Souza & de Oliveira Magalhães, 2010). Optimization of chemical and physical parameters during fermentation may increase economic and practical viability of the isolate to produce amylase (Francis et al., 2003).

A quantitative assay of the production of protease by the *Cladosporium* spp. (isolates BRIP 71173c, BRIP 71364a, BRIP 71372a) also has potential for biotechnological use. Most fungal protease used in industry comes from *Aspergillus* spp. and *Penicillium* spp. and increasing the commercial production of fungal protease is critical for the advancement of a range of industries (de Souza et al., 2015).

A more accurate qualitative assay investigating cellulase and mannanase production by the obtained isolates, involves staining with Congo red, Grams iodine or 1% hexadecyltrimethyl ammonium bromide following incubation (Coronado-Ruiz et al., 2018; Florencio et al., 2012; Kasana et al., 2008; Soni et al., 2017; Y. Wang & Easteal, 1999). The isolates that grew on agar containing cellulose and galactomannan are appropriate isolates for further study.

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APPENDICES

Appendix A. Fungal species reported on leaves of cycads (Cycadales) that are verifiable by specimens and molecular data

Species	Order (Class)	Culture accession no.	Host	Location	GenBank Accession no. ITS	Reference
Acrocalymma cycadis	Pleosporales (Dothideomycetes)	CBS H-21683^{T} (CPC 17345 = CBS 137972)	*Cycas calcicola*	Australia	KJ869124	Crous et al. (2014a)
Alloconiothyrium encephalarti	Pleosporales (Dothideomycetes)	CBS H-24161^{T} (CPC 35980 = CBS 146012)	*Encephalartos* sp.	South Africa	MN562102	Crous et al. (2019a)
Boeremia exigua var. *exigua*	Pleosporales (Dothideomycetes)	PEXC	*Cycas circinalis*	India	MK584540	Banerjee and Panja (2020)
Circinotrichum cycadis	Xylariales (Sordariomycetes)	CBS H-21680 ^{T} (CPC 17285 =CBS 137969)	*Cycas* sp.	Australia	KJ869121	Crous et al. (2014a)
Cladosporium cycadicola	Capnodiales (Dothideomycetes)	CBS H-21681 ^{T} (CPC 17251 = CBS 137970)	*Cycas media*	Australia	KJ869122	Crous et al. (2014a)
Colletotrichum cycadis	Glomerellales (Sordariomycetes)	BRIP 71326a	*Cycas revoluta*	Australia	MT439915	Crous et al. (2020a).
Colletotrichum siamense	Glomerellales (Sordariomycetes)	DBSTTJB-9	*Cycas debaoensis*	China	MN305712	Han et al. (2021)
Corynespora encephalarti	Pleosporales (Dothideomycetes)	CBS H-23951^{T} (CPC 35867 = CBS 145555)	*Encephalartos* sp.	South Africa	MK876383	Crous et al. (2019b)
Diaporthe ceratozamiae	Diaporthales (Sordariomycetes)	CBS H-20757^{T} (CPC	*Ceratozamia robusta*	Australia	JQ044420	Crous et al. (2011a)

		17205 = CBS 131306)				
Discosia macrozamiae	Amphisphaeriales (Sordariomycetes)	CBS H-23593^{T} (CPC 32113 = CBS 144436)	*Macrozamia miquelii*	Australia	MH327819	Crous et al. (2018)
Exophiala encephalarti	Chaetothyriales (Eurotiomycetes)	CBS-H 20491$^{\mathbf{T}}$ (CPC 16282, 16281 = CBS 128210)	*Encephalartos transvenosus*	South Africa	HQ599588	Crous and Groenewald (2010)
Gyrothrix encephalarti	Incertae sedis (Pezizomycotina)	CBS H-24364^{T} (CPC 35966 = CBS 146684)	*Encephalartos* sp.	South Africa	MT373376	Crous et al. (2020b)
Hansfordia pulvinata	Xylariales (Sordariomycetes)	CBS H-23581^{T} (CPC 32119 = CBS 144422)	*Macrozamia miquelii*	Australia	MK442587	Crous et al. (2019c)
Muyocopron zamiae	Muyocopronales (Dothideomycetes)	CBS H-23882^{T} (CBS 203.71); CBS H-24348^{T} (CPC 37461 = CBS 146636)	*Zamia fisheri; Zamia integrifolia*	USA	MW883424	Hernández-Restrepo et al. (2019)
Neocatenulostroma abietis	Capnodiales (Dothideomycetes)	CPC14996	*Encephalartos altensteinii*	South Africa	FJ372387	Crous et al. (2008)
Neodevriesia cycadicola	Capnodiales (Dothideomycetes)	CBS H-23949^{T} (CPC 35833 = CBS 145553)	*Cycas* sp.	Italy	MK876397.1	Crous et al. (2019b)
Devriesia hilliana	Capnodiales (Dothideomycetes)	CBS H-20340^{T} (CPC	*Macrozamia communis*	New Zealand	MH863277	Crous et al. (2009)

