University of Southern Queensland

THE TAXONOMY, ECOLOGY AND BIOACTIVE PROPERTIES OF SOUTH-EAST QUEENSLAND RUSSULACEAE

A Thesis submitted by

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Abstract

The Russulaceae are a cosmopolitan family of basidiomycetous fungi. They have great ecological importance as ectomycorrhizas, forming mutualistic associations with a large number of plant species. The family also includes edible species, and species possessing bioactive secondary metabolites.

Southeast Queensland (SEQ), Australia has a diverse geography, supporting a significant biodiversity, which includes Russulaceae fungi. However, few formal studies have been conducted on this group in the region, leaving many taxa undescribed or poorly documented, a situation that needs to be addressed.

Morphologically, members of the two main genera, *Russula* and *Lactarius*, are readily identifiable in the field, however the determination of species is difficult. Microscopic examination may improve species diagnosis, but the results are often inconclusive or incorrect. Therefore molecular taxonomy was utilised to collect additional information from specimens collected from 23 sites within SEQ so as to identify known and unknown species.

From the 149 specimens genetically studied, 94 sequences of the basidiomycete-ITS region were obtained. 15 of these sequences were considered to be identical to sequences held in GenBank, the majority of these being Australian. Another two were from an order unrelated to this study. However, it was revealed the remaining 83 sequences were potentially members of up to 33 new species. Four new taxa which contained the highest number of collected specimens, were subsequently described using both molecular and morphological taxonomic approaches.

Russulaceae species are known to associate with Australian obligate mycoheterotrophic orchids. To further document the Russulaceae of SEQ, the fungi that associate with roots of the orchid, *Dipodium roseum* were investigated for the first time. Both Sanger and Next Generation sequencing of extracted and amplified DNA showed the presence of both *Russula* and *Lactarius* spp, in roots of plants. This is the first time *Lactarius* spp. have been identified as mycobionts in an Australian orchid and this may have conservation implications for threatened species within the *Dipodium* genus.

As the development of antimicrobial resistance and rates of cancer continue to be of societal concern, there is a need to continue the search for new or more effective drugs. Bioactive metabolites produced in nature are one of the most extensive sources of these compounds, and it has been shown species of the Russulaceae produce antioxidant, anticancer and antimicrobial compounds. This study provided the opportunity for investigation of the bioactive properties of some of the new Russulaceae specimens obtained.

Although extracts of the *Russula* fungi obtained here had little antimicrobial activity there was some impact on the growth of the two cancer cell lines studied which warrants further study.

This study has thus shown that the SEQ region in Australia contains a substantial number of undescribed Russulaceae fungi. Further investigation of these taxa may reveal additional novel aspects of Australian ecosystem functioning as well as potentially provide useful bioactive compounds for humankind.

Certification of Thesis

This Thesis is entirely the work of Morwenna Boddington except where otherwise acknowledged. The work is original and has not previously been submitted for any other award, except where acknowledged.

Principal Supervisor: Assoc. Prof. John Dearnaley

Associate Supervisor: Patrick Leonard

Student and supervisors signatures of endorsement are held at the University.

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This project details work I have undertaken on a part-time basis since 2012. It has been an interesting and generally enjoyable experience, but would not have occurred without the support and encouragement received from numerous quarters over this time.

First of all, I wish to thank my principal supervisor, Dr John Dearnaley, primarily for the opportunity to undertake this "little" project. In addition, his experience, advice, encouragement and support have been invaluable in my pursuit of this research and in the preparation of this document.

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Members of my PhD Candidature panel deserve some recognition - it was a somewhat daunting process, but their suggestions and advice certainly contributed to a much stronger research project. As colleagues they continued their support and interest, which at times was most beneficial!

I need to thank and acknowledge Dr Karren Beattie. As a chemist she was invaluable as she knew everything about Preparative HPLC! She extracted and fractionated the samples used in chapter 5 with my assistance, and taught me a lot about the process.

I sincerely thank property owners Del Wham and Ian Milinovitch (Tully Park), Judy Lethbridge (Lodbroke), Robert Gratwick and Catherine Burton (Hell Hole Creek) and Paul Stevens and Diane Guthrie for their interest, support and access to their properties for the collection of many of the specimens studied.

Members of the QMS were, and remain, incredibly supportive of this work, and I am sure they will encourage its continuation. Their collections of Russulaceae specimens contributed greatly to this project, and I am indebted to them for that.

My team mates in the Technical area of the Faculty of HES not only provided genuine interest and support, but also massive amounts of support in making sure everything at work continued to run as smoothly as possible - a large enough task as it is! Particular thanks go to Lynn Rose, Tracey Tyers and Sharon Seaniger, who take care of a lot of "the little" yet important stuff, and Andrea Schilf whom provided a useful sounding board for the more technical discussions and told me I could eat all the chocolate I wanted.

I reserve particular thanks for my friend, colleague and fellow minion, Amanda Wakefield.

We have been through a lot together over the last several years, and I am much stronger for it.

I would also take the opportunity to mention Laurie and the regulars of the Sunday morning 80m net, Ivan, Bill and Mick. This is a lovely group of amateur radio operators with whom I have occasionally chatted. Their genuine interest in my research has been humbling and encouraging, and I thank them for that. Ivan, in particular, was looking forward to calling me "Doctor", but sadly, he passed away just months before this submission.

Finally, I wish to thank my family and friends for their support and inspiration. My mother provided additional proofreading of my thesis; my sister provided faith in my abilities; my father provided inspiration and shared a love of photography. My husband James not only provided a great deal of technical assistance, but has been a constant provider of encouragement, comfort and chocolate. He also developed a very keen eye in finding specimens! I owe much of this result to him.

I should also thank the fungi and Mother Nature for providing me with many excellent seasons for the collection of fungal specimens. Fungi are amazing organisms, but so easily and so often overlooked. We really do need to take the time to stop and see the fungi. I wouldn't necessarily smell them.

Thank you one and all for believing in me.

While the investigation in to the anti-cancer properties of these fungi comprises only a small proportion of this work, I would like to "dedicate" it to my friend Genevive Burney and my uncle David Smith. The lives of both of these incredible people were cut short because of this insidious disease, but they also did what they could to help others in their position.

Gen was so close to finalising her PhD, using bioinformatics in the study of ovarian cancer. She was young, intelligent, the mother of 3 beautiful children and supportive of other post-graduate students in her area. But the cancer was unrelenting and despite her best efforts, it took her amazing life.

David was twice a survivor of cancer, but felt badly treated, both mentally and physically, by the medical system. He dedicated his life to learning as much as he could about the disease and how to fight it, helping as many people as he could along the way. He was a passionate fighter, but despite his zest for a healthy life, the damage was already done.

I miss them both. This is only a small gesture, but I dedicate this thesis to them...

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Chapter 1

Introduction

The Kingdom Fungi contains eukaryotic organisms characterised by the presence of chitin in cell walls, storage of glycogen and lipids as food reserves and a non-photosynthetic, absorptive feeding method. Originally placed in the Plant Kingdom, they actually share a common ancestral group, the Opisthokonts, with the Animalia, Metazoa and Choanozoa (Shalchian-Tabrizi et al. 2008). The two largest fungal groups, the Ascomycota and Basidiomycota, are combined into the Subkingdom Dikarya, and represent approximately 95% of all described fungi (Stajich et al. 2009).

The smaller Division, Basidiomycota, is further divided into three subphyla (and 2 classes *incertae sedis*) which could be broadly described as the Rusts, Smuts and Agarics. Members of the latter subphylum, Agaricomycotina, include easily recognisable examples such as mushrooms, puffballs and stinkhorns, and currently represents approximately 70% of the Division's species. These are separated across 3 Classes which are further divided into 19 formally-named Orders (Hibbett et al. 2007, Hibbett 2007). The Order Russulales contains over 3000 species across 13 Families and another group of 42 species currently not assigned to a family. Approximately two thirds of the species reside in the largest family, the Russulaceae (Kirk 2019).

The Russulaceae are a cosmopolitan family of basidiomycetous fungi with strong representation in Australia. Members of this group are common in temperate woodlands where they form important ectomycorrhizal associations with plant species (Bougoure & Dearnaley 2005, Dearnaley & Le Brocque 2006, Miller et al. 2006, Lebel & Tonkin 2007, Li et al. 2013a). The family contains four genera including *Russula, Lactarius, Multifurca*

and *Lactifluus* (Buyck et al. 2008, Lebel et al. 2013). The fruiting bodies of Russulaceae come in a number of forms including agaricoid and pleurotoid within the epigeous species, while hypogeous forms include secotioid and gasteroid (Miller et al. 2006, Buyck et al. 2008). These forms are not confined to any particular genus (Buyck et al. 2008).

Found during the cooler months, the epigeous members of the family are readily identified in the field due to their bright red-purple colours, lack of annulus (stem ring) and volva (stem cup) and their brittle structure. The genus *Lactarius* can be further identified by the production of latex from the gills when damaged (Figure 1.1). However, identifying a specimen to species in the field is difficult, relying on many subtle and subjective characteristics and subsequent extensive microscopic examination. At times, even this does not produce sufficient data to identify a specimen to species. It has therefore become necessary to include molecular examination to assist in the definition of each Russulaceae species.

(a) *Russula* (b) *Lactarius*

Figure 1.1: Examples of *Russula* and *Lactarius* species. Scale bars = 10mm

Molecular analysis of highly conserved regions of DNA provides a more accurate and standardised means of fungal identification than morphological characteristics alone. It has been found that the ITS region of the nuclear ribosomal DNA, located between the 18S small subunit and 28S large subunit, is a suitable DNA barcode marker for fungi (Nugent & Saville 2004, Manassila et al. 2005, Bellemain et al. 2010, Avis 2012, Izumitsu et al. 2012, Osmundson et al. 2012) and is considered to provide better species discrimination than other rDNA regions (Schoch et al. 2012). This area incorporates the non-coding ITS1, 5.8S and ITS2 regions (Osmundson et al. 2012), which are variable in sequence composition and length between species (Kennedy & Clipson 2003), highly conserved and have a high-copy number (Kennedy & Clipson 2003, Bellemain et al. 2010). Due

to a high frequency of occurrence, only a small amount of DNA is required for ultimate successful sequencing (Bellemain et al. 2010).

Historically, the morphologically distinctive corticioid and pleurotioid forms of the Russulaceae belonged in the Corticiaceae or Gloeocystidiellaceae families, whilst the sequestrate Russulaceae belonged to the genera *Macowanites, Gymnomyces, Martellia, Cystangium, Arcangeliella* and *Zelleromyces*. As a result of molecular analysis, Larsson (2007) found the Corticiaceae could be distributed across all major families of the Agaricales; the genera *Gymnomyces, Martellia* (Calonge & Martin 2000, Miller et al. 2001) *Cystangium* (Miller et al. 2001) and *Macowanites* (Miller et al. 2001, Lebel & Tonkin 2007) could be included under *Russula*; and *Zelleromyces* (Calonge & Martin 2000, Miller et al. 2001) and *Arcangeliella* (Miller et al. 2001) could be included in the genus *Lactarius*. Molecular analysis was also used to identify a new monophyletic genus, *Multifurca* into which *Lactarius furcatus* and the *Russula* subsection *Ochricompactae* were moved (except for one species) (Buyck et al. 2008). Subsequently, phylogenetic analysis revealed the genus *Lactarius* contained two well defined clades. Ultimately, the smaller of the two was moved to the genus *Lactifluus* (Verbeken & Nuytinck 2010).

These significant alterations to the scientific classification of these fungi came as a result of examining the ITS and large subunit (LSU) regions of the fungal DNA. The polymerase chain reaction (PCR) was used to amplify a specific region of DNA extracted from fungal specimens with the use of appropriately designed primer pairs (Kennedy & Clipson 2003). As mentioned, the ITS region is particularly important, as it is supported as a primary fungal barcode marker (Schoch et al. 2012). A number of primers have been developed to amplify this region. Early primers included ITS1 and ITS4 which could amplify the ITS region of most fungi (White et al. 1990) but also some plant material. Subsequently, primers ITS1-F and ITS4-B were developed in order to preferentially amplify the ITS region of basidiomycetes (Gardes & Bruns 1993).

Accurate identification of species has a number of uses. For example, as an ectomycorrhizal family, knowledge regarding the interaction of Russulaceae species with neighbouring plant species may be important for conservation efforts; the family includes a small number of edible species, though there may be more; the identification of novel bioactives is becoming increasingly important in the discovery of antibiotics; and species which can contribute to the remediation of environmental damage need to be identified. It has also been estimated that barely 8% of all fungi have been named (Hawksworth & Lücking 2017). Research into fungal diversity is a priority, hence this study of the taxonomy, ecol-

ogy and bioactive properties of SEQ Russulaceae. Ultimately, there are four primary aims of this research:

1. Examine the diversity of Russulaceae species in SEQ using molecular/genetic analysis
2. Use molecular/genetic analysis and morphological methods to describe any new species found
3. Examine the potential relationship between species of Russulaceae and the orchid species *Dipodium roseum*
4. Evaluate the Russulaceae species found as a potential source of biologically-active compounds

Chapter 2

Genetic Diversity of SEQ Russulaceae

2.1 Introduction

The Atlas of Living Australia online database lists approximately 63 species of Russulaceae currently known in Australia. The largest genus within the family, *Russula*, currently represents 41 of these Australian species (ALA 2018). Cleland (1976) morphologically described 15 species from South Australia (Table 2.1) and Grgurinovic (1997) updated or described 20 mostly epigeous species predominantly located in South Australia and New South Wales (Table 2.2). However, only 23 *Russula* spp. have so far been genetically described (Table 2.3) (Lebel & Tonkin 2007).

Of the 4365 Russulaceae specimens held in Australian herbariums, just 14% were found in Queensland, with the majority of the remaining specimens collected in the southern states. The Queensland herbarium is small, currently holding 190 specimens of Russulaceae, 50 of which have been identified to species (The Council of Heads of Australasian Herbaria 2013), and is not a reflection of the richness of Russulaceae fungi observed in south-east Queensland woodlands (Leonard unpublished.) According to Queensland Mycological Society (QMS) records, to date 41 species found in Queensland have been named while a further 10 have been identified as new species but not formally recognised. Another 48 groups of specimens have been recorded and characterised, but have only been assigned a species number (Table 2.5) (QMS 2011).

Table 2.1: Russulaceae species of South Australia described by Cleland (1976)

Russula adusta	*Russula flocktonae*	*Russula purpureo-flava*
Russula cheelii	*Russula fragilis*	*Russula viridis*
Russula cyanoxantha	*Russula mariae*	*Russula xerampelina*
Russula delica	*Russula pectinatoides*	*Lactarius clarkei*
Russula erumpens	*Russula persanguinea*	*Lactarius serifluus*

Table 2.2: Species of Australian Russulaceae described by Grgurinovic (1997)

Russula cheelii	*Russula marangania*	*Cystangium balpineum*
Russula erumpens	*Russula neerimea*	*Lactarius clarkeae*
Russula flocktonae	*Russula persanguinea*	*Lactarius clelandii*
Russula ingwa	*Russula purpureoflava*	*Lactarius mea*
Russula iterika	*Russula viridis*	*Lactarius necator*
Russula kalimna	*Russula wollumbina*	*Lactarius subclarkeae*
Russula lenkunya		*Lactarius wirrabara*

Table 2.3: Australian Russulaceae as listed in GenBank

Species	Accession Number
Cystangium seminudum[1]	EU019947
C. sessile[1]	EU019948
Lactarius eucalypti[1]	EU019923
Lactifluus cf. *wirrabara*[2,4]	GU258289 to GU258293, GU258303, GU258305 to GU258307, GU258309, JF731000, JF731001
L. clarkeae[1,3]	HQ318282 to HQ318284, EU019924
L. genevievae[2]	GU258294
L. leonardii[2]	GU258295, GU258304
L. sepiaceus[1]	EU019926
Macowanites luteiroseus[1]	EU019946
Multifurca sp. (australis)[6]	MH063871
M. stenophylla[5,6]	JX266627 to JX266632, MH063864 to MH063868
Russula aff. *adusta*[1]	EU019918
R. aff. *compacta*[5]	JX266625
R. aff. *pilosella*[1]	EU019932
R. albobrunnea[1]	EU019933, EU019939
R. brunneonigra[1]	EU019945
R. cheelii[5]	JX266623
R. clelandii[7]	DQ328136
R. flocktonae[5]	JX266621, JX266622
R. galbana[1]	EU019936
R. ingwa[1,5]	EU019919, JX266624
R. iterika[1]	EU019929

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Species	Accession Number
R. kalimna[1]	EU019927
R. marangania[1]	EU019930
R. neerimea[1]	EU019915
R. persanguinea[1]	EU019916
R. pilosella[1]	EU019941
R. pumicoidea[1]	EU019931
R. purpureoflava[1,5]	EU019914, EU019917, JX266626
R. reddellii[1]	EU019944
R. rostraticystidia[1]	EU019938
R. sinuata[1]	EU019943
R. variispora[1]	EU019934
R. wollumbina[1]	EU019921

[1]Lebel & Tonkin (2007)

[2]Stubbe et al. (2010)

[3]de Putte et al. (2010)

[4]Stubbe et al. (2011)

[5]Lebel et al. (2013)

[6]Wang et al. (2018)

[7] Francis A.A. unpublished (2005)

Table 2.4: List of Russulaceae species recorded in Australia (ALA 2018)

Russula adusta	*Russula mariae*	*Gastrolactarius hepaticus*
Russula albidoflava	*Russula multicolor*	*Lactarius clarkeae* *
Russula albobrunnea *	*Russula neerimea* *	*Lactarius clelandii*
Russula albonigra	*Russula nigricans* *	*Lactarius deliciosus*
Russula australiensis	*Russula pectinata*	*Lactarius eucalypti* *
Russula brunneonigra	*Russula pectinatoides* *	*Lactarius helvus*
Russula cheelii *	*Russula persanguinea* *	*Lactarius mea* *
Russula clelandii *	*Russula pilosella* *	*Lactarius necator*
Russula compacta	*Russula pumicoidea*	*Lactarius piperatus* *
Russula cyanoxantha *	*Russula purpureoflava**	*Lactarius plumbeus*
Russula delica	*Russula reddellii* *	*Lactarius pubescens*
Russula emetica	*Russula rosacea*	*Lactarius sepiaceus* *
Russula erumpens *	*Russula rostraticystidia**	*Lactarius stenophyllus*
Russula foetens *	*Russula rubra* *	*Lactarius subclarkeae* *
Russula galbana *	*Russula sinuata*	*Lactarius subdulcis* *
Russula ingwa *	*Russula sororia*	*Lactarius torminosus*
Russula iterika *	*Russula variispora* *	*Lactarius wirrabara* *
Russula kalimna *	*Russula viridis* *	*Zelleromyces malaiensis* *
Russula lenkunya *	*Russula wollumbina* *	*Lactifluus clarkeae*
Russula luteirosea	*Russula xerampelina* *	*Lactifluus leonardii*
Russula marangania *	*Arcangeliella daucina*	*Multifurca furcata* *

* Recorded in Queensland

Table 2.5: List of Russulaceae species currently known in QLD (QMS 2011)

Russula albobrunnea	*Lactarius deliciosus*	*Russula* aff. *griseoviolacea*
Russula cheelii	*Lactarius eucalypti*	*Russula* aff. *luteotacta*
Russula clelandii (Purple form)	*Lactarius leonardii*	*Russula* aff. *pseudointegra*
Russula clelandii (Red form)	*Lactarius sepiaceus*	*Russula* aff. *vesca*
Russula erumpens	*Lactarius subclarkeae*	*Lactarius* aff. *lignyotus*
Russula galbana	*Lactarius wirrabara*	*Lactarius* aff. *ochrogalactus*
Russula ingwa	*Lactifluus aurantiorubra*	*Lactarius* aff. *ruginosus*
Russula iterika	*Lactifluus clarkeae*	*Russula* Browning WA
Russula kalimna	*Lactifluus mea*	*Lactarius* 'austropiperatus'
Russula lenkunya	*Lactifluus queenslandicus*	*Lactarius* 'lactifuscus'
Russula marangania	*Lactifluus subclarkeae*	*Lactarius* 'lactiglaucus'
Russula minipes	*Multifurca eucalypti*	*Lactarius* 'luteocremeus'
Russula neerimea	*Russula* aff. *adusta*	*Lactarius* 'pallidoaurantiaca'
Russula persanguinea	*Russula* aff. *albonigra*	*Lactarius* 'tasmanicus'
Russula purpureoflava	*Russula* aff. *cyanoxantha*	*Lactifluus* Bald Rock
Russula viridis	*Russula* aff. *densifolia*	*Lactifluus* 'corvinidum'
Russula wollumbina	*Russula* aff. *foetens*	*Lactifluus* 'petruscalvus'

