THE DIVERSITY AND PHYLOGENY OF PHYLLOPLANE YEASTS ON *BANKSIA INTEGRIFOLIA* IN SOUTH-EASTERN QUEENSLAND

A thesis submitted by

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ABSTRACT

Phylloplane yeasts are an important part of microbial communities in tropical and temperate regions. In Australia, the diversity of phylloplane yeasts on crops and indigenous plant species has received little attention. The aim of this study was to isolate and identify phylloplane yeasts from *Banksia integrifolia* in south-eastern Queensland. This study showed that leaves of *B. integrifolia* harboured a high diversity of yeasts, including putatively novel species in *Bannoa*, *Hannaella* and *Vishniacozyma*. Further, several phylloplane yeast species found on *B. integrifolia* in south-eastern Queensland had not been previously reported from Australia.

Banksia integrifolia is an iconic native Australian tree, which is widespread throughout coastal heathlands from Tasmania to southern Queensland. Leaves of *B. integrifolia* at different canopy heights were collected from undisturbed ecosystems at three locations in south-eastern Queensland. Phylloplane yeasts were isolated, cultured and preserved for morphological, physiological and molecular examination. DNA of the yeast was extracted, amplified and their sequences analysed by comparison with the sequences of reference isolates in GenBank.

Molecular phylogenetic analyses showed that most of the yeasts belonged to basidiomycetous genera, especially *Bannoa*, *Hannaella* and *Vishniacozyma*. Five putative novel species were identified from *Bannoa*, *Hannaella*, and *Vishniacozyma*. This study shows that *B. integrifolia* has a rich diversity of phylloplane yeast species, including some that are apparently endemic to Australia.

CERTIFICATION OF THESIS

This thesis is entirely the work of Diman Aswad Latef Krwanji, except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

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ABBREVIATIONS AND ACRONYMS

ITS	internal transcribed spacer
PCR	polymerase chain reaction
BRIP	Queensland Plant Pathology Herbarium
AGRF	Australian Genome Research Facility
UV	ultraviolet
sp.	indicates a single species
spp.	indicates several species
IGS	intergenic spacer
TEF	translation elongation factor
TUB	ß-tubulin gene
HIS	histone gene
CAL	calmodulin gene
ACT	actin gene
CYTB	cytochrome b
RPB	ribonucleic acid polymerase
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
LSU	large subunit region
SSU	small subunit region
QDAF	Queensland Department of Agriculture and Fisheries
ML	maximum likelihood
BI	Bayesian inference
MCMC	Markov chain Monte Carlo
MSC	multispecies coalescent model
YMA	yeast-extract malt-extract agar
PDA	potato dextrose agar
TAE	Tris-acetate buffer with EDTA
NCBI	National Centre for Biotechnology Information

CHAPTER ONE. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Australia is an isolated continent with many endemic animal, plant, and fungal communities. The number of accepted published species that are endemic to Australia represents about 8 % of world's known species (1,900,000) (Chapman, 2009). The number of fungi worldwide is estimated at between 2.2 and 3.8 million (Hawksworth & Lücking, 2017) of which more than 90 % have yet to be discovered (Cheek et al., 2020). There is much evidence to show that Australia has a significant level of endemism in its fungal species (Warcup, 1981; MacArthur & McGee, 2000; Vánky & Shivas, 2008; Vánky & Lutz, 2011; Crouch, 2014; Shivas et al., 2016; Tan et al., 2019). Currently, Australia accounts for about 7 % of the global number of new species described each year (Cheek et al., 2020).

Amongst the understudied fungi are yeasts, with estimate that only 1 % of yeast species worldwide have been discovered (Li et al., 2020). Metagenomic studies are further revealing an enormous diversity of uncultured yeast species in little studied habitats (Tagirdzhanova et al., 2021). Very little is known about the diversity and endemicity of yeasts in Australia, with most taxonomic studies focused on phylloplane species (Shivas & de Miranda, 1983a, b; Lachance et al., 1999; Crous et al., 2019).

1.2. Introduction to yeasts

The word yeast is derived from an old English and Indo-European word that means froth or foam, in reference to the fermentative ability of many well-known species (Sonia & Anil, 2013). In mycology, the term yeast refers to unicellular fungi. Yeasts form a polyphyletic assemblage occurring in both the phyla Ascomycota and Basidiomycota, that spend most of their life cycle in a unicellular form (Kurtzman et al., 2011a; Nagy et al., 2014; Sohlberg et al., 2015). Worldwide, about 1,500 yeast species have been described in 85 ascomycetous genera and 61 basidiomycetous genera (Wang et al., 2015a). Some yeasts are pleomorphic, having the ability to produce both a unicellular budding stage as well as a filamentous stage.

Yeasts are found in a diverse range of natural environments including water bodies, soil, plant surfaces, inside plants and on human and animal skin (Montes de Oca et al., 2016; Aljohani et al., 2018). Yeasts have been used widely in food preparation, alcoholic beverage fermentation, antibiotic production and in many biotechnical applications (Montes de Oca et al., 2016). For example, *Saccharomyces cerevisiae* and *Pichia pastoris*, have been used to produce recombinant proteins at low cost (Demain & Vaishnav, 2009; Cherry et al., 2012). *Saccharomyces cerevisiae* (commonly known as baker's yeast) has long been used as a model organism in many biological studies, and was one of the first eukaryotic organisms to have its genome sequenced (Hanson, 2018). Yeasts are important and inexpensive model organisms for biological research (Hanson, 2018). More biological studies have focused on *S. cerevisiae* than any other eukaryotic organism (Stewart, 2014). Yeasts have been utilised in the production of biopharmaceutical proteins, which have beneficial roles in the treatment of several diseases (Kim et al., 2015).

1.3. Characteristics of yeasts

1.4. Yeast cell ultrastructure

Yeasts are unicellular fungi that have ultrastructural features similar to those of other eukaryotes (Walker, 1998; van der Klei et al., 2011). Yeast cells contain a nucleus, mitochondria, endoplasmic reticulum, cell wall, Golgi apparatus, microbodies, vacuoles and vesicles (Stewart & Russell, 1998; Taddei & Gasser, 2012). Yeast cells usually have one nucleus bounded by a double membrane that encloses the genetic material of the cell (Walker, 1998; Taddei & Gasser, 2012).

1.4.1. Life cycle of yeasts

1.4.2. Vegetative reproduction

Yeasts reproduce vegetatively by budding, fission or bud-fission (Lachance & Walker, 2001) as well as sexually and parasexually. Some yeasts grow as simple irregular filaments (Phale, 2018) or form pseudomycelia, e.g. *Candida* and *Brettanomyces* (Skinner, 1947). The yeasts that reproduce by fission belong to *Taphrinomycotina*, which is an early evolving lineage of ascomycete that has been an important model for studying eukaryote biology (Rhind et al.,

2011). Many yeasts are dimorphic, with both unicellular and filamentous modes of growth (Sipiczki et al., 1998). Dimorphic yeasts lack true hyphae, and the main components of the yeast cell wall are glucan and mannoproteins (Montes de Oca et al., 2016). Most yeasts grow under aerobic conditions; at a temperature between 25–30 ºC; in a moist environment; with available sugars and organic materials (van Dijken et al., 1993; Ginovart et al., 2018). The most common form of cell development in the yeast is multilateral budding, where the daughter cells grow from different positions on the mother cell. In some yeast species, the daughter cell detaches from the mother cell (Figure 1.1) and in other species the daughter cell and subsequent buds remain attached to each other (Steffen et al., 2009; Juanes & Piatti, 2016).

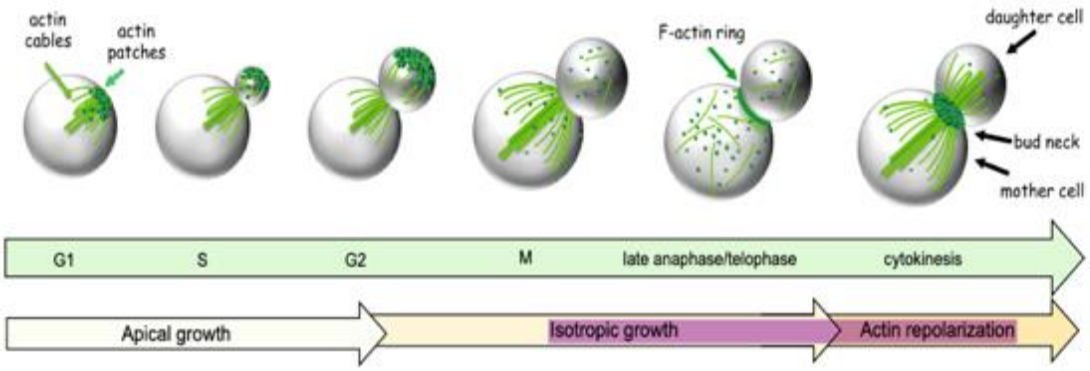


Figure 1.1 Cell division in the budding yeast (Juanes & Piatti, 2016).

Some of the ascomycetous yeast genera have bipolar budding (Figure 1.2), where the daughter cells form repeatedly at the tips of lemon shaped mother cells, e.g. *Hanseniaspora* and *Saccharomycodes* (Lachance & Walker, 2001; Jindamorakot et al., 2009).

Vegetative growth in the fission yeasts occurs by septum formation. An example of a fission yeast is *Schizosaccharomyces pombe*, which produces septa to form new daughter cells (Chang, 2017) (Figure 1.3).

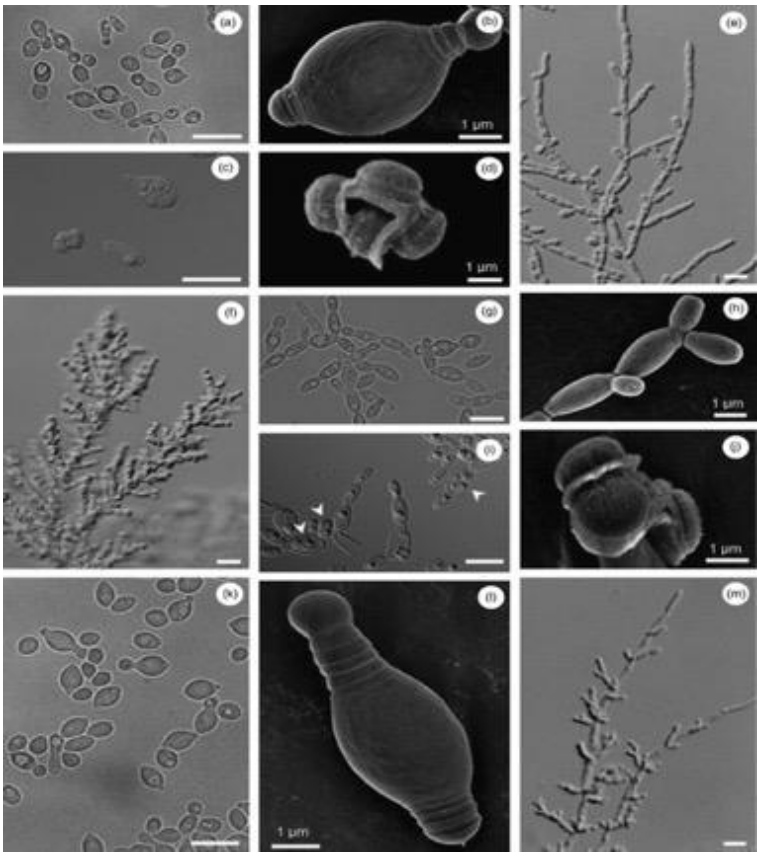


Figure 1.2 Bipolar budding, pseudohyphae and ascospores of *Hanseniaspora thailandica* (Jindamorakot et al., 2009).

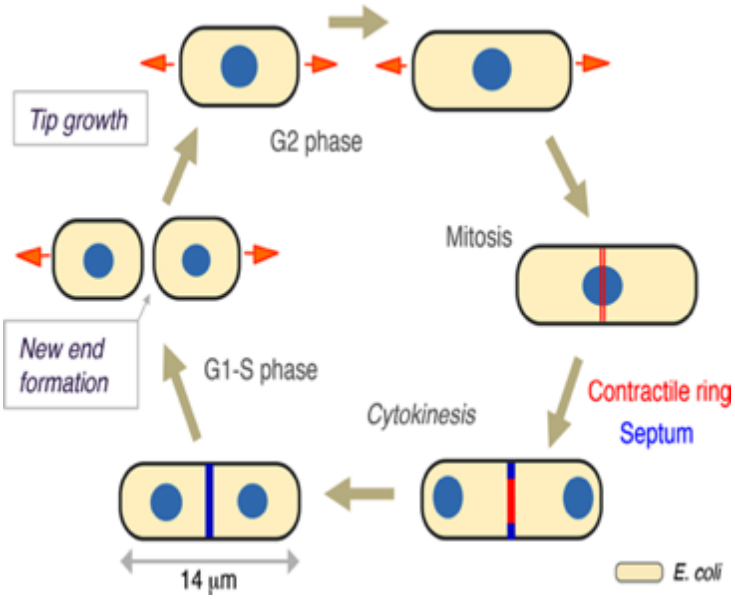


Figure 1.3. Cell cycle fission in *Schizosaccharomyces pombe* (Chang, 2017)

1.4.2.1. Sexual reproduction

The process of sexual reproduction in yeasts occurs via mating and fusion of two haploid yeast cells to produce a diploid zygote. There are usually three phases during sexual reproduction, (i) plasmogamy that occurs when the cytoplasm of two parent cells fuse and form secondary structures, (ii) karyogamy that occurs when the two nuclei fuse and form a diploid zygote, and (iii) meiosis that occurs when the diploid zygote forms four haploid cells or four haploid nuclei (ascospores or basidiospores) (Sandhir et al., 1999) (Figure 1.4).

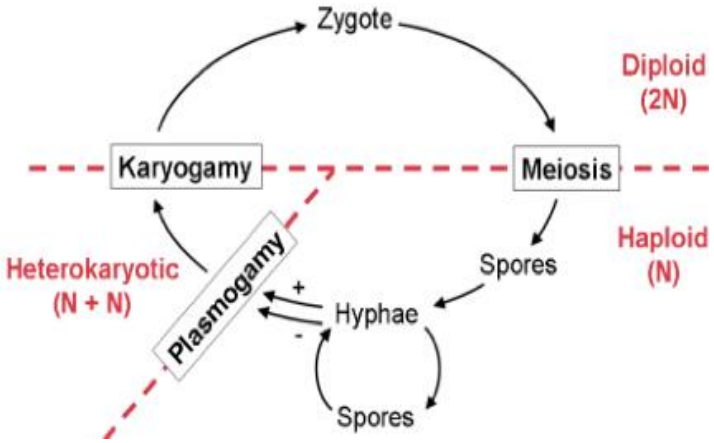


Figure 1.4. Sexual reproduction of a yeast (Sandhir et al., 1999) .

1.5. The phylloplane

The above-ground parts of a plant (phyllosphere) may be divided into the anthosphere (flowers), carposphere (fruits), caulosphere (stems) and the phylloplane (leaves) (Figure 1.5) (Ruinen, 1961; Lindow & Brandl, 2003). The term phylloplane is most often used in reference to the surface of leaves when considered as a habitat for micro-organisms (Leveau, 2006). Worldwide, the total leaf surface area available for microbial colonization has been calculated to be about 2–6 x 10^8 km^2 (Morris et al., 2004). Since the 1950s, the phylloplane has been documented as a major habitat for undescribed microbial taxa (Lindow & Brandl, 2003; Fonseca & Inácio, 2006; Andrews & Hirano, 2012).

The phylloplane can harbour a complex community of epiphytic microorganisms, including bacteria, filamentous fungi, yeasts, algae, protozoa and nematodes (Lindow & Brandl, 2003). Yeasts are a significant component of the phylloplane ecosystem (Glushakova & Chernov, 2004; Inácio et al., 2005). These fungi survive on endogenous and exogenous nutrient sources (Freeman & Beattie, 2008). Endogenous nutrients are leached from leaves by the action of rain, dew and fog as well as by secretory glands (Tukey, 1970; Griffiths et al., 2008). Exogenous nutrient sources include dust, pollen, dead microorganisms and insect excrement (Andrews, 1992). These nutrient sources provide carbohydrates, amino acids, organic acids, sugar alcohols and salts for microbial metabolism (Leveau & Lindow, 2001; Jespersen et al., 2015; Li et al., 2016).

The surfaces of leaves are usually covered with a cuticle composed of hydrophobic waxes (Holloway, 1971; Zeisler-Diehl et al., 2018). The cuticle prevents nutrients from being leached from the leaf (Tukey, 1970; Rodríguez-Sánchez et al., 2020). The abundance of nutrients on the leaf surface is affected by the species of plant, environmental conditions and leaf age (Mercier & Lindow, 2000). Plant leaves in their natural environment are exposed to abrupt changes in temperature, humidity and ultraviolet (UV) radiation, which all influence the ability of yeasts to survive on the leaf surface (Lindow & Brandl, 2003; Fonseca & Inácio, 2006). The survival of phylloplane yeasts depends on their ability to respond and adapt to both changes in microclimate as well as the physical environment (Inácio et al., 2002).

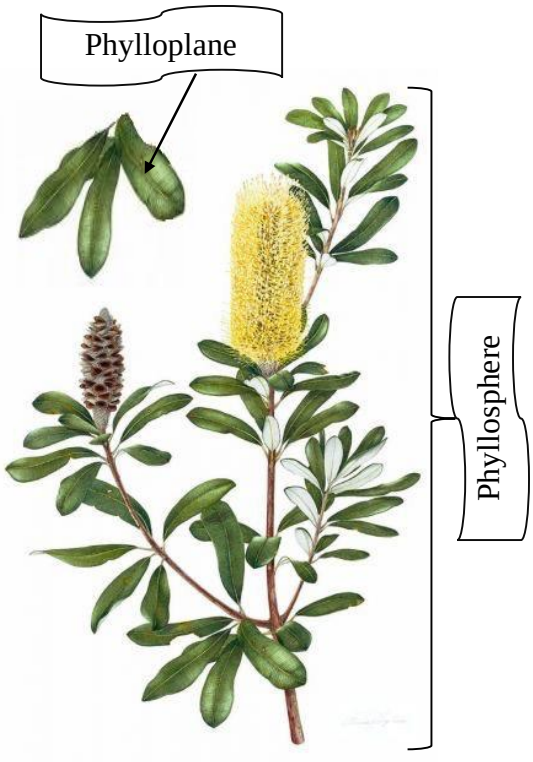


Figure 1.5. Position of the phylloplane and phyllosphere on *Banksia integrifolia*.