		15382 = CBS 123187)				
Neothyrostroma encephalarti	Pleosporales (Dothideomycetes)	CBS H-24162^{T} (CPC 35999, CPC 35998 = CBS 146037)	*Encephalartos* sp.	South Africa	MN562105 MN567613	Crous et al. (2019a)
Ochroconis macrozamiae	Incertae sedis (Pezizomycotina)	CBS H-21682^{T} (CPC17262 = CBS 137971)	*Macrozamia* sp.	Australia	KJ869123	Crous et al. (2014a)
Paraphaeomoniella capensis	Phaeomoniellales (Eurotiomycetes)	CBS H-20159^{T} (CPC 15416; CBS 123535)	*Encephalartos altensteinii*	South Africa	FJ372391	Crous et al. (2008)
Parateratosphaeria altensteinii	Mycosphaerellales (Dothideomycetes)	CPC 15133; CBS 123539	*Encephalartos altensteinii*	South Africa	FJ372394	Crous et al. (2008)
Phaeosphaeria cycadis	Pleosporales (Dothideomycetes)	KIB022 (KIB022A= KIB022B)	*Cycas* sp.	China	MK356378	Phookamsak et al. (2019)
Phyllosticta encephalarticola	Botryosphaeriales (Dothideomycetes)	CBS H-24160^{T} (CPC 35970 = CBS 146014)	*Encephalartos* sp.	South Africa	MN562101	Crous et al. (2019a)
Pseudocercospora encephalarti	Mycosphaerellales (Dothideomycetes)	YMMA78	*Encephalartos barteri*	Benin	MK397016	Meswaet et al. (2019)
Pseudocladosporium proteae	Chaetothyriales (Eurotiomycetes)	CPC14902	*Encephalartos altensteinii*	South Africa	FJ372388	Crous et al. (2008)
Ramopenidiella cycadicola	Capnodiales (Dothideomycetes)	CBS H-21684 **T** (CPC 17291 = CBS 137973)	*Cycas calcicola*	Australia	KJ869125	Crous et al. (2014a)
Saccharata kirstenboschensis	Botryosphaeriales (Dothideomycetes)	CPC 15275 = CBS 123537	*Encephalartos princeps*	South Africa	FJ372392	Crous et al. (2008)
Sclerostagonospora cycadis	Pleosporales (Dothideomycetes)	CBS H-20161^{T} (CPC	*Cycas revoluta*	Japan	FJ372393	Crous et al. (2011b)

		12388 = CBS 123538)				
Teratosphaeria encephalarti	Mycosphaerellales (Dothideomycetes)	CPC 14886, CBS 123540; CPC 15281, CBS 123544; CPC 15362, CBS 123541; CPC 15413, CBS 123545; CPC 15464, CBS 123546; CPC 15465; CPC 15466	*Encephalartos altensteinii; Encephalartos lebomboensis*	South Africa	FJ372395 – FJ372401	Crous et al. (2008)
Triposporium cycadicola	Helotiales (Leotiomycetes)	CBS H-21679 **T** (CPC 17215 = CBS 137968)	*Cycas* sp.	Australia	KJ869119	Crous et al. (2014a)
Xenocylindrosporium kirstenboschense	Pezizomycotina (Incertae sedis)	CBS H-20346^{T} (CPC 16311, 16312 = CBS 125545)	*Encephalartos friderici-guilielmi*	South Africa	NR132841	Crous et al. (2011b)
Zygosporium pseudogibbum	Xylariales (Sordariomycetes)	CBS H-23580^{T} (CPC 32120 = CBS 144442)	*Macrozamia miquelii*	Australia	MK442633	Crous et al. (2019c)