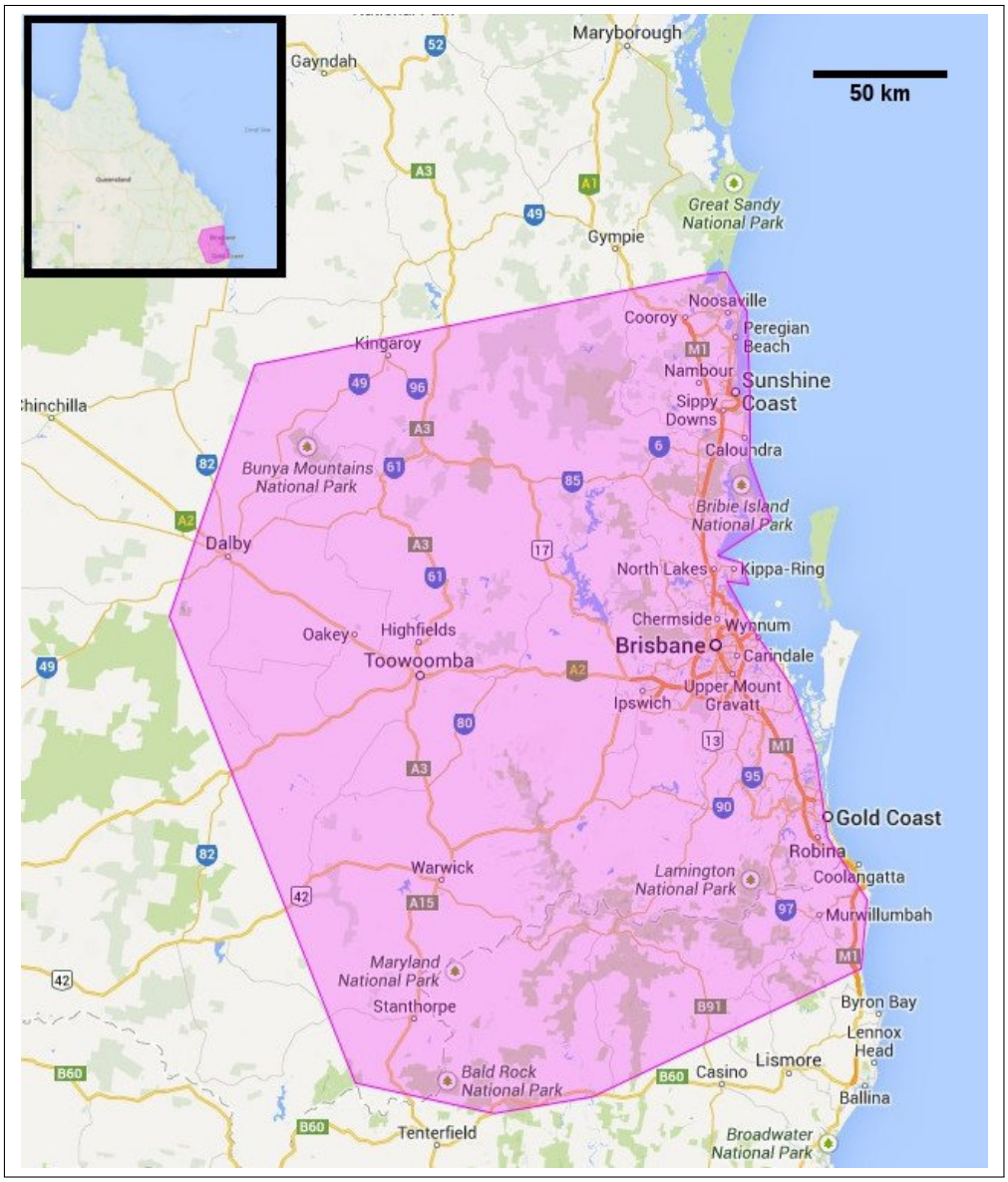

Figure 2.1: Map of the study area in SEQ

SEQ (Figure 2.1) has a diverse geography ranging from coastal sands and swamps, alluvial plains, to hills and basalt ranges. It is a region of diverse ecosystems, including mangroves, open heaths, dry to wet open forests and vine forests (Neldner et al. 2017). From the coast, the land gradually climbs westward with the highest point being Mount Superbus at 1372m. The climate ranges from temperate to sub-tropical (Department of Environment and Science 2018). Despite hosting almost three-quarters of the state's population, the area maintains a significant biodiversity (The State of Queensland, Department of Infrastructure, Local Government and Planning 2017).

As mentioned, SEQ is rich in Russulaceae fungi (Leonard unpublished) but few formal studies have been conducted on this group in the region with the result that many taxa are undescribed or poorly documented. Given this background, it would suggest there is much to learn about the Russulaceae of SEQ. Therefore, the aims of this initial study include to:

- collect Russulaceae specimens from multiple locations in SEQ over 5 years
- collect sequencing data of these taxa through the amplification of the ITS region
- compare this data with that of known Australian species (Table 2.3)

2.2 Materials and Methods

2.2.1 Collection sites

Five sites within SEQ were predominantly used for collection of fresh samples (Table 2.6). Common to each site were areas of dry eucalypt forest, populated with native trees and shrubs including genera known to associate with Russulaceae fungi. Ground cover ranged from sparse native grasses to thick patches of exotic kikuyu (*Pennisetum clandestinum*). Members of the QMS provided dried specimens from an additional 29 locations. Details regarding the sites are listed in Table 2.6. The number on the left hand side refers to the numbers on the maps in Figures 2.2 and 2.3. An additional 4 specimens from SEQ were provided, however specific location details were omitted. The QMS holds a permit for the collection of fungal material from all of the national parks listed.

2.2.2 Sample collection

Sites were accessed following periods of rain and cool temperatures in order to locate fungal specimens. When located, a specimen number was assigned and written on a tag, photographs were taken of the fruiting body *in situ* and location and habitat details recorded. A blunt knife or small trowel was used to carefully lever the fruiting body out of the ground which was then placed into a paper bag or plastic container for transport.

Subsequently, basic morphological characteristics of each specimen were noted and additional photographs were taken. A small amount of tissue (approximately 0.5cm^2) was transferred to a tube and stored at -20^{o}C for future DNA analysis. A spore sample was collected by either removing the cap and placing it gill-side down, or lying the entire specimen down on an open petri dish. A toothpick was used to transfer a small number of the spores into a drop of Meltzer's iodine placed on a glass microscope slide which was examined at 400x magnification and the reaction recorded. Meltzer's reagent had been previously prepared by dissolving 20g chloral hydrate in 22ml distilled water, after which 0.5g iodine and 1.5g potassium iodide were added.

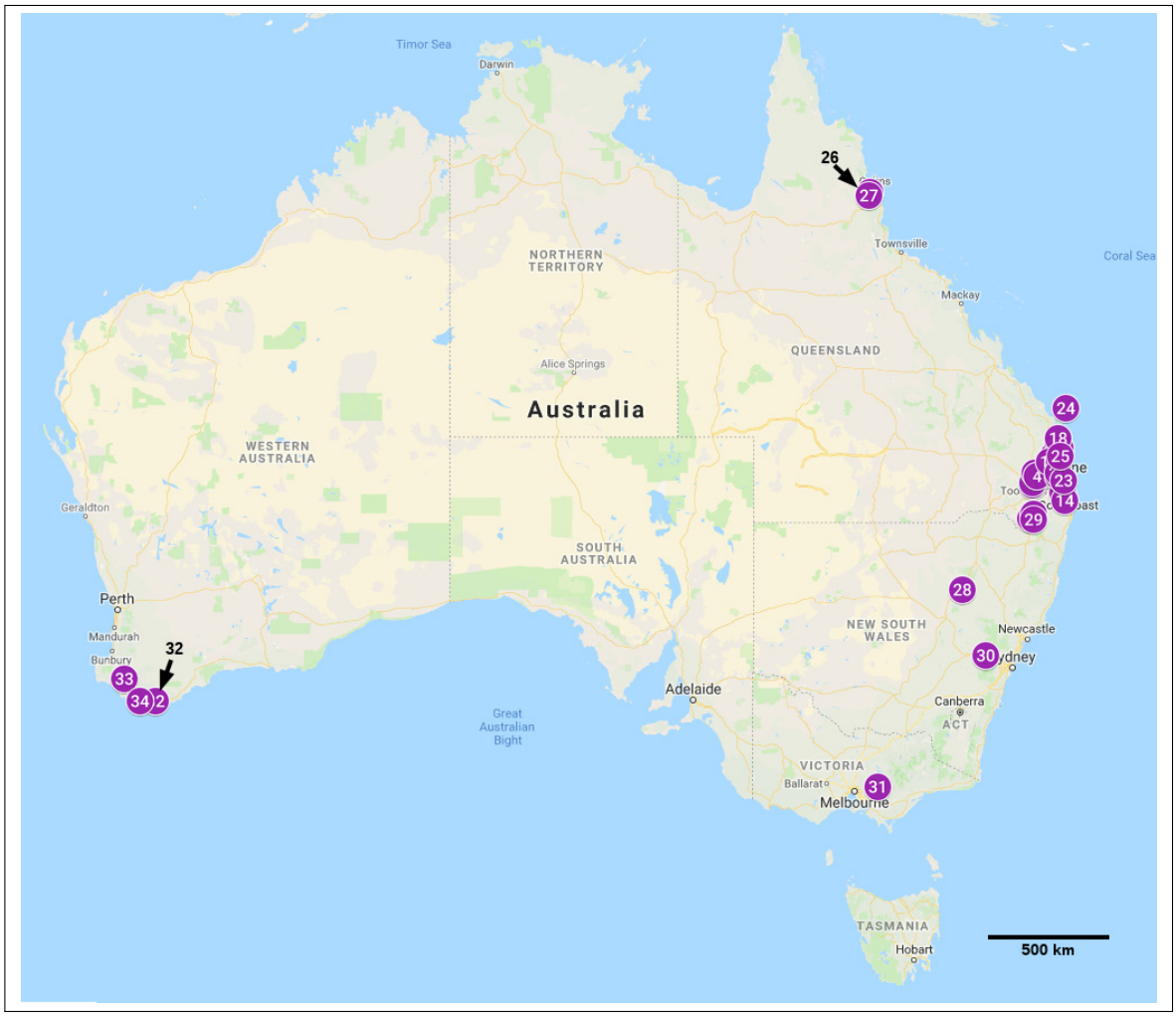

Figure 2.2: Australian locations of collection sites. The numbers refer to the site names as listed in Table 2.6.

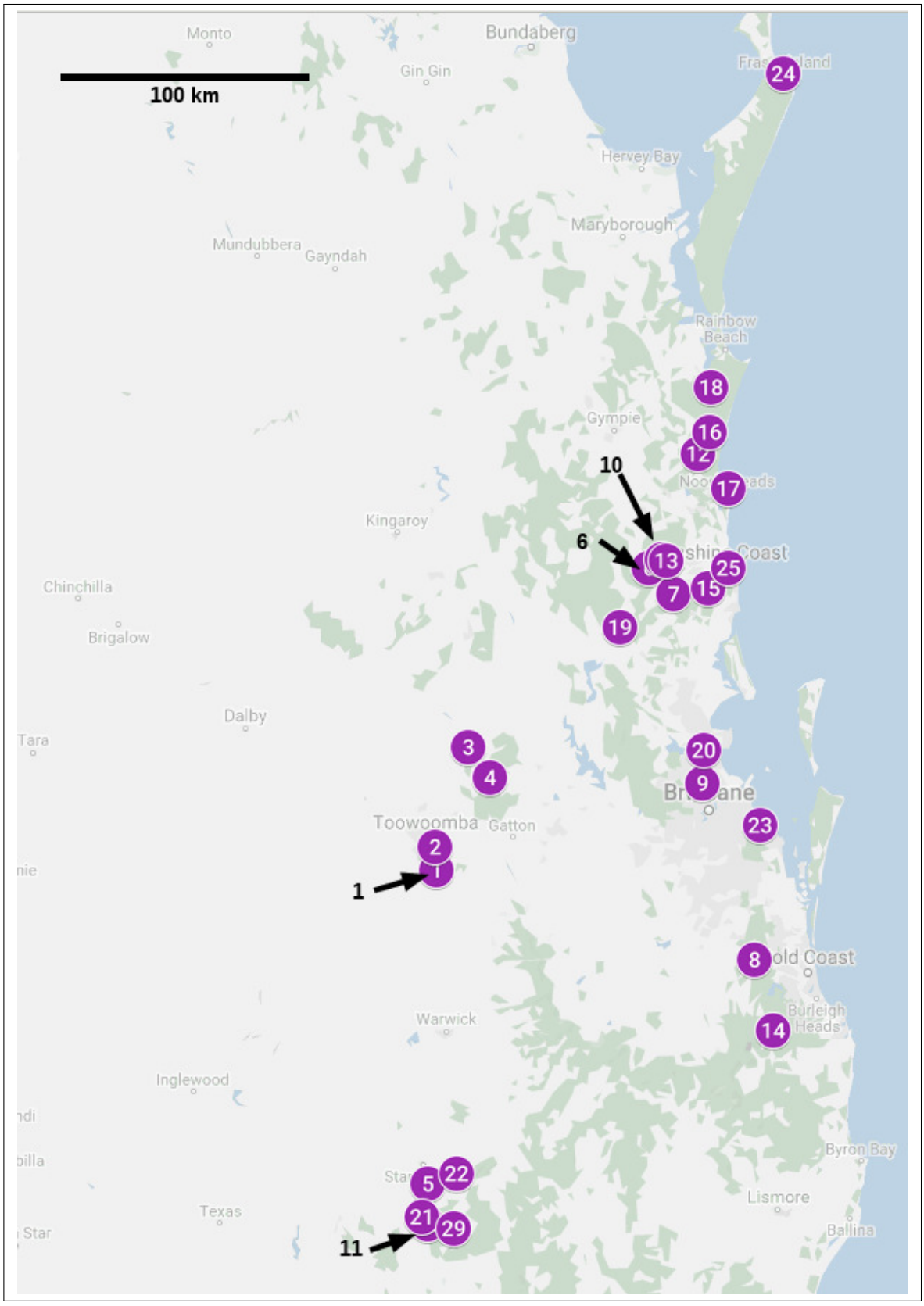

Figure 2.3: Locations of collection sites within SEQ. The numbers refer to the site names as listed in Table 2.6.

Table 2.6: Collection sites of samples in study.

No. on map.	Site	General Habitat	Collections
1	Lodbroke	Dry eucalypt forest	141*
2	Toowoomba	Parkland	10*
3	Crows Nest	Dry eucalypt forest	21* + 2
4	Ravensbourne	Rainforest	7* + 6
5	Tully Park	Dry eucalypt forest	26* + 1
6	Obi Obi	Rainforest	7
7	Dilkusha, Maleny	Rainforest	4
8	Mt Tamborine	Rainforest	1
9	Chermside Hills Reserve and Brisbane	Dry eucalypt forest	4
10	Linda Garrett Reserve	Wet eucalypt forest	15
11	Mt Norman Track	Dry eucalypt forest	3
12	Cootharaba	Wet eucalypt forest	3
13	Mapleton	Wet eucalypt forest	4
14	Springbrook	Wet eucalypt forest	2
15	Maroochy BBG	Wet eucalypt forest	6
16	Harry's Hut	Wallum heathland	1
17	Noosa NP	Wallum heathland	1
18	Cooloola	Dry eucalypt forest, wallum heathland	4
19	Bellthorpe NP	Wet eucalypt forest	3
20	John Oxley Reserve	Areas of dry and wet eucalypt forest	1
21	Girraween NP	Dry eucalypt forest	6

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No. on map.	Site	General Habitat	Collections
22	Wilson's Downfall	Wet eucalypt forest	13
23	Alexandra Hills	Dry eucalypt forest	5
24	Fraser Island	Dry eucalypt forest, wallum heathland	3
25	Maroochydore	Dry eucalypt forest	4
26	Davies Creek and Dinden State Forest	Areas of dry and wet eucalypt forest	8
27	Tinaroo Forest Reserve	Areas of dry and wet eucalypt forest	2
28	Coonabarabran, NSW	Wet eucalypt forest	4
29	Bald Rock NP, NSW	Dry eucalypt forest	10
30	Lithgow, NSW	Dry eucalypt forest	1
31	Yarra State Forest, VIC	Wet eucalypt forest	1
32	Denmark, WA	Dry eucalypt forest	2
33	Manjimup, WA	Wet eucalypt forest	2
34	Walpole, WA	Wet eucalypt forest	2
An additional 4 specimens from SEQ were provided, without a location specified			

* collected by author

Total = 340

If dehydration was not immediately possible, each specimen was stored in a paper bag at 4^{o}C for up to two days, or at -20^{o}C for longer term. Two methods were used for specimen dehydration:

Food dehydration A Sunbeam Food Dehydrator, Model DT5600 was used to dehydrate up to 25 fruiting bodies over 5 drying trays. Specimens and their tags were placed so that overlap of specimens was minimal. Heat setting 2 was selected, which had an approximate drying temperature of 55^{o}C, and the dehydrator allowed to run until the specimens had dried.

Freeze-drying Specimens were placed into freeze-dryer flasks and placed at -80^{o}C to freeze. The freeze drier was prepared, the flask attached, and the system allowed to run until the specimen had dried.

Both methods took up to 2 days to dry the specimens, depending on the size and fleshiness of the sample. A combination of specimen weight, texture and temperature (moisture conducts temperature whereas dried specimens do not) was used to determine when specimens had dried. After drying, specimens were placed into clip-seal bags along with a silica gel sachet, and kept at ambient temperature.

A number of forms were designed and utilised for recording of relevant specimen information. These can be found in Appendix A.

2.2.3 DNA extraction and PCR

Reagent preparation

Stock solutions of 5M sodium chloride (NaCl), 1M Tris-HCl pH 8.0 and 0.1M EDTA (sodium salt) were prepared by mixing the appropriate weight of salt in a portion of distilled water. The pH of the Tris and EDTA were adjusted to 8.0 using 1M hydrochloric acid or 1M sodium hydroxide. Once the salts had dissolved, the solutions were made up to the fnal volume and autoclaved to sterilise (15 minutes at 121^{o}C). Once cooled, the solutions were kept at ambient temperature. Working solutions prepared from the stocks were pH adjusted as required, autoclaved and stored as above.

10% (w/v) sodium dodecyl sulphate (SDS) was prepared by stirring 10g SDS into 90ml distilled water until dissolved. 0.5M sodium hydroxide (NaOH) was prepared by dissolving 0.2g NaOH in distilled water and making the volume up to 10ml. These solutions were not autoclaved.

0.8mg/ml bovine serum albumin (BSA) was prepared by dissolving 8mg BSA in 10ml water. The solution was filter sterilised into a sterile container through a 0.22μm syringe filter, and dispensed into 1ml aliquots for storage at -20^{o}C.

Lyophilised primers were obtained from GeneWorks (Thebarton, SA, Australia) and rehydrated with 10mM Tris-HCl pH 8.0 to a concentration of 50μM. Working concentrations

were prepared by diluting the stock 1:4 in the same buffer, so each primer was available as a 10μM solution. Concentrated stocks were stored at -80^{o}C and working solutions stored at -20^{o}C. Fresh working solutions were prepared every 6 months.

Trial fungal samples

For each initial experiment, material was provided from a single fruiting body of *Agaricus bisporus*. This ensured a plentiful supply of material with which to test. Once a procedure was determined not to be detrimental to the resultant DNA extract, the procedure was repeated using samples from three *Russula* specimens: MB28, MB31 and MB99. DNA of the latter specimen had previously been extracted and amplified successfully. The other two specimens were selected because of their numerous amplification failures. Unless otherwise specified, approximately 100mg of fungal tissue was used as the starting material.

Ascertaining extraction success

In order to ascertain the success of DNA extraction, spotting plates were prepared by pouring 15 to 20ml of 2% agarose in TAE (Tris-acetate-EDTA) buffer and RedSafe DNA dye into petri dishes and allowing these to set. Spots of 1μl volumes of DNA extract were placed on the surface and allowed to absorb before examination under UV. A photographic example of one spot plate is shown in Figure 2.4.