1.6. Yeasts as inhabitants of the phylloplane

Although the most abundant phylloplane inhabitants are bacteria and yeasts, their ecological roles are not well understood (Wang et al., 2016; Limtong & Nasanit, 2017). Yeasts have been reported as the main microbial components of the phylloplane of plants from temperate (McBride & Hayes, 1977; Glushakova & Chernov, 2004) and tropical regions (Ruinen, 1963; De Jager et al., 2001). Phylloplane yeasts represent a diverse mycobiota with representatives from both the ascomycetes and basidiomycetes (Phaff, 1987; Limtong et al., 2014). Phylloplane yeasts have received some attention because of their antagonistic activity to fungal plant pathogens in natural and agricultural environments (Stirling et al., 1995; Lindow & Brandl, 2003; Spadaro & Droby, 2016; Ferraz et al., 2019; Into et al., 2020a).

Many phylloplane yeasts belong to the basidiomycetous genera *Rhodotorula* and *Sporobolomyces* in the *Sporidiobolaceae,* which are referred to as pink or red yeasts because of their ability to produce carotenoid pigments in culture (Inácio et al., 2005; Cobban et al., 2016). In Indonesia, 82 yeast species in 16 genera were identified on the leaves of paper

mulberry (*Broussonetia papyrifera*), with the majority identified as *Rhodotorula* and *Cryptococcus* spp. (Sukmawati et al., 2015).

Common non-pigmented phylloplane yeasts include species of *Cryptococcus, Candida* and *Dioszegia* spp. (Wang et al., 2016; Buzzini et al., 2017). For example, two species of phylloplane yeast, *Candida aechmeae* and *Ca. vrieseae* were reported on the leaves of the bromeliads *Aechmea recurvata* and *Billbergia nutans* in south Brazil (Landell et al., 2010). Also in Brazil, new methylotrophic (use methanol as a carbon source) species of *Candida* and *Ogataea* were isolated from mango (*Mangifera indica*) leaves (Santos et al., 2015). Wang et al. (2016) documented *Cryptococcus* spp. from the phylloplane of *Arabidopsis thaliana*.

1.7. Ecology of phylloplane yeasts

Yeasts are the dominant component of phylloplane microbial communities in temperate, tropical and subtropical regions (Nakase, 2000). The association between phylloplane yeasts and plants may be one of parasitism, competition, mutualism, or commensalism (Wells & Varel, 2011). The composition of phylloplane yeast communities is influenced by environmental factors, leaf age and competing microorganisms (Murashige & Skoog, 1962; Gainvors et al., 1994; Cervone et al., 1996; Schisler et al., 2011).

Very few yeasts are plant pathogens. Schisler et al. (2011) provided a review of plant pathogenic ascomycetous yeasts in the genus *Taphrina*, which can cause significant fruit crop losses. *Protomyces* are early diverging ascomyceteous yeasts that cause disease on plants in the Asteraceae and Apiaceae (Kurtzman et al., 2001). *Protomyces* and *Taphrina* have similar lifestyles, spreading as budding yeasts through the air and landing on leaf surfaces (Kurtzman et al., 2011a). *Protomyces macrosporus* was reported to produce galls on the stems, petioles, leaves and fruits of many genera assigned to the Apiaceae throughout Asia, North Africa and Europe (Reddy & Kramer, 1975). Plant pathogenic basidiomycetous yeasts include species of *Itersonilia* that cause flower blights on china aster and sunflower plants (McGovern et al., 2006). Other basidiomycetous species in the genus *Tilletiopsis* cause postharvest disease in apple.

Phylloplane yeasts may also directly benefit their host plants. For instance, the endophytic yeast *Williopsis saturnus*, produced the plant growth hormone indole-3-acetic acid (IAA) (Nassar et

al., 2005; Xin et al., 2009). Some nectivorous yeasts attract pollinators that benefit both the plant host and the fungus (Herrera et al., 2009). In this case the relationship between plant and fungus is mutualistic as both benefit through enhanced reproduction and access to nutrients (Raguso, 2008).

Temporal and spatial factors impact the structure of phylloplane yeast communities (Kemler et al., 2017). Seasonal leaf drop is a particular challenge for phylloplane yeasts. Phylloplane yeast communities generally increase in size from spring to autumn in temperate regions (Fonseca & Inácio, 2006; Glushakova et al., 2007). Some ascomycete taxa varied in frequency throughout the year, e.g., *Torulaspora delbrueckii* and *Saccharomyces paradoxus* were dominant on leaf surfaces throughout the summer, while *Candida oleophila* and *Kazachstania barnettii* were more common during autumn and winter (Glushakova & Chernov, 2004). In the same study, an increase in the number of basidiomycetous *Vishniacozyma* yeasts was found during summer. Phylloplane yeasts are often well-adapted to dry conditions and exhibit high tolerance to aridity by forming extracellular polysaccharides (Glushakova & Chernov, 2010; Buzzini et al., 2017).

Phylloplane yeasts are an important part of microbial communities in tropical regions (Into et al., 2020b), where the leaf surface of tropical rainforests is estimated as 140 x 10^6 km^2 (Morris et al., 2004). Jager et al. (2001) found that in South Africa, yeast densities on mango leaves varied according to the leaf position within the tree canopy. They also found that the prevalent yeasts in the mango phylloplane belonged to *Aureobasidium*, *Sporobolomyces* and *Cryptococcus*. The phylloplane yeasts in tropical and subtropical regions are highly diverse and many novel yeast species have been found by culture-dependent methods (Nakase & Suzuki, 1985; de Azeredo et al., 1998; Limtong et al., 2014; Limtong & Kaewwichian, 2015). The discovery of new species of phylloplane yeasts is most likely to occur in tropical regions (Limtong & Nasanit, 2017).

1.8. Phylloplane yeasts as biological control agents

Phylloplane yeasts may control plant diseases (Sharma et al., 2009). An example is *Torulaspora indica* that reduced sheath blight (*Pyricularia oryzae*, *Rhizoctonia solani*, *Fusarium moniliforme*) on rice (Into et al., 2020a; Limtong et al., 2020). *Saccharomyces cerevisiae*, *Candida albicans*, and *Ca. sake* significantly reduced powdery mildew (*Blumeria graminis*)

and *Cercospora* leaf spot disease on sugar beet (De Curtis et al., 2012). *Tilletiopsis* spp. exhibit potential as biocontrol agents against powdery mildew, particularly in the Cucurbitaceae (Kiss, 2003). Antagonistic yeasts might increase resistance against post-harvest diseases of fruits (Ippolito et al., 2000; Janisiewicz et al., 2008; Spadaro & Droby, 2016).

1.9. Phylogeny of phylloplane yeasts

Molecular phylogenetic studies aim to discover evolutionary relationships and histories based on similarities of genetic loci (barcodes). Phylogenetic approaches are most commonly used for the identification and classification of yeasts. These methods compare multiple DNA sequence alignments of homologous regions, particularly the two rDNA loci (ITS including the 5.8S rRNA gene; and D1/D2 region of the 26S rRNA gene) together with secondary loci, e.g. translation elongation factor 1α, and the β-tubulin gene.

The common ancestors of unicellular yeasts evolved early in fungal evolution around tens to hundreds of millions of years ago (Nagy et al., 2014; Diepeveen et al., 2017). Nagy et al. (2014) developed a computational pipeline that identified orthologous genes as well as mapped gene losses/duplication across the yeast phylogeny (Figure 1.7). The repeated evolution of yeasts with similar phenotypic traits in distinct clades was common in nature and occurred in most groups of fungi, although the underlying molecular and genomic mechanisms were unknown (Nagy et al., 2014; Sun et al., 2019) (Figure 1.6).

Several studies have shown that most of the yeast species isolated from leaf surfaces were basidiomycetes, often accounting for more than 90 % of the species detected, with ascomycetes making up the remainder (Sláviková et al., 2009; Wang et al., 2016; Into et al., 2020a). Further, many phylloplane basidiomycetous yeasts are ballistosporogenous, i.e. form asexual spores known as ballistospores that are forcibly discharged (Kurtzman et al., 2011a). Typical basidiomycete inhabitants of the phylloplane belong to the ballistosporogenous genera *Bullera* and *Sporobolomyces* (Cobban et al., 2016; Li et al., 2020).

Ballistospores are produced on sterigmata that protrude from asexual cells and are forcibly discharged into the air by the Buller's drop mechanism (Kluyver & van Niel, 1924; Kurtzman et al., 2011c). Spore discharge in the majority of the 30,000 described species of Basidiomycota is powered by the rapid motion of a fluid droplet (Buller's drop) over the spore surface (Stolze-

Rybczynski et al., 2009). For example, in basidiomycete yeasts as well as plant pathogenic rusts and smuts, spores are directly discharged into the air around the fungal colony. The distances for the maximum discharge is reported as 1–2 m (Stolze-Rybczynski et al., 2009).

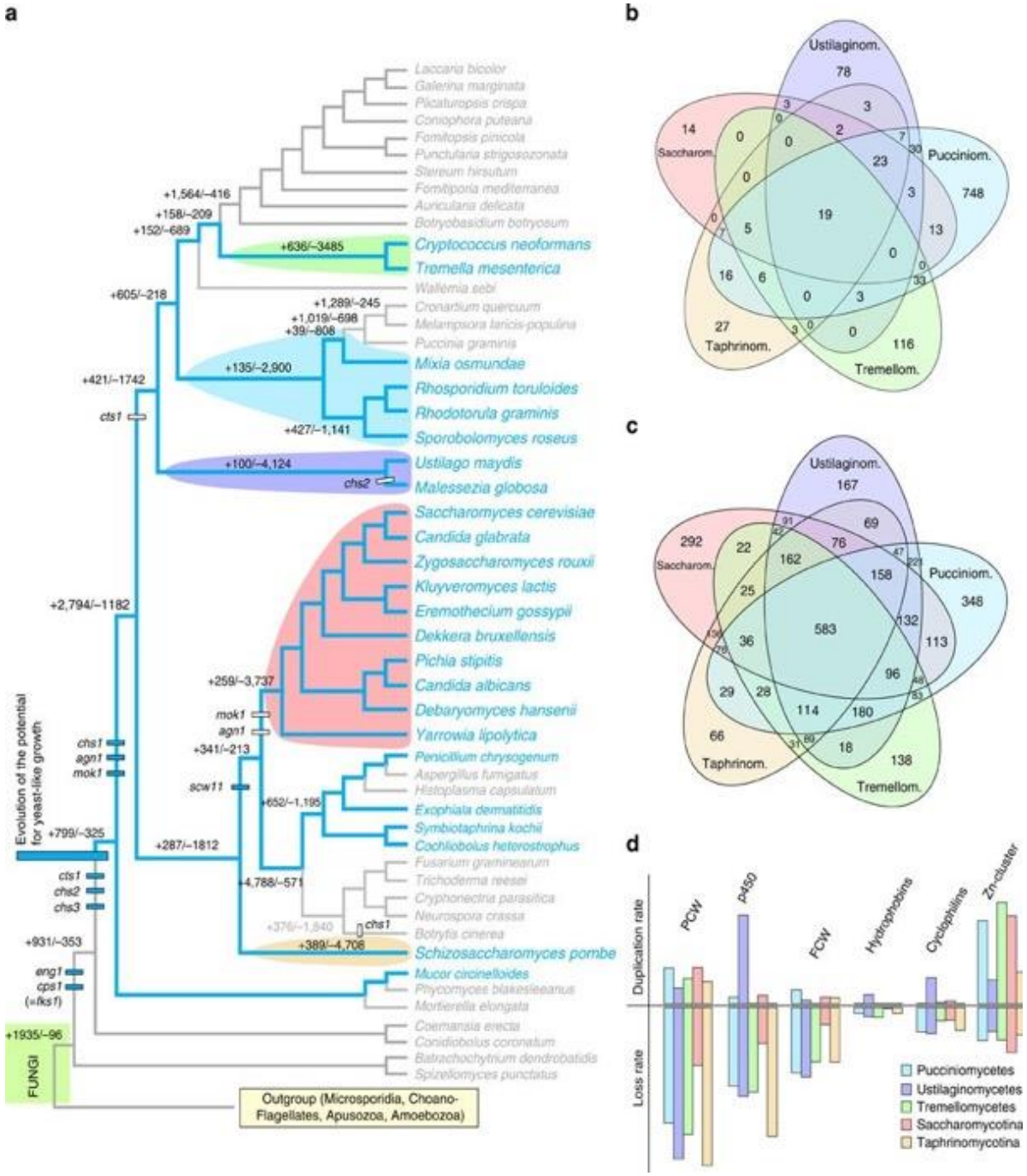


Figure 1.6 The origin of yeasts in five major yeast lineages (shaded clades) inferred from genome studies. (Nagy et al., 2014).

1.10. Taxonomy of phylloplane yeasts in Australia

In Australia, the diversity of phylloplane yeasts on either crop or indigenous plant species has received little attention. Two phylloplane yeast species, *Pseudomicrostroma phylloplanum* (syns *Cryptococcus phylloplanus* and *Cryptococus hinnuleus*) and *Symmetrospora foliicola* (syn. *Sporobolomyces foliicola*), were first described from *Banksia collina* (Proteaceae) (Shivas & de Miranda, 1983a). Another two yeasts, *Buckleyzyma armeniaca* (syn. *Rhodotorula armeniaca*) and *Erythrobasidium elongatum* (syn. *Sporobolomyces elongatus*), were first described from *Callistemon viminalis* (Myrtaceae) (Shivas & de Miranda, 1983a, b) (Table 1.1). Southwell et al. (1999) found that the pink yeast *Rhodotorula glutinis* was the dominant taxon on the phylloplane of wheat and barley in Australia. One further species of phylloplane yeast in Australia, *Saitozyma wallum*, was described from *Banksia aemula* in south-eastern Queensland (Crous et al., 2019; Gogorza Gondra et al., 2019). Two species, *Candida tolerans* and *Kodamaea kakaduensis*, were isolated from the flowers of *Hibiscus* plants in eastern Australia and the Northern Territory (Lachance et al., 1998; Lachance et al., 1999) (Table 1.1). These early studies indicated that Australian phylloplane yeasts likely represent a significant portion of the unclassified and poorly understood fungi known only from sequence data (Grossart et al., 2016).

Table 1.1 Yeast species described from plants in Australia

Species	Basionym	Ex-type culture	Substrate	Host	State	Reference
Candida tolerans	This name is invalid as two specimens were designated as type (Art. 40.3)	UWO (PS) 98-117.5 CBS 8613	flowers	*Hibiscus* sp.	NT	Lachance et al. (1999)
Erythrobasidium elongatum	*Sporobolomyces elongatus*	CBS 8080	leaf	*Callistemon viminalis*	NSW	Shivas & de Miranda (1983b)
Kodamaea kakaduensis	This name is invalid as two specimens were designated as type (Art. 40.3)	UWO (PS) 98-119.2 CBS 8611	flowers	*Hibiscus meraukensis*	NT	Lachance et al. (1999)
Metschnikowia hibisci	–	UWO (PS) 95-747.4	flowers	*H. heterophyllus*	QLD	Lachance et al. (1998)
Pseudomicrostroma phylloplanum	*Cryptococcus phylloplanus*	CBS 8073	leaf	*Banksia collina*	NSW	Shivas & de Miranda (1983a)
Saitozyma wallum	–	–	leaf	*B. aemula*	QLD	Crous et al. (2019)
Symmetrospora foliicola	*Sporobolomyces foliicola*	CBS 8075	leaf	*B. aemula*	QLD	Shivas & de Miranda (1983b)

1.11. Endemic fungi in Australia

Australia is an isolated continent with many endemic animal, plant and fungal communities. The number of endemic organisms in Australia is estimated to be almost 150,000 accounting for about 8 % of the world's published species (1,900,000) (Chapman, 2009). There is much evidence to suggest that Australia also has a significant level of endemism in its fungal species. Chapman (2009) estimated that Australian endemic fungal species represented about 12 % of the world's total. Some examples of endemicity in Australia's fungi follow.

- Australian smut fungi. These are one of the largest fungal groups, rich in endemic species, containing over 300 species in 43 genera (Vánky & Shivas, 2008; Vánky & Lutz, 2011). The life history of most smut fungi includes both an asexual yeast stage and a sexual stage that produces spore in either roots, stems, leaves, inflorescences or flowers. The yeast stage is unknown for most Australian smut fungi. Nomenclatural issues around the naming of these fungi have been resolved mostly in favour of the sexual name by priority.
- Australian mycorrhizal fungi. These have evolved symbiotic partnerships with native Australian plants. For example, orchids often associate with *Serendipita* spp. (Warcup, 1981). These fungi assist seed germination and early seedling growth by providing inorganic nutrients to adult plants. A recent study has shown that Australia has a diversity of unique *Serendipita* spp. (Oktalira, Dearnaley, May and Linde in press).
- Australian endophytic fungi. Studies have shown that some Australian endemic plants harbour endophytic fungi. For example, leaves of *B. integrifolia* harboured several species of endemic endophytes (MacArthur & McGee, 2000).
- Australia plant pathogenic ascomycetes. *Colletotrichum* and members of the Botryosphaeriaceae are important genera of plant pathogens that can cause severe disease, especially if plant hosts are under stress (Shivas et al., 2016; Tan et al., 2019). In south-eastern Queensland, *Colletotrichum alcornii* is only known from two endemic Australian grass species (Crouch, 2014). Tan et al. (2019) reported many species in the Botryosphaeriaceae (*Diplodia, Dothiorella, Lasiodiplodia*, and *Neofusicoccum*) for the first time from Australia.

1.12. Classification of yeasts

1.12.1. Taxonomy of yeasts

Fungal taxonomy may be complicated by intra-specific physiological and morphological variation as well as an inadequate number of morphological indicators (Yang et al., 2014). The use of stable genomic DNA-based characteristics has revolutionised taxonomic studies and produced accurate and robust classifications for yeasts (Kurtzman et al., 2011b). The frequency of DNA mutation is usually low and the percentage of the genome affected over short periods is extremely small (Rodriguez et al., 2004). Gassmann et al. (2000) stated that the mutation rate occurs at a frequency of about one in every million individuals in a population, although this can vary across genes and organisms.

It has been 25 years since the first genome of *Saccharomyces cerevisiae* was sequenced (Goffeau et al., 1996). Yeast research has advanced exponentially with the publication of this genome. Phylogenetic analyses that compare genetic loci or whole genes between isolates have become a cornerstone of fungal taxonomy (Oliver, 2007; Raja et al., 2017). New species are frequently and easily recognized from gene sequence comparisons of isolates (Kurtzman et al., 2011b). It is notable that almost half of yeast species that have been described are based on a single strain or still lack any DNA sequence information in public databases (Kurtzman et al., 2015; Xu & Adamowicz, 2016).