Appendix B. DNA extraction and amplification

Fungal mycelium was harvested with a fine needle and placed in cell lysis solution. Genomic DNA was extracted using Gentra Puregene Kits (Qiagen, Australia), following the manufacturers' protocols. The ITS region of the nuclear ribosomal DNA (rDNA) was used to identify all cultures. Additional sequences of BTUB and RPB2 were obtained for BRIP 71434a. The ITS region was amplified using Polymerase Chain Reaction with primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). Additionally, fragments of nuc 28S rRNA (28S) were amplified with primer LROR and LR7 (Vilgalys & Hester, 1990) and fragments of nuc 18S rRNA (18S) were amplified with the primers NS1 and NS4 (White et al., 1990). The primer T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) were used to amplify part of the BTUB gene. The partial region of the RPB2 gene was amplified with the primers RPB2-5F2 (Sung et al., 2007) and RPB2-7cR (Liu et al., 1999).

Appendix C. Culture and GenBank accession numbers of sequences used in phylogenetic trees

Species	Sequence source	GenBank accession number (s)[1]					Reference
		ITS	LSU	SSU	BTUB	RPB2	
Acrocalymma ampeli	MFLU 19-2734	MW063150	MW079342	MW079341			Tennakoon et al. (2021)
Acrocalymma aquatica	MFLUCC 11-0208^{T}	JX276951					Schoch et al. (2014)
Acrocalymma cycadis	CBS 137972^{T}	KJ869124					Crous et al. (2014a)
Acrocalymma fici	CBS 317.76^{T}	KP170619					Trakunyingcharoen et al. (2014)
Acrocalymma hongheense	HKAS 111907	MW424763					Mortimer et al. (2021)
Acrocalymma medicaginis	CPC 24340^{T}	KP170620					Trakunyingcharoen et al. (2014)
Acrocalymma pterocarpi	MFLUCC 17-0926^{T} C233	MK347732					Jayasiri et al. (2019)
Acrocalymma vagum	NYN8G01 NYN8C05	KY548388 KY548387					Jin et al. (2018)
Acrocalymma walkeri	CBS 257.93 DAOM 198791a	MH862398 AF383965					Vu et al. (2019) Liew et al. (2002)
Aspergillus fumigatus	ATCC 1022 ^{T}; MUT ITA 1658; ATHUM 5013	HQ026746			KU935623	EU982097	Schoch et al. (2014); Bovio et al. (2017); Krimitzas et al. (2013)
Bambusistroma didymosporum	MLFU 15-0057	KP761733					Dai and Hyde (2015, unpublished)
Beauveria bassiana	ARSEF 1564^{T}	HQ880761					Schoch et al. (2014)
Boeremia var. *exigua*	CBS 431.74	FJ427001					Aveskamp et al. (2009)
Brycekendrickomyces acaciae	CBS 124104^{T}	FJ839606					Crous et al. (2009)
Ceramothyrium chiangraiense	MFLUCC 18-1354^{T}	MN481190					Wijesinghe et al. (2019)
Ceramothyrium exiguum	VTCC F-1209^{T}	LC360297					Yen et al. (2018)
Ceramothyrium fici	MFLUCC 15-0228^{T}	KT588601					Hongsanan et al. 2014)
Ceramothyrium melastoma	CPC 19837^{T}	KC005771					Crous et al. (2012, p. 162)
Ceramothyrium podocarpi	CPC 19826^{T}	KC005773					Crous et al. (2012, p. 166)
Ceramothyrium thailandicum	MFLUCC 10-0008^{T}	HQ895838					Chomnunti et al. (2012a)
Cercospora beticola	CBS 116456^{T}	AY840527					Groenewald et al. (2005)
Cladosporium angustiterminale	CBS 140480 = CPC15564 ^{T}	KT600379					Bensch et al. (2015)
Cladosporium asperulatum	CPC 14040 ^{T}	HM147998					Bensch et al. (2010)