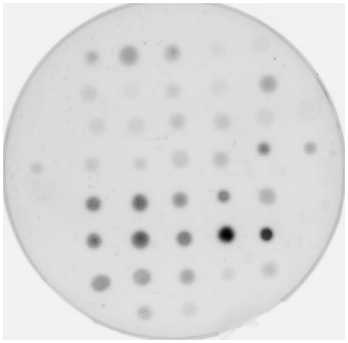

Figure 2.4: DNA visualisation spot plate. This example demonstrates how a spot plate can be used to confirm successful extraction of DNA from each sample. The intensity of the spot indicates the relative level of DNA.

DNA concentration determination

The rationale behind the measurements at each wavelength is outlined in Table 2.7. Concentration of the DNA in solution was calculated by multiplying the A260 value by the dilution factor, and multiplying by 50. At 260nm, an absorbance of 1.0 is equivalent to a double-stranded DNA concentration of 50μg/ml (0.15mM).

Table 2.7: Spectrophotometric measurements of DNA solutions (after Desjardins & Conklin (2010))

Measurement wavelength	Rationale
A230	Influenced by a number of potential PCR inhibitors such as polysaccharides, phenols and salts
A260	Maximum absorbance of nucleic acids
A280	Maximum absorbance of many proteins
A320	Measures turbidity of the sample which may influence other measurements
A260/A230	Provides an indication of contamination by potential PCR inhibitors. A solution containing a pure sample of nucleic acid is expected to be within the range of 1.8 - 2.2.
A260/A280	Provides an indication of contamination by proteins, but also phenols and chaotropic salts. A pure DNA solution in water is expected to have a ratio of 1.8.

Extraction and PCR Methods

As initial PCR attempts were unsuccessful, a number of protocols were trialled to improve DNA extraction or to enhance the PCR process.

Extraction methods trialled

1: Effect of pre-soaking, buffer choice and extraction temperature. The stock solutions were used to prepare a 3M sodium chloride solution, and a modified Edwards buffer (Edwards et al. 1991) containing 250mM Tris-HCl pH 8.0 (instead of 200 mM at pH 7.5), 25mM EDTA, 200mM NaCl and 0.5% SDS. Four samples were initially prepared, with each tube containing 100mg of *A. bisporus*; 100μl sterile water was added to two tubes and 100μl 3M NaCl added to the other two. One tube of each solution was placed at 4^{o}C and allowed to soak overnight, whilst the remaining two were soaked for 60 minutes at 65^{o}C. After soaking, the liquid was removed and replaced with 100μl of modified Edwards buffer containing 1μl of β-mercaptoethanol. DNA was extracted from these samples over 60 minutes at 65^{o}C.

An additional four samples were prepared for extraction without pre-soaking. One tube contained 100μl of modified Edwards buffer with 1μl of β-mercaptoethanol; two contained 100μl of modified Edwards buffer without β-mercaptoethanol; the final tube contained 50μl extraction buffer obtained from the Extract-N-Amp Plant PCR kit (Sigma-Aldrich, Castle Hill, NSW, Australia). DNA was extracted over 10 minutes at 95^{o}C (Sigma kit tube and one Edwards buffer tube) or 60 minutes at 65^{o}C.

At the end of extraction, 50μl dilution buffer was added to the Sigma kit tube, bringing the volume of each tube up to 100μl. 55μl of each extract was transferred to a clean tube and an equal volume of ice-cold iso-propanol added. After 10 minutes incubation at ambient temperature, the samples were centrifuged at 15,000g for 5 minutes. The supernatant was discarded, and the alcohol wash repeated. After discarding the second volume of supernatant, the pellet was allowed to air-dry before being resuspended in 55μl of distilled water. A spectrophotometer was used to measure the absorbance of each solution at 260, 280 and 320nm.

Subsequently, the modified Edwards buffer without β-mercaptoethanol was used to extract DNA without pre-soaking from samples MB28, MB31 and MB99. Following extraction of DNA from samples MB28 and MB31, PCR was performed as described below using the primer pair ITS1-F and ITS4-B (White et al. 1990).

2: Effect of salt concentration and alcohol precipitation 100ml of modified Edwards buffer was prepared without sodium chloride, which was subsequently weighed separately in different amounts to obtain a range of molarities including 0.2, 0.5, 1.0, 1.5, 2.0 and

3.0M NaCl. The final volume of 10ml contained no sodium chloride (0M). 10g of *A. bisporus* tissue was roughly macerated, and 1g added to each tube of buffer. DNA was extracted over 15 minutes at 95^{o}C, the extra time being allowed for the increased volume.

Following extraction, a 2ml aliquot of each solution was combined with 4ml of iso-propanol, and a second aliquot with 6ml of 95% ice-cold ethanol. The tubes were incubated for 15 minutes at either room temperature (tubes containing iso-propanol) or on ice (tubes containing ethanol) after which they were centrifuged at 15,000g for 5 minutes. The supernatant was discarded, and the tubes inverted to dry. After resuspension of the DNA in 2ml distilled water, the samples were diluted and measured spectrophotometrically as before, with the addition of a measurement at 230nm.

3: Effect of sodium hydroxide Three tubes were prepared with each containing 500mg of freshly macerated test material, and either 2ml of 0.5M sodium hydroxide, 2ml modified Edwards solution with 3M NaCl (EdNa) or 1ml EdNa plus 0.25M sodium hydroxide solution. DNA was extracted over 10 minutes at 95^{o}C. Following extraction, 1ml of 200mM Tris pH 5 was added to the latter sample, and the tubes allowed to cool on ice. A 400μl aliquot of each extract was transferred to a clean tube and 800μl ice-cold ethanol added. After 15 minutes incubation on ice, the tubes were centrifuged and the supernatant discarded as before. The pellet was resuspended in 600μl of 70% ethanol, and the incubation and centrifugation step repeated. The tubes were inverted to dry before the DNA was resuspended in 200μl distilled water. DNA was subsequently extracted from samples MB28 and MB31 with 0.5M NaOH and EdNa.

4: Effect of sodium source and ethanol precipitation Modified Edwards buffer was prepared with a final concentration of 3M of one of the sodium salts - either sodium chloride or sodium acetate. 2ml of each buffer was added to 500mg of the MB28 and MB31 tissue samples and DNA extracted over 10 minutes at 95^{o}C. Four aliquots of 400μl were transferred to fresh tubes - tube 1 was centrifuged at 2500g for 5 minutes and the supernatant transferred to a clean tube. Tube 1 and tube 2 were precipitated and washed as described above, whilst tubes 3 and 4 were precipitated only. Subsequently DNA in tube 3 was resuspended in 240μl distilled water prior to the addition of 560μl ethanol (final concentration 70%) followed by centrifugation. Tube 4 was not washed. After drying, the DNA in each tube was resuspended in 200μl of 10mM Tris-HCl pH 7.5. Following spectrophotometric determination of concentration and purity, amplification was performed using the two best DNA extractions from each salt/wash combination.

5: Effect of pH and sodium ions 200mM solutions of sodium hydroxide, potassium hydroxide and Tris were prepared and the pH adjusted to 8.5. EDTA and SDS were added to a final concentration of 25mM and 0.5% respectively. Each solution was halved and the sodium salts added to final concentrations of 3M sodium chloride or 0.1M tri-sodium citrate. DNA was extracted in 250μl volumes of each buffer from samples MB28 and MB31 over 10 minutes at 95^{o}C. After cooling, 250μl of 50mM Tris pH 5 was added to each tube, followed by centrifugation at 2000g for 5 minutes. 400μl of each extract was transferred to a clean tube for ethanol precipitation as described above.

6: Comparison of several commercial DNA extraction methods A DNeasy Plant Mini Kit (QIAGEN, Chadstone, VIC, Australia) was used as per the manufacturer's Quick Start protocol. Essentially, the tissue was briefly vortexed in a solution of 400μl Buffer AP1 and 4μl RNase A, then incubated with occasional mixing for 10 minutes at 65^{o}C. 130μl Buffer P3 was added and mixed, and the tube placed on ice for 5 minutes. The mixture was centrifuged for 5 minutes at 20,000g and the supernatant transferred to a QIAshredder spin column seated in a 2ml collection tube. After centrifugation for 2 minutes at 20,000g the flow through was transferred to a fresh tube with 1.5x volume of Buffer AW1. Up to 650μl was transferred to a DNeasy mini spin column in a collection tube and centrifuged for 1 minute at 6000g. The flow through was discarded and the process repeated with any remaining sample. The spin column was transfered to a fresh collection tube, and 500μl of Buffer AW2 added to the column. After centrifugation, the flow-through was discarded and another 500μl of Buffer AW2 was added. After centrifugation for 2 minutes at 20,000g the spin column was transferred to a sterile 1.5ml microcentrifuge tube. 100μl of elution buffer (Buffer AE) was added to the column and incubated for 5 minutes at room temperature. After centrifugation for 1 minute at 6000g, this was repeated to obtain 200μl of eluate.

An Extract-N-Amp Plant PCR kit (Sigma-Aldrich) was used according to the manufacturer's instructions. Approximately 0.1g of fungal tissue was macerated in 100μl of extraction buffer, followed by incubation for 10 minutes at 95^{o}C. Subsequently 100μl diluent was added, resulting in 200μl of crude DNA extract.

PCR methods trialled

1: Alterations to PCR master mix Three partial master mixes were prepared from an Extract-N-Amp Plant PCR kit and KAPA2G Robust HotStart PCR Kit (Sigma-Aldrich). Using the Extract-N-Amp kit, 12.5μl of the PCR Ready Mix was combined with 1.25μl

of each primer per reaction. Two mixes were prepared from the KAPA2G kit. Both contained 0.5μl of dNTP mix and 1.25μl of each primer per reaction. A number of 5x buffers were provided in the kit, including Buffer A which was optimized for high yield, specificity, and sensitivity, and Buffer B which was recommended for use with inhibitor contaminated template samples. One mix was prepared from each buffer, using 5μl per reaction.

Each of these mixes were divided into 4 equal volumes. BSA and DMSO were added to two of each mix, to a final concentration of 1.25μl per reaction. Magnesium chloride (MgCl) was also added to one of the mixes, using the MgCl solution supplied in the KAPA2G kit. All mixtures were brought up to a final reaction volume of 23.9μl with DNase-free water.

Each mix received 0.1μl of the relevant kit-supplied Taq. After dispensing into 24μl volumes into 0.2ml thin-walled PCR tubes, 1μl of sample was added. One set of reactions without additives received iso-propanol purified sample, whilst the non-purified, non-diluted sample was used in the remainder.

2: Alterations to thermalcycle parameters A touchdown cycle was utilised for some runs which replaced the 35 cycle temperature sequence of 94^{o}C for 1 minute, 30 seconds at 50^{o}C (ITS4) or 55^{o}C (ITS4-B), and 72^{o}C for 1 minute, with 15 cycles of 94^{o}C for 1 minute, 30 seconds starting at 63^{o}C dropping to 45^{o}C, and 72^{o}C for 1 minute, followed by 20 cycles of the same sequence with annealing at 45^{o}C.

3: Nested PCR Nested PCR was also trialled. Initial amplification was performed using the primer pair ITS1-F and ITS4-B, after which 1μl of product was re-amplified with the primers ITS1 and ITS4. The concentration of all primers used was 10μM.

Following amplification, 2 to 3μl of each sample was combined with 1 to 2μl of 10x loading dye (ThermoFisher) and electrophoresed on a gel containing 2% agarose in TAE buffer and 5μl/100ml Red Safe dye (Scientifix, South Yarra, VIC, Australia). A one to three μl volume of MassRuler Express LR Forward DNA Ladder (ThermoFisher) was included in each well flanking the samples. The gel was run at 250V for 15 minutes, before being viewed and photographed on a Quantum ST4 1100 Gel Doc system using Quantum-Capt software Version 15.18 (Fisher Biotech, Wembley, WA, Australia).

All PCR products were stored at -20^{o}C until sequencing submission.

2.2.4 Sequencing and BLAST Analysis

DNA purification methods trialled

A number of techniques were used to purify the PCR products prior to sequencing submission. These included:

1: Macherey-Nagel NucleoSpin Gel and PCR Clean-Up Kit (Scientifix, Cheltenham, VIC, Australia) PCR products were mixed with a 2x volume of binding buffer and transferred to a spin column. After centrifugation for 1 minute at 11,000g, 700μl of wash buffer was added to the column and cenrifugation repeated. The flow through was discarded and the column dried by cenrifugation at 11,000g for 2 minutes. The column was transferred to a fresh collection tube, and up to 30μl elution buffer added. After incubation for at least 1 minute at room temperature, the centrifugation step was repeated to elute the DNA.

2: Diffinity RapidTips (Sigma-Aldrich) PCR products were made up to 25 or 50μl and transferred to a 0.6ml microcentrifuge tube. After ensuring the solid matter (porous beads) was available in the bottom of the tip, the contents of each tube were slowly drawn up through the matter and gently expelled. This was repeated approximately 15 times over 1 minute, ensuring the solution was mixing well through the porous beads.

Following electrophoretic and spectrophotometric confirmation of product presence, concentration and purity, 10μl volumes of suitable products were sent to the Australian Genome Research Facility (AGRF) at a concentration of approximately 1.5 to 2.5ng/μl along with 2μl of either ITS1F or ITS4B, depending on the original primer pair used and direction of sequence required. The concentration of later products was made up in 9μl, and 1μl of DMSO added.

Sanger sequencing was performed with the Applied Biosystem 3730 capillary sequencer utilising Big Dye Terminator (BDT) chemistry. The cycling conditions used are shown in Table 2.8.

Sequence chromatograms were manually checked for accuracy and corrected if necessary, using the software FinchTV version 1.3.1 (Geospiza Inc). Corrected sequences greater than 300 bases were combined into a single text file, formatted and saved in FASTA format. This was used as data input to perform nucleotide Basic Local Alignment Search Tool (BLASTn) searches on GenBank.

Table 2.8: Cycling conditions used at the AGRF for Sanger sequencing of purified PCR products

Temperature	Time	Number of cycles
96^{o}C	2 min	1
96^{o}C	10 secs	
50^{o}C	5 secs	30
60^{o}C	4 min	
4^{o}C		Hold

Search results were used in the selection of several sequences closely related to each specimen (query) sequence. Sequences of fully named, preferably vouchered specimens aligning to the query sequence with a displayed E-value of 0 was used as the initial basis for sequence selection.

From these results, sequence alignments having a percent identity of 96 or greater with 80% or higher query coverage were considered as having a high likelihood of being identical. This process was subsequently repeated but limited to the Australian sequences as listed in Table 2.3.

In order to validate both sets of results, a phylogenetic tree was constructed with specimen and matched sequence pairs, along with four additional sequences as an outgroup. The sequences were aligned using the MUSCLE algorithm in MEGA X with the default settings and the resulting alignment used to generate a phylogenetic tree using the Maximum Likelihood method based on the Kimura 2-parameter model and bootstraping with 500 replications. Gaps of 5% or less were deleted.

2.3 Results

2.3.1 DNA Extraction, PCR and DNA purification methods

Extraction methods trialled

1: Effect of pre-soaking, buffer choice and extraction temperature (Table 2.9) In this experiment it was found, pre-soaking of the material did not appear to remove sufficient amounts of inhibitors to improve the success rate of subsequent PCR; the addition of β-mercaptoethanol improved the results slightly, though the toxicity of the chemical may preclude its use; the use of the modified Edwards buffer appeared to result in a higher quality but lower concentration of DNA than the Sigma kit.

Table 2.9: Selected results of extraction trial 1

Test condition	A260/A280*	DNA conc. (ng/μl)
Sigma kit (baseline)	0.84	29
Edwards buffer, extract over 10 min at 95^{o}C	2.67	12
Edwards buffer, extract over 60 min at 60^{o}C	2.25	9
Extraction as above following overnight pre-soak at 4^{o}C	2.15	14

*Ideally this figure should be 1.8 or higher

2: Effect of salt concentration and alcohol precipitation (Table 2.10) Results of this trial showed the highest levels of purity were achieved by including 3M sodium chloride in the extraction buffer followed by ethanol precipitation. However, a large amount of DNA was lost in the process.

Table 2.10: Selected results of extraction trial 2

Test condition	A260/A230[1]	A260/A280[2]	DNA conc. (ng/μl)
Ethanol precipitation			
No NaCl	0.45	1.65	23.8
200mM NaCl	0.37	1.53	27
3M NaCl	1.17	3.5	7
Iso-propanol precipitation			
No NaCl	0.45	1.71	14
200mM NaCl	0.59	1.67	13
3M NaCl	0.66	1.67	5

[1] Ideally this figure should be within the range of 1.8 - 2.2

[2] Ideally this figure should be equal to or greater than 1.8

Table 2.11: Selected results of extraction trial 3

Test condition	A260/A230[1]	A260/A280[2]	DNA conc. (ng/μl)
0.5M NaOH	0.5	1.27	35
EdNa buffer	0.5	1.5	15
50:50	1.0	2.0	10

[1] Ideally this figure should be within the range of 1.8 - 2.2

[2] Ideally this figure should be equal to or greater than 1.8

3: Effect of sodium hydroxide (Table 2.11) In this experiment it was found the use of 0.5M sodium hydroxide appeared to increase the amount of DNA extracted, whilst the EdNa buffer seemed to produce an extract with slightly less contamination. A 50:50 mix

of the two buffers appeared to further reduce the contamination levels, but also reduced the amount of DNA extracted.

4: Effect of sodium source and ethanol precipitation (Table 2.12) In general it was found the use of NaCl provided a higher quantity of DNA containing less impurities than the use of tri-sodium citrate, however, it was not a consistent result.

Table 2.12: Selected results of extraction trial 4

Test condition	A260/A230[1]	A260/A280[2]	DNA conc. (ng/μl)
Specimen MB28			
Sodium chloride	0.79	1.93	40.5
Tri-sodium acetate	0.82	1.93	40.5
Specimen MB31			
Sodium chloride	0.67	1.64	27
Tri-sodium acetate	0.68	1.63	19.5

[1] Ideally this figure should be within the range of 1.8 - 2.2

[2] Ideally this figure should be equal to or greater than 1.8

5: Effect of pH and sodium ions Source of the alkaline environment (NaOH, KOH or Tris) did not appear to have an impact on the DNA extract - similar results were obtained for each salt regardless of the alkali source. However, as in the method above, the use of NaCl appeared to provide a higher quantity and quality of DNA than the use of tri-sodium citrate.

6: Comparison of several commercial DNA extraction methods In this instance, the Extract-N-Amp kit outperformed the DNeasy Plant Mini Kit in terms of successful PCR. It also had the advantage of being a faster procedure, producing an extract in approximately 10 minutes rather than 45, and requiring substantially less components, thereby reducing the cost of each extraction.

PCR methods trialled

1: Alterations to PCR master mix The most consistent results in obtaining PCR products were achieved using the KAPA2G kit incorporating Buffer B and BSA. The addition of extra MgCl did not improve the results in any mixture tested.

2: Alterations to thermalcycle parameters Touchdown thermalcycling did not appear to improve the success of PCR amplification, though again, the results were inconsistent.

3: Nested PCR Nested PCR using the primer combinations trialled did not appear to improve the success of PCR amplification.

DNA purification methods trialled

Both purification systems worked well, however the Macherey-Nagel PCR clean-up kit seemed to be more reliable than the Diffinity tips. On occasion the matrix within the Diffinity tip would draw higher up the tip than required so the sample was unable to be adequately recovered.

2.3.2 Specimen collection

A total of 340 fruiting body specimens were collected, comprising 221 fresh and 119 dried samples (see Appendix B). DNA was successfully extracted from all specimens, however the quantity of DNA extracted varied as determined by visualisation of the DNA spot plates. Sequences were obtained for 149 of these specimens (Table 2.13 and 2.14). 94 sequences (63%) were informative, generating alignments with basidiomycete ITS sequences within GenBank. Another 29 sequences (19.5%) were non-descriptive being either too short or too unique to form meaningful alignments, though it was possible to confirm the genus. Ten sequences matched to non-target species, including Ascomycetes and an enenterid (flatworm); eight sequences were too difficult with which to work, showing multiple traces in the chromatogram. The remaining 8 sequences returned the error message “No significant matches found”, which was most likely due to the poor quality of the sequences used.