In the last decade, many yeast species have been revealed by phylogenetic analyses of DNA sequences (Into et al., 2020a). Species based on multiple isolates are preferred as this accounts for specific variation. The internal transcribed spacer (ITS) region was established as the official DNA barcode gene for fungi (Schoch et al., 2012; Hoang et al., 2019). The ITS region includes two non-coding and variables regions (ITS1 and ITS2) and the intervening highly conserved 5.8S gene, which are located between the 18S (SSU) and 28S (LSU) nuclear ribosomal DNA (White et al., 1990; Hoang et al., 2019; Nilsson et al., 2019). An advantage of the ITS region is that it can be amplified easily from most fungal taxa using several primers, e.g. ITS1, ITS2, ITS3, ITS4 and ITS5 (White et al., 1990). Increasingly, phylogenomic studies have been used to unravel the enormous evolutionary diversity of yeasts (Kurtzman et al., 2011a; Dujon & Louis, 2017).

1.12.2. Description of new taxa

The descriptions of new species must incorporate essential characters and diagnoses that distinguish these taxa from previously described species. The *International Code of Nomenclature for algae, fungi, and plants* (Turland et al., 2018; May et al., 2019) provides rules for new species' descriptions. An important requirement for a proposed new species is the deposition of a type specimen in a publicly accessible herbarium. The Queensland Plant Pathology Herbarium (BRIP) has the largest collection of fungal type specimens in Australia. Lyophilised specimens may be used as type material (Greuter et al., 1994). BRIP holds a culture collection of approximately 23,000 living cultures of bacteria and fungi that are preserved in a metabolically inactive state (Shivas et al., 2019). These cultures are preserved either freeze dried, frozen at -80 °C or retained underwater (QDAF, 2013).

1.13. *Banksia integrifolia*

Banksia is an iconic Australian genus in the Proteaceae (Weston & Barker, 2006). *Banksia* spp. are widespread throughout coastal heathlands from Tasmania to southern Queensland (Mast & Givnish, 2002; He et al., 2011). *Banksia integrifolia* was known to indigenous Australians before naming and discovering by the Europeans (Lim, 2014). *Banksia integrifolia* is one of 173 *Banksia* species that are endemic to Australia (ANBG, 2015). Trees of *B. integrifolia* can live for over 100 years, grow up to 10 m in height and have thick bark that provides protection from bushfires. The leaves of *B. integrifolia* are smooth, leathery and the lower surface is covered in white hairs. Mature leaves of *B. integrifolia* are 6–10 cm long and 15–25 mm wide, with a rectangular shape and pointed edge (Figure 1.8). *Banksia integrifolia* has three subspecies, i.e. *compar*, *monticola* and *aquilonia* (Thiele & Ladiges, 1994). Woody seed follicles have helped *B. integrifolia* adapt to bushfires, predation and severe drought for millions of years (Lamont & He, 2012).

Three yeasts, *Pseudomicrostroma phylloplanum*, *Saitozyma wallum* and *Symmetrospora foliicola* have been described from the phylloplane of *Banksia* spp. in eastern Australia (Table 1.1). The discovery of these three yeasts indicates that *Banksia* spp. may harbour undiscovered yeast biodiversity.

Figure 1.7. *Banksia integrifolia* subsp. *compar* at Noosa Heads, Sunshine Coast

1.14. Phylogenetic species recognition approaches

The accurate identification and classification of fungi is challenging (Lücking et al., 2020). The identification and classification of individual yeast species is currently determined from the nucleotide sequence divergence of the ITS rDNA region (Schoch et al., 2012) compared to those of ex-type cultures of known species (Buchan et al., 2002) (Figure 1.8) using a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of deposited sequences. Multilocus sequence analysis employs secondary genes, e.g. large subunit (LSU) of the ribosomal DNA, intergenic spacers (IGS) of the ribosomal DNA, ITS of the ribosomal DNA, translation elongation factor 1-α (*TEF1*) gene, ß-tubulin (*TUB*) gene, histone (*HIS*) gene, calmodulin (*CAL*) gene, actin (*ACT*) gene, DNA-lyase (*APN2*) gene, 60s ribosomal protein L37 (FG1093) gene and mating type genes (MAT-1-1-1 and MAT-1-2-1) (Santos et al., 2017). Genealogical concordance phylogenetic species recognition has been the most widely used approach for species determination. However single gene coalescent approaches that incorporate gene tree uncertainty into species recognition may more accurately and objectively describe species (Stewart et al., 2014; Lücking et al., 2020). The gold standard for fungal identification and classification is a combination of genealogy

(phylogeny), phenotype (including autecology), and reproductive biology (when feasible) (Lücking et al., 2020).

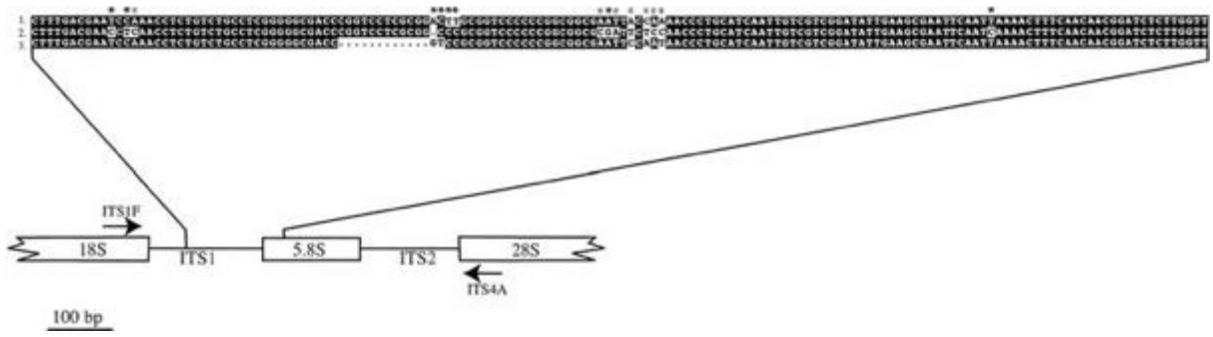


Figure 1.8 Primer location for amplification of ascomycete ITS regions and comparison of sequences of variable ITS1 (Buchan et al., 2002).

1.14.1. Phylogenetic reconstruction methods

1.14.1.1. Maximum parsimony

Maximum parsimony is a phylogenetic reconstruction method that summarises the number of evolutionary steps through minimizing distance and branches on the phylogenetic tree (Kannan & Wheeler, 2012). Maximum parsimony methods do not account for the different characters on the tree such as reversals, convergence, and homoplasy. Maximum parsimony approaches usually underestimate the actual evolutionary change. Maximum parsimony does not always generate the actual species tree with high probability given sufficient data (Hoang et al., 2018).

1.14.1.2. Minimum evolution (incl. neighbour joining)

Minimum evolution or neighbour joining method of phylogenetic inference is based on the assumption that the tree with the smallest sum of branch length estimates is most likely to be the true one (Rzhetsky & Nei, 1993). Although, this method creates a phylogenetic tree quickly and easily, it is not reliable and cannot be used for a final decision about species recognition because

it only produces one tree and neglects other possible trees, errors in distance estimates are exponentially large for long distance and will yield a biased tree (Bruno et al., 2000).

1.14.1.3. Maximum likelihood

Maximum likelihood (ML) is often used to build phylogenetic trees in evolutionary studies of fungi (Whelan & Morrison, 2017). The ML approach provides probabilities for the sequences given a specific model of their evolution, i.e. parameters for nucleotide substitions (Tamura et al., 2011). Determining the most suitable model for phylogenetic reconstruction constitutes a fundamental step in ML analysis. A disadvantage of this approach is that large datasets require considerable computational time to generate phylogenetic trees.

1.14.1.4. Bayesian inference

Bayesian inference (BI) and ML methods are similar in that both use a model of evolution to analyse sequence data. BI is a statistical method that uses probability distributions to describe the uncertainty of all unknowns. It combines the prior probability of a tree with the likelihood of the data to produce a posterior probability distribution on trees (Huelsenbeck et al., 2001). BI methods have become very popular in molecular phylogenetics due to the availability of user-friendly computer programs that apply sophisticated evolution models. BI methods uses Markov chain Monte Carlo (MCMC) to calculate posterior probabilities. The downside of these approaches that they are computationally expensive and require time to create reliable trees.

1.14.1.5. Coalescent methods

Traditional phylogenetic approaches infer species trees from the alignments concatenated across genes (de Queiroz & Gatesy, 2007). The multispecies coalescent model was developed to deal with gene tree heterogeneity observed in multilocus sequence data (Rannala & Yang, 2003; Edwards et al., 2007; Liu & Pearl, 2007). Coalescent methods allow multilocus sequences with different genes to have different histories. Unlike concatenation, the multispecies coalescent model (MSC) explains the evolutionary history of multilocus sequences through two levels of biological hierarchy, the gene tree and the species tree (Liu et al., 2019).

1.15. Significance of the project

Accurately documenting biodiversity is important for conservation purposes and to preserve undiscovered species before they become extinct from habitat destruction. Undiscovered species of fungi may have future unrecognised potential benefits as sources of novel compounds as well as biological properties, e.g., antagonism to plant pathogens. The use of yeasts in biotechnology is an area of rapid advancement in biotechnology and biological research (Żymańczyk-Duda et al., 2017). It has been increasingly recognised that the full potential of yeasts in biotechnological applications has not been realised. Current applications of yeasts include traditional fermentation (bread and beer), in food (enzymes and pigments), biofuels, pharmaceuticals, protein production (hormones, vaccines), fundamental biological research (a yeast was the first fungus to be fully sequenced), drug discovery, biocontrol (crop protection) and bioremediation (heavy metal sequestration) (Kim et al., 2015; Gonçalves et al., 2016; Azhar et al., 2017). There are scientific and economic imperatives to accurately document yeasts in Australia before they disappear from threats such as climate change and habitat destruction.

Sequencing methods and molecular phylogenies have provided evidence of a vast hidden diversity of fungi in many ecosystems (Dujon & Louis, 2017). With knowledge about the real diversity of yeasts still in infancy, there is an opportunity to study ecosystems such as the phylloplane of Australian native plants to discover new species of yeasts (Hawksworth & Lücking, 2017; Lücking et al., 2020).

1.16. Research aims and objectives

This study will address the following research questions:

Do leaves of *B. integrifolia* at three sites in south-eastern Queensland support yeast communities with unrecognised species richness? *Banksia integrifolia* was chosen as the source plant because it is an endemic Australian species that had not previously been studied as a substrate for yeasts. The leaves of *Banksia integrifolia* were considered to have a high likelihood as a substrate for novel yeasts, as new yeast species were previously described from the phylloplane of other *Banksia* spp. in eastern Australia (Table 1.1).

Do the phylloplane yeasts on *B. integrifolia* include putative novel taxa (genera and species)?

- Are the species of yeasts found on leaves of *B. integrifolia* endemic or introduced species?

The specific objectives of this study are to:

- isolate the phylloplane yeasts on *B. integrifolia* in three natural habitats; and permanently preserve these cultures in an internationally recognised culture collection;
- reconstruct accurate molecular phylogenies for the yeasts isolated;
- identify known yeast species on leaves of *B. integrifolia*; and classify putative novel taxa.

1.17. Thesis overview

Chapter 1: Introduction and Literature Review

This chapter reviews the essential characteristics of yeasts as well as the ecology of phylloplane yeasts. The research aims and objectives of this study are presented.

Chapter 2: Diversity of yeasts on leaves of *Banksia integrifolia* in south-eastern Queensland

This chapter describes the research methods used to determine the diversity of yeasts on leaves of *B. integrifolia* in south-eastern Queensland. It describes how specimens were collected and processed. It explains the methods that were used to isolate and preserve yeasts cultures; to extract DNA; and to amplify DNA by the polymerase chain reaction (PCR). It also describes how sequences were analysed; how phylogenetic trees were constructed; and how yeast species were identified. The diversity of phylloplane yeasts on leaves of *Banksia integrifolia* in south-eastern Queensland are presented and discussed.

Chapter 3: Diversity and phylogeny of *Vishniacozyma* yeasts on leaves of *Banksia integrifolia* in south-eastern Queensland

This chapter explains how isolates of *Vishniacozyma* from *B. integrifolia* were identified and classified using morphology, phylogeny and physiology.

Chapter 4: Diversity and phylogeny of *Hannella* yeasts on leaves of *Banksia integrifolia* in south-eastern Queensland.

This chapter explains how isolates of *Hannaella* spp. from *B. integrifolia* were identified and classified using morphology, phylogeny and physiology.

Chapter 5: Diversity and phylogeny of *Bannoa* yeasts on leaves of *Banksia integrifolia* in south-eastern Queensland.

This chapter explains how isolates of *Bannoa* spp. from *B. integrifolia* were identified and classified using morphology, phylogeny and physiology.

Chapter 6: General Discussion

This chapter interprets the results of this study in the context of what is known about the biodiversity of phylloplane yeasts worldwide. Recommendations for future research are discussed.

CHAPTER TWO: DIVERSITY OF YEASTS ON LEAVES OF *BANKSIA INTEGRIFOLIA* IN SOUTH-EASTERN QUEENSLAND

2.1. Introduction

The diversity of phylloplane in yeasts in Australia has received little attention despite the enormous diversity of taxa reported worldwide (Limtong & Kaewwichian, 2015; Into et al., 2020b). Only seven species of phylloplane yeasts had been reported from Australia prior to this study (Table 1.1, Chapter 1). These species were *Candida tolerans*, *Kodamaea kakaduensis* (Lachance et al., 1999), *Metschnikowia hibisci* (Lachance et al., 1998), *Erythrobasidium elongatum*, *Symmetrospora foliicola* (Shivas & de Miranda, 1983b), *Pseudomicrostroma phylloplanum* (Shivas & de Miranda, 1983a) and *Saitozyma wallum* (Crous et al., 2019). MacArthur & McGee (2000) also found that *B. integrifolia* harboured many endophytic fungal species. The aim of this study was to determine whether there was hidden diversity of yeasts on leaves of *B. integrifolia* at three sites in south-eastern Queensland.

2.2. Materials and Methods

2.2.1. Sample collection

Plants of *B. integrifolia* were targeted at three sites (Esk, Highfields and Noosa Heads) that represented different ecosystems in south-east Queensland. The sites represented, respectively, undisturbed natural open woodland, a peri-urban environment and undisturbed coastal heathland. In 2020, three leaves of *B. integrifolia* subsp. *compar* were randomly excised from each of three mature trees at three canopy positions (lower, middle, upper). Samples were collected once on (different days) from each of the three sites (Figs 2.1–2.3). Only green and healthy leaves with no visual symptoms of disease or signs of stress were sampled. The leaves were stored in sealable plastic bags and labelled with the name of the site, canopy position, date of collection and

geographical coordinates. The leaf samples were stored at 8 °C in an icebox to keep the leaves fresh during transportation. Yeasts were isolated from the samples on the same day of collection.

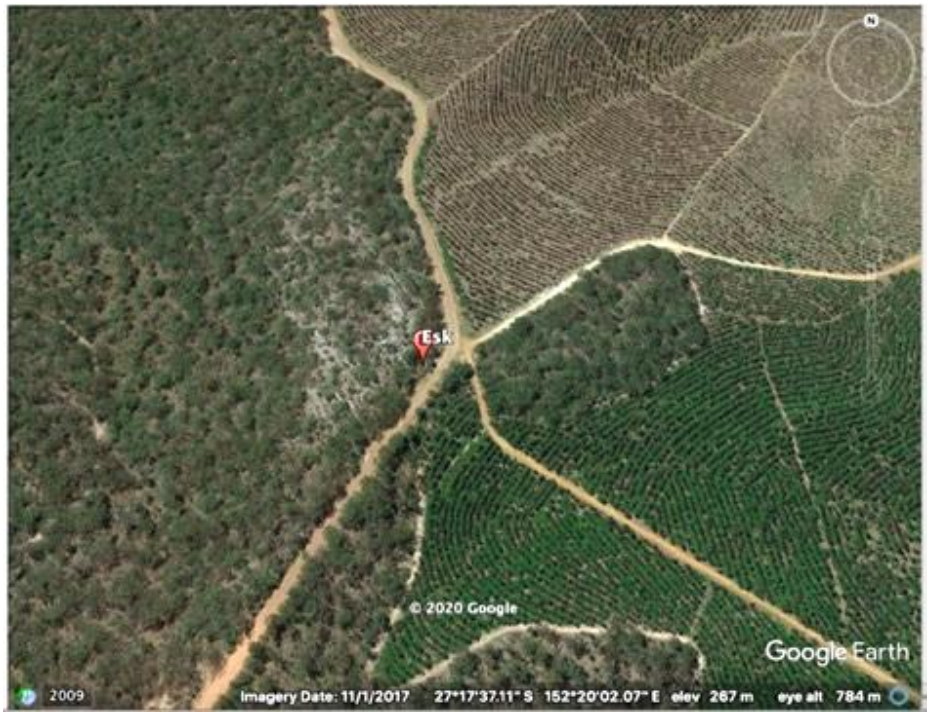


Figure 2.1. Google Earth map of location the sample site for the collection of leaves on *Banksia integrifolia* at Esk (27° 17'33.0"S, 152°19'59.0"E), Queensland.

Figure 2.2. Google Earth map of location the sample site for the collection of leaves on *Banksia integrifolia* at Highfields (27°26'21.3"S 151°59'21.3"E), Queensland.

Figure 2.3. Google Earth map of location the sample site for the collection of leaves on Banksia integrifolia at Noosa Heads (26°22'59.1"S 153°04'49.3"E), Sunshine Coast, Queensland.

Figure 2.4. *Banksia integrifolia* tree at Noosa Heads, Sunshine Coast, Queensland.

2.2.2. Yeast isolation

The isolation of phylloplane yeasts followed the methods of Into et al. (2020a). The leaf samples were cut into 1 cm pieces and washed in a 250 mL sterilized conical flask containing 70 mL of 0.85 % saline solution. The samples were shaken for 1 hr on an orbital shaker at room temperature. 100 µL of the saline solution was pipetted and spread onto 9 cm diam. plates of yeast-extract malt-extract agar (YMPA; 2 % agar, 0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 0.09 %

tetracycline) (Sukmawati et al., 2015). The plates were incubated at 25 ˚C for 3 d to allow for yeast growth. Plates contaminated by bacteria and filamentous fungi were discarded. Yeast colonies were then sub-cultured twice onto modified YMPA plates (without tetracycline) to ensure isolate purity. A total of 107 yeast isolates from all samples at all sites were spread inoculated.