Cladosporium australiense	CPC 13226 [T]	HM147999	Bensch et al. (2010)
Cladosporium austroafricanum	CBS 140481 = CPC 16763 [T]	KT600381	Bensch et al. (2015)
Cladosporium cladosporioides	CBS 112388[T]	HM148003	Bensch et al. (2010)
Cladosporium colombiae	CBS 274.80B [T]	FJ93615	Schubert et al. (2009)
Cladosporium exasperatum	CPC 14638 [T]	HM148090	Bensch et al. (2010)
Cladosporium funiculosum	CBS 122129 [T]	HM148094	Bensch et al. (2010)
Cladosporium ipereniae	CBS 140483 = CPC 16238[T]	KT600394	Bensch et al. (2015)
Cladosporium montecillanum	CBS 140486 = CPC 17953 [T]	HM148094	Bensch et al. (2015)
Cladosporium pini-ponderosae	CBS 124456 = CPC 13980 [T]	FJ936160	Schubert et al. (2009)
Cladosporium pseudochalastosporoides	CBS 140490 = CPC 17823 [T]	KT600415	Bensch et al. (2015)
Isaria farinosa	CBS 240.32 CBS 262.58	AY624178 AY624179	Luangsa-ard et al. (2005)
Jahnula aquatica	R68-1	JN942354	Campbell et al. (2007)
Muyocopron alcornii	CBS 141314[T]	MK487735	Hernandez-Restrepo et al. (2019)
Muyocopron atromaculans	BPI GB1369[T]	MK487736	Hernandez-Restrepo et al. (2019)
Muyocopron castanoposis	MFLUCC 14-1108[T]	MT137784	Mapook et al. (2020a)
Muyocopron chromolaenae	MFLUCC 17-1513[T]	MT137777	Mapook et al. (2020b)
Muyocopron chromolaenicola	MFLUCC 17-1470[T]	MT137778	Mapook et al. (2020b)
Muyocopron coloratum	CBS 720.95[T]	MH862554	Vu et al. (2019)
Muyocopron dipterocarpi	MFLUCC 14-1103[T]	MT137785	Mapook et al. (2020a)
Muyocopron geniculatum	CBS 721.95[T]	MK487737	Hernandez-Restrepo et al. (2019)
Muyocopron heveae	MFLU 18-1382[T]	MH986836	Senwanna et al. (2019)
Muyocopron laterale	CBS 141029[T]	MK487738	Hernandez-Restrepo et al. (2019)
Muyocopron lithocarpi	MFLUCC 14-1106[T]	MT137786	Mapook et al. (2020a)

Muyocopron zamiae	CBS 203.71[T] CPC:37461 = CBS146636 CBS 202.71	MW883426 MW883424 MW883425			Hernandez-Restrepo et al. (2019)
Mycoleptodiscus endophytica	MFLUCC 17-0545[T]	MG646961			Tibpromma et al. (2017)
Mycoleptodiscus indicus	-	MK773899			Maboni et al. (2019)
Mycoleptodiscus suttonii	CBS 276.72[T]	MK487753			Hernandez-Restrepo et al. (2019)
Mycoleptodiscus terrestris	CBS 231.53[T]	JN711860			Hernandez-Restrepo et al. (2019)
Mycoleptodiscus variabilis	CBS 719.95	MH862553			Vu et al. (2019)
Neomycoleptodiscus venezuelense	CBS 100519[T]	MK487756			Hernandez-Restrepo et al. (2019)
Paecilomyces hepiali	Ph-4Qinghai	EF555097.3			Gao et al. (2012)
Paramycoleptodiscus albizziae	CBS 141320[T]	KX228279			Crous et al. (2016, ppp. 370)
Penicillium adametzii	CBS 209.28 [T]; MUT ITA 2107	JN714929	MG832165	JN121455	Schoch et al. (2014); Marchese et al. (2020)
Penicillium ardesiacum	CBS 497.73 [T]; DTO 037-D4	MH860758	KJ834433	KM089477	Vu et al. (2019); Visagie et al. (2014)
Penicillium chalabudae	CBS 219.66 [T]	KP016811	KP016748	KP064572	Visagie et al. (2016)
Penicillium choerospondiatis	HMAS 248813 [T]	KX885063	KX885043	KX885034	Wang et al. (2017)
Penicillium herquei	CBS 336.48 [T]	JN626101	JN625970	JN121494	Rivera and Seifert (2011)
Penicillium improvisum	CBS 140994 [T]; KAS 2393	KT887867	KT887829	MN969169	Visagie et al. (2016)
Penicillium malachiteum	CBS 647.95 [T]	KC773838	KC773794	JN121543	Schoch et al. (2014)
Penicillium sclerotiorum	FRR 2074 [T]; NRRL 2074; ATHUM 5021	AY373930	JN626001	FJ004491	Schoch et al. (2014); River and Seifert (2011); Krimitzas et al. (2008, unpublished)
Penicillium thomii	FRR 2077 [T]; CBS 347.59; CV905	AY373934	JX271577	JN121655	Haugland et al. (2004); Houbraken and Samson (2011);Visagie et al. (2014)