Interestingly, of the 135 PCR products successfully sequenced 46 (34%) were sourced from dried specimens and 89 (66%) were from fresh or frozen specimens.

Morphologically, 10 specimens were found to not be members of the Russulaceae, and therefore not sequenced. Another 40 specimens failed to successfully amplify. The remaining DNA extracts are yet to be processed.

BLASTn was used to compare the 94 informative sequences with the Australian sequences as listed previously (Table 2.3). Based on the criteria of a displayed E-value of 0, and percent identity of 96% or greater over 80% of the query sequence, 15 specimen ITS-sequences were considered as having a high probability of being identical to an Australian sequence (Table 2.15). Using the same criteria, but including the requirement of fully named vouchered specimens, 9 specimen ITS sequences were considered as having a high likelihood of being identical to non-Australian Russulaceae ITS sequences held in GenBank (Table 2.16). Seven of these specimens also appear in the Australian-match list.

Table 2.13: Sequencing results of the ITS-PCR products sourced from fresh specimens. BLASTn was used to determine the closest match to sequences held in GenBank, whilst BLAST 2 Sequences was used to find the closest match to an Australian sequence as listed previously in Table 2.3.

Collection			Closest match			Closest Australian match	
Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB01	12/02/12	Lodbroke	*R. violeipes*	KF361797.1	95.7	*R. rostraticystidia*	94.6
MB02	12/02/12	Lodbroke	*R. tawai*	JX178491.1	90.7	*R. clelandii*	89.0
MB03	26/02/12	Lodbroke	*R. tawai*	JX178491.1	89.9	*R. clelandii*	88.0
MB04	17/03/12	Crows Nest NP	*R. kanadii*	KJ866936	91.3	*R. iterika*	86[2]
MB05	18/03/12	Lodbroke	*R. tawai*	JX178491.1	91[1]		
MB06	18/03/12	Lodbroke	*R. tawai*	JX178491.1	92.1	*R. clelandii*	91.2
MB08	12/03/12	Lodbroke	*R. delica*	KX812842.1	92.4	*R. pumicoidea*	93.1
MB09	23/03/12	Duggan Park	*R. tawai*	JX178491.1	90.5	*R. clelandii*	89.1
MB10	23/03/12	Lodbroke	*R. tawai*	JX178491.1	90.6	*R. clelandii*	89.1
MB11	23/03/12	Lodbroke	*R. tawai*	JX178491.1	90.5	*R. clelandii*	89.0
MB12	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.3	*R. clelandii*	89.7
MB13	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.7	*R. clelandii*	89.0
MB14	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.3	*R. clelandii*	89.7
MB15	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.3	*R. clelandii*	89.7

...continued on next page

Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB16	24/03/12	Lodbroke	*R. tawai*	JX178491.1	86[1]		
MB17	24/03/12	Lodbroke	*R. tawai*	JX178491.1	92.2	*R. clelandii*	90.9
MB18	24/03/12	Lodbroke	*R. tawai*	JX178491.1	92.4	*R. clelandii*	91.4
MB19	04/05/12	Lodbroke	*Melanoleuca strictipes*	JF908359.1	93.4		
MB20	04/05/12	Lodbroke	*Melanoleuca strictipes*	JF908359.1	93.4		
MB23	17/06/12	Ravensbourne	*R. tawai*	JX178491.1	90.4	*R. clelandii*	88.6
MB24	17/06/12	Ravensbourne	*R. acrolamellata*	MF461612	94.8	*R. neerimea*	96.2
MB25	02/03/13	Lodbroke	*R. tawai*	JX178491.1	90.2	*R. clelandii*	88.7
MB32	04/03/13	Tully Park	*R. archaea*	EU598165.1	87.0	*R. galbana*	84[2]
MB35	04/03/13	Tully Park	*R. subfoetens*	KF002757.1	89.7	*R. neerimea*	90.6
MB37	04/03/13	Tully Park	*R. tawai*	JX178491.1	88[1]		
MB38	04/03/13	Tully Park	*R. foetens*	KX095018.1	87[1]		
MB39	09/03/13	Crows Nest NP	*R. acrolamellata*	MF461612	79[1]		
MB40	09/03/13	Crows Nest NP	*R. virescens*	MK782587	80[1]		
MB41	09/03/13	Crows Nest NP	*R. atroviridis*	GU222285.1	79[1]		
MB43	09/03/13	Crows Nest NP	*R. roseostipitata*	GU222324.1	94.0		
MB44	09/03/13	Crows Nest NP	*R. subfoetens*	KF002757.1	93.0	*R. neerimea*	94.0
MB45	09/03/13	Crows Nest NP	*R. delica*	KX812842.1	93.1	*R. sinuata*	91.8
MB46	09/03/13	Crows Nest NP	*R. delica*	KX812842.1	93.6	*R. sinuata*	93.4
MB47	09/03/13	Crows Nest NP	*R. acrolamellata*	MF461612	80[1]		
MB48	10/03/13	ANWR	*R. tawai*	JX178491.1	93.9		
MB49	10/03/13	ANWR	*R. tawai*	JX178491.1	93.8		
MB50	12/03/13	ANWR	*R. tawai*	JX178491.1	93.8		
MB52	14/04/13	Lodbroke	*R. tawai*	JX178491.1	92.2	*R. clelandii*	91.2
MB54	21/04/13	Crows Nest NP	*R. redolens*	KT933950.1	75[1]		
MB55	20/04/13	Ravensbourne	*R. purpureoflava*	EU019914	85[1]		
MB59	21/04/13	Crows Nest NP	*R. redolens*	KT933950.1	94.0	*R. galbana*	87[2]
MB60	21/04/13	Crows Nest NP	*R. foetens*	KF245487.1	94.0	*R. galbana*	91.6
MB61	21/04/13	Crows Nest NP	*R. virescens*	MG680184	92.2	*R. iterika*	85[2]

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB62	21/04/13	Crows Nest NP	*R. kanadii*	KM275230.1	92.0	*R. iterika*	88[2]
MB63	19/04/13	Ravensbourne	*R. atroviridis*	JX178493.1	96.4	*R. wollumbina*	95.5
MB64	21/04/13	Crows Nest NP	*R. virescens*	LC008519	88[1]		
MB68	21/04/13	Crows Nest NP	*R. virescens*	KX267657	82[1]		
MB69	14/07/13	Lodbroke	*R. tawai*	JX178491.1	90.6	*R. clelandii*	89.1
MB70	29/07/13	Lodbroke	*R. tawai*	JX178491.1	92.4	*R. clelandii*	91.4
MB71	15/04/14	Lodbroke	*R. tawai*	JX178491.1	92.2	*R. clelandii*	91.2
MB72	17/04/14	Lodbroke	*R. tawai*	JX178491.1	91.8	*R. clelandii*	91.1
MB73	17/04/14	Lodbroke	*R. tawai*	GU222263.1	89.2	*R. clelandii*	88.5
MB75	18/04/14	Lodbroke	*R. delica*	KX812842.1	94.1	*R. sinuata*	93.7
MB77	24/04/14	Brisbane	*R. crustosa*	EU598193.1	90.0	*R. marangania*	86[2]
MB78	24/04/14	Brisbane	*R. delica*	KX812842.1	92.8	*R. sinuata*	91.8
MB79	26/04/14	Lodbroke	*R. violeipes*	AY061726.1	95[1]		
MB80	26/04/14	Lodbroke	*R. violeipes*	AY061726.1	95[1]		
MB82	26/04/14	Lodbroke	No significant similarity found				
MB84	27/04/14	Lodbroke	*R. tawai*	GU222263.1	92.7	*R. clelandii*	91.4
MB87	27/04/14	Lodbroke	*R. violeipes*	AY061726.1	93.0	*R. rostraticystidia*	94.0
MB88	03/05/14	Lodbroke	*R. tawai*	JX178491.1	92.2	*R. clelandii*	91.1
MB89	03/05/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.2
MB90	03/05/14	Lodbroke	*R. tawai*	JX178491.1	94.3	*R. clelandii*	93.4
MB91	03/05/14	Lodbroke	*R. tawai*	JX178491.1	94.3	*R. clelandii*	93.3
MB92	04/05/14	Lodbroke	*R. tawai*	JX178491.1	92.6	*R. clelandii*	91.7
MB94	04/05/14	Lodbroke	No significant similarity found				
MB96	27/05/14	Lodbroke	*R. tawai*	JX178491.1	92.3	*R. clelandii*	91.4
MB97	01/06/14	Lodbroke	*R. tapawera*	EU019942	80[1]		
MB99	12/02/15	Lodbroke	*R. violeipes*	AY061726.1	94.0	*R. rostraticystidia*	94.4
MB106	22/02/15	Lodbroke	Multiple traces				
MB107	22/02/15	Lodbroke	Multiple traces				
MB111	09/04/15	Tully Park	*R. purpureoflava*	JX266626	92[1]		

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB112	09/04/15	Tully Park	*Lactifluus volemus*	HQ318222	79[1]		
MB116	12/04/15	Wilson's Downfall	*R. kanadii*	KJ866936.1	92.0	*R. iterika*	85[2]
MB117	12/04/15	Wilson's Downfall	*R. crustosa*	EU598193.1	90.0	*R. marangania*	86[2]
MB119	12/04/15	Wilson's Downfall	*R. tawai*	JX178491.1	89.4	*R. clelandii*	88.1
MB121	12/04/15	Wilson's Downfall	*R. kanadii*	KJ866936.1	92.0	*R. iterika*	85[2]
MB127	22/04/15	Lodbroke	*R. violeipes*	JF908655.1	94.0	*R. rostraticystidia*	94.4
MB130	03/06/15	Tully Park	*R. marangania*	EU019930	95.0	*R. marangania*	95.0
MB131	03/06/15	Tully Park	*R. tapawera*	EU019935.1	94[1]		
MB132	03/06/15	Tully Park	*R. atrovirens*	GU222260.1	97.3	*R. wollumbina*	98.9
MB134	03/06/15	Tully Park	*R. wollumbina*	EU019921.1	97.7	*R. wollumbina*	97.7
MB146	31/12/15	Lodbroke	No significant similarity found				
MB151	05/01/16	Lodbroke	*R. tawai*	JX178491.1	83[1]		
MB155	05/01/16	Lodbroke	*R. violeipes*	JF908655	94.6	*R. rostraticystidia*	94.7
MB157	07/01/16	Lodbroke	No significant similarity found				
CW1	08/02/16	Coonabarabran	*R. delica*	KX812842.1	75[1]		
CW2	08/02/16	Coonabarabran	*R. delica*	KX812842.1	89.9		

[1] Short specimen sequence, so percent identity provides an indicator of the genus and clade only

[2] Matched sequence contains large numbers of gaps and/or mismatches

Table 2.14: Sequencing results of the ITS-PCR products sourced from dried specimens. BLASTn was used to determine the closest match to sequences held in GenBank, whilst BLAST 2 Sequences was used to find the closest match to an Australian sequence as listed previously (Table 2.3)

Collection			Closest match			Closest Australian match	
Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
DS01	17/07/12	Maroochy BBG	*R. tricholomopsis*	JX178492.1	89.9	*R. clelandii*	92.6
DS02			*R. tawai*	JX178491.1	94	*R. clelandii*	94.2
DS03	17/07/12	Maroochy BBG	*R. tricholomopsis*	GU222261.1	94.4	*R. clelandii*	94.2
DS04	21/04/12	Obi Obi	*R. virescens*	KU552087	89.8	*R. marangania*	86[2]
DS05	07/01/10	Dilkusha, Maleny	*R. violeipes*	KR673669.1	91[1]		
DS06	13/07/12	Dilkusha, Maleny	*R. tawai*	JX178491.1	90.0	*R. clelandii*	88.9
DS07	20/02/12	Dilkusha, Maleny	*R. cerolens*	KX095042	91.9	*R. pilosella*	91.8
DS08	07/04/12	Mt Tamborine	*R. foetens*	KF245487.1	93.5	*R. neerimea*	93.2
DS09	16/04/12	Linda Garrett	*R. shingbaensis*	KM386692.1	96.7	*R. cheelii*	97.8
DS10	17/04/12	Linda Garrett	*R. tapawera*	EU019942.1	82[1]		
DS11	16/04/12	Linda Garrett	*R. tapawera*	EU019942.1	90[1]		
DS12	31/03/12	Chermside Hills	*R. senecis*	KX574699.1	94.8	*R. galbana*	92
DS14	24/03/12	Mt Norman, Girraween	*R. brevipes*	FJ845429.1	93.1	*R. pumicoidea*	94.3
DS15	21/04/12	Obi Obi	*R. senecis*	KX574699.1	92.8	*R. galbana*	88.5
DS27	16/03/13	Linda Garrett	*R. ingwa*	EU019919.1	94.1	*R. ingwa*	94.1
DS28	17/04/11	Tully Park, Girraween	*R. griseobrunnea*	MF461604.1	98.1	*R.* aff. *adusta*	98.2
DS36	2013	Bald Rock NP, NSW	*Lactarius volemus*	LC176757.1	88.0	*L. clarkeae*	82[2]
DS42	26/02/13	Alexandra Hills	No significant similarity found				
DS63	2011	Manjimup, WA	*R. subfoetens*	KY681430.1	94.0	*R. neerimea*	94.7

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
DS67	2013	Ravensbourne	*Lactifluus luteolus*	KR364016	90.6	*R. flocktonae*	83[2]
DS69	2013	Bald Rock NP, NSW	*Lactarius piperatus*	DQ422035.1	94.4	*M. stenophylla*	80[2]
DS71	2008	Obi Obi	*Lactarius clarkeae*	HQ318283.1	99.4	(Australian match only)	
DS73	2012	Obi Obi	*Lactarius sepiaceus*	EU019926.1	99.6	*L.* cf. *wirrabara*	100
DS74	2013	Cooloola	*Lactarius leonardii*	GU258288.1	97.4	(Australian match only)	
DS75	2013	Cooloola	*Lactarius* cf. *wirrabara*	JF731001	99.7	(Australian match only)	
DS76	2013	Ravensbourne	*L. longipilus*	HQ318256	80[1]		
DS77	2011	Manjimup, WA	*R. flavida*	KX267651.1	87[1]		
DS81	11/04/15	N. Dr Roberts Girraween	*R. acrolamellata*	MF461612.1	94.1	*R. brunneonigra*	98.2
DS82	11/04/15	N. Dr Roberts Girraween	*Lactarius decipiens*	KT165312.1	97.0	*L. eucalypti*	98.4
DS83	12/04/15	Wilson's Downfall	*Lactifluus luteolus*	KR364016	87[1]		
DS84	10/04/15	Bald Rock NP, NSW	*Lactarius glaucescens*	AB509515	93[1]		
DS85	10/04/15	Bald Rock NP, NSW	*L. volemus*	HQ318273	73[1]		
DS86	10/04/15	Bald Rock NP, NSW	*L. crocatus*	JN388985.1	94.0	*L. clarkeae*	83[2]
DS87	10/04/15	Bald Rock NP, NSW	*L. volemus*	HQ318273	78[1]		
DS88	10/04/15	Bald Rock NP, NSW	*R. cheelii*	JX266623	97.9	(Australian match only)	
DS89	16/04/15	Mt Norman, Girraween	*R. pseudoareolata*	JX178488.1	96.6	*R.* aff. *compacta*	99
DS90	14/06/14	Linda Garrett	*Multifurca stenophylla*	MH063867	99.9	(Australian match only)	

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
DS92	10/04/15	Bald Rock NP, NSW	*R. vesca*	HM189956	98.9	*R. galbana*	86[2]
DS93	29/03/11	Bellthorpe	*R. crustosa*	EU598193.1	90	*R. marangania*	86[2]
DS94	20/04/15	Davies Creek Falls Circuit	No significant similarity found				
DS96	20/04/15	Davies Creek	No significant similarity found				
DS97	21/04/15	Davies Creek	*R. chloroides*	KF432954.1	86[1]		
DS98	21/04/15	Davies Creek	*R. aeruginea*	HQ604837.1	92.8	*R. iterika*	86[2]
DS99	21/04/15	Davies Creek	No significant similarity found				
DS102	24/04/15	Tinaroo Forest Reserve	*R. virescens*	DQ422014.1	88	*R. iterika*	86[2]

[1] Short specimen sequence, so percent identity provides an indicator of the genus and clade only

[2] Matched sequence contains large numbers of gaps and/or mismatches

Table 2.15: Samples matched with Australian sequences as listed in Table 2.3

Specimen ID	Name	Accession Number	Percent Identity	Percent coverage
DS09	*R. cheelii*	JX266623	97.8	90.7
DS28	*R.* aff. *adusta*	EU019918	98.2	84.7
DS71	*L. clarkeae*	HQ318283	99.5	100
DS73	*L.* cf. *wirrabara*	GU258309	100.0	100
DS74	*L. leonardii*	GU258295	97.4	100
DS75	*L.* cf. *wirrabara*	JF731001	99.7	100
DS81	*R. brunneonigra*	EU019945	98.3	84.1
DS82	*L. eucalypti*	EU019923	98.4	80.3
DS88	*R. cheelii*	JX266623	97.9	99.0
DS89	*R.* aff. *compacta*	JX266625	99.2	80.6
DS90	*M. stenophylla*	MH063867	99.9	99.9
MB24	*R.* aff. *pilosella*	EU019932	97.1	80.9
MB130	*R. marangania*	EU019930	98.6	99.7
MB132	*R. wollumbina*	EU019921	99.3	79.6
MB134	*R. wollumbina*	EU019921	97.7	99.8

Table 2.16: Samples matched with non-Australian Russulaceae sequences in GenBank

Specimen ID	Name	Accession Number	Percent Identity	Percent coverage	Country of Origin
DS09*	*R. shingbaensis*	KM386692	96.7	98	India
DS28*	*R. griseobrunnea*	MF461604	98.1	99	New Zealand
DS73*	*L. sepiaceus*	EU019926	99.6	92	New Zealand
DS74*	*L. leonardii*	GU258288	97.4	99	New Zealand
DS82*	*L. decipiens*	KT165312	97.0	99	Slovakia
DS89*	*R. pseudoareolata*	JX178488	96.6	99	New Zealand
DS92	*R. vesca*	HM189956	98.9	100	Germany
MB63	*R. atroviridis*	JX178493	96.4	97	New Zealand
MB132*	*R. atrovirens*	GU222260	97.3	94	New Zealand

*Also appear on the list of matches to Australian sequences

A phylogenetic tree (Figure 2.5) was generated incorporating the sequences of the 17 specimens, 13 Australian specimens and 9 international specimens appearing in the tables above. Another 4 sequences were included as an outgroup.

Colour plates of the fresh specimen collection can be found in Appendix B, and sequencing results of all specimens in Appendix C. Sequences generated are provided in Appendix D.1 and D.2.