2.2.3. DNA extraction

Genomic yeast DNA was extracted from colonies grown on modified YMPA at 25 ˚C for 3 d using a commercial kit (Bioline Isolate II Plant DNA Kit) following the manufacturers' instructions. About 50 mg of cells were lysed by vortexing (ISG® Vortex mixer) in 400 µL of lysis buffer in 1.5 ml microcentrifuge tubes for 10 secs; followed by adding 10 µL of RNase A solution and incubation for 10 min at 65 °C on a dry block heater (Ratek, Australia). A crude lysate filter was placed into a 2 mL collection tube and centrifuged for two min at 11,000 g (Eppendorf Centrifuge 5415D, Hamburg, Germany). The clear supernatant was collected carefully to avoid pellet disturbance and placed into a 1.5 ml micro-centrifuge tube. 450 µL of binding buffer PB were added to the solution and mixed thoroughly by vortexing. Around 700 µL of the mixture were placed into the spin column that was attached to a 2 mL collection tube and centrifuged for one min at 11,000 g. The spin column tube was retained and the flow-through discarded. The DNA was washed and dried sequentially by adding 400 µL of Wash Buffer PAW1 to the spin column tube, centrifuging for one min at 11,000 g and discarding the flow through. 700 µL of wash buffer were added to the silica membrane spin column tube; the tube was centrifuged for one min at 11,000 g and the flow-through discarded. This step was repeated by adding 200 µL of wash buffer and centrifuging for two min. For DNA elution, the spin column tube was placed into a 1.5 ml micro-centrifuge tube; 50 µL of preheated elution buffer PG (65 °C) were added and the tube was incubated for 5 min at 65 °C. The tube was then centrifuged for one minute at 11,000 g. This step was repeated and the extracted DNA was stored at 5 °C.

2.2.4. Polymerase chain reaction (PCR)

ITS1 and ITS4 primers (White et al., 1990) were used to amplify the ITS rDNA. PCRs were performed as described in Lorenz (2012) was as follows. Four µl of sterile distilled H_2O were added into the PCR tubes; 10 µl of PCR ReadyMix was then added, and the solution mixed with the pipette tip. One µl of ITS1 was added to the PCR tube and stirred with the pipette tip; followed by the addition of 1 µl of ITS4 into the same PCR tube. Four µl of extracted DNA were then added to the PCR tube and mixed in well. For a negative control, 8 µl of distilled water were added into another PCR tube instead of extracted DNA. The PCR tubes were placed into the thermocycler (Thermo Hybaid PCR Express), and the samples amplified with 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final incubation at 72 °C for 10 min.

2.2.5. Gel electrophoresis

PCR products were visualized in 1 % agarose gels stained with RedSafe Nucleic Acid Staining Solution (Fisher Biotech, Wembley, WA). Gels were prepared by adding 1 g agarose to 100 ml of Tris/Acetic acid/EDTA (TAE) buffer; microwaving for 30 sec; stirring and microwaving again for 30 sec. After cooling, 5 µl of Red Safe was added into the gel mixture and gently stirred in a conical flask. Gels were prepared by pouring into electrophoresis trays (Select BioProducts) with plastic combs and left for 20 min to set. The set gel was placed into the electrophoresis tank and 300 mL of TAE buffer was added. Running dye (0.5 µl) and 2 µl of PCR amplified sample was mixed in a centrifuge tube. Two µl of each sample were loaded into the gel, which was run at 100 mV for 30 min and visualized under UV light in a gel documentation system to determine the quality and purity of extracted DNA.

2.2.6. Sequencing and phylogenetic analyses

Samples containing between 17 ng and 30 ng of DNA (estimated optically from the mass ruler) were sent to Macrogen (Seoul, South Korea) or the Australian Genome Research Facility (AGRF) for purification and sequencing of the ITS in both directions using the same primers used for PCR. Raw Chromatograms were viewed in the Chromas® 2.0 program (Technelysium, Tewantin,

Australia) or 4Peaks program (Griekspoor & Groothuis, 2017) to check for contamination, and to trim low-quality regions. Consensus sequences were constructed in 4Peaks software as well as using Geneious Prime. A BLAST search against the National Centre for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/) was used to determine close matches and comparison between two sequence alignments. Sequences of the type isolates of known yeast species were obtained from NCBI GenBank. MEGA-10 (Tamura et al., 2007) was used to explore the phylogenetic context of the sequenced taxa.

2.2.7. Morphology

The colonies and cells of putative novel yeast species were described from colonies grown for 6 d on PDA plates incubated in the dark at 25°C (Barnett et al., 2000). The texture and colour of colonies was defined by comparison with Rayner's colour charts (Rayner, 1970). The shapes and sizes of cells were visualised using a Leica DM5500B compound microscope. Measurements were made of cells mounted on glass slides in 100 % lactic acid. Images of cells were taken under Nomarski interference with a Leica DFC500 camera.

2.2.8. Physiology

The physiological properties of putative novel species were determined by Biolog YT kits (Biolog Inc., Hayward, CA, USA), which determine their fermentative and oxidative properties. Yeasts were grown on PDA plates for 3 d at 25 °C. A moistened sterile cotton swab was used to remove active yeast cells and mix in 20 mL of distilled water, which made a slightly turbid suspension. A micropipette was used to inoculate 100 µL of yeast suspension into each of the test wells on the Biolog YT microplates. After 3 d at 25 °C the Biolog YT microplates were read with an ELISA plate reader. Positive results were also verified visually. The wells in the top 3 rows A1—C12 of the Biolog YT microplates contain tetrazolium chloride that produces a colour reaction if positive, while a positive reaction in the remaining rows is indicated by an increase in turbidity. The individual tests are shown on the YT Microplate Test Panel.

2.3. Results

More than 70 isolates representing at least 27 species in 15 genera were identified from the leaf surfaces of *B. integrifolia* in south-eastern Queensland (Table 2.2). Of these, 10 were assigned species names based on high (98 %) sequence identity to ex-type sequences on GenBank (Table 2.2). Twelve isolates could not be confidently assigned to species.

Several isolates of *Bannoa*, *Hannaella* and *Vishniacozyma* appeared to represent novel species. Isolates that were identified as belonging to these three genera are discussed separately in Chapters 3, 4 and 5, respectively. The number basidiomycetous yeasts (58 %) was higher than ascomycetous yeasts (42 %). Isolates of *Aureobasidium* were most frequently identified (28 %).

Species of *Vishniacozyma* and *Hannaella* were the next most frequently identified yeasts on *B. integrifolia* with 20 % and 11 % of total isolates, respectively. *Pseudosydowia* and *Symmetrospora* each accounted for 7 % of isolates; *Sporidiobolus* (6 %), *Dioszegia* and *Pezicula* (each 4 %), and *Bannoa*, *Zalaria and Sporobolomyces* (each 3 %). Isolates of *Aspergillus*, *Filobasidium*, *Pseudomicrostroma* and *Tremella* were rarely identified.

Table 2.1. Yeasts isolated from leaf surfaces of *Banksia integrifolia* in south-eastern Queensland.

Species	BRIP	Location	ITS sequence with closest match on GenBank	Similarity (%)
Aspergillus peyronelii	71277	Highfields	MH857717.1	98
Aureobasidium leucospermi	71260	Highfields	KT693727.1	99
A. melanogenum	71032; 71041; 71045; 71048; 71050; 71053; 71055; 71060; 71061; 71036; 71261; 71262; 71264-67; 71272; 71940	Esk; Highfields; Noosa Heads	NR_159598.1	99
Aureobasidium sp.	71272	Highfields	NR_159598.1	93
A. subglaciale	71254	Highfields	MH863294.1	98
Bannoa sp.	71965; 71967	Noosa Heads	NR_154870.1	95
Dioszegia sp.	71936; 71939; 71961	Noosa Heads	NR_155061.1	97
Filobasidium magnum	71276	Highfields	KY103433.1	99
Hannaella sinensis	71278	Highfields	KY103510.1	98
Hannaella sp.	71253; 71259	Highfields	NR_111065.1	96
Hannaella sp.	71047	Esk	EU252551.1	95
Hannaella sp.	71051; 71256	Esk	NR_155180.1	97
Hannaella sp.	71058	Esk	KR859211.1	96
Hannaella sp.	71270	Highfields	NR_144771.1	95
Pseudomicrostroma sp.	71280	Highfields	NR_148081.1	97
Pseudosydowia sp.	71031; 71035; 71052; 71059; 71268	Esk; Highfields	NR_165231.1	97
Pezicula fagacearum	71057	Esk	NR_155654.1	97
Pezicula sp.	71054; 71062	Esk	KR859211.1	96
Sporidiobolus pararoseus	71037; 71044	Esk	NR_155770.1	99
Sporidiobolus sp.	71255; 71258	Highfields	NR_155770.1	96
Sporobolomyces beijingensis	71263	Highfields	SNR_137663.1	99
Sporobolomyces sp.	71271	Highfields	NR_137663.1	94
Symmetrospora pseudomarina	71800; 71941; 71953; 71960; 71964	Noosa Heads	KJ701216.1	99
Tremella sp.	71038	Esk	NR_155907.1	87
Vishniacozyma sp.	71030; 71043; 71273; 71788; 71034; 71281; 71932; 71933, 71951; 71959; 71269; 71279; 71946; 71963	Esk; Highfields; Noosa Heads	NR_144810.1	92-94
Zalaria sp.	71040	Esk	NR_153466.1	96
Z. obscura	71042	Esk	NR_153466.1	99

2.4. Discussion

The phylloplane is an understudied habitat for microbial communities, especially yeasts. This chapter shows that the yeasts isolated from the phylloplane of *B. integrifolia* belonged to diverse and rarely collected genera. Further, the species identity of the isolates comprised almost entirely novel taxa. As the leaves were cut prior to washing, there is the possibility that some of the yeasts were endophytes and/or epiphytes. Most yeasts reported in this chapter have been found on the phylloplane of other plant species (Inácio et al., 2002; Kurtzman et al., 2011a; Limtong & Nasanit, 2017; Into et al., 2020b) and some as endophytes, e.g. *Aureobasidium* spp. (Bolívar-Anillo et al., 2020). Species of *Aureobasidium*, *Sporobolomyces, Rhodotorula* are often the dominant phylloplane species globally (Nakase, 2000; Inácio et al., 2002; van Nieuwenhuijzen, 2014). MacArthur & McGee (2000) also isolated 41 isolates of *Aureobasidium* on the leaves of *B. integrifolia* from various locations of New South Wales, Australia. *Aureobasidium* is also commonly found on other parts of plants such as stem, root and flower and fruits (Shivas & Brown, 1989; Ippolito et al., 2000; van Nieuwenhuijzen, 2014).

Vishniacozyma and *Hannaella* yeasts were commonly identified in this study (see Chapters 3 and 4). These yeasts exist on different habitats (soil, water, dead leaves, rotten wood, tephra, pasture plants and shrimp) including the phylloplane (Hamamoto & Nakase, 1996; Wang & Bai, 2008; Wang et al., 2011; Landell et al., 2014; Liu et al., 2015a; Yurkov & Kurtzman, 2019; Li et al., 2020). *Symmetrospora* has been previously isolated from the phylloplane of *B. integrifolia* (Shivas & de Miranda, 1983b). *Pseudosydowia* has only been isolated from phylloplane of endemic *Eucalyptus* spp. (Myrtaceae) in Australia and South Africa (Crous et al., 2019). *Sporidiobolus* has been reported from several habitats, including the phylloplane and soil (Valério et al., 2008).

Dioszegia and *Pezicula* are common yeasts on organic substrates. Most species of *Dioszegia* have been isolated from plant leaves, roots and soil (Takashima & Nakase, 2001; Kurtzman et al., 2011a). *Pezicula* is one of the most widespread yeasts that lives on different organic substrates.

Zalaria spp. have been previously isolated from house dust in the United States of America and Canada (Humphries et al., 2017). This study is the first record of *Zalaria* from the phylloplane of any plant, as well as the first record of the genus in Australia.

Bannoa spp. are rare plant-associated yeasts (Hamamoto et al., 2002; Wang et al., 2015b; Parra & Aime, 2019). *Bannoa* has also not been reported from the phylloplane of any plant, as well as in Australia (see Chapter 5).

An isolate of *Pseudomicrostroma* was found on leaves of *B. integrifolia* in this study. *Pseudomicrostroma* spp. have been recorded from air (*Pseudomicrostroma glucosiphilum,* Kijpornyongpan & Aime (2017)); leaves of walnut seedlings (*Pseudomicrostroma juglandis*, Lutz et al. (2018)); and leaves of *Banksia collina* (*Pseudomicrostroma phylloplanum,* Shivas & de Miranda (1983a)).

The findings of this chapter show that the phylloplane of *B. integrifolia* harbours a diverse and rarely collected genera comprised almost entirely of novel species. This shows that the phylloplane of Australian native plants may be a source of diverse and novel fungi.

CHAPTER THREE: DIVERSITY AND PHYLOGENY OF *VISHNIACOZYMA* YEASTS ON LEAVES OF *BANKSIA INTEGRIFOLIA* IN SOUTH-EASTERN QUEENSLAND

3.1. Introduction

The Tremellomycetes (Agaricomycotina, Basidiomycota) contains many yeasts and yeast-like fungi with dimorphic stages in their life cycles (Liu et al., 2015b). Amongst these tremellomycetous yeasts, the genus *Vishniacozyma* (Bulleribasidiaceae, Tremellales) was established (Liu et al., 2015a) for the phylogenetic clades previously recognised in *Cryptococcus* as the *victoriae* clade (Fonseca et al., 2011) and the *dimennae* clade (Boekhout et al., 2011) (Table 3.1). *Vishniacozyma* takes its name from the American microbiologist Helen S. Vishniac in recognition for her work on the diversity and ecology of yeasts of cold climates (Liu et al., 2015a). Liu et al. (2015a) recognised 11 species, namely *Vishniacozyma carnescens*, *V. dimennae*, *V. foliicola*, *V. globispora*, *V. heimaeyensis*, *V. nebularis*, *V. penaeus*, *V. psychrotolerans*, *V. taibaiensis*, *V. tephrensis*, and *V. victoriae*. Of these, *V. psychrotolerans* and *V. taibaiensis* (Liu et al., 2015b) are invalid names (Art. 40.7, Melbourne Code). Since 2015, eight further species have been named, i.e. *V. alagoana* (Félix et al., 2020), *V. ellesmerensis* (Tsuji et al., 2019), *V. europaea* (Li et al., 2020), *V. changhuana* (Chang et al., 2021), *V. kurtzmanii* (Yurkov & Kurtzman, 2019), *V. psychrotolerans* (Li et al., 2020), *V. taibaiensis* (Li et al., 2020) and *V. taiwanica* (Chang et al., 2021). Some species of *Vishniacozyma* were originally classified in other genera, i.e., *Bullera*, *Cryptococcus*, *Rhodotorula*, *Trichosporum*, *Trimorphomyces* (Verona & Luchetti, 1936; Phaff et al., 1952; Bandoni, 1984; Kirschner & Chen, 2008) (Table 3.1).

The aim of this Chapter was to identify the species of *Vishniacozyma* isolated from leaves of *B. integrifolia* in Chapter 2. The work was part of a study to determine if novel species of yeast inhabited the leaf surface of *B. integrifolia*, and whether these yeasts were more closely related to other Australian species than to overseas species.

Table 3.1 Collection records for ex-type cultures of *Vishniacozyma* species used in the phylogenetic analysis

Species	Ex-type culture	Basionym	Host/substrate	Location (country)	Reference
Vishniacozyma alagoana	CBS 15966	-	Bromeliaceae	Brazil	Félix et al. (2020)
V. carnescens	CBS 973	*Torulopsis carnescens*	Muscatel grapes	Italy	Verona & Luchetti (1936), Liu et al. (2015a)
V. changhuana	CBS 16556	-------	in mangrove forest	Taiwan	Chang et al. (2021)
V. dimennae	CBS 5770	*Cryptococcus dimennae*	pasture plants	New Zealand	Liu et al. (2015a) Fell & Phaff (1967)
V. ellesmerensis	JCM 32573	-------	sediments and soil	Canada	Tsuji et al. (2019)
V. europaea	CBS 15464	-------	leaf	Germany	Li et al. (2020)
V. foliicola	CBS 9920	*C. foliicola*	leaves	China	Liu et al. (2015a) Wang et al. (2011)
V. globispora	CBS 6981	*Bullera globispora*	rotten wood	Canada	Bandoni (1984) Liu et al. (2015a)
V. heimaeyensis	CBS 8933	*Cryptococcus heimaeyensis*	Tephra	Iceland	Vishniac (2002) Liu et al. (2015a)
V. kurtzmanii	CBS 12229	-------	maize and rice	USA	Yurkov & Kurtzman (2019)
V. nebularis	CBS 12283	*Trimorphomyces nebularis*	dead branch	Taiwan	Liu et al. (2015a) Kirschner & Chen (2008)
V. penaeus	CBS 2409	*Rhodotorula penaeus*	shrimp	Gulf of Mexico	Phaff et al. (1952) Liu et al. (2015a)
V. psychrotolerans	CBS 6578	*C. psychrotolerans*	water	Patagonia and Svalbard	De Garcia et al. (2012) Liu et al. (2015a)
V. taibaiensis	CBS 9919	*C. taibaiensis*	leaves	China	Wang et al. (2011) Liu et al. (2015a)
V. taiwanica	CFC-2020C	-------	mangrove forest	Taiwan	Chang et al. (2021)
V. tephrensis	CBS 8935	*C. tephrensis*	tephra	Iceland	Vishniac (2002) Liu et al. (2015a)
V. victoriae	CBS 8685	*C. victoriae*	mosses, lichens, and soils	Australia	Montes et al. (1999) Liu et al. (2015a)

3.2. Materials and methods

3.2.1. Yeast isolates

Leaf samples were collected from three different locations in south-eastern Queensland (see Chapter 2). Phylloplane yeasts were isolated from leaf surfaces and were grown on YMPA for 7 d at 22–25 ºC following the method of Into et al. (2020a). For the molecular studies, 14 isolates (BRIP 71030, 71034, 71043, 71269, 71273, 71279, 71281, 71788, 71932, 71933, 71946, 71951, 71959, 71963) were identified as putative *Vishniacozyma* species based on their ITS sequences in BLAST searches.