Penicillium verrucisporum	HMAS 248819 [T]	KX885069	KX885049	KX885040	Wang et al. (2017)
Periconia aquatica	HKAS 92754[T]	KY794701			Hyde (2017, unpublished)
Periconia byssoides	CBS 685.70	MH859902			Vu et al. (2019)
Periconia cookei	MFLUCC 17-1399	MG333490			Hyde et al. (2018)
Periconia cyperacearum	CPC 32138[T]	MH327815			Crous et al. (2018)
Periconia digitata	CBS 510.77	LC014584			Tanaka et al. (2015)
Periconia echinochloae	9Y-G56	MT138587			Yu and Luo (2020, unpublished)
Periconia elaeidis	MFLUCC 17-0087	MG742713			Hyde et al. (2018)
Periconia homothallica	HHUF 29105[T]	AB809645			Tanaka et al. (2015)
Periconia minutissima	MUT ITA 2887	MG813227			Bovio et al. (2018)
Periconia neobrittanica	CPC 37903[T]	MN562149			Crous et al. (2019a)
Periconia prolifica	CBS 209.64[T]	MH858422			Vu et al. (2019)
Periconia pseudobyssoides	MUT ITA 6279	MN944517			Spina (2020, unpublished)
Periconia pseudodigitata	HHUF 29370[T]	LC014591			Tanaka et al. (2015)
Periconia salina	MFLU 19-1235	MN047086			Dayarathne et al. (2020)
Periconia submersa	HKAS 92738[T]	KY794702			Hyde (2017, unpublished)
Periconia thailandica	NBHC3-2	MN398986			Ngo et al. (2019, unpublished)
Periconia verrucosa	MFLUCC 17-2158[T]	MT310617			Phukhamsakda et al. (2020)
Samsoniella alboaurantium	BBC5	MT004827			Kovac et al. (2020)
Samsoniella aurantia	TBRC 7271[T]	MF140764			Mongkolsamrit et al. (2018)
	TBRC 7272	MF140763			
Samsoniella coleopterorum	A19501	MT626376			Chen et al. (2020)
	A19502	MT626625			
Samsoniella hepiali	CGMCC 3.7103[T]	KF757339			Wang et al. (2015)
Samsoniella hymenopterorum	A19521	MN128224			Chen et al. (2020)
	A19522	MN128081			
Samsoniella inthanonensis	TBRC 7915[T]	MF140761			Mongkolsamrit et al. (2018)
Samsoniella lepidopterorum	DL10071	MN128076			Chen et al. (2020)
	DL10072	MN128084			
Samsoniella sp.	SZMC 24494	MW301427			Donát Magyar et al. (2021)
Samsoniella sp.	SZMC 24349	MW301426			Donát Magyar et al. (2021)
Trichomerium camporesii	MFLU 19-2251[T]	MN644590			Marasinghe (2019, unpublished)
Trichomerium chiangmaiensis	SQUCC 12166[T]	MH345731			Maharachchikumbura (2018)

Trichomerium cicatricatum	CGMCC 3.17307[T]	KP174854	Su et al., (2014, unpublished)
Trichomerium deniquelatum	MFLUCC 10-0884[T]	JX313654	Chomnunti et al. (2012b)
Trichomerium dioscoreae	CBS 138870 = CPC 24259[T]	KP004468	Crous et al. (2014b)
Trichomerium eucalypti	CBS 143443 = CPC 32199[T]	MG386068	Crous et al., (2017, unpublished)
Trichomerium flexuosum	CGMCC 3.17988[T]	KX348464	Sun et al. (2020)
Trichomerium foliicola	MFLUCC 10-0078[T]	JX313655	Chomnunti et al. (2012b)
Trichomerium lapideum	CGMCC 3.17311[T]	KP174850	Sun et al. (2020)
Trichomerium leigongense	CGMCC 3.17983[T]	KX348467	Sun et al. (2020)
Trichomerium syzygii	CPC 37184[T]	MT223865	Crous et al. (2020c)

[T] indicate ex-type (sampled from Type material)

[1]ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: partial large subunit (28S) of the nrRNA gene operon; RPB2: partial DNA-directed RNA polymerase II second largest subunit gene; SSU: partial small subunit (18S) of the nrRNA gene operon; BTUB: partial beta-tubulin gene.

Note: unpublished references were direct submissions to the GenBank database.