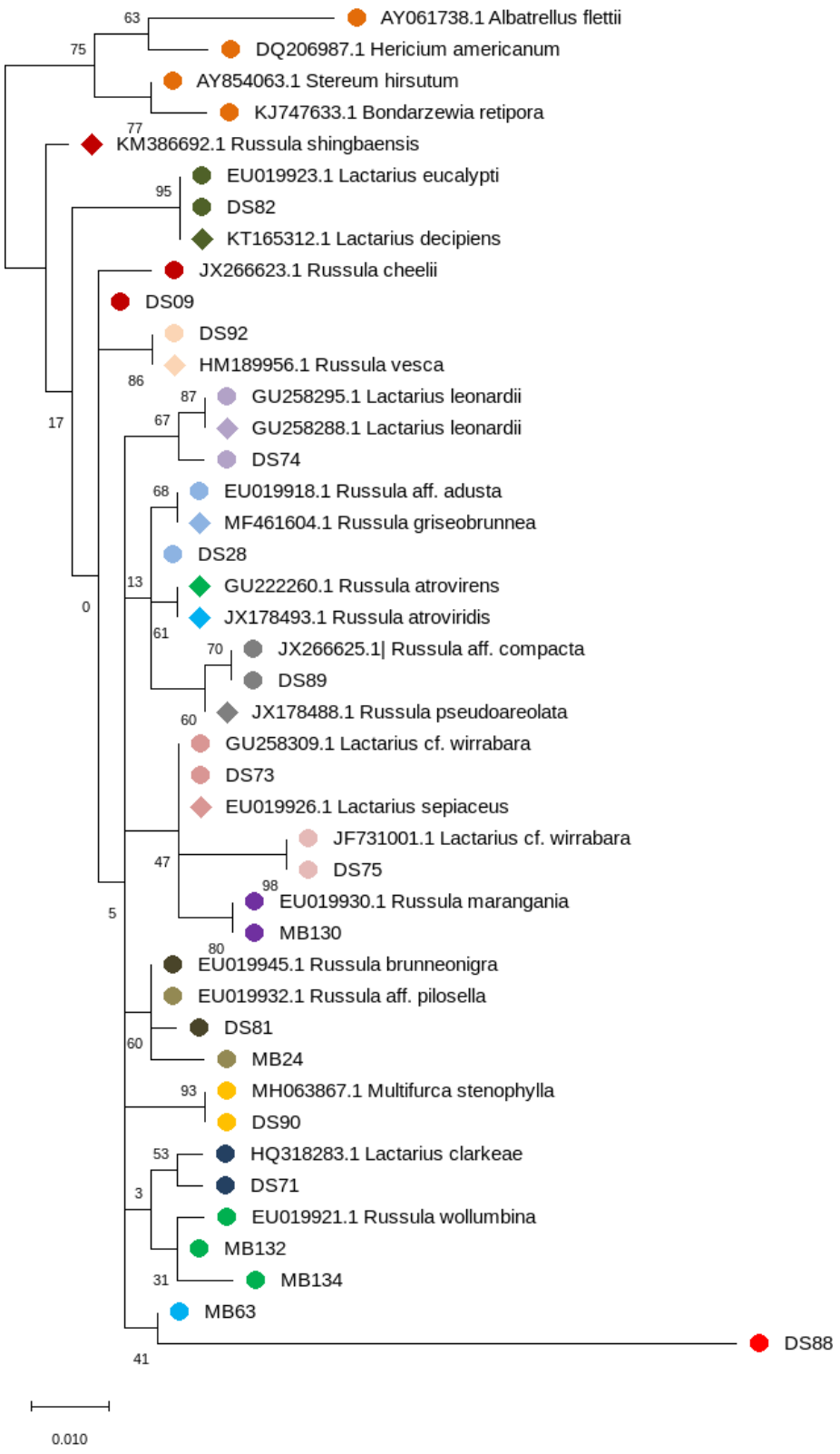


Figure 2.5: Phylogenetic tree of sequences determined to be identical following BLASTn searches. Orange markers indicate the outgroup sequences; diamond markers indicate international sequences and circular markers indicate Australian ones. Seperate colours are used for each set of matches.

2.4 Discussion

2.4.1 Procedure optimisation

The successful extraction of DNA from the cells of any organism relies on very few, non-complex steps (Ihase et al. 2016). Essentially, cells are ruptured, generally with the aid of detergents and/or enzymes and the DNA is released, along with other cellular contents. These contents vary in form and concentration from organism to organism, and can have multiple undesired effects on downstream applications. The DNA extraction buffers themselves may also contain inhibitory substances. Therefore, extraction procedures need to be optimised for each organism and purpose of extraction.

Throughout the course of obtaining PCR products suitable for sequencing, it was found a large number of specimens either failed to produce a product at all, or the product was not able to be successfully sequenced. Whilst an acceptable concentration of DNA was extracted from the bulk of the fungal samples, it was determined the presence of PCR inhibitors was the most likely cause of these PCR failures.

Inhibitors can interfere in a number of ways including binding with the DNA template thereby limiting template availability, binding to the polymerase thereby limiting its activity, or by adverse interactions during the extension stage of PCR (Opel et al. 2010). PCR inhibitors may be neutralised via chemical means, be physically removed from the DNA extract prior to PCR, and/or diluted to a non-inhibitory concentration (Kreader 1996, Opel et al. 2010, Azmat et al. 2012, Schrader et al. 2012). In cases where this does not resolve the problem, alternative primers or loci may need to be investigated (Opel et al. 2010), though this may not always be feasible. Examples of potential inhibitors, their source and counter measures are listed in Table 2.17.

In the case of the Russulaceae, it is possible polysaccharides, lipids and polyphenolic compounds were the most likely contributors to PCR failure (Samarakoon et al. 2013). Similar to many plants, fungal DNA extracts can also contain PCR inhibitors, but there are many more reports of extract optimisation for plants than fungi.

Table 2.17: Examples of PCR inhibitors, their source and potential counter measures

Source	Examples	Counter measure
Environmental contaminants	Tannic and humic acids, melanins	Neutralise by incorporation of additives such as BSA and DMSO in to the PCR master mix[1,2]
Specimen tissue	Polysaccharides	Precipitation of the DNA and removal of the contaminant in the supernatant[3,4]
	Polyphenolics, dyes, enzymes	Neutralise by incorporation of additives such as β-mercaptoethanol to the DNA extract[3]
Buffers	EDTA, salts, detergents, alcohols	Dilute to non-inhibitory concentrations[1,5]

[1]Kreader (1996)
[2]Farell & Alexandre (2012)
[3]Azmat et al. (2012)
[4]Fang et al. (1992)
[5]Schrader et al. (2012)

Extraction methods trialled

1: Effect of pre-soaking, buffer choice and extraction temperature For the optimal extraction of polysaccharides from the basidiomycete *Tricholoma giganteum*, Mo et al. (2013) soaked the dried, powdered product in water at 100^{o}C for several hours. It was therefore wondered if a pre-soak of the fungal material would be useful for the removal of polysaccharides prior to DNA extraction.

Tamari et al. (2013) found DNA extracted from the garden petunia, (*Petunia hybrida*) in Edwards buffer generally outperformed yield and subsequent PCR performance than DNA extracted using the CTAB method (Murray & Thompson 1980). Azmat et al. (2012) included β-mercaptoethanol in the extraction buffer to prevent oxidative damage to DNA extracted from mature leaves of the mango (*Magnifera indica*) caused by some polyphenolic compounds.

The use of high temperatures may eliminate the need to include potentially inhibitory com-

ponents in a lysis buffer. The lysis buffer used/assessed by Zhang et al. (2010) contained 50mM sodium phosphate, 5% glycerol and 1mM EDTA. Whilst EDTA at that concentration in PCR may be inhibitory, a 1:10 dilution eliminates that concern. Incubation of fungal hyphae at 85^{o}C for 20 to 30 minutes was found to produce a suitable amount and quality of genomic DNA to be used in subsequent PCR.

2: Effect of salt concentration and alcohol precipitation. Sodium ions present in the extract from the addition of sodium chloride, neutralise the charge of the DNA. When combined with 70% alcohol, this causes the DNA to form a precipitate which can be pelleted through centrifugation.

Iso-propanol and ethanol are the alcohols generally used for the precipitation of DNA out of high-salt solutions. Iso-propanol requires the use of less volume than ethanol, but results in a softer pellet, which is easier to dislodge and accidentally discard. It can also co-precipitate simple sugars along with the DNA. Ethanol has the disadvantage of needing to be cold, but appears to be easier with which to work (Bag et al. 2016).

An appropriate concentration of NaCl is required in order to supply the sodium ions required to neutralise the charge on the DNA molecule to allow it to precipitate. Fang et al. (1992) found 2M NaCl was optimal in the extraction of DNA from their samples, and that 3M precipitated out of solution and was hard to wash out. In this study, 3M NaCl was found to produce an extract containing less contaminants than other salt concentrations as determined by spectrophotometric methodology. These differences in result may have been due to different DNA sources (plant versus fungi) or different methods for discerning success (enzymatic digestion of the DNA and subsequent PCR versus spectrophotometry).

3: Effect of sodium hydroxide DNA degradation and presence of PCR inhibitors are also problematic in DNA extracts of forensic samples. Bourke et al. (1999) found DNA extract treated with a sodium hydroxide wash after extraction just prior to PCR improved the success of amplification. It was thought PCR inhibitors collected from the environment with the DNA sample intercalated with the double-stranded DNA. As an alkaline environment denatures the DNA, it was theorised the inhibitors would have reduced affinity for the DNA as a single strand. Bourke et al. (1999) found 50% of failed amplifications were successful following treatment with NaOH. However, care was required to avoid subsequent DNA degradation.

4: Salt selection and ethanol precipitation As the pH of the extraction solution has an

effect on the efficacy of the PCR reaction, it was decided to compare the type of salt used. Once disassociated, the chloride ions from the sodium chloride form hydrochloric acid, a strong acid whereas the acid by-product from sodium acetate is the far weaker acetic acid. There was concern the strong acid could reduce the pH of the solution to the point the DNA could hydrolyse. This did not appear to be an issue in this study.

5: Effect of pH and sodium ions In extracting DNA from the plant species *Arabidopsis thaliana* and *Brassica napus*, Wang et al. (1993) determined an alkaline environment (equal to or greater than pH 8.0) was critical to the success of PCR. This was initially supplied by 100mM Tris at pH 8, but subsequently by 0.5M sodium hydroxide. However, in this study, the use of different alkalis did not have a major impact on PCR success.

6: Comparison of several commercial DNA extraction kits DNA was successfully extracted from the fungal material by both of the commercial kits used here. However, the Sigma kit was preferred due to its simplicity and speed.

PCR methods trialled

A number of additives were trialled in the PCR mix in an attempt to counter the influence of potential inhibitors or issues. Winship (1989) found the addition of 10% DMSO improved sequencing results by minimising the reannealing of the template and/or reducing the formation of secondary structures. However, it was subsequently found DMSO could halve the efficacy of the *Taq* polymerase. A number of studies found the addition of bovine serum albumin (BSA) was beneficial to PCR, including Kreader (1996) and Iotti & Zambonelli (2006). However, Farell & Alexandre (2012) found BSA on its own did not improve results in the amplification of GC-rich templates, but significantly increased the yield when used in conjunction with DMSO or formamide. They also reported no detrimental effects from the inclusion of BSA in the PCR mix.

The choice of polymerase was also examined in this study. Nilsson et al. (2016) reported some *Thermus aquaticus*-based Taqs were susceptible to a number of inhibitors. They subsequently found second generation genetically engineered polymerases, such as the KAPA2G Robust polymerase, performed more reliably in the amplification of degraded, contaminated DNA than others. These polymerases were developed specifically to improve amplification consistency, improve tolerance to common inhibitors, and decrease the need for optimisation across sample source and amplicon types (Biosystems 2018). The use of this polymerase in this study certainly improved the success rate of PCR com-

pared with a "wild type" Taq, but is still dependent on the concentration and types of inhibitors present in the DNA extract. Third generation polymerases have now been developed, including one with improved tolerance to the polysaccharides and polyphenolics found in plants. However, it would be interesting to see if the amplification of Russulaceae DNA could be improved with this polymerase as well.

Standard PCR proved to be suitable for the amplification of Russulaceae DNA, with no improvements seen using touchdown or nested PCR. Theoretically, the primer combination ITS1-F/ITS4-B should produce the largest sequence of the primer combinations, whilst ITS1/ITS4 the shortest. Initial amplification using the crude DNA extract and primers ITS1-F and ITS4-B may result in the generation of undetectable concentrations of PCR product even in the presence of inhibitors. However, using this product as the source of DNA for subsequent amplification with the ITS1 and ITS4 primers should minimise the concentration of inhibitors and thereby eliminate inhibitor interference, whilst also providing a more targeted DNA template. This was not the case in this study, though the use of alternative primer combinations may improve this outcome.

Far greater amplification success seemed to be achieved using DNA extracted from fresh specimens rather than dried. However, this was not always feasible or convenient - fungal specimens tend to appear for a short period of time and degrade quickly. Conversely, apart from extending the time available for the processing of specimens, dried tissues have a number of other advantages - in the absence of water, the DNA is less likely to shear; if rehydrated in DNA extraction buffer, degradation is minimised due to the presence of detergents and EDTA (Murray & Thompson 1980); the dried material may also be easier to grind. However, the manner of drying, (or use of other preservation methods) and subsequent storage can have a major impact on the future success of molecular analysis. This would be an ideal area for further optimisation, to maximise the sequencing of future material.

Ultimately, the following molecular approaches were developed:

DNA extraction Where possible, DNA should be extracted from fresh tissue. Where this is not possible, tissue for DNA extraction should be selected, excised, preserved and stored following an optimally described process. This has yet to be determined, and may be dependent on the genus/family/order of interest. An excess of extraction buffer is preferable, particularly if the tissue is dry. This allows for full rehydration, and appears to reduce PCR inhibition. Maximum maceration of the tissue is required, and following the extraction,

the extract should be removed from the tissue debris either through centrifugation or filtration. One should carefully consider whether ethanol precipitation is necessary - while it improves the purity of the DNA, it can substantially decrease the concentration.

PCR Include BSA and DMSO in the PCR mix. Use a polymerase and buffers designed for the amplification of difficult templates.

Unfortunately, these processes were developed over several years, and with improved knowledge a number of issues have since become apparent. For example, where a high concentration of DNA is produced, the effect of dilution on PCR was not examined. Assuming any inhibition is not due to physical damage to the DNA, it is quite possible the inhibitor could be diluted to a point where it no longer posed a problem.

2.4.2 Specimen identification

In order to use sequencing as an aide to specimen identification, a set of criteria needs to be established to filter out highly unlikely matches. The E-value provides an important starting point, as it provides a measure of the number of hits (matches) that could be found by chance. The closer the value to 0, the less likely the match is random. It does not provide a constant value, as it is affected by the size of the database and lengths of the sequences searched as the GenBank database expands, repeat searches will alter the E-value.

Percent identity provides a measure of similarity between two sequences. A value of 97% or greater is frequently used for declaration of specimen identification, allowing 3% variability for natural mutation or PCR or sequencing error (Taylor & Houston 2011). This value was based on the weighted intraspecific ITS variability in the fungal kingdom, the average of which was calculated to be 2.51% (SD= 4.57). However, as the value specifically calculated for the Basidiomycota was 3.33% (SD= 5.62) (Nilsson et al. 2008), the percent identity value of 96% or greater was selected for this study. It should be noted this is an arbitrary value, as the calculations are based on a very small proportion of fungi (estimated to be 0.28% of the currently estimated 1.5 million fungal species) (Nilsson et al. 2008) and the ITS regions of different fungal clades evolve at differing rates (Taylor & Houston 2011) meaning any threshold value selected may be too generous or too strict.

The percent query coverage is also used in the selection criteria as it shows how much

of the query's sequence was used to produce an alignment with a sequence in GenBank. It has been suggested at least 80% query coverage (Raja et al. 2017) be used or 90% for smaller sequences such as those obtained with Next Generation Sequencing (Xu 2016).

The final sequence selection is restricted to those matching the above criteria and being obtained from fully named voucher specimens. This provides useful information such as the species name and specimen details, but also an element of "robustness". In 2003 it was estimated 20% of sequences in one publicly accessible database were unreliable, due to misidentification or the inability to independently verify (lack of voucher specimens or publications) (Bridge et al. 2003).

Finally, by placing the selected sequences in to a phylogenetic tree it can be seen how these specimens are hypothetically related. It is assumed a number of changes occur in DNA over time, leading to an increased divergence and phylogenetic distance between the sequences of different species and their ancestors. Algorithms used to generate phylogenetic trees utilise these variables to infer the optimal and most likely branches (Mitchell 2010). Given the appropriate sequences and parameters in sequence alignment and tree generation, the visual depiction of the relationships can be used to strengthen or weaken an argument for the declaration of species identification.

2.4.3 Descriptions of identified Russulaceae in SEQ

A total of 17 specimen ITS sequences were considered as having a high probability of being identical to ITS sequences currently held in GenBank, the majority of these being Australian. Matches to non-Australian species may suggest the presence of cosmopolitan species, but may also be simply due to high matches over short lengths of DNA.

Five of these specimens showed good matches to *R.* aff. *adusta*, *R.* aff. *compacta*, *R.* aff. *pilosella* and *L.* cf. *wirrabara*. However, these Russulaceae species are yet to be described, a process to which the specimens from this collection will contribute. Currently, there is a lack of descriptions, so they are not covered here.

The Atlas of Living Australia (ALA) is an online database used for the collection and distribution of information related to Australia's biodiversity. As a collaboration between a number of universities, museums and biodiversity-data providers it provides an up-to-date and as accurate as possible source of taxonomic information and has been used in the

generation of the information following. Individual links have not been provided due to potentially frequent changes in the url's for different taxa.

The phylogenetic tree referred to below is shown in Figure 2.5. Relevant matches, and percent identities and query coverage can be found in Table 2.15.

***Russula marangania* (Specimen MB130)**

(a) Specimen MB130

(c) *R. marangania*

(b) Distribution map

Figure 2.6: Appearance and distribution of *R. marangania* in Australia. (a) Specimen MB130 *in situ* (Photograph taken by I. Milinovitch) (c) *R. marangania* (QMS 2011). Scale bar (a) = 15mm (b) = 1000km

Originally described by Cleland as *R. delica* (Cleland 1976), it was renamed in 1976 to *R. marangania* (Grgurinovic 1997). There are 41 records currently held in the ALA database, including the specimen to which MB130 matched.

The pileous of *R. marangania* is described as being a dull milky-white which does not darken upon drying, though it does develop patches of "rusty" stains. These features were also found in specimen MB130.

The taxonomic description, high percent identity and query coverage and adjacent positions on the phylogenetic tree suggest MB130 is an example of *R. marangania*.

The holotype and specimen of the original description were both found in South Australia in the Mount Lofty Ranges (located in the Adelaide suburb of Cleland). The sequence-matching specimen was found at Mount Richmond in Victoria, and specimen MB130 was found in Stanthorpe, Queensland.

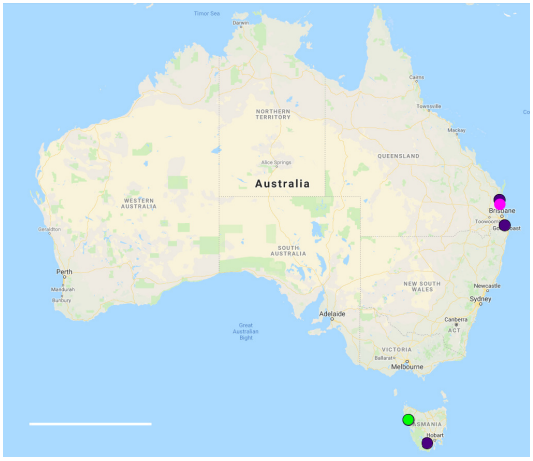
***Multifurca stenophylla* (Specimen DS90)**

Multifurca stenophylla was first described as *Lactarius stenophyllus* in 1860, renamed in 1891 as *Lactifluus stenophyllus*, before obtaining its current name in 2012 (Lebel et al. 2013). Currently there are 6 records of the genus in the ALA (Figure 2.7), one of which was found in the same location as the holotype. The sequence of that specimen matched the study specimen, with a 99.9% identity over 99.9% of the query sequence.

Species of *Multifurca* possess a distinctive concentrically zoned cap, rendering them easily identifiable in the field. They also have comparatively small spores, averaging 6.2 x 4.6μm compared with other Russulaceae species which don't appear to be smaller than 5 x 5μm. These features were both noted on this specimen.

The taxonomy and genetic analysis including the placement on the phylogenetic tree, suggests specimen DS90 is a member of the *M. stenophylla* species.

(a) *M. stenophylla*



(b) Distribution map

Figure 2.7: Appearance and distribution of *M. stenophylla* in Australia. (a) Epitype from Tasmania (Lebel et al. 2013). Scale bar (b) = 1000km

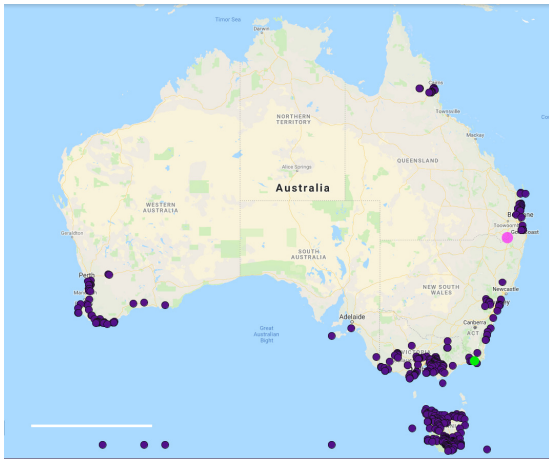
L. eucalypti (Specimen DS82)

L. eucalypti is a small, red-brown species commonly found in Australia, with almost 2400 records held in the ALA database (Figure 2.8). The specimen source of the matching ITS sequence was located in a forest of stringybarks with a dense understorey, and was considered to be uncommon in that area. The holotype was collected in 1979 in Western Australia (WA) and the species named in 2000 (Miller & Hilton 1987). At the time it was noted as the only species of *Lactarius* known in WA.