3.2.2. Morphology

Eight *Vishniacozyma* isolates (BRIP 71269, 71788, 71043, 71034, 71959, 71030, 71963, 71946) were sub-cultured on PDA for morphological studies (Figure 3.1). Descriptions of the colony colours were made with Rayner's colour charts (Rayner, 1970). Cells of the isolates were examined under Nomarski interference microscopy and the size and shape of cells were recorded (n > 30). Yeast cells were mounted on glass slides in 100 % lactic acid for microscopic examination after 10 d incubation in the dark at 25 °C. Measurements were taken from at least 30 cells and expressed as min.–max. Images were captured with a Leica DFC 500 camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

3.2.3. DNA extraction, amplification and sequencing

DNA was extracted using Bioline Isolate II Plant DNA Kits (London, UK) following the manufacturer's instructions. DNA was amplified using the polymerase chain reaction (PCR) with primers ITS1 and ITS4, with 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final incubation at 72 °C for 10 min. The (PCR) samples sent to Macrogen (Seoul, South Korea) and Australian Genome Research Facility (AGRF) for purification and Sanger sequencing. (See Chapter 2 for more detailed explanation of the methods used).

3.2.4. Phylogenetic analysis

Sequences were aligned using MUSCLE (Edgar, 2004) embedded in Geneious Prime (2021.1.1). The evolutionary relationships of *Vishniacozyma* isolates with reference species in the Tremellomycetes was based on analysis of the ITS1 sequences. The maximum likelihood tree was constructed using MEGAX (Kumar et al., 2016) with bootstrap support values >50 given at nodes based on the 1000 replications. Gaps or missing data were treated as partial deletion using a site coverage cut-off of 95%. *Derxomyces schimicola* (CBS 9144) was used as an outgroup for analysing 14 *Vishniacozyma* isolates (Wang & Bai, 2008).

3.2.5. Physiology

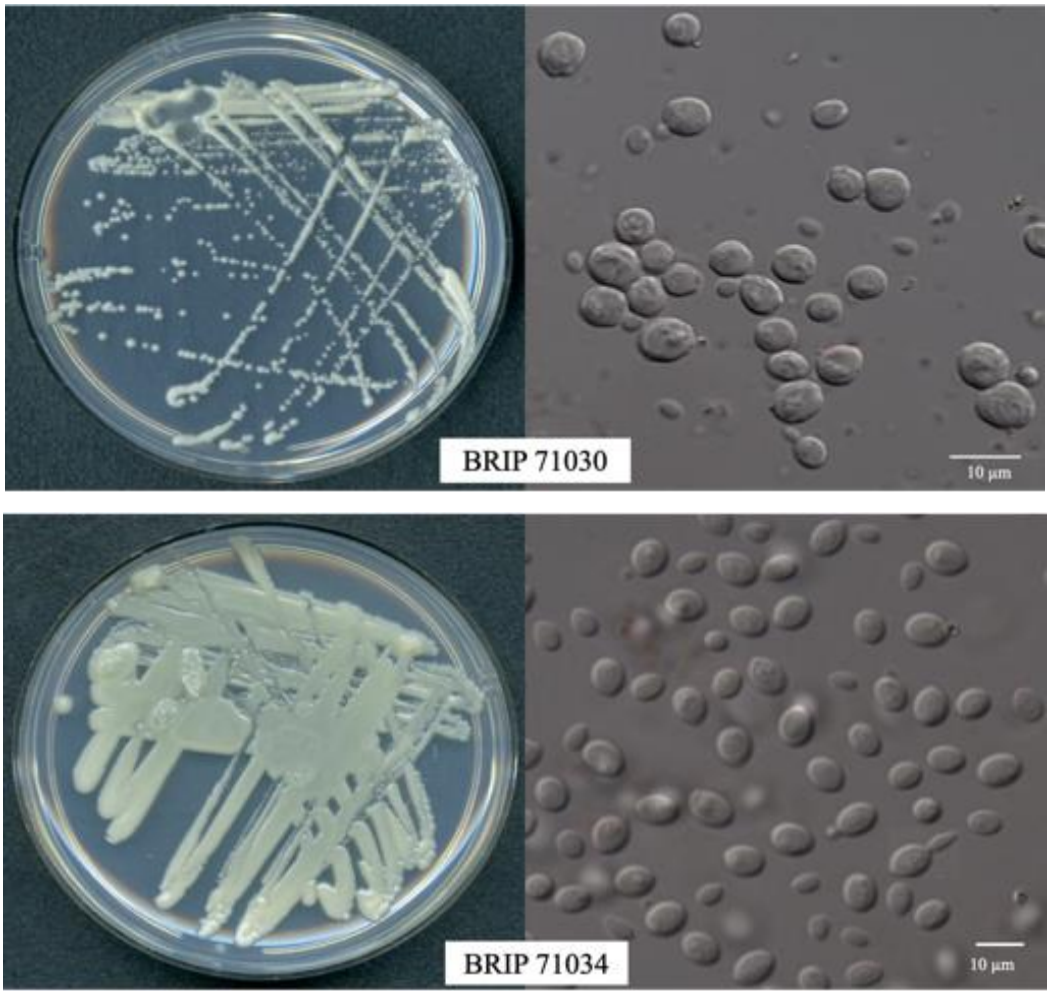
Eight isolates of *Vishniacozyma* (BRIP 71030, 71034, 71043, 71269, 71788, 71946, 71959, 71963) were grown on PDA for 7 d at 22–25 ºC for phenotypic characterisation. A suspension of yeast cells in sterile water (1 µl) was transferred into wells on Biolog YT microplates and incubated for 4 d at 25 ºC. An ELISA microplate reader was used to determine whether the isolates were reactive in assimilation and oxidation tests. A value ≥ 0.15 was considered as a positive reaction (+) and ≤ 0.15 was considered a negative reaction (–) (Table 3.3). Double distilled water was used as a negative control. A positive reaction in the oxidative tests was indicated by a blue colour change in wells of the Biolog YT microplates.

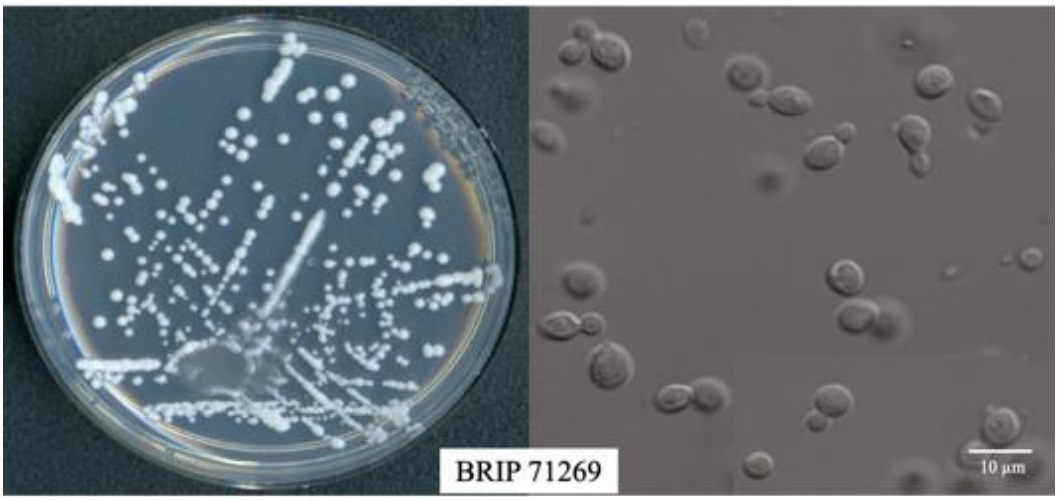
3.3. Results

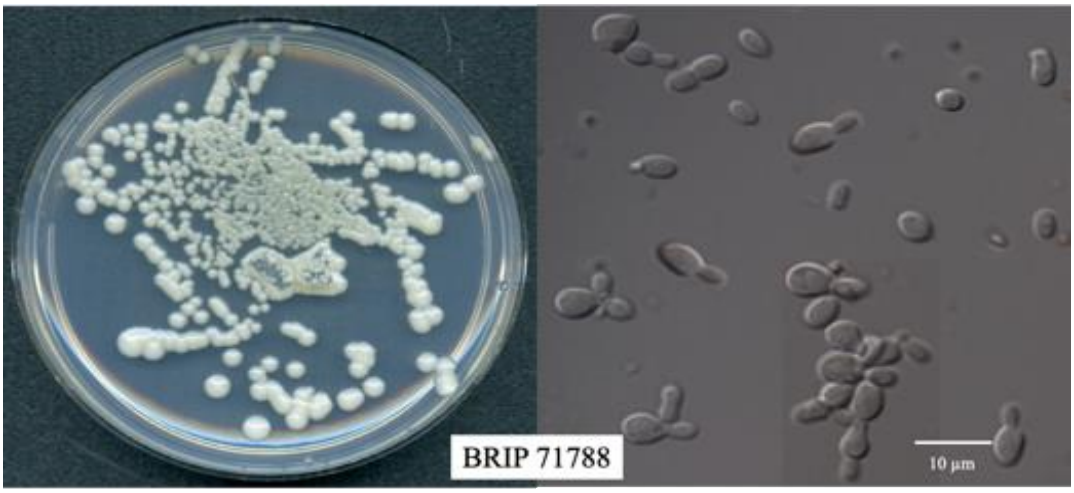
3.3.1. Description of Vishniacozyma isolates

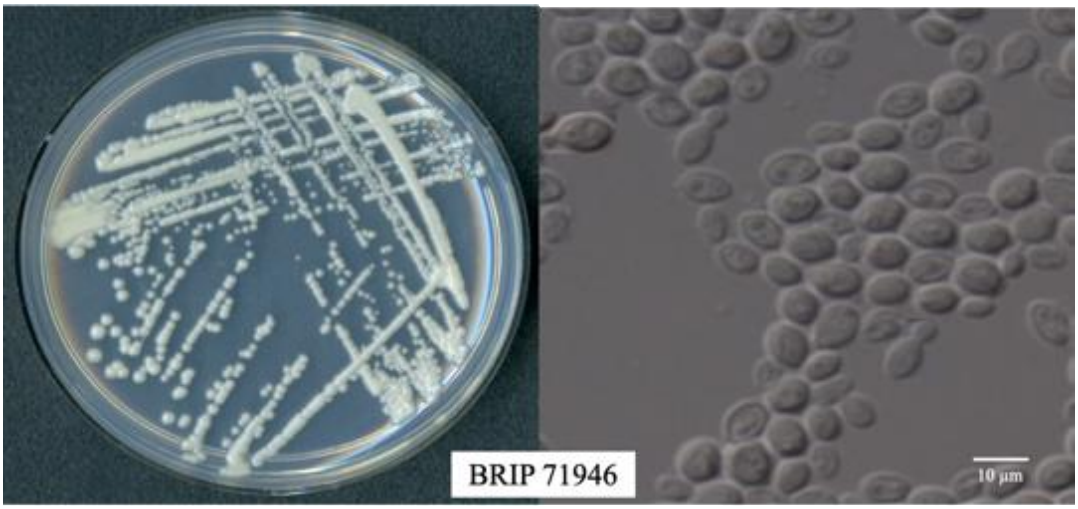
In this Chapter, 14 isolates of *Vishniacozyma* were obtained and deposited as reference cultures in the culture collection at the Queensland Plant Pathology Herbarium (BRIP), Dutton Park. Based on MegaBLAST searches of NCBI's GenBank nucleotide database using the ITS sequence for ex-type specimens, these isolates were identified as putative novel species of *Vishniacozyma* (Table 3.2).

The colonies of eight *Vishniacozyma* isolates (BRIP 71030, 71034, 71043, 71269, 71788, 71946, 71959, 71963) were round with entire margins, butryous, glistening, cream to buff. The cells were subglobose to ovoid, measured 3–8 µm, with polar buds.




BRIP 71269
10 µm


BRIP 71788
10 µm


BRIP 71946
10 µm

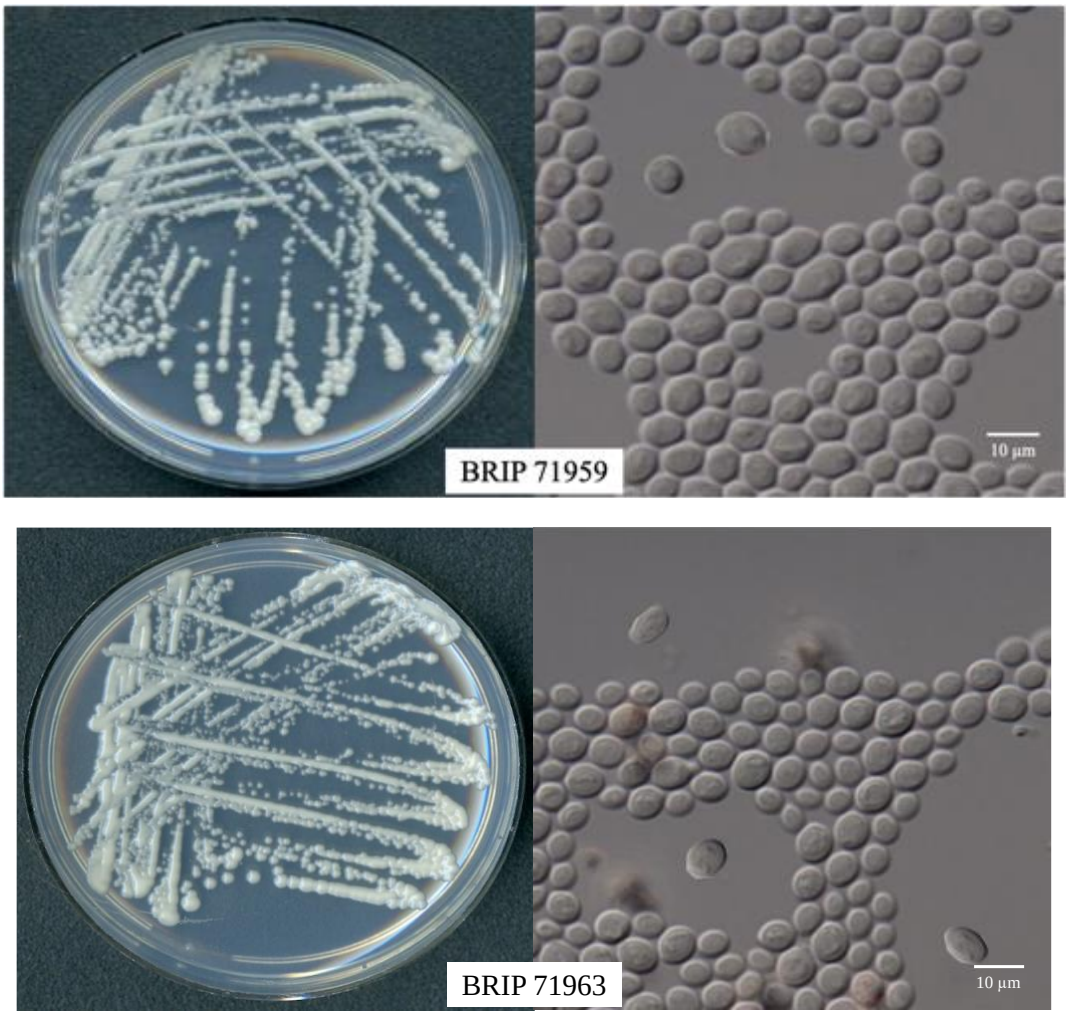


Figure 3.1 PDA cultures and cell of Vishniacozyma sp. of BRIP 71946, 71959,71963, 71030, 71034, 71269 and 71788 morphologies isolates phylloplane yeasts.

3.3.2. Phylogeny

The sequences generated from this study were analysed with reference sequences from ex-type cultures of all known species of *Vishniacozyma* (Vu et al., 2019) through searches in GenBank (Table 3.2). The isolated yeasts had closest ITS sequence identity to the ex-type culture **(CGMCC 2.2444)** of *Vishniacozyma taibaiensis* (GenBank NR_144810.1) (Table 3.2). In the phylogenetic analysis, all 14 of the *Visnhniacozyma* isolates from the phylloplane of *B. integrifolia* clustered in one clade sister to *V. taibaiensis* (Figure 3.3).

Table 3.2 *Vishniacozyma* spp. isolated from leaves of *Banksia integrifolia* in south-eastern Queensland.

Culture accession	Location	ITS sequence identity (%) compared with GenBank NR_144810.1
BRIP 71030	Esk	93
BRIP 71034	Esk	94
BRIP 71043	Esk	95
BRIP 71273	Highfields	94
BRIP 71269	Highfields	94
BRIP 71279	Highfields	94
BRIP 71281	Highfields	94
BRIP 71788	Highfields	94
BRIP 71932	Noosa Heads	95
BRIP 71933	Noosa Heads	94
BRIP 71946	Noosa Heads	94
BRIP 71951	Noosa Heads	94
BRIP 71959	Noosa Heads	94
BRIP 71963	Noosa Heads	94

Ex-type isolates no.CBS:9919

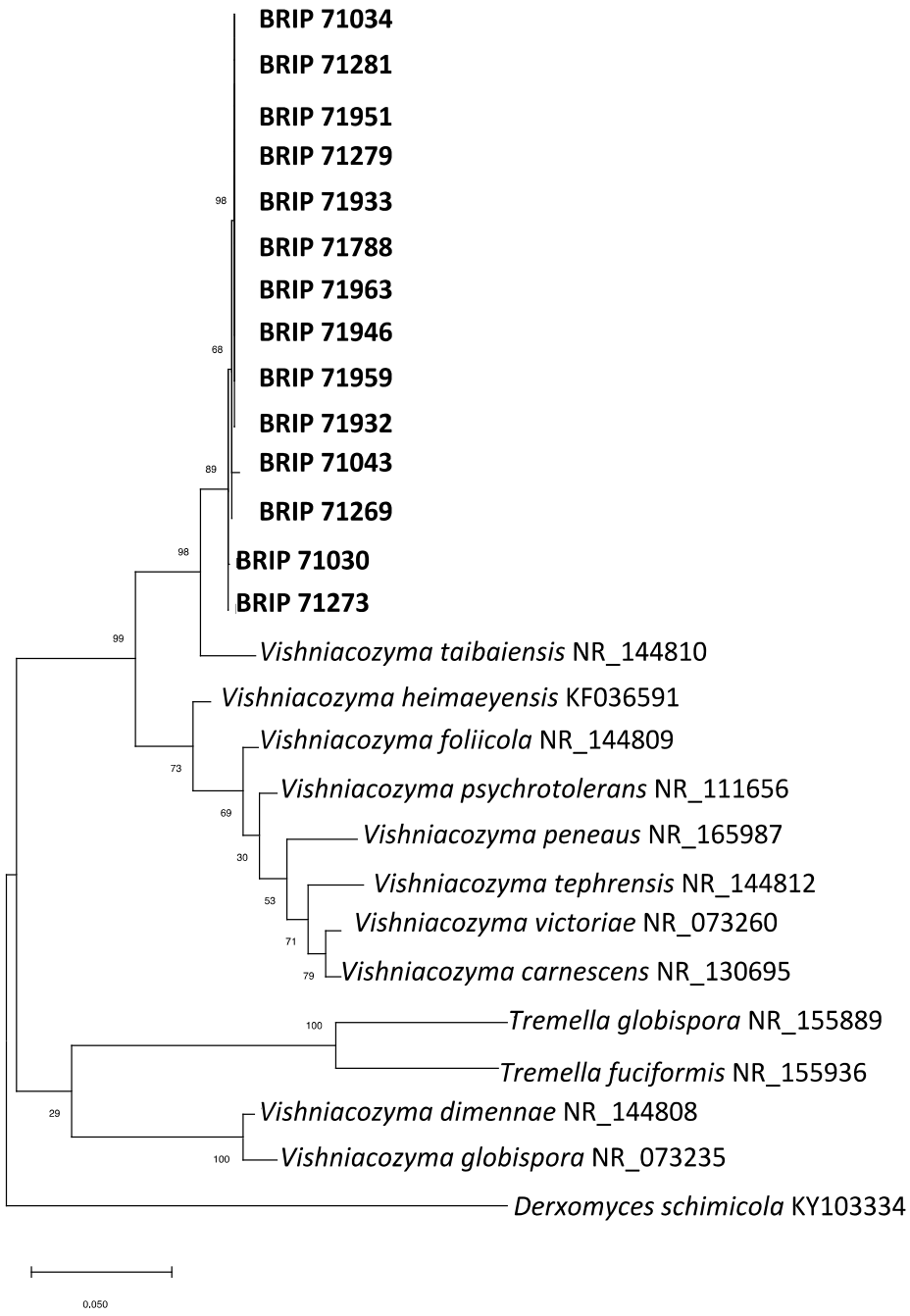


Figure 3.2 Maximum likelihood phylogenetic tree of *Vishniacozyma* isolates based on ITS. Bootstrap values from 1,000 replicates are shown at the branches. Branch lengths are measured in the number of substitutions per site (scale bar = 0.05).

3.3.3. Physiology

The physiological characteristics of *Vishniacozyma* isolates were determined by Biolog YT microplate tests (Table 3.3). The Biolog YT microplate test results showed that all Australian isolates assimilated the following carbon compounds succinic acid, L-aspartic acid, L-glutamic acid, D-gluconic acid, dextrin, D-cellobiose, maltose, maltotriose, D-melezitose, D-melibiose, palatinose, D-raffinose, stachyose , sucrose, turanose , N-acetyl-D- glucosamine, α-D-glucose, D-galactose, L-sorbose, salicin, D-mannitol, D-sorbitol, D-arabitol, for most of the isolates are positive (+).

The following nitrogen compounds were assimilated 2-keto-D- gluconic acid, D-gluconic acid, dextrin, D-cellobiose, gentiobiose, maltose, D-melezitose, D-melibiose, palatinose, D-raffinose, stachyose, sucrose, N-acetyl-D– glucosamine, D-glucosamine, α-D-glucose , D-galactose, maltitol, D-arabitol, i-erythritol L-arabinose N-acetyl-L- glutamic acid plus D-xylose, ouinic acid plus D-xylose, α-D-lactose plus D-xylose, D-melibiose plus D-xylose, m-inositol plus D-xylose,acetoin plus D-xylose (Table 3.3). The physiological results for isolated *Vishniacozyma* BRIP 71043, 71946, 71788, and 71959 were identical in carbon and nitrogen assimilations while BRIP 71030, 71034, 71269 and 71963 had different physiological profiles (16 out of 94 tests). *Vishniacozyma* BRIP 71034, 71269, 71963 isolated from *B. integrifolia* did not assimilated ribitol as also known in only two other species, *V. taibaiensis* and *V. psychrotolerans* (Félix et al., 2020).

Table 3.3 Physiological characteristics of *Vishniacozyma* yeasts isolated from leaves of *Banksia integrifolia* in south-eastern Queensland.

Characteristics	BRIP accession no.							
	71030	71034	71043	71269	71788	71946	71959	71963
Oxidation test								
Water	–	–	–	–	–	–	–	–
Acetic Acid	–	+	+	–	–	–	–	–
Formic Acid	–	–	–	–	–	–	–	–
Propionic Acid	–	–	–	–	–	–	–	–
Succinic Acid	+	+	+	+	+	+	+	+
SuccinicAcid Mono-Methyl Ester	–	+	+	–	–	+	+	+
L-Aspartic Acid	+	+	+	–	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+	+	+
L-Proline	+	–	+	–	+	+	+	+
D-Gluconic Acid	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	–	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Maltotriose	+	+	+	+	+	+	+	+
D-Melezitose	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+
Palatinose	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	+	+	+	+	+
Stachyose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	–	+	+	+	+
Turanose	+	+	+	+	+	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+
D-Psicose	+	+	+	–	+	+	+	+
L-Sorbose	–	+	–	–	+	+	+	+
Salicin	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	–	+	+	+	+
D-Arabitol	+	+	+	+	+	+	+	+
Xylitol	–	+	+	–	+	+	+	+
Glycerol	–	–	–	–	–	–	–	–
Tween 80	–	–	+	–	+	–	–	–
Assimilation Tests								
Water	–	–	–	–	–	–	–	–
Fumaric Acid	+	+	+	+	+	+	+	+
L-Malic Acid	+	+	+	–	+	+	+	+

Table 3.3 (Ctd).								
Succinic Acid Mono-Methyl Ester	+	+	–	–	–	+	+	+
Bromo succinic Acid	+	+	+	–	+	+	+	+
L-Glutamic Acid	+	+	+	–	+	+	+	+
γ-Aminobutyric Acid	–	+	+	–	+	+	+	+
α-Ketoglutaric Acid	+	+	+	–	+	+	+	+
2-Keto-D- Gluconic Acid	+	+	+	+	+	+	+	+
D-Gluconic Acid	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Maltotriose	+	+	+	–	+	+	+	+
D-Melezitose	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+
Palatinose	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	+	+	+	+	+
Stachyose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	–	+	+	+	+
N-Acetyl-D–Glucosamine	+	+	+	+	+	+	+	+
D-Glucosamine	+	+	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+	+	+
D -Galactose	+	+	+	+	+	+	+	+
D-Psicose	+	+	+	–	+	+	+	+
L-Rhamnose	+	+	+	–	+	+	+	+
L-Sorbose	–	–	–	–	+	+	+	–
α-Methyl-D-Glucoside	+	+	+	–	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	–	+	+	+	+
Amygdalin	–	–	–	–	–	–	–	–
Arbutin	+	+	+	–	+	+	+	+
Salicin	+	+	+	–	+	+	+	+
Maltitol	+	+	+	+	+	+	+	+
D-Mannitol	+	+	+	–	+	+	+	+
D-Sorbitol	+	+	+	–	+	+	+	+
Adonitol	+	–	+	–	+	+	+	–
D-Arabitol	+	+	+	+	+	+	+	+
Xylitol	–	+	+	–	+	+	+	+
i-Erythritol	+	+	+	+	+	+	+	+
Glycerol	–	–	–	–	–	–	–	–
Tween 80	–	+	–	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	+
D-Arabinose	+	+	+	–	+	+	+	+
N-Acetyl-L-Glutamic Acid plus D-Xylose	+	+	+	+	+	+	+	+
Quinic Acid plus D-Xylose	+	+	+	+	+	+	+	+
D-Glucuronic Acid plus D-Xylose	+	+	+	–	+	+	+	+
α-D-Lactose plus D-Xylose	+	+	+	+	+	+	+	+
D-Melibiose plus D-Xylose	+	+	+	+	+	+	+	+
D-Galactose plus D-Xylose	+	+	+	–	+	+	+	+
m-Inositol plus D-Xylose	+	+	+	+	+	+	+	+
1,2Propanediol plus D-Xylose	+	–	+	+	+	+	+	+
Acetoin plus D-Xylose	+	+	+	+	+	+	+	+

3.4. Discussion

The results of this Chapter showed that a putatively novel species of *Vishniacozyma* was widespread on the leaves of *B. integrifolia* in south-eastern Queensland. This putatively novel species of *Vishniacozyma* was phylogenetically close to the ex-type of culture of *V. taibaiensis* (CGMCC 2.2444) (Liu et al., 2015a). Fourteen isolates of this *Vishniacozyma* sp. were collected.

This is the first record of *Vishinacozyma* in Australia as well as the first record of a *Vishinacozyma* yeast having been found on *B. integrifolia*. The literature shows that *Vishniacozyma* have been isolated from around the world on fruits, dead leaves, dead branches, in soil and water (Liu et al., 2015a; Félix et al., 2020; Chang et al., 2021). There are 19 known species of *Vishniacozyma* worldwide. The *Vishniacozyma* sp. isolated from Australia on *B. integrifolia* represents a putative novel species that warrants further taxonomic investigation. This discovery lends weight to the hypothesis that Australia has a rich diversity of undiscovered and endemic yeast species found nowhere else in the world.

CHAPTER FOUR: DIVERSITY AND PHYLOGENY OF *HANNAELLA* YEASTS ON LEAVES OF *BANKSIA INTEGRIFOLIA* IN SOUTH-EASTERN QUEENSLAND

4.1. Introduction

The genus *Hannaella* (Basidiomycota, Tremellales) contains anamorphic yeasts (Wang & Bai, 2008; Into et al., 2020b) formerly classified in the *Cryptococcus luteolus* lineage (Scorzetti et al., 2002; Wang & Bai, 2008). *Hannaella* was named in honour of the Canadian scientist William Fielding Hanna for his pioneering study on ballistoconidium forming yeasts (Wang & Bai, 2008). *Hannaella* is closely related to *Diozegia* and *Derxomyces* (Wang & Bai, 2008).

Seven species, namely *H. coprosmaensis*, *H. kunmingensis*, *H. luteola*, *H. oryzae*, *H. sinensis*, *H. surugaensis* and *H. zeae*, have been transferred from *Bullera* and *Cryptococcus* (Wang & Bai, 2008). A further six species have been discovered and formally named, viz. *H. dianchiensis* (Han et al., 2017), *H. pagnoccae* (Landell et al., 2014), *H. phetchabunensis* (Kaewwichian et al., 2015), *H. phyllophila* (Surussawadee et al., 2015), *H. siamensis* (Kaewwichian et al., 2015), and *H. taiwanensis* (Kachalkin et al., 2019) (Table 4.1).

The aim of this Chapter was to identify the species of *Hannaella* isolated from leaves of *B. integrifolia* in Chapter 2. The work was part of a study to determine if putative novel species of yeast inhabited the leaf surface of *B. integrifolia*, and whether these yeasts were more closely related to other Australian species than to overseas species.

Table 4.1 Collection records for ex-type cultures of *Hannaella* species used in the phylogenetic analysis

Species	Ex-type culture accession no.	Basionym	Host/substrate	Location (country)	References
Hannaella coprosmae	CBS 8284	*Bullera coprosmae*	dead leaf and fruit of mountain currant	New Zealand	Hamamoto & Nakase (1996); Wang & Bai (2008)
H. dianchiensis	-------	-------	lake water	China	Han et al. (2017)
H. kunmingensis	CBS 8960	*B. kunmingensis*	dried leaf of *Parthenocissus*	Yunnan	Bai et al. (2001); Wang & Bai (2008)
H. luteola	CBS 943	*Cryptococcus luteola*	Air	Japan	Wang & Bai (2008); Skinner (1950)
H. oryzae	CBS 7194	*B. oryzae*	leaves and stems of corn	Honshu Japan	Wang & Bai (2008); Nakase & Suzuki (1985)
H. pagnoccae	CBS 11142	-------	plant and soil	Brazil	Landell et al. (2014)
H. phetchabunensis	DMKU-CP 430	-------	leaf surfaces of rice and corn leaf	Thailand, Hungary	Kaewwichian et al. (2015)
H. phyllophila	CBS 13921	-------	leaves	Thailand, Taiwan	Surussawadee et al. (2015)
H. siamensis	DMKU-RP 72	-------	leaf surfaces of rice and corn leaf	Thailand, Hungary	Kaewwichian et al. (2015)
H. sinensis	CBS 7238	*B. sinensis*	wheat	China	Wang & Bai (2008); Li (1982)
H. surugaensis	CBS 9426	*C. surugaensis*	sea	Honshu Japan	Wang & Bai (2008); Nagahama et al. (2003)
H. taiwanensis	BCRC 23252	*C. taiwanensis*	leaf surface	Taiwan	Kachalkin et al. (2019); Huang et al. (2011)
H. zeae	CBS 10801	*C. zeae*	maize	Austria	Molnár & Prillinger (2006); Wang & Bai (2008)

4.2. Materials and methods

4.2.1. Yeast isolates

Leaf samples were collected from three different locations in south-eastern Queensland (see Chapter 2). Yeasts were isolated from leaf surfaces and were grown on YMPA for 7 d at 22–25 ºC following the method of Into et al. (2020a). Eight isolates (BRIP 71047, 71051, 71058, 71253, 71256, 71259, 71273, 71269) that had been identified as putative *Hannaella* species based on their ITS sequences (the detailed methodology is described in Chapter 2).

4.2.2. Morphology

Two isolates of *Hannaella* (BRIP 71047 and 71259) were sub-cultured onto plates of PDA for morphological examination. Descriptions of the colonies were made with the aid of Rayner's colour charts (Rayner, 1970). Cells of these isolates were examined under Nomarski interference microscopy and the size and shape of cells were recorded (n > 30). Yeast cells were mounted on glass slides in 100 % lactic acid for microscopic examination after 10 days of incubation in the dark at 25 °C. Measurements were taken from at least 30 cells and expressed as min.–max. Images were captured with a Leica DFC 500 camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

4.2.3. DNA extraction, amplification and sequencing

DNA was extracted using Bioline Isolate II Plant DNA Kits (London, UK,) following the manufacturer's instructions. DNA was amplified using the polymerase chain reaction (PCR) with primers ITS1 and ITS4, with 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final incubation at 72 °C for 10 min. The (PCR) samples sent to Macrogen (Seoul, South Korea) for purification and Sanger sequencing (See Chapter 2 for more detailed explanation of the methods used).

4.2.4. Phylogenetic analysis

Sequences were aligned using MUSCLE (Edgar, 2004) embedded in Geneious Prime (2021.1.1). The evolutionary relationships of *Hannaella* isolates with reference species in the Tremellomycetes was based on analysis of the ITS1 sequences. The maximum likelihood tree was constructed using MEGA7 (Kumar et al., 2016) with bootstrap support values >50 given on branches based on 1000 replications. Gaps or missing data were treated as partial deletion using a site coverage cut-off of 95 %. *Bulleribasidium setariae* (AB118875.1:1857-2207) was used as an outgroup (Liu et al., 2015a).

4.2.5. Physiology

Two isolates of *Hannaella* (BRIP 71047 and 71259) were grown on PDA for 7 d at 22–25 ºC for phenotypic characterisation. A suspension of yeast cells in sterile water (1 µl) was transferred into wells on Biolog YT microplates and incubated for 4 d at 25 ºC. An ELISA microplate reader was used to determine whether the isolates were reactive in assimilation and oxidation tests. A value ≥ 0.15 was considered as a positive reaction (+) and ≤ 0.15 was considered a negative reaction (–) (Table 4.5). Double distilled water was used as a negative control. A positive reaction in the oxidative tests was indicated by a blue colour change in wells of the Biolog YT microplates.

4.3. Results

4.3.1. Description of Hannaella isolates

In this study, eight yeast isolates of *Hannaella* were obtained and deposited as reference cultures in the culture collection at the Queensland Plant Pathology Herbarium (BRIP), Dutton Park, Queensland. Based on MegaBLAST searches of NCBI's GenBank nucleotide database using the ITS sequence for ex-type specimens, these isolates were identified as species of *Hannaella* (Table 4.2).

- The cells of *Hannaella* isolate BRIP 71047 were subglobose, broadly ellipsoidal or ovoid, 4-6 x 2.5-5 µm, single or with polar buds. The colonies were mucoid, slimy, buff.

- The cells of *Hannaella* isolate BRIP 71259 were subglobose, broadly ellipsoidal or ovoid, 3-5 x 3-6 µm, single or with polar buds. The colonies are round with entire margins, butryous to mucoid, glistening, buff to pale luteus (Figure 4.1).

The morphological characteristics of the Australian *Hannaella* isolates were similar to overseas *Hannaella* spp., i.e. *H. siamensis* (3–5 x 5–7 µm), *H. phetchabunensis* (2–3 x 3–6 µm) (Kaewwichian et al. (2015), *H. pagnoccae* (3.5–7.1 x 2.0–5.3 µm) (Landell et al. (2014), *H. phyllophila* (2–5.5 x 4–11 µm) (Surussawadee et al. (2015), and *H. dianchiensis* (5–7 x 3–5 µm) (Han et al., 2017).