The initial BLASTn search resulted in the sequence of Specimen DS82 matching to a Slovakian specimen of *Lactarius decipiens*, a species found throughout Europe. The match with the Australian sequence had a higher percent identity but only 80.3% query coverage. However, all three sequences appear together in the phylogenetic tree.

The base of the fruiting body of *L. eucalypti* is generally adorned with short, dark stiff hairs, but these were not evident when examining the dried fruiting body of DS82. The lamellulae present are described as alternating, whilst those of DS82 aren't as numerous. Therefore, it is felt DS82 is closely related to *L. eucalypti* and *L. decipiens*, but is not either species.

(a) Specimen DS82



(b) Distribution map

(c) *L. eucalypti*

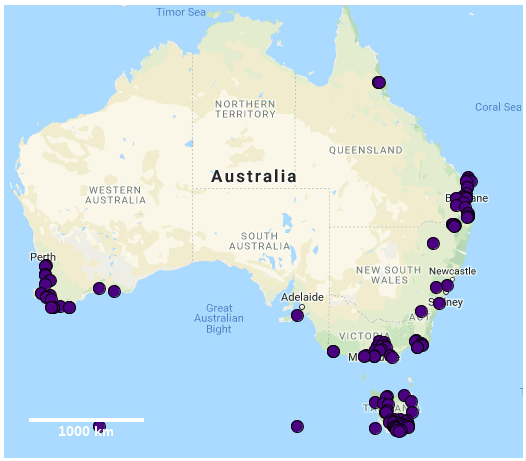
Figure 2.8: Appearance and distribution of *L. eucalypti* in Australia. (c) Rudman (2012) Scale bars (a) = 15mm, (b) = 1000km.

L. clarkeae (Specimen DS71)

Initially described by Cleland (1976) as *Lactarius clarkei*, it was renamed to *Lactifluus clarkeae* by Verbeken et al. (2012). There are currently 552 records of this species in the ALA, 514 of which are Australian (Figure 2.9). The remainder of these records are from New Zealand.

(a) *L. clarkeae*, QMS

(c) *L. clarkeae*



(b) Distribution map

Figure 2.9: Appearance and distribution of *L. clarkeae* in Australia. (a) QMS specimen similar to specimen DS71. QMS (2011) (c) Ridgeway (2016). Scale bar (b) = 1000km

The fruiting body resembles that of *R. flocktonae*, but the production of latex (milk) by *L. clarkeae* immediately discounts it being a species of *Russula*. The pileous surface is described as being finely felted and an ochraceous shade of buff or salmon, with similar colouration often seen in the stipe (Cleland 1976). The description provided for specimen DS71 is much the same, though the colouration is described as shades of orange (QMS 2011). It has been noted that *L. carkeae* has a fishy odour, but this does not appear in all references.

Specimen DS71 was found in Obi Obi, QLD, and the sequence to which it was matched was found in Tasmania. It was a very strong match (99.5%) over the entire sequence, and both sequences are located together in the phylogenetic tree. It is a fairly distinctive species, which suggests DS71 is an example of *L. clarkeae*.

Lactarius leonardii (Specimen DS74)

Lactarius leonardii was first described by Stubbe & Verbeken in 2011, and renamed the following year to *Lactifluus leonardii* (Stubbe et al. 2012). One of its distinguishing features is the pink bruising occuring on its gills, a feature which was also noted in the Queensland Fungal Record prepared for this specimen by Leonard (2015). There are 5 Australian records in the ALA, all occurring in Tasmania, and 2 in New Zealand (Figure 2.10). However, the ALA records do not include the type specimen to which this specimen matched. Interestingly, both specimen DS74 and the type specimen were found in Queensland. DS74 also matched to a sequence from a New Zealand specimen, and these three sequences appear together in the phylogenetic tree. Given specimen DS74 also displayed the distinctive pink bruising, it is most likely a member of this species.

(a) *L. leonardii*, QMS

(b) Distribution map

(c) *L. leonardii*

Figure 2.10: Appearance and distribution of *L. leonardii* in Australia. (a) QMS specimen similar to specimen DS74 (QMS 2011) (c) Image taken from A Field Guide to Tasmanian Fungi (Gates & Ratkowsky 2016). Scale bar (b) = 1000km

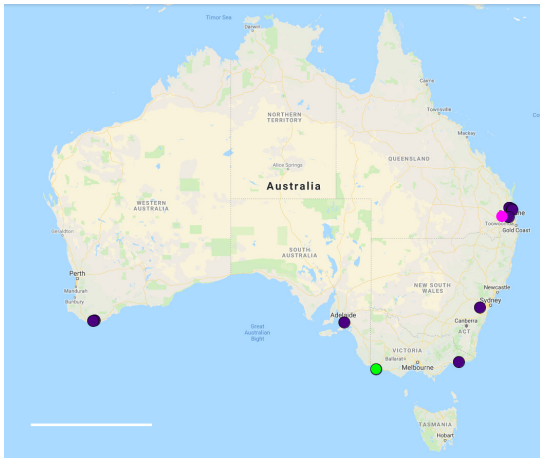
R. wollumbina (Specimens MB132 and MB134)

R. wollumbina was first described in 1997 from a specimen located in South Australia by Grgurinovic. Currently, there are 15 records from Australia (Figure 2.11).

There are a number of inconsistencies between the descriptions of the original, the one provided by the QMS and these two specimens. Firstly, the original description describes

the cap as being pallid whitish in colour, with bright brownish vermilion blotches. At first glance, specimen MB132 appears to be devoid of any bright colour, whereas specimen MB134 appears to be predominantly shades of pink, as does the QMS specimen in Figure 2.11. The stipe is described as strongly tapering ((QMS 2011)), or attenuated downwards ((Grgurinovic 1997)), whilst the stipe of MB132 and MB134 could be described as cylindrical. Microscopic features of the QMS specimen are described as thick walled, while those of the others are described as thin.

(a) Specimen MB132 (b) Specimen MB134



(c) Distribution map

(d) *R. wollumbina*

Figure 2.11: Appearance and distribution of *R. wollumbina* in Australia. Specimens (a) MB132 and (b) MB134 *in situ* (Photographs taken by I. Milinovitch) (d) *R. wollumbina* (QMS 2011). Scale bar (a) and (b) = 15mm (c) = 1000km

Specimens MB132 and MB134 matched with the sequence of a specimen found in Victoria, and all three sequences are placed in the same area of the phylogenetic tree. MB134 also matched to *R. atrovirens*, a species found in New Zealand. This match had a lower percent identity than the match with *R.wollumbina* (97.3 compared with 99.3) but a higher percent coverage (94 compared with 79.6). However, the *R. atrovirens* ITS sequence placed in a different section of the tree.

At this stage, it is felt additional specimens and analysis are required in order to determine the exact relationship between specimens MB132, MB134 and *R. wollumbina*.

R. cheelii (Specimens DS09 and DS88)

The ALA currently lists 32 records for *Russula cheelii*, which was first described by Cleland in 1934. All occurrences are currently known only from Australia (Figure 2.12), and it is common in SEQ where it can be found in wet or dry eucalypt forests. Morphologically, it has a brown-coloured cap and white stipe and gills. The cap is convex with a central depression and is often split at the margin. The specimen descriptions provided by the collector of specimens DS09 and DS88 are consistent with the original.

In a description for a third Queensland specimen collected and described by Leonard (unpublished) it was noted while the spore size of it was within the expected range they tended to fall on the smaller side of the scale. The spores of the originally described specimen measured 8.5 x 6.5μm (Cleland 1976), those measured by Grgurinovic (1997) averaged 6.7 x 10.3μm whereas the Queensland specimens averaged 7.3 x 6.45μm. However, a consensus of the range of spore size cannot be located, but may indicate a variation based on location.

(a) Specimens DS09 and DS88

(c) *R. cheelii*

(b) Distribution map

Figure 2.12: Appearance and distribution of *R. cheelii* in Australia. (a) Dried specimens of DS09 (left) and DS88 (right). (c) Collection from Noosa Botanic Gardens (QMS 2011). Scale bars (a) = 10mm, (b) = 1000km

Despite a strong match (97.5% identity and 100% query coverage) to *R. cheelii* specimen DS88 does not appear with DS09 or *R. cheelii* in the phylogenetic tree. This may be due to the size of the sequences (the sequence of DS88 is 429 basepairs (bp) compared with

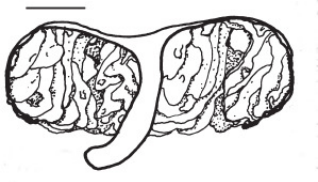
DS09's 492bp and GenBank's JX266623.1 *Russula cheelii* sequence of 558bp) and how they have aligned. A longer sequence for specimen DS88 would be required to clarify its position in relation to DS09 and *R. cheelii*.

R. brunneonigra Specimen DS81

R. brunneonigra was first described by Lebel in 2007, named for its brownish-black coloured cap. It was the ITS sequence from the holotype to which this specimen was matched. To date, there are 2 records available in the ALA (Figure 2.13), both from Northern NSW. However, *R. brunneonigra* is a sequesterate species whilst specimen DS81 is agaricoid. The selection criteria were as previously described - the percent identity was 98.3% over 84.1% query coverage, and both sequences appear in the same area of the tree. This area also includes specimen MB24, an agaricoid specimen, which matched most highly with *R.* aff. *pilosella* (97.1% identity over 80.9% coverage), another sequesterate species. Taxonomically, these are not the same species, but are related as members of the Ingratae section, along with other agaricoid species such as *R. neerimea, R. foetens* and *R. galbana*.

(a) Specimen DS81

(b) Distribution map



(c) *R. brunneonigra*

Figure 2.13: Appearance and distribution of *R. brunneonigra* in Australia. The line drawing of *R. brunneonigra* taken from (Lebel & Tonkin 2007). Scale bars (a) and (b) = 10mm (c) = 1000km

These results highlight some of the difficulties in the identification of the Russulaceae - macroscopic characteristics may or may not be definitive and strict adherance to genetic/sequence criteria (particularly query coverage) may or may not eliminate appropriate matches. This is an area which requires a significant amount of work, and may not be easily resolved.

However, what of the remaining 81 sequences? They formed alignments with other sequences in GenBank, but did not meet the criteria for being "matched", i.e. their sequences share some commonalities, but not sufficient to be classed as identical. This would suggest there are a number of new species potentially to be described.

Chapter 3

A description of new *Russula* species in SEQ

3.1 Introduction

The data described in Chapter 2 demonstrated a large number of ITS sequences of the Russulaceae specimens did not match to any known sequences held in GenBank, including Australian Russulaceae ITS sequences. This suggests that a number of new species exist in the region that can be formally described.

The requirements for the naming of fungi are governed by the current version of the International Code of Nomenclature for algae, fungi and plants (Turland et al. 2018). This document specifies the requirements for publication of a species being named, including the citation of a unique identifier issued by one of the three recognised repositories - Fungal Names, Index Fungorum or MycoBank. Sequencing data and morphological analysis (including illustrations of distinguishing features) are both considered to be "descriptions" and currently only one is required. However, for the publication of new taxa many journals require phylogenetic trees and/or DNA sequence accession numbers as well as a physical description of the species. Therefore it seems prudent to have both.

When describing new fungal taxa, morphological descriptions based on multiple specimens is preferred, as this allows for the development of characteristic-ranges to account for variability due to age, locality and intraspecific variations (Jörger & Schrödl 2013),

though there have been exceptions for rare specimens (Seifert & Rossman 2010). Of the Russulaceae macroscopic characteristics, it would appear no combination is sufficient to determine species, though specimens can be divided into groups or clades within a genus based on these criteria (Romagnesi 1967, Buyck et al. 2008, Crop et al. 2017). Spore print colour and taste of the flesh have been most useful in this regard (Romagnesi 1967), as well as colour changes of the context (flesh) and/or latex presence (Buyck et al. 2008, Crop et al. 2017). However, morphological features have limitations. Growth habit (agaricoid, pleurotioid, secotioid and gasteroid) does not delineate a genus (Buyck et al. 2008). Spore colour range tends to include white, cream, ochre or yellow (Romagnesi 1967) and is not always easy to determine. Environmental factors, specimen age or condition and subjectivity can impact the descriptions of many morphological characteristics. This passage from a description of *Russula emetica* (*Agaricus emetica*) by Hussey (1847) illustrates the difficulties with features such as taste:

> "It gives no warning by its scent or by any other external circumstance of its deleterious quality; if the ignoramus should be tempted to *taste*, for a few moments all appears harmless, for it is *tardily* acrid: but it fully makes up for the delay, as the tortured investigator, with burning lips and fancies, and tearful eyes, seeks in vain for alleviation."
>
> - Mrs T. J. Hussey

Microscopic characteristics, particularly of the spores, may be more valuable in delineating species rather than macroscopic descriptions. Whilst spores vary in shape and size, there is generally insufficient variety to delimit species. However, in species with exceptionally sized spores, it can be useful (Buyck 1991, Shimono et al. 2004). Height and description of spore ornamentation appears to be beneficial (Buyck 1991, Melera et al. 2016), as is the degree of amyloidity of the plage (also referred to as the suprahilar region) (Buyck 1991, Shimono et al. 2004), though the latter can differ with the age and condition of the fruiting body providing the spore sample (Shimono et al. 2004). Descriptions of basidial shape may be valuable, but few descriptions or illustrations are currently available (Romagnesi 1967, Buyck 1991). Examination of the pileipilis can provide useful information, particularly in the description of its layers and cellular forms.

As many morphological characteristics are subjective or dependent on the age and condition of the specimen, molecular taxonomy provides objectivity, ignoring unimportant variations and biases (Buyck et al. 2008). The results outlined in Chapter 2 have demonstrated

the benefit of molecular analysis by utilising sequences of the ITS DNA region in the separation of specimens into species. Subsequently by comparing the sequences obtained, phylogenetic trees can be created to determine evolutionary relationships between multiple species (Kennedy & Clipson 2003, Nugent & Saville 2004, Mitchell 2010). However, many factors influence the usefulness and accuracy of this approach, including sequence and alignment quality (Jörger & Schrödl 2013), the selection and number of DNA regions included (Nugent & Saville 2004, Gadagkar et al. 2005) and ingroup- and outgroup-taxa selection (Moncalvo et al. 2000, Crop et al. 2017).

Possibly due to its status as universal fungal barcode, ITS remains the most frequently used DNA region in fungal phylogenetic analysis (Geml et al. 2010, Avis 2012, Li et al. 2013a, Maba et al. 2014, Kropp 2016, Melera et al. 2016). However, analysis using two (Nuytinck et al. 2007, Lebel et al. 2013), three (Buyck et al. 2008, de Putte et al. 2010, Stubbe et al. 2011, Verbeken et al. 2014, Wang et al. 2018) or more DNA regions (James et al. 2006) are now common place.

The large subunit (LSU) is located adjacent to the ITS-2 region and is used by a number of Russulaceae taxonomists in conjunction with ITS sequences (Buyck et al. 2008, Lebel & Tonkin 2007, Stubbe et al. 2011, Lebel et al. 2013, Verbeken et al. 2014, Wang et al. 2018). It is generally less variable than the ITS region (Nugent & Saville 2004), but contains three highly divergent domains, D1, D2 and D3 (Vilgalys 2018) (Figure 3.1). LSU sequences covering these domains are sufficient in delimiting species (Nugent & Saville 2004, Sonnenberg et al. 2007).

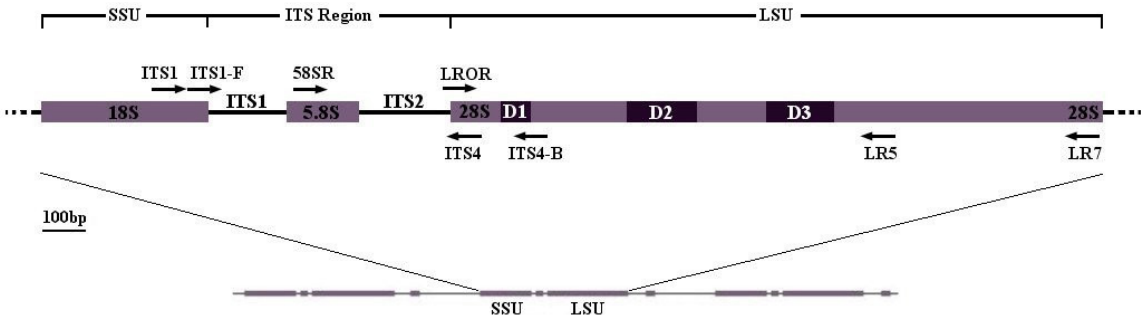


Figure 3.1: Primer map showing approximate positions of ITS and LSU primer annealing sites on a single tandem repeat of RNA (Adapted from Nilsson et al. (2018)).

As a third selection, the second largest subunit of the RNA polymerase II gene, *rpb2* is gaining popularity (Buyck et al. 2008, Stubbe et al. 2011, Verbeken et al. 2014, Wang

et al. 2018). Unlike the ITS and LSU, this region is a single-copy protein-encoding gene (Větrovský et al. 2015). It contains 12 highly conserved regions (Liu et al. 1999, Liu & Hall 2004, Malkus et al. 2006) and a low rate of evolutionary change (Liu & Hall 2004), but shows sufficient variability to resolve phylogenies (Malkus et al. 2006). Other potentially useful barcoding regions include translation elongation factor 1-α (*TEF1*α) (Stielow et al. 2015, Li et al. 2018) and glycoside hydrolase 63 (Pérez-Izquierdo et al. 2017).

The phylogenetic trees subsequently generated can be based on either a single region, or multiple regions through consensus or concatenation. The latter requires formation of a "super-gene" sequence, by sequentially combining the individual sequences into one large sequence. A consensus tree is prepared by comparing phylogenetic trees generated from individual regions, forming a consensus, and a new tree generated from this information (Gadagkar et al. 2005).

Inclusion of an outgroup is crucial in the generation of phylogenetic trees as it grounds or roots the tree and aids in its structure. Outgroup sequences are from taxa related to those being studied, but have some evolutionary distance (Mitchell 2010). The level of relationship appears to be determined by the breadth of taxa being studied. So, if this is confined to specimens within a single clade, outgroups may be sourced from alternative clades (Nuytinck & Verbeken 2003, Das et al. 2018), while a broader study, such as this one of the family Russulaceae, may source outgroup sequences from other families within the Order Russulales. A list of outgroup sequences utilised by researchers of the Russulaceae is provided in Table 3.1.

Whilst it appears few key morphological characteristics are available for identifying the Russulaceae to species, there is still a need for the provision of sufficient morphological taxonomic information (Jörger & Schrödl 2013). For example, in the case of rare or old specimens, the extraction and amplification of DNA may not be feasible, leaving morphology as the sole means for classification (Wiens 2004). It also serves to strengthen molecular-based results, by providing a physical link to phylogenetic hypotheses (Lee & Palci 2015). Conversely phylogenetic analysis has proven useful in the determination of what constitutes a useful morphological taxonomic characteristic (Shimono et al. 2004, Crop et al. 2017).

In this chapter, four of the new species of Russulaceae found in SEQ will be described. Using a combination of ITS and LSU sequences and morphological analysis, it is intended

Table 3.1: Outgroup sequences used by Russulaceae taxonomists.

Family	Species	ITS	LSU	Reference
Albatrellaceae	*Albatrellus skamanius*		AF393044	Buyck et al. (2008)
	A. flettii	AY061738		Miller & Buyck (2002), Lebel et al. (2013), Li et al. (2013a), Kropp (2016)
	A. ovinus	AY198202		Lebel & Tonkin (2007), Li et al. (2012, 2013a)
Amylostereaceae	*Amylostereum laevigatum*	AY781246	AF287843	Buyck et al. (2008), Crop et al. (2017)
Auriscalpiaceae	*Auriscalpium vulgare*	DQ911613	DQ911614	
Bondarzewiaceae	*Bondarzewia montana*	DQ200923	DQ234539	
	B. mesenterica			Li et al. (2012)
	Heterobasidion annosum			Crop et al. (2017)
Echinodontiaceae	*Echinodontium tinctorium*	AY854088	AF393056	Buyck et al. (2008), Crop et al. (2017)
Hericiaceae	*Hericium americanum*	DQ206987		Lebel et al. (2013)
Hybogasteraceae				
Lachnocladiaceae	*Scytinostroma alutum*		AF393075	Buyck et al. (2008), Crop et al. (2017)
Peniophoraceae	*Peniophora nuda*	DQ411533	AF287880	Buyck et al. (2008)
Gloeocystidiellaceae	*Gloeocystdiellum porosum*	AY048881	AY048881	Buyck et al. (2008)
	G. aculeatum	AY061739		Miller & Buyck (2002), Li et al. (2013a)
Stereaceae	*Stereum hirsutum*	AY854063		Lebel et al. (2013)

to determine their phylogenetic placement within the Russulaceae. This analysis may also contribute to understanding the morphological characteristics important for their identification in the field.

Table 3.2: Accession numbers of the ITS and LSU sequences of Australian Russulaceae currently held in GenBank

Species	Accession Number	
	ITS	LSU
Lactarius eucalypti[1]	EU019923	EU019923
Lactifluus cf. *wirrabara*[2,4]	GU258289 to GU258293, GU258303, GU258305 to GU258307, GU258309, JF731000, JF731001	GU265652 to GU265656, GU265663 to GU265667, GU265669, JF731003, JF731002
L. clarkeae[1,3]	HQ318282 to HQ318284, EU019924	EU019924
L. genevievae[2]	GU258294	GU265657
L. leonardii[2]	GU258295, GU258304, GU258308	GU265658, GU265664, GU265668
L. sepiaceus[1]	EU019926	EU019926
Macowanites luteiroseue[1]	EU019946	MF503292
Multifurca sp. (australis)[6]	MH063871	MH063836
M. stenophylla[5,6]	JX266627 to JX266632, MH063864 to MH063868	JX266633 to JX666636, MH063833, MH063864 to MH063867
Russula aff. *adusta*[1]	EU019918	EU019918
R. aff. compacta[5]	JX266625	JX266639
R. aff. pilosella[1]	EU019932	EU019932

...continued on next page

Species	ITS	LSU
R. albobrunnea[1]	EU019933, EU019939	EU019933, EU019939
R. brunneonigra[1]	EU019945	
R. cheelii[5]	JX266623	JX266638
R. clelandii[7]	DQ328136	
R. flocktonae[5]	JX266621, JX266622	JX266637
R. galbana[1]	EU019936	EU019937
R. ingwa[1,5]	EU019919, JX266624	JX266640
R. iterika[1]	EU019929	EU019919
R. kalimna[1]	EU019927	EU019927
R. marangania[1]	EU019930	EU019930
R. neerimea[1]	EU019915	EU019915
R. persanguinea[1]	EU019916	EU019916
R. pilosella[1]	EU019941	
R. pumicoidea[1]	EU019931	EU019931
R. purpureoflava[1,5]	EU019914, EU019917, JX266626	EU019914, EU019917, JX266641
R. reddellii[1]	EU019944	
R. rostraticystidia[1]	EU019938	EU019938
R. sinuata[1]	EU019943	
R. variispora[1]	EU019934	EU019934
R. wollumbina[1]	EU019921	EU019921

[1]Lebel & Tonkin (2007)

[2]Stubbe et al. (2010)

[3]de Putte et al. (2010)

[4]Stubbe et al. (2011)

[5]Lebel et al. (2013)

[6]Wang et al. (2018)

[7] Francis A.A. unpublished (2005)

3.2 Materials and Methods

3.2.1 Sequence grouping

The novel Russulaceae sequences generated in the initial study (see Chapter 2) were compared with each other using the BLAST 2 Sequence feature in order to divide the specimens into any potential species groups. Alignments displaying an E-value of 0 and having a percent identity of 96 or greater with 80% or higher query coverage were classed as highly likely to be of the same species.

3.2.2 Morphological analysis

A comprehensive examination of the morphological characteristics was performed on specimens within each species group. This included examination of microscopic characteristics.

Macroscopic detail

A combination of fresh and dried fruit bodies and photographs of the fresh specimens were used to assess a number of characteristics. Morphology and colour of the cap (pileus), stem (stipe), gill (lamellae) and flesh (context) were noted. The number of gills per half cap was an important feature as it allowed the definition of gill spacing (defined by number of gills per half cap, so that 50 to 60 gills was classed as sub-distant, whilst below that was distant and above was classed as crowded) and allowed quantification of bifurcations and lamellulae (Tables 3.3 and 3.4).

Table 3.3: Description of gill bifurcation quantity. Bifurcations are forks in the gills (circled), where one gill splits into two. In *Russula* spp., they often occur very near the stipe, but can occur anywhere within the length of the gill. If present, the quantity is being defined by the number of bifurcations per gill, per half-cap. Scale bar = 1mm

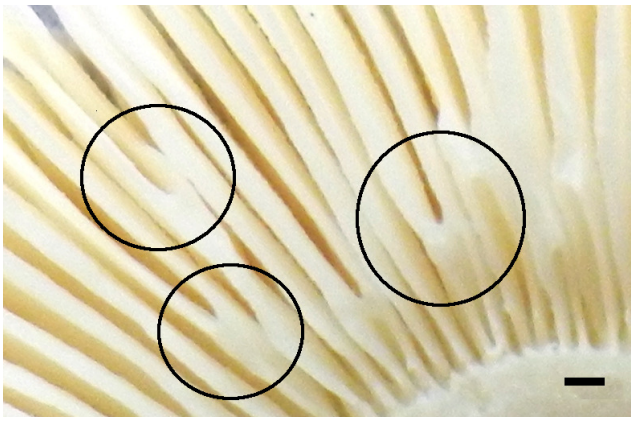
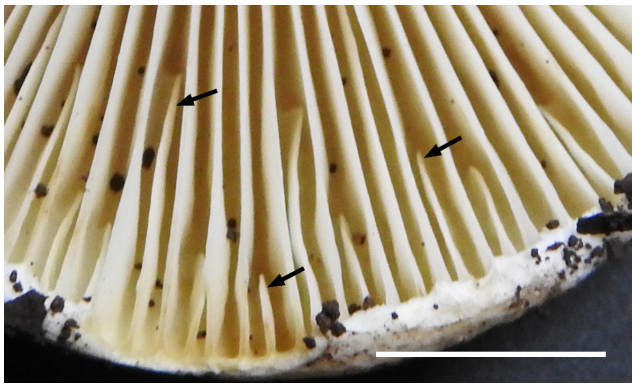
	Quantity (Number of bifurcations per gill)
	None
	Few or sparse (Approximately 1 per 16)
	Medium (illustrated) (Approximately 1 per 6)
	Abundant (Approximately 1 per 4)

Table 3.4: Description of lamellulae quantity. Lamellulae are ”short” gills (indicated by arrows). They range in length from short (less than one third the full length of the gill), to long extending almost to the stipe, but not making contact. If present, the quantity is being defined as the number of lamellulae per full-length gill, per half-cap. Scale bar = 10mm

	Quantity (Number of lamellulae per gill)
	None
	Few or sparse (Approximately 1 per 4)
	Medium (Illustrated) (Approximately 1 per 2)
	Abundant (Approximately 1 for every gill)

Microscopic detail

An OMAX 9.0MP USB Digital Camera inserted in an eyepiece tube of an Olympus microscope and ToupView software were used for obtaining photographs of microscopic features. Measurements were made within the software, having been initially calibrated with a micrometer.

Spores

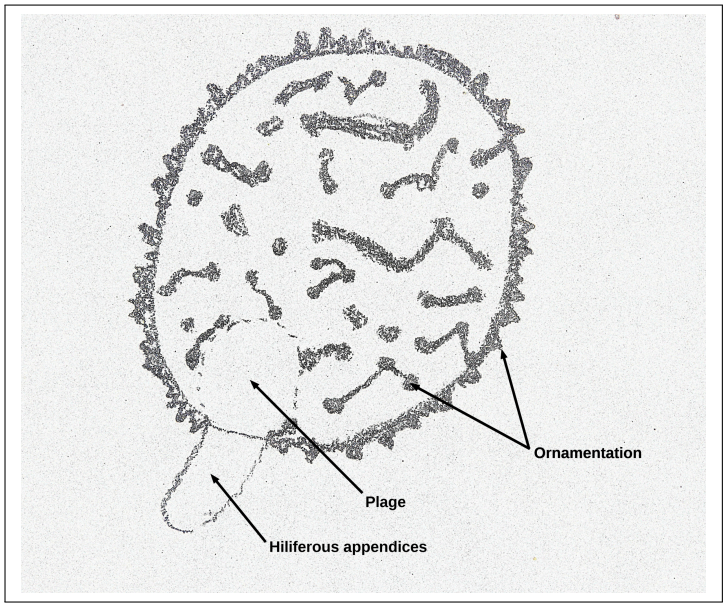


Figure 3.2: Line drawing of a typical Russulaceae spore showing the descriptive features.

A small number of spores were collected from the spore print and suspended in a drop of Meltzer's iodine. Slides were examined under 400 and 1000 x magnification and the following features noted (Figure 3.2):

Amyloid ornamentation The shape and abundance of warts and ridges were described as per Rayner (1968).

Spore dimensions The length and width of 25 randomly selected spores were measured within the ToupView software, which had been previously calibrated with an optical and stage micrometer. Dimensions did not include ornamentation.

Hiliferous appendices Length was measured where possible.

Plage Level of amyloidity where possible.

Where spores failed to deposit in a print, a small amount of tissue from the gill edge was mounted in a drop of Meltzer's iodine and observed as described above.

Tissues

Dried fruiting bodies provided the bulk of the source material. A small amount of tissue was removed and rehydrated in Clemencon's solution or 5% KOH. The tissue was stained with 1% aqueous Congo Red, rinsed in water to remove excess stain, and observed under 400 and 1000 x magnification. The following features were then investigated:

Gill edge Basidia and cystidia size and shape. Number of sterigmata per basidium. Location and relative abundance of cystidia.

Cap surface Description of any unusual cell types present, hyphae and hyphal-termination.

Scanning Electron Microscopy (SEM)

A small drop of water was placed on the centre of a carbon disk attached to a specimen mount, and a number of spores emulsified within. After natural evaporation, the specimens were placed into a desiccator containing fresh silica to ensure no moisture was present. The samples were coated with gold, placed into the holder of a Benchtop SEM (JCM-6000) and photographed. Magnification was determined at the time for each sample.

3.2.3 Genetic analysis

Primers to amplify the LSU were obtained from GeneWorks (Australia) as per Table 3.5. A trial was set up to determine the optimal annealing temperature for each primer pair, and effectiveness with amplifying the fungal DNA extract. A PCR master mix for each primer combination was prepared by combining 120μl ReadyMix (Sigma), 12μl of each 10μM forward and reverse primer, 48μl nuclease-free water and 48μl crude diluted DNA extract from specimen MB09. After mixing, this was dispensed in 20μl aliquots into 12 thin-walled, 0.2ml PCR tubes. Primer combinations included 58SR/LR5, 58SR/LR7, LROR/LR5, and LROR/LR7.

Table 3.5: Primers obtained for amplification of the LSU (after Vilgalys & Hester (1990), Vilgalys (2018))

Primer ID	Sequence (5' – 3')
58SR	TCG ATG AAG AAC GCA GCG
LR7	TAC TAC CAC CAA GAT CT
LR5	TCC TGA GGG AAA CTT CG
LROR	ACC CGC TGA ACT TAA GC

A temperature gradient covering a 15^{o}C range across the 12 tubes was included in the 35 cycles of the temperature sequence 94^{o}C for 1 minute, 40 to 55^{o}C for 30 seconds and 72^{o}C for 1 minute followed by a final extension of 7 minutes at 72^{o}C.

PCR products were visualised on a 2% agarose gel, using 1μl of each product and 0.5% loading dye. The two strongest reactions from each primer combination were combined and made up to 50μl with water. After purification with Daffinity Tips, the samples were adjusted to a concentration of 2.5ng/μl and 10μl sent to the AGRF with 2μl of an appropriate primer for sequencing.

For final species LSU amplification, components of the KAPA2G Robust HotStart PCR Kit (Sigma-Aldrich) were used to prepare a PCR master mix by combining 5.0μl 5x KAPA2G Buffer B, 0.5μl 10mM dNTP Mix, 1.25μl each of primers 58SR and LR5, and 0.1μl of 5U/μl KAPA2G Robust HotStart DNA Polymerase. In addition to the kit components, 1.25μl each of BSA (0.8mg/ml) and pure DMSO, and 13.4μl nuclease-free water were added per 25μl reaction. 24μl aliquots were dispensed into thin-walled, 0.2ml PCR tubes, and 1μl crude DNA extract added. A negative control was also prepared, where nuclease-free water was used in place of the DNA. Thermalcycle parameters comprised of one cycle at 94^{o}C for 3 minutes, 35 cycles of the temperature sequence 95^{o}C, 40.5^{o}C and 72^{o}C, 1 minute for each temperature and final extension of 10 minutes at 72^{o}C.

Following amplification, 2μl of each sample was combined with 1μl of 10x loading dye and electrophoresed on a gel containing 2% agarose in TAE buffer and 5μl/100ml Red Safe dye. A one to three μl volume of MassRuler Express LR Forward DNA Ladder was included in each well flanking the samples. The gel was run at 250V for 15 minutes, before being viewed and photographed on a Quantum ST4 1100 GelDoc system using Quantum-Capt software Version 15.18 (Fisher Biotech).

Following visualisation and confirmation of product presence, concentration and purity, 10μl volumes of suitable products were sent to AGRF at a concentration of approximately 30ng/ml, along with 2μl of primer 58SR or LR5.

3.2.4 Phylogenetic analysis

The ITS sequences described in Chapter 2 were aligned using the MUSCLE algorithm in MEGA X with the default settings. The alignment file (file extension .meg) was imported into Mesquite (Version 3.51) and a consensus sequence obtained for each species group. These sequences were used in a BLASTn search on GenBank, and the highest matched sequence belonging to a vouchered, fully named species obtained. These were combined with the closest Australian ITS sequence matches and four outgroup sequences as detailed below. Sequence alignment was repeated, and used to generate a phylogenetic tree using the Maximum Likelihood method based on the Kimura 2-parameter model and bootstraping with 500 replications. Gaps of 5% or less were deleted.

For the four new species being described, Australian and outgroup specimens with LSU sequences were selected. The sequences were aligned using the MUSCLE algorithm and phylogenetic tree generated using the ML method as described above.

3.3 Results

3.3.1 Sequence grouping

The initial study (see Chapter 2) resulted in 94 basidiomycete-ITS sequences. Two of these were related to *Melanoleuca strictipes* and, as members of the order Agaricales, were not analysed further. Another 12 sequences matched sequences of vouchered, named species, and were described in Chapter 2. The remainder were compared against each other using BLAST 2 Sequence analysis. Based on an identity of 97% or greater, each sequence fell in to one of 10 groups, as shown in Table 3.6.

As they contained the largest number of sequences, specimens from groups A, B, C and D were further documented and their descriptions follow.

Table 3.6: Sequence groups obtained following a BLAST 2 Sequence comparison of the ITS sequences

Number of sequences	Group
7	Group A
13	Group B
6	Group C
16	Group D
3	Group E
3	Group F
3	Group G
4	Group H
4	Group J
24	Ungrouped

3.3.2 Morphological descriptions of new species

Species A

Figure 3.3: Species A, specimen MB87, *in situ*. Scale bar = 10mm

Cap:

3–7cm in diameter, broadly convex with a gradual to sharp depression in the centre (Figure 3.3). Pale yellow when immature (Figure 3.4), becoming mottled with age in shades of pale pink and yellow, darker in the centre than the edges. Surface smooth, dry and no peeling; often split. Smooth to striate, plane margin.

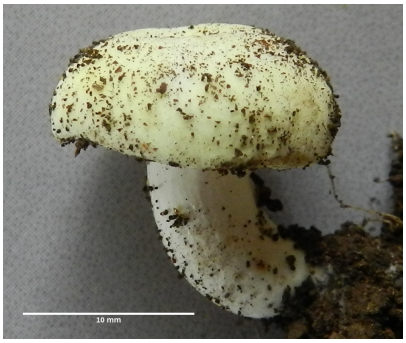


Figure 3.4: Immature specimen of Species A. Scale bar = 10mm.

Stipe:

1.5–2cm diameter by 5–5.5cm in height, central to off-central attachment. Cylindrical with a blunt end; off-white in colour, though a pale pink flush may also be present. Texture fine to smooth.

Gills:

Adnexed to adnate attachment, thick, sub-distant spacing, bifurcations, medium quantity in close proximity to stipe (Figure 3.5) sparse lamellulae of varying lengths. Off-white or cream in colour.

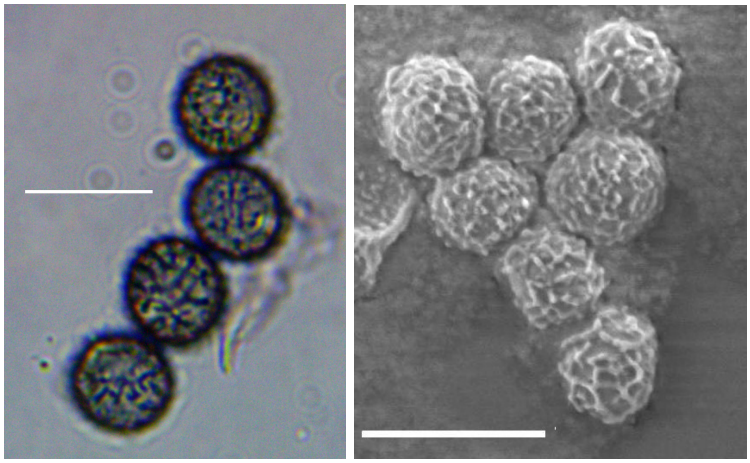
Figure 3.5: Gills of specimen MB87. Scale bar = 5mm.

Flesh:

White, brittle

Spores:

Spore print colour pale cream; spores almost globose (Figure 3.6), 6.8-8.8 x 7.6-9.7 μm. Amyloid ornamentation consisting predominantly of fine ridges and very few individual warts.



(a) Spores stained with Meltzer's iodine (b) SEM view of unstained spores

Figure 3.6: Spores of species A. Scale bar = 10μm

Habit and Habitat:

Generally found in small groups. Dry eucalypt forest on black clay soil covered with leaf litter and grasses. Growth not prominent above ground, tending to be hidden under soil or leaf litter (Figure 3.7). Associated with *Eucalyptus tereticornis*.

Figure 3.7: Typical growth habit of Species A. On the left, a slight mound suggests the presence of a fruiting body. Careful removal of the soil and leaf matter reveals the cap surface. Scale bar = 10mm

Notes: To date, specimens have only been found in a single locality, at Lodbroke near Toowoomba

Collections examined:

GPS location: -27.6750, 151.9927

- MB87, 27 April 2014, Lodbroke
- MB99, 12 Feb 2015, Lodbroke
- MB127, 22 April 2015, Lodbroke
- MB155, 5 Jan 2016, Lodbroke
- MB79, 26 April 2014, Lodbroke
- MB212, Dec 2017, Lodbroke

Species B

Figure 3.8: Species B, specimen MB25 *in situ*. Scale bar = 10mm

Cap:

2.5–5cm. Initially convex and a single shade of reddy-pink (Figure 3.8), becoming flat with a shallow depression and variable shades of colour (reddy-pink) - the pigment appears to fade when exposed to sunlight. Surface margin smooth, becoming striate in dry and older specimens. Cap margin plane and receeded when mature. Surface texture is smooth and peels readily.

Stipe:

Smooth, cylindrical or becoming slightly larger towards the base; generally white developing a pale to dark pink flush after collection. Base blunt to rounded. Generally longer than the cap diameter, ranging from 3–6cm.

Gills:

Adnate to adnexed, distant, thick, very neat. Lamellulae absent, few bifurcations near the stipe (Figure 3.9). White, becoming cream with age, occasionally with a dark pink tinge to the edge.