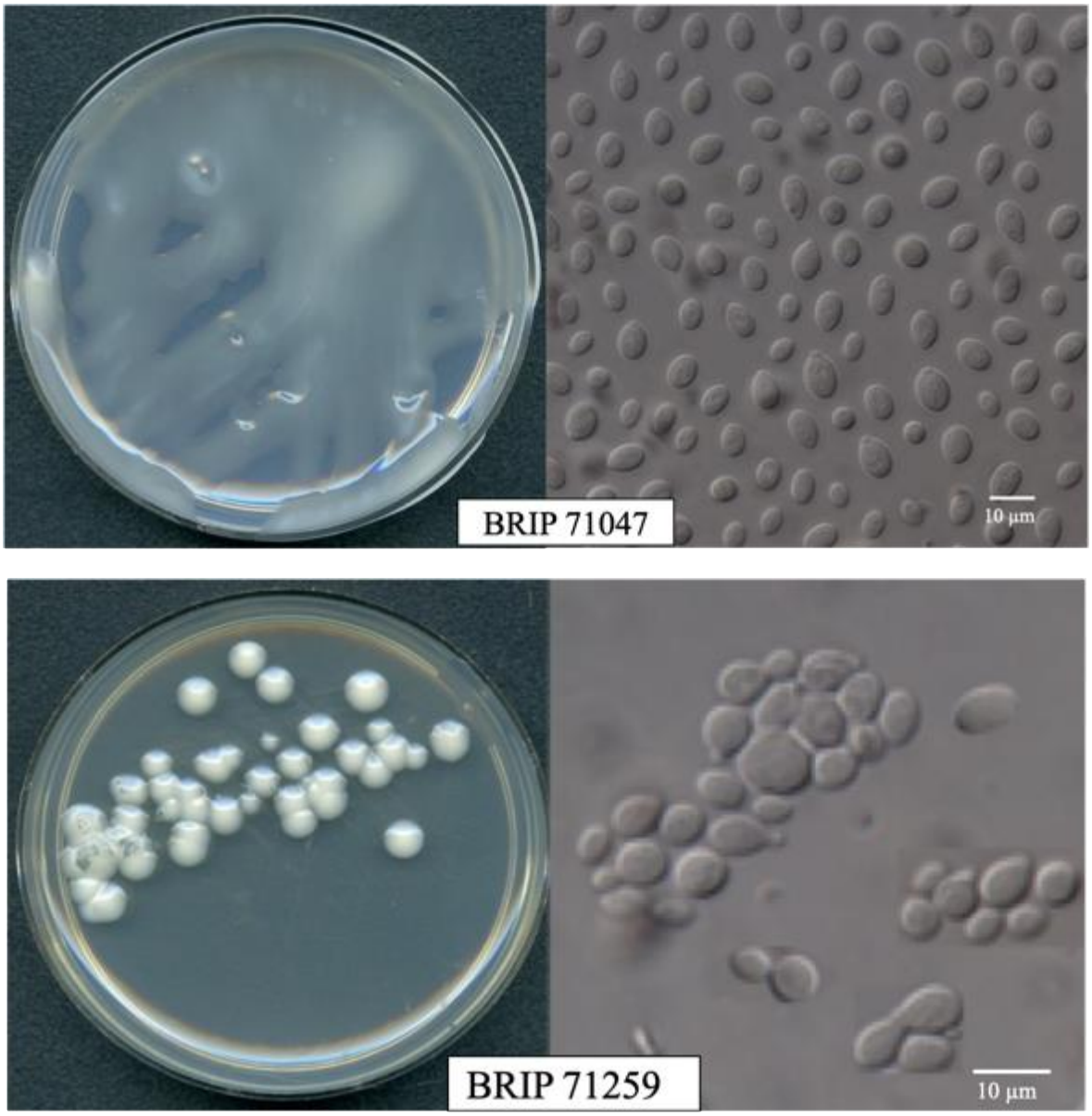


Figure 4.1 Cultures and cells of *Hannaella* sp. (BRIP 71047, 71259) on PDA after 7 d. Leica DFC 500 camera attached to a Leica DM5500B compound microscope was used to capture the images.

Table 4.2 *Hannaella* spp. isolated from leaves of *Banksia integrifolia* in south-eastern Queensland.

Culture accession	Location	ITS sequence identity (%) compared on GenBank	Ex-type isolates no.	GenBank accession* ITS
BRIP 71047	Esk	98	**CBS:943**	EU252551.1
BRIP 71051	Esk	99	**CBS:11142**	NR_155180.1
BRIP 71058	Esk	98	**CBS:11142**	NR_155180.1
BRIP 71253	Highfields	98	**CBS:8960**	NR_111065.1
BRIP 71256	Highfields	98	**CBS:11142,**	NR_155180.1
BRIP 71259	Highfields	98	**CBS:8960**	NR_111065.1
BRIP 71273	Highfields	96	**CBS:10801**	NR_144771.1
BRIP 71269	Highfields	99	**CBS:7238**	NR_111075.1

4.3.2. Phylogeny

The sequences generated from this study were analysed together with reference sequences from ex-type cultures of all known species of *Hannaella* in GenBank (Schoch et al., 2012; Vu et al., 2019) (Table 4.2). The *Hannaella* yeasts had high ITS1 sequence identity (>98 %) to *Hannaella* ex-type cultures, specifically *Hannaella kunmingensis* (CBS 8960), *H. luteola* (CBS 943), *H. pagnoccae* (CBS 11142), *H. sinensis* (CBS 7238)*, H. zeae* (CBS 10801), in a BLAST search (Table 4.2). All eight of the *Hannaella* isolates from the phylloplane of *B. integrifolia* clustered in a clade sister to *Derxomyces* (Figure 4.2).

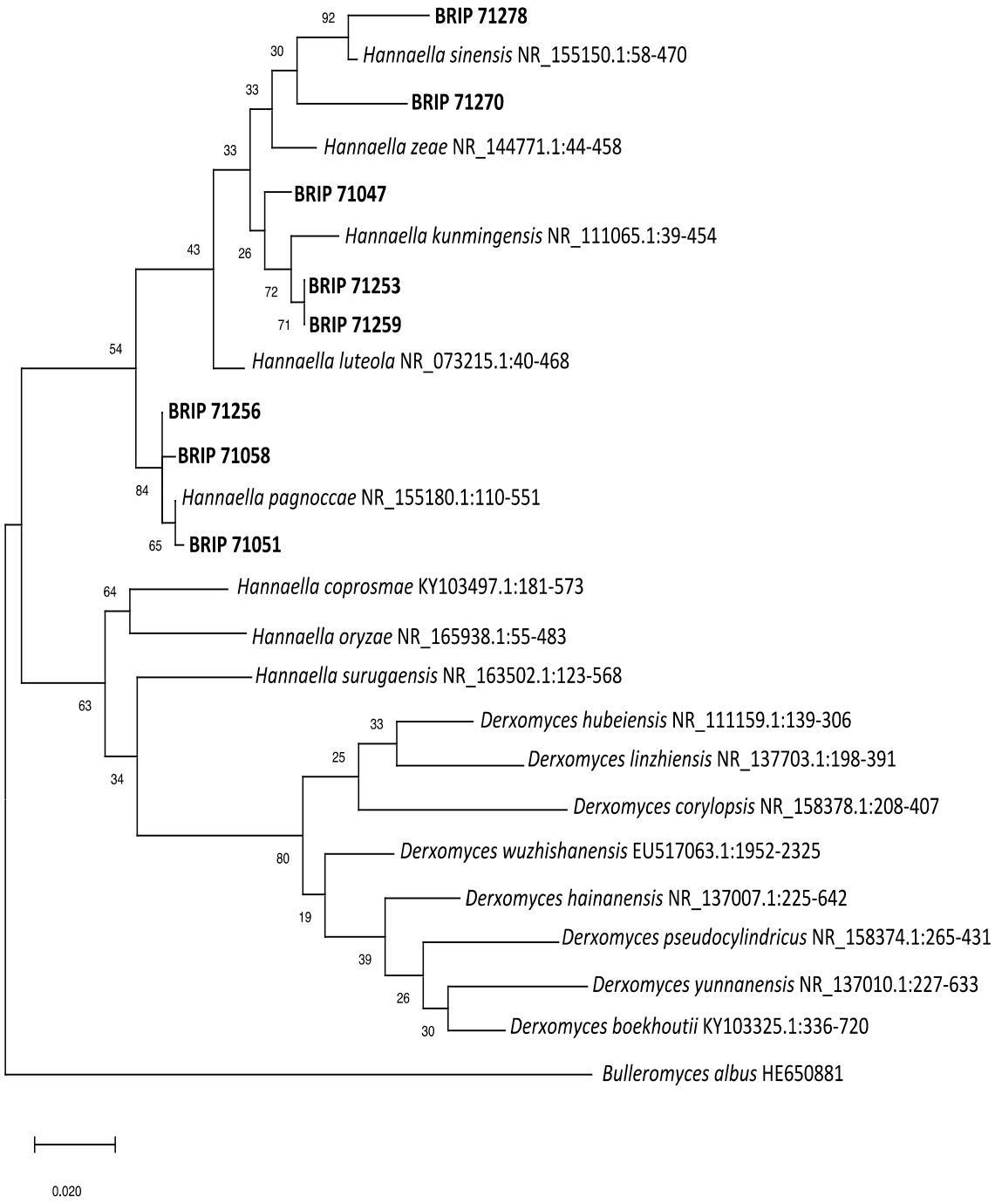


Figure 4.2 Maximum likelihood phylogenetic tree of *Hannaella* isolates based on ITS. Bootstrap values from 1,000 replicates are shown at the branches. Branch lengths are measured in the number of substitutions per site (scale bar = 0.02).

4.3.3. Physiological characteristics of Hannaella isolates

Physiological test reactions for the two *Hannaella* isolates showed that two isolates (BRIP 71047 and 71259) had dissimilar profiles for carbon and nitrogen compounds in both assimilation and oxidation tests (Table 4.3). The Biolog YT microplate tests results showed that the two Australian *Hannaella* isolates assimilated the following carbon compounds succinic acid, L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, dextrin, D-cellobiose, gentiobiose, maltose, maltotriose, D-melezitose, D-melibiose, palatinose, D-raffinose, stachyose, sucrose, D-trehalose, turanose, N-acetyl-D-glucosamine, α-D-glucose, D-galactose, L-sorbose, salicin, D-mannitol, D-sorbitol, D-arabitol and glycerol were positive for both *Hannaella* isolates (Table 4.3).

The following nitrogen compounds were assimilated, L-Malic Acid, Succinic Acid, Mono-Methyl Ester, Bromo succinic Acid, L-Glutamic Acid, γ-Aminobutyric Acid, 2-Keto-D- Gluconic Acid, D-Gluconic Acid, Dextrin, D-Cellobiose, Gentiobiose, Maltose, Maltotriose,D-Melezitose, Palatinose, D-Raffinose, Stachyose, Sucrose, D-Trehalose,N-Acetyl-D– Glucosamine, D-Glucosamine, α-D-Glucose, D-Galactose, D-Psicose, L-Rhamnose, L-Sorbose, α-Methyl-D-Glucoside, β-Methyl-D- Glucoside, Arbutin, Salicin, Maltitol D-Mannitol, D-Sorbitol D-Arabitol, Xylitol, i-Erythritol, Glycerol, L-Arabinose, D-ArabinoseQuinic Acid plus D-Xylose, D-Glucuronic Acid plus D-Xylose, α-D-Lactose plus D-Xylose, D-Melibiose plus D-Xylose and D-Galactose plus D-Xylose (Table 4.3). The physiological results for isolated *Hannaella* (BRIP 71047) from *B. integrifolia* did not assimilated D-Xylose, Melibiose, Inositol, Xylitol, which is assimilated with other discovered *Hannaella* species *H. dianchiensis* (Han et al., 2017), *H. phyllophila* and *H. siamensis* (Surussawadee et al., 2015).

Table 4.3. Physiological characteristics of *Hannaella* isolates BRIP 71047, 71259

Characteristics	71047	71259	Characteristics (Ctd.)	71047	71259
Oxidation test					
Water	–	–	Dextrin	+	+
Acetic Acid	–	–	D-Cellobiose	+	+
Formic Acid	–	–	Gentiobiose	+	+
Propionic Acid	–	–	Maltose	+	+
Succinic Acid	+	+	Maltotriose	+	+
SuccinicAcid Mono-Methyl Ester	–	+	D-Melezitose	+	+
L-Aspartic Acid	+	+	D-Melibiose	–	+
L-Glutamic Acid	+	+	Palatinose	+	+
L-Proline	+	+	D-Raffinose	+	+
D-Gluconic Acid	+	+	Stachyose	+	+
Dextrin	+	+	Sucrose	+	+
D-Cellobiose	+	+	D-Trehalose	+	+
Gentiobiose	+	+	N-Acetyl-D– Glucosamine	+	+
Maltose	+	+	D-Glucosamine	+	+
Maltotriose	+	+	α-D-Glucose	+	+
D-Melezitose	+	+	D -Galactose	+	+
D-Melibiose	+	+	D-Psicose	+	+
Palatinose	+	+	L-Rhamnose	+	+
D-Raffinose	+	+	L-Sorbose	+	–
Stachyose	+	+	α-Methyl-D- Glucoside	+	+
Sucrose	+	+	β-Methyl-D- Glucoside	+	+
D-Trehalose	+	+	Amygdalin	–	–
Turanose	+	+	Arbutin	+	+
N-Acetyl-D- Glucosamine	+	+	Salicin	+	+
α-D-Glucose	+	+	Maltitol	+	+
D-Galactose	+	+	D-Mannitol	+	+
D-Psicose	–	+	D-Sorbitol	+	+
L-Sorbose	+	–	Adonitol	–	+
Salicin	+	+	D-Arabitol	+	+
D-Mannitol	+	+	Xylitol	+	+
D-Sorbitol	+	+	i-Erythritol	+	+
D-Arabitol	+	+	Glycerol	+	+
Xylitol	–	+	Tween 80	–	–
Glycerol	+	+	L-Arabinose	+	+
Tween 80	–	–	D-Arabinose	+	+
Assimilation Tests					
Water	–	–	N-Acetyl-L-GlutamicAcidplusD-Xylose	–	+
Fumaric Acid	+	+	Quinic Acid plus D-Xylose	+	+
L-Malic Acid	+	+	D-Glucuronic Acid plus D-Xylose	+	+
SuccinicAcidMono-Methyl Ester	+	+	α-D-Lactose plus D-Xylose	+	+
Bromosuccinic Acid	+	+	D-Melibiose plus D-Xylose	+	+
L-Glutamic Acid	+	+	D-Galactose plus D-Xylose	+	+
γ-Aminobutyric Acid	+	+	m-Inositol plus D-Xylose	–	+
α-Ketoglutaric Acid	–	+	1,2Propanediol plus D-Xylose	–	+
2-Keto-D- Gluconic Acid	+	+	Acetoin plus D-Xylose	–	+
D-Gluconic Acid	+	+			

4.4. Discussion

All of the eight Australian isolates of *Hannaella* clustered in a monophyletic clade with known species of *Hannaella*. In the phylogenetic analysis, five of these isolates were identified, specifically *H. kunmingensis* (2 isolates) and *H. pagnnoccae* (3 isolates) (Figure 4.2). Three isolates (BRIP 71047, 71270 and 71278) may represent putative novel species. Overseas, *Hannaella* spp. have been isolated from the phylloplane of several plant species in the families Poaceae, Fabaceae and Vitaceae, as well as from water and soil (Table 4.1). *Hannaella* has not been previously found in Australia. The present study is the first to show that *Hannaella* spp. are relatively common on *B. integrifolia* in south-eastern Queensland. Further, this study reports putative novel species of *Hannella* that will be formally described and named elsewhere. It is be anticipated that the ongoing studies in Australia will discover further putative novel *Hannaella* spp. on the phylloplane of native and introduced plants.

CHAPTER FIVE: DIVERSITY AND PHYLOGENY OF *BANNOA* YEASTS ON LEAVES OF *BANKSIA INTEGRIFOLIA* IN SOUTH-EASTERN QUEENSLAND

5.1. Introduction

The Cystobasidiomycetes (Pucciniomycotina, Basidiomycota) contains many yeast like fungi with dimorphic stages in their life cycle (Wijayawardene et al., 2020). *Bannoa* was introduced as a monotypic genus in the Cystobasidiomycetes based on the dimorphic yeast *Bannoa hahajimensis* (Hamamoto et al., 2002). *Bannoa* was first recognised in the *Sporobolomyces* clade (Hamamoto et al., 2002) and was named after the Japanese microbiologist I. Banno, in honour of his pioneering work on the teleomorphic life cycle of basidiomycetous yeasts (Hamamoto et al., 2002). Wang et al. (2015a) listed three species, namely *Bannoa bischofiae*, *B. ogasawarensis* and *B. syzygii* that had been transferred from *Sporobolomyces*. Since 2015, three further species have been discovered and named, i.e. *B. guamensis*, *B. rosea* and *B. tropicalis* (Parra & Aime (2019). Since 2002, the total number of *Bannoa* spp. has increased to seven (Table 5.1).

The aim of this Chapter was to identify the species of *Bannoa* isolated from leaves of *B. integrifolia* in Chapter 2. The work was part of a study to determine if novel species of yeast inhabited the leaf surface of *B. integrifolia*, and whether these yeasts were more closely related to other Australian species than to overseas species.

Table 5.1 Collection records for ex-type cultures of *Bannoa* species

Species	Ex-type culture accession no.	Basionym	Host/substrate	Location (country)	References
Bannoa bischofiae	CBS 9041	*Sporobolomyces bischofiae*	Dead leaves of bishop wood	Japan	Wang et al. (2015a) Hamamoto et al. (2002)
B. guamensis	PUL F21058	------	Diseased and healthy leaf surface Euphorbiaceae, Asteraceae, Poaceae	Guam and Guyana islands, South America	Parra & Aime (2019)
B. hahajimensis	CBS 9039	------	Dead leaves of *Bryophyllum pinnatum*	Japan	Hamamoto et al. (2002)
B. ogasawarensis	CBS 9038	*S. ogasawarensis*	Dead leaves of *Schima mertensiana*	Japan	Wang et al. (2015a) Hamamoto et al. (2002)
B. rosea	PUL F21059	------	Diseased and healthy leaf surface Euphorbiaceae, Asteraceae, Poaceae	Guam and Guyana islands, South America	Parra & Aime (2019)
B. syzygii	CBS 9040	*S. syzygii*	Dead leaves of *Syzygium buxifolium*	Japan	Wang et al. (2015a) Hamamoto et al. (2002)
B. tropicalis	PUL F21060	------	Diseased and healthy leaf surface Euphorbiaceae, Asteraceae, Poaceae	Guam and Guyana islands, South America	Parra & Aime (2019)

5.2. Materials and methods

5.2.1. Yeast isolates

Leaf samples were collected from three different locations in south-eastern Queensland. Phylloplane yeasts were isolated from leaf surfaces and grown on YMPA for 7 d at 22–25 ºC following the method of Into et al. (2020a). For the molecular studies, two isolates (BRIP 71965 and 71967) were identified as putative *Bannoa* species by their ITS sequences in BLAST searches.

5.2.2. Morphology

Two *Bannoa* isolates (BRIP 71965 and 71967) were sub-cultured on PDA for morphological studies. Descriptions of the colonies were made with the colony colour recorded using Rayner's colour charts (Rayner, 1970). Cells of these isolates were examined under Nomarski interference microscopy and the size and shape of cells were recorded (n > 30). Yeast cells were mounted on glass slides in 100 % lactic acid for microscopic examination after 10 days of incubation in the dark at 25 °C. Measurements were taken from at least 30 cells and expressed as min.–max. Images were captured with a Leica DFC 500 camera attached to a Leica DM5500B compound microscope with Nomarski differential interference contrast.

5.2.3. DNA extraction, amplification and sequencing

DNA was extracted using Bioline Isolate II Plant DNA Kits (London, UK) following the manufacturer's instructions. DNA was amplified using the polymerase chain reaction (PCR) with primers ITS1 and ITS4, with 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final incubation at 72 °C for 10 min. The (PCR) samples were sent to Macrogen (Seoul, South Korea) for purification and sanger sequencing. (See Chapter 2 for more detailed explanation of the methods used).

5.2.4. Phylogenetic analysis

Sequences were aligned using MUSCLE (Edgar, 2004) embedded in Geneious Prime (2021.1.1). The evolutionary relationships of *Bannoa* isolates with reference species in the Cystobasidiomycetes was based on analysis of the ITS1 sequences. The maximum likelihood tree was constructed using MEGAX (Kumar et al., 2016) with bootstrap support values >50 given at nodes based on the 1000 replications. Gaps or missing data were treated as partial deletion using a site coverage cut-off of 95 %. *Bannozyma arctica* (KY101707) was used as an outgroup for analysing two *Bannoa* isolates (Wang et al., 2015a).

5.3. Results

5.3.1. Description of Bannoa isolates

In this study, two isolates of *Bannoa* were obtained and deposited as reference cultures in the culture collection at the Queensland Plant Pathology Herbarium (BRIP), Dutton Park. Based on MegaBLAST searches of NCBI's GenBank nucleotide database using the ITS sequence for ex-type specimens, these isolates were identified as a putative novel species of *Bannoa*.

The colonies of two of *Bannoa* isolates (BRIP 71965 and 71967) on 1/2 PDA at 25 ºC after 10 d were apricot, smooth, domed, glossy, butryous with an entire margins. The conidia were sublobose to narrowly ellipsoidal or obovoid, 3-5 x 3.5 - 9.5 µm, single or in pairs. (Figure 5.1).

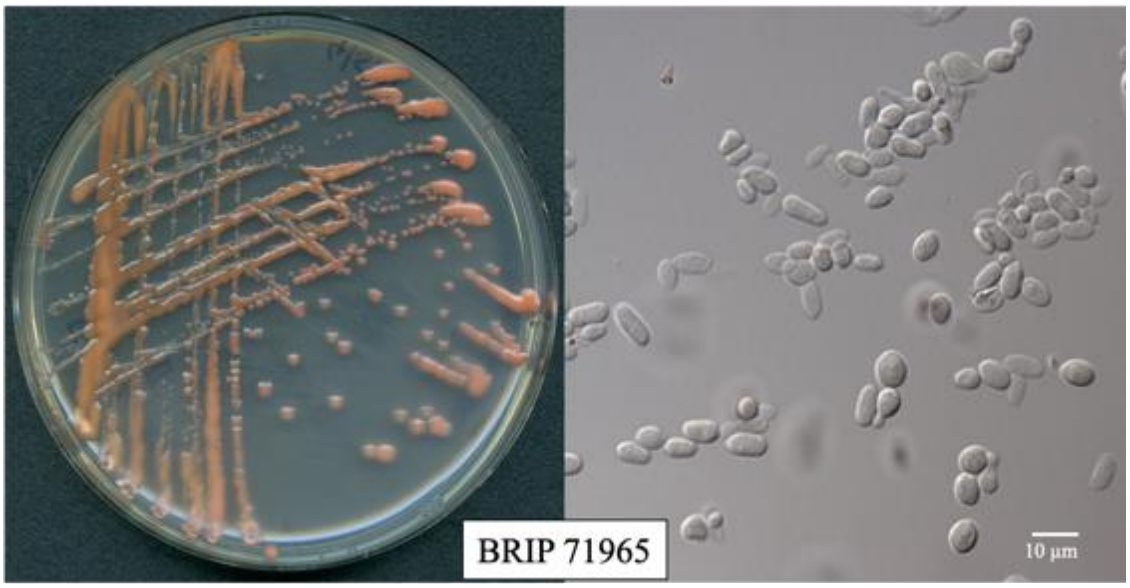


Figure 5.1 Cultures and cells of Bannoa sp. (BRIP 71965) on PDA after 7 d.

5.3.2. Phylogeny

The sequences generated from this study were analysed with reference sequences from ex-type cultures of all known species of *Bannoa* (Vu et al., 2019) through searches in GenBank. The isolated yeasts had closest ITS sequence identity to the ex-type culture of *Bannoa syzygii* (CBS 9040) in a BLAST search (GenBank: NR_154870.1, 526/548 base pairs 96%, 527/549 base pairs 96%). Two of the *Bannoa* isolates from the phylloplane of *B. integrifolia* clustered in a clade sister to other known species of *Bannoa* (Figure 5.2).

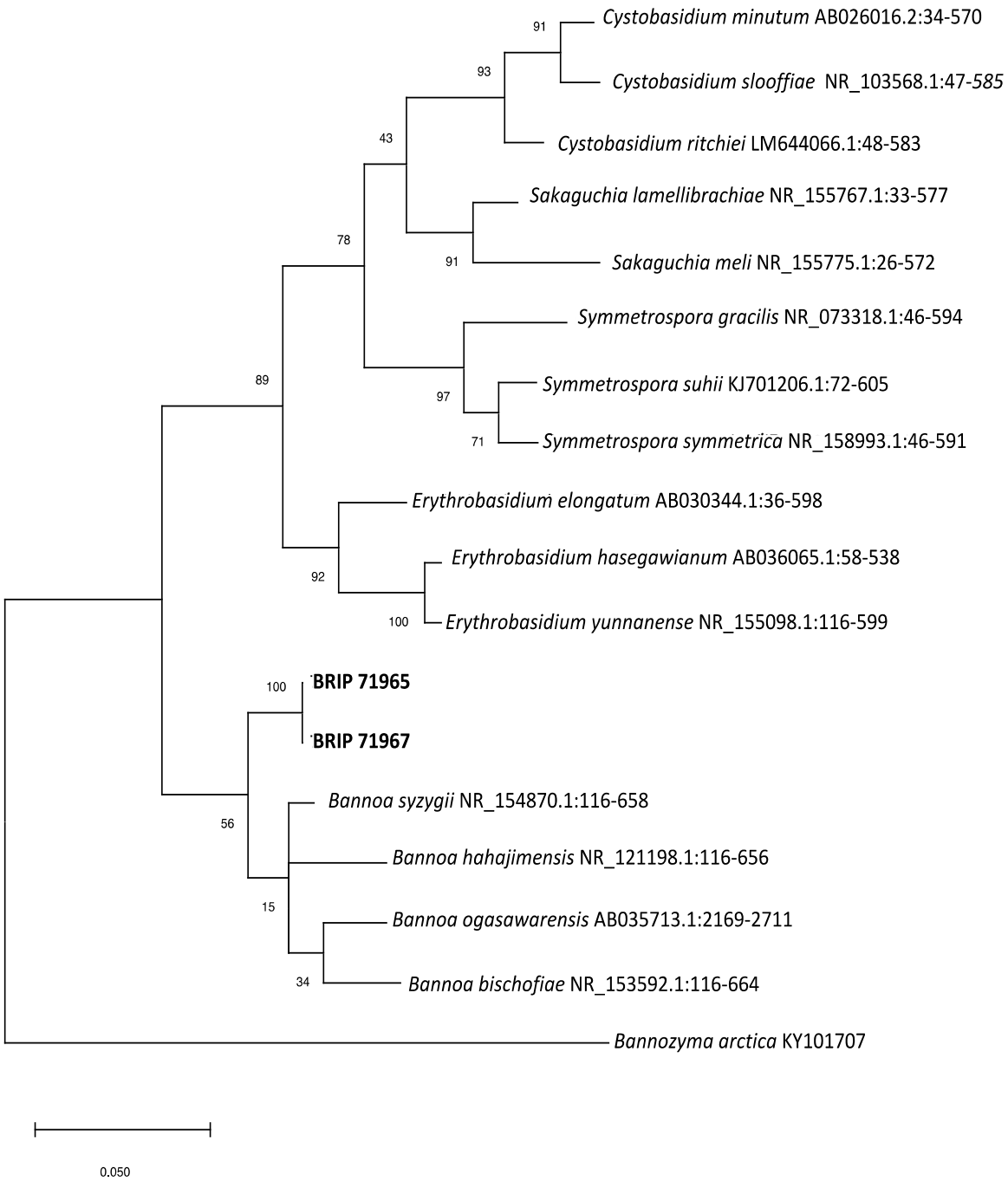


Figure 5.2 Maximum likelihood phylogenetic tree of *Bannoa* isolates based on ITS. Bootstrap values from 1,000 replicates are shown at the branches. Branch lengths are measured in the number of substitutions per site (scale bar = 0.05).

5.4. Discussion

Australian plants are host of many unique fungal species (Chapman, 2009). The present study discovered a putative novel species of *Bannoa* from the phylloplane of *B. integrifolia* in south-eastern Queensland. In the ML phylogenetic analysis, the Australian *Bannoa* sp. was sister to all other known species of *Bannoa*. The literature indicates that *Bannoa* spp. are often isolated from the phylloplane of plants, e.g., in Japan and South America (Hamamoto et al., 2002; Wang et al., 2015a; Parra & Aime, 2019). The present study reports the first record for *Bannoa* in Australia as well as the first record for *Bannoa* on *B. integrifolia*. Further, the Australian species of *Bannoa* represents a putative novel species that will be formally described and named elsewhere. This discovery supports the hypothesis that the phylloplane of Australian plants has a rich diversity of undiscovered yeast species.

CHAPTER SIX: GENERAL DISCUSSION

The findings of this thesis show that the phylloplane of *B. integrifolia* in south-east Queensland harbours a diversity of rarely collected genera that are comprised almost entirely of novel species. Three such genera, namely *Bannoa* (8 species known worldwide), *Hannaella* (7) and *Vishniacozyma* (18), are represented in Australia by only one species each. This study found and recognised 9 species as novel in *Bannoa* (1 novel species), *Hannaella* (1) and *Vishniacozyma* (7).

Previous studies have shown that Australian native plants harbour many unique fungi. There have been very few studies in Australia about the diversity of phylloplane yeasts on native and cultivated plants. Prior to this study, only seven species of phylloplane yeasts had been reported from Australia (Table 1.1, Chapter 1). These species were *Candida tolerans*, *Kodamaea kakaduensis* (Lachance et al., 1999), *Metschnikowia hibisci* (Lachance et al., 1998), *Erythrobasidium elongatum*, *Symmetrospora foliicola* (Shivas & de Miranda, 1983b), *Pseudomicrostroma phylloplanum* (Shivas & de Miranda, 1983a) and *Saitozyma wallum* (Crous et al., 2019). MacArthur & McGee (2000) also found that *B. integrifolia* harboured many endophytic fungal species. In the present study, numerous phylloplane yeast species were isolated from the leaf surfaces of *B. integrifolia*. Based on molecular phylogenetic analyses, the phylloplane yeasts were Basidiomycetous and Ascomycetous species. The majority were Basidiomycetous species from the genera *Vishniacozyma*, *Hannaella*, *Bannoa*, *Dioszegia*, *Filobasidium*, *Sporidiobolus*, *Sporobolomyces*, *Symmetrospora*, and *Tremella*. The remainder for Ascomycetous from the genera *Aureobasidium*, *Aspergillus*, *Pezicula*, *Pseudosydowia*, *Pseudomicrostroma*, and *Zalaria* (Figure 2.8). Some of these isolates may represent new Australian records of known species as well as putative novel taxa (Table 2.3).

In this study molecular approaches were used together with morphological and physiological methods for identifying and classifying phylloplane yeast isolates from the leaf surfaces of *B. integrifolia*. Conventional methods that rely on only morphological and physiological characters are no longer sufficient for species classification. The results presented in this thesis show that

some of the yeast isolates shared morphological and physiological characters, although they represented different taxa based on their DNA profiles.

In the last decade, many yeasts have been reclassified by multilocus sequence analyses of the ITS rDNA, the small subunit (SSU) rDNA, RNA polymerase II (RPB1 and RPB2), translation elongation factor 1-α (TEF1) and cytochrome b (CYTB) (Wang et al., 2015a). ITS sequences are particularly useful for identifying most fungal species (Schoch et al., 2012). This region includes the gene for 5.8S rRNA, and the ITS1 and ITS 2 regions. The ITS region is now accepted by the mycology community as the DNA barcode for most of fungal species (Xu & Adamowicz, 2016; Vu et al., 2019). The ITS region was amplified for most isolates from *B. integrifolia*, with the exception of two isolates of *Sporidiobolus*. Although the ITS region is the standard barcode region for fungi, it may not separate closely related species. In this case ITS can be used in combination with other DNA barcode regions (Liu et al., 2017; Hoang et al., 2019; Vu et al., 2019).

Species of *Vishniacozyma* are widespread around the world, having been found in association with many different hosts. In the last decade, several species of *Vishniacozyma* have been discovered and some transferred from other genera based on their DNA profiles (Table 3.1, Chapter 3).

The *Vishniacozyma* isolates from the phylloplane of *B. integrifolia* represented a single, putative novel species. This *Vishniacozyma* sp. was found at several locations in south-eastern Queensland. Further surveys for yeasts in Australia are needed to determine whether the *Vishniacozyma* sp. found on *B. integrifolia* is restricted to this host plant.

Two isolates of *Hannaella kunmingensis*, and three of *H. pagnoccae* were isolated and identified from the phylloplane of *B. integrifolia* in south-eastern Queensland. Further, three putative novel species of *Hannaella* (BRIP 71047, 71270 and 71278) were also isolated. *Hannaella* is mostly comprised of species that inhabit plant surfaces. Based on DNA profiles, several species of *Hannaella* have been discovered and some transferred from other genera (Chapter 4). For instance, *Hannaella luteola* (Wang & Bai, 2008) was first isolated from the air in Japan by Skinner (1950). *Hannaella sinensis* (Wang & Bai, 2008) was isolated from wheat in China by (Li, 1982). *Hannaella oryzae* (Wang & Bai, 2008) was isolated from the leaf and stem of corn in Japan by Nakase & Suzuki (1985). *Hannaella surugaensis* (Wang & Bai, 2008) was first isolated from the

sea in Japan and (Nagahama et al., 2003). *Hannaella taiwanensis* (Kachalkin et al., 2019) was isolated from the surface of leaf in Taiwan by Huang et al. (2011). *Hannaella zeae* (Wang & Bai, 2008) was first isolated from corn in Austria (Molnár & Prillinger, 2006). *Hannaella coprosmae* (Wang & Bai, 2008) was isolated from a dead leaf and fruit in New Zealand (Hamamoto & Nakase, 1996).

Since 2014, five more species of *Hannaella* have been discovered from different countries. For instance *H. pagnoccae* was isolated from plant and soil in Brazil by Landell et al. (2014), *H. phetchabunensis* and *H. siamensis* were isolated from leaf surfaces of rice and corn in Thailand and Hungary by Kaewwichian et al. (2015), *H. phyllophila* was isolated from plant leaves in Thailand and Taiwan by Surussawadee et al. (2015), and *H. dianchiensis* was isolated from lake water in China by Han et al. (2017).

All of the eight Australian isolates of *Hannaella* clustered in a monophyletic clade with other described *Hannaella* spp. These *Hannaella* isolated from the leaf surface were collected from Esk and Highfields in south-eastern Queensland. The phylogenetic analysis revealed that five of the Australian *Hannaella* isolates were identified specifically three of isolates were represent *Hannaella pagnoccae*, two of isolates were represent *H. kunmingensis*, and the remainder of three isolates represent putative novel species (Figure 4.2, Chapter 4)

In this study, two isolates of *Bannoa* that represent putative novel species (Figure 5.2, Chapter 5) were isolated from *B. integrifolia* in south-eastern Queensland. In recent years several species of *Bannoa* have been revealed and transferred from other genera based on their DNA profiles (Table 5.1, Chapter 5). For instance, *Bannoa bischofiae* (Wang et al., 2015a) was first isolated from the dead leaves of bishop wood in Japan (Hamamoto et al., 2002). *Bannoa hahajimensis* was isolated from the dead leaves of *Bryophyllum pinnatum* in Japan by Hamamoto et al. (2002). *Bannoa ogasawarensis* (Wang et al., 2015a) was isolated from the dead leaves of *Schima mertensiana* in Japan (Hamamoto et al., 2002). *Bannoa syzygii* (Wang et al., 2015a) was first isolated from the dead leaves of *Syzygium buxifolium* in Japan by Hamamoto et al. (2002). *Bannoa guamensis, B. rosea, and B. tropicalis* were isolated from the diseased and healthy leaves surface of the Euphorbiaceae, Asteraceae, Poaceae in South America (Guam and Guyana islands) by Parra & Aime (2019).

This study reports the first records of the three genera, *Bannoa, Hannaella* and *Vishniacozyma* in Australia. Further, five putative novel species in these genera, *Bannoa* (1)*, Hannaella* (3) and *Vishniacozyma* (1) were isolated from the phylloplane of *B. integrifolia*. *Vishniacozyma* sp. was widespread in south-eastern Queensland. The three putative novel *Hannaella* spp. were only found at Esk and Highfields. The *Bannoa* sp. was found at Noosa Heads National Park.

This study showed that most phylloplane yeasts on *B. integrifolia* isolated in south-eastern Queensland were either putative novel species or first records for Australia, e.g., *Hannaella kunmingensis* and *H. pagnoccae* (Table 6.1, 6.2 and 6.3). The results of the ML phylogenetic analysis showed that *Vishniacozyma* isolates from Australia belong in a separate clade sister to *V. taibaiensis*, which are together sister to all other *Vishniacozyma* species. This was not the case for Australian isolates of *Hannaella* that were distributed throughout the *Hannaella* clade in the ML phylogenetic analysis. In addition, a new Australian species of *Bannoa* was sister to overseas *Bannoa* species. Further sampling is needed to determine whether these yeasts are endemic to Australia.

This thesis has provided an insight into the diversity of phylloplane yeasts of *B. integrifolia* in south-eastern Queensland, Australia. It has shown that leaf surface of *B. integrifolia* harbours a substantial diversity of taxa of ascomycetes and basidiomycetes, with many unclassified species.

As a result of the work reported in this thesis, suggestions for future research have been identified as follows.

- Isolate phylloplane yeasts on *B. integrifolia* from around Australia to determine whether there are further undiscovered yeasts.
- Isolate phylloplane yeasts from other Australian plants such as macadamia (*Macadamia integrifolia*), eucalyptus (*Eucalyptus argophloia*), acacia (*Acacia pycnantha*) and casuarina (*Casuarina equisetifolia*).
- Investigate the potential of Australia phylloplane yeasts as biological control agents for other pathogenic fungi on economically important plants.

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