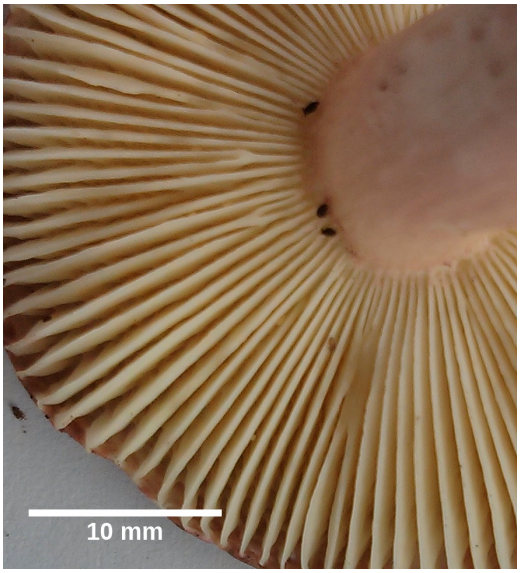


Figure 3.9: Species B, specimen MB69 illustrating the gill pattern.

Flesh:

White and very thin.

Spores:

Spore print white. Spores subglobose, 5.5-6.0μm. Ornamentation strongly amyloid, consisting of small warts and partial mesh (Figure 3.10).

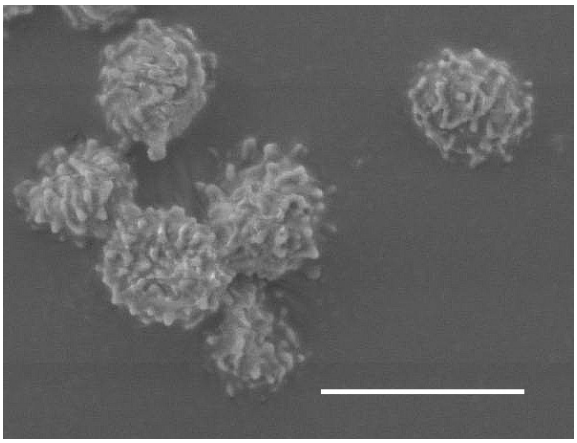


Figure 3.10: Species B, SEM. Scale bar = 20μm

Habit and Habitat:

Individual to scattered group around the base of *Eucalyptus crebra* (Figure 3.11).

Figure 3.11: Species B, specimens MB69 prior to collection. Scale bar = 10cm

Notes:

Appears to be very attractive to a small beetle species making it difficult to find specimens without damage.

Collections examined:

Lodbroke GPS: -27.6749, 151.9933

- MB02, 12 February 2012, Lodbroke
- MB03, 26 February 2012, Lodbroke
- MB09, 23 March 2012, Toowoomba
- MB10 to MB15, 23 March 2012, Lodbroke
- MB23, 17 June 2012, Ravensbourne
- MB25, 2 March 2013, Lodbroke
- MB69, 14 July 2013, Lodbroke
- MB119, 13 April 2015, Wilson's Downfall
- DS01, 17 July 2012, Maroochy BBG
- DS06, 13 July 2012, Dilkusha, Maleny

Species C

Figure 3.12: Species C, specimen MB116 after collection.

Cap:

Convex to broadly flat with a central depression (Figure 3.12). 3.5–8cm, off-white to pale pink with orange to pink areolate squames (Figure 3.13). Striate margin.

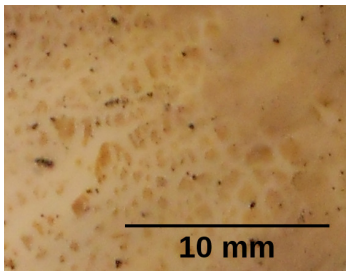


Figure 3.13: Aerolate squames of the cap surface. Specimen MB116.

Stipe:

Cylindrical, white to off-white. 4-6cm in length by 2cm.

Gills:

Adnate, cream (dark), crowded. Lamellulae and bifurcations not present.

Flesh:

White, unchanging on exposure to air

Spores:

Cream spore print. Spores almost globose (Figure 3.14), 5-7 x 6-7μm. Amyloid ornamentation consisting of broad warts mostly connected with cattenate ridges, giving the ornamentation a chunky appearance.

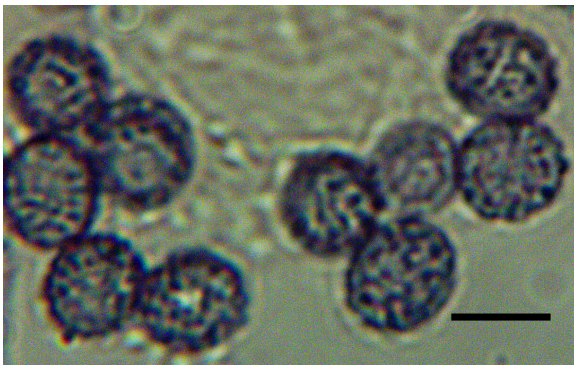


Figure 3.14: Spores of Species C. Spores from specimen MB04 mounted in Meltzer's iodine. Scale bar = 10μm

Habit and Habitat:

Dry eucalypt forest associated with *Allocasuarina* or *Eucalyptus* species.

Notes:

Specimen DS102 was identified in the field as *Russula kalimna* based on the presence of the pale pink areolate cap. However, the size of the spores suggested otherwise. The QMS (2011) describes the spores of *R. kalimna* as being broadly ellipsoid with an average size of 7.3 x 6.3μm. In contrast, none of the spores measured for this species exceeded 7.0μm in size, and were closer in shape to globose rather than ellipsoid.

Collections examined:

Crows Nest GPS: -27.2588, 151.1108

- MB04, 17 March 2012, Crows Nest NP, QLD
- MB61, 21 April 2013, Crows Nest NP, QLD
- MB62, 21 April 2013, Crows Nest NP, QLD
- MB116, 12 April 2015, Wilson's Downfall GPS: -28.7312, 152.0831
- MB121, 12 April 2015, Wilson's Downfall
- DS102, 24 April 2015, Tinaroo Dam Site

Species D

Figure 3.15: Species D, specimen MB73 *in situ*. Scale bar = 10mm.

Cap:

Pale to dark pinks (Figure 3.15), though changeable with environmental conditions and age. 5-11cm, flat with a broad, gradual central depression. Pileus often with cracks or wrinkles, peels readily. Surface edge striate; cap margin plane with gills often exposed.

Stipe:

Cylindrical to tapered with a slightly larger, rounded end. White with a pale pink flush which darkens with age or damage. 4-7cm x 1.5-3cm.

Gills:

Pale cream, adnate with sparse bifurcations and lamellulae.

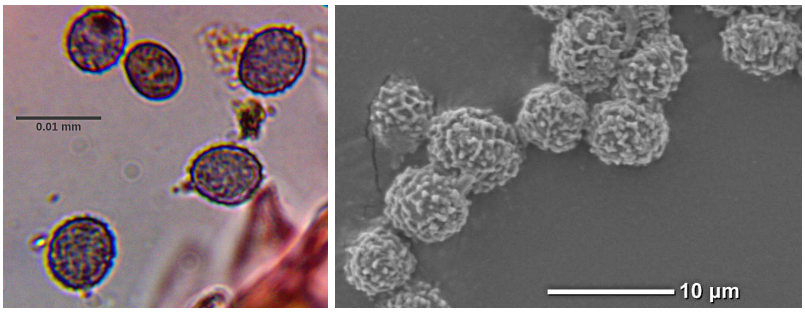
Flesh:

Flesh white to pale yellow, darkening with age or damage

Spores:

White spore print; Spores measuring 6-7 x 7-8μm (Figure 3.16). Sub-globose to ellipsoid.

Ornamentation strongly amyloid, appearing punctate under light microscopy. Amyloid plage; hiliferous appendices 0.1μm.



(a) Stained with Meltzers iodine

(b) SEM

Figure 3.16: Spores of Species D, specimen MB70. Scale bars = 10μm.

Habit and Habitat:

Lawn or on bare, dry soil. Single to several grouped specimens extending from the base of several *Eucalyptus tereticornis*.

Collections examined:

GPS: -27.6750, 151.9927 and -27.6749, 151.9933

- MB05-MB06, 18 March 2012, Lodbroke
- MB17-MB18, 24 March 2012, Lodbroke
- MB52, 15 April 2013, Lodbroke
- MB70, 29 July 2013, Lodbroke
- MB71, 15 April 2014, Lodbroke
- MB72-MB73, 17 April 2014, Lodbroke
- MB84, 27 April 2014, Lodbroke
- MB88-MB92, 3 May 2014, Lodbroke
- MB96, 27 May 2014, Lodbroke

3.3.3 Phylogenetic analysis - ITS

The consensus sequences obtained for each species group were compared with sequences held in GenBank, and the highest matched vouchered, fully-named ITS sequences noted (Table 3.7) and included in the following phylogenetic trees (Figures 3.17 and 3.18).

As Figure 3.17 shows, species A forms a clade with *R. rostraticystidia* and *R. violeipes*, whilst species B, D and G form a clade with *R. tawai* and *R. clelandii*. Species E and F form a cluster along with *R. pumicoidea, R. sinuata* and *R. delica*, species J clusters with *R. virescens, R. iterika* and *R. crustosa* and species H closely groups with *R. atroviridis* and *R. wollumbina*. For species A, B and D these placements are replicated with the Australian species in Figure 3.18. However, there is disagreement between the two ITS trees with the placement of species C. In Figure 3.17 species C has grouped with *R. rostraticystidia* and *R. violeipes*, whilst in Figure 3.18 it has grouped with *R. iterika*.

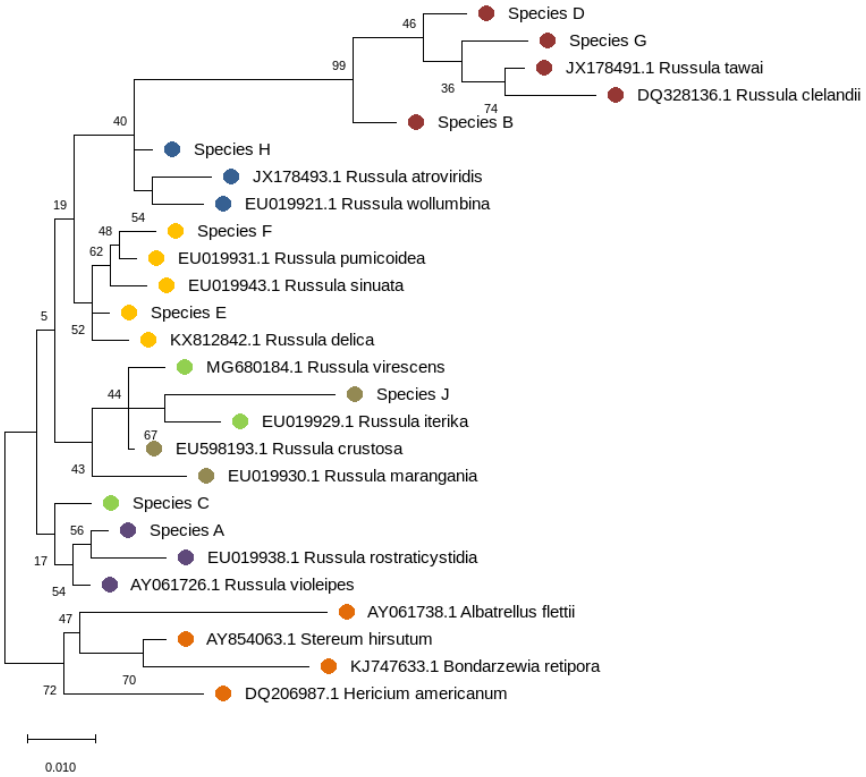


Figure 3.17: Phylogenetic tree including the ITS sequences of the nine new *Russula* spp. from this study. Orange symbols indicate outgroup sequences; remaining colours indicate new species and their matches.

Table 3.7: Species groups and closest match (global) currently in GenBank and closest Australian match.

Species Group	Match	Accession Number	Percent Identity	Australian match	Accession Number	Percent Identity
A	*R. violeipes*	AY061726	91	*R. rostraticystidia*	EU019938	94
B	*R. tawai*	JX178493	90	*R. clelandii*	DQ328136	89
C	*R. virescens*	MG680184	89	*R. iterika*	EU019929	86*
D	*R. tawai*	JX178493	88	*R. clelandii*	DQ328136	88
E	*R. delica*	KX812842	87	*R. pumicoidea*	EU019931	93
F	*R. delica*	KX812842	90	*R. sinuata*	EU019943	89
G	*R. tawai*	JX178493	94	*R. clelandii*	DQ328136	95
H	*R. atroviridis*	JX178493	91	*R. wollumbina*	EU019921	95
J	*R. crustosa*	EU598193	90	*R. marangania*	EU019930	86

* E value greater than 0

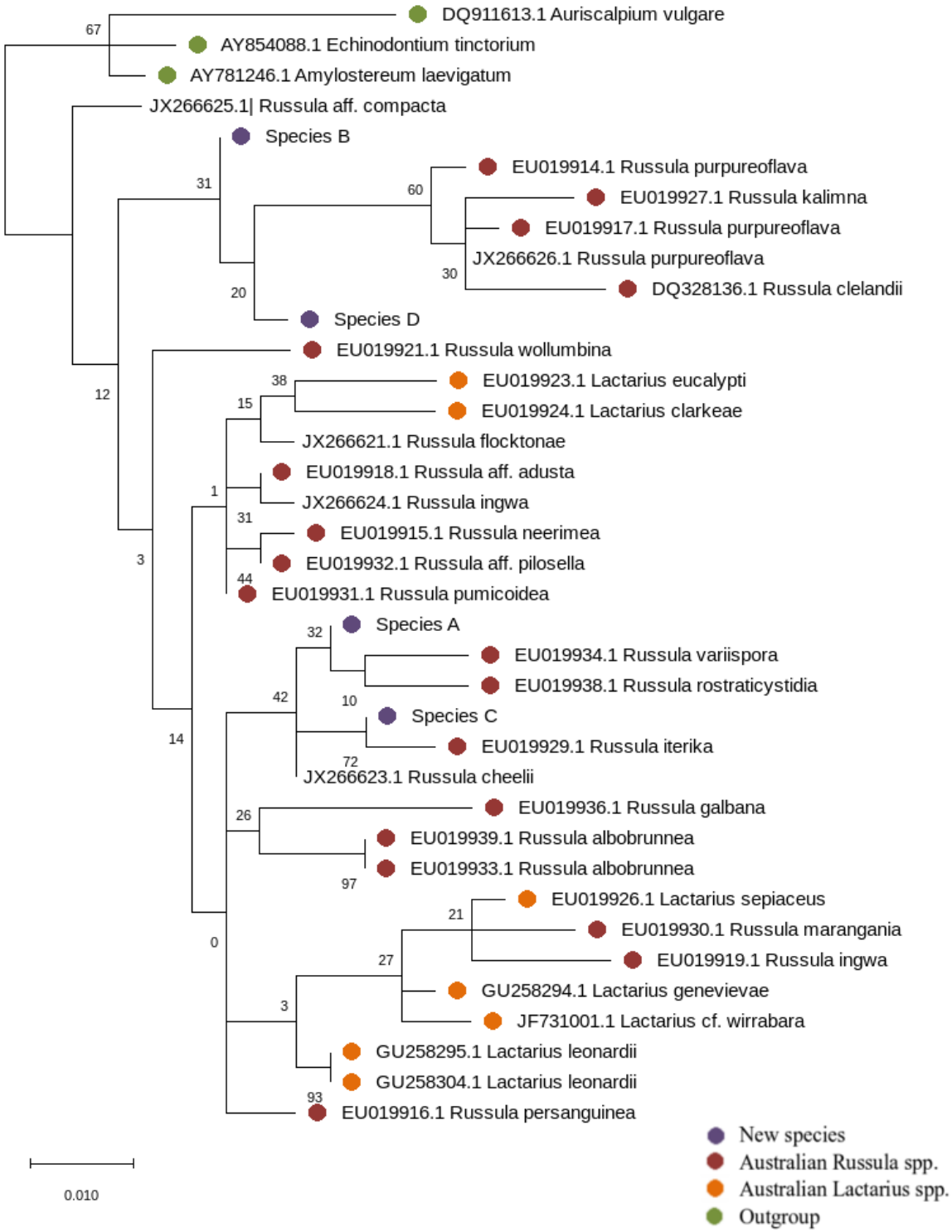


Figure 3.18: Phylogenetic tree comprising ITS sequences of the 4 new species described in this chapter, and Australian *Russula* and *Lactarius* species.

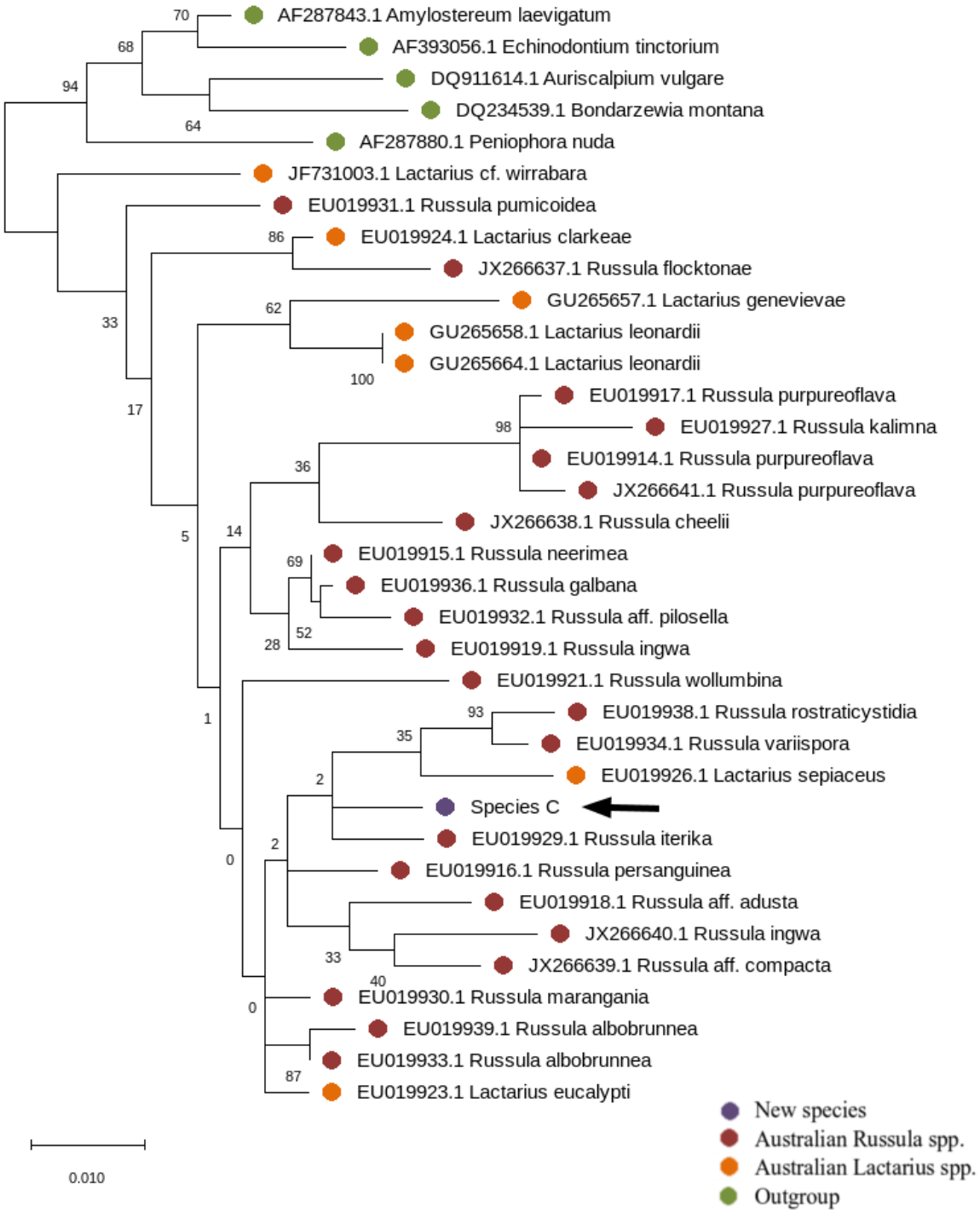


Figure 3.19: Phylogenetic tree comprising 35 LSU sequences, including Species C (indicated by the arrow). The tree with the highest log likelihood is shown (-1877.09) and covers 503 base positions

3.3.4 Phylogenetic analysis - LSU

LSU sequences were obtained for the four new species documented in this chapter. However, the LSU sequences obtained for species A, B and D failed to group with any of the Australian or international species. This is most likley due to poor sequence quality. Species C was subsequently placed near *R. iterika* (Figure 3.19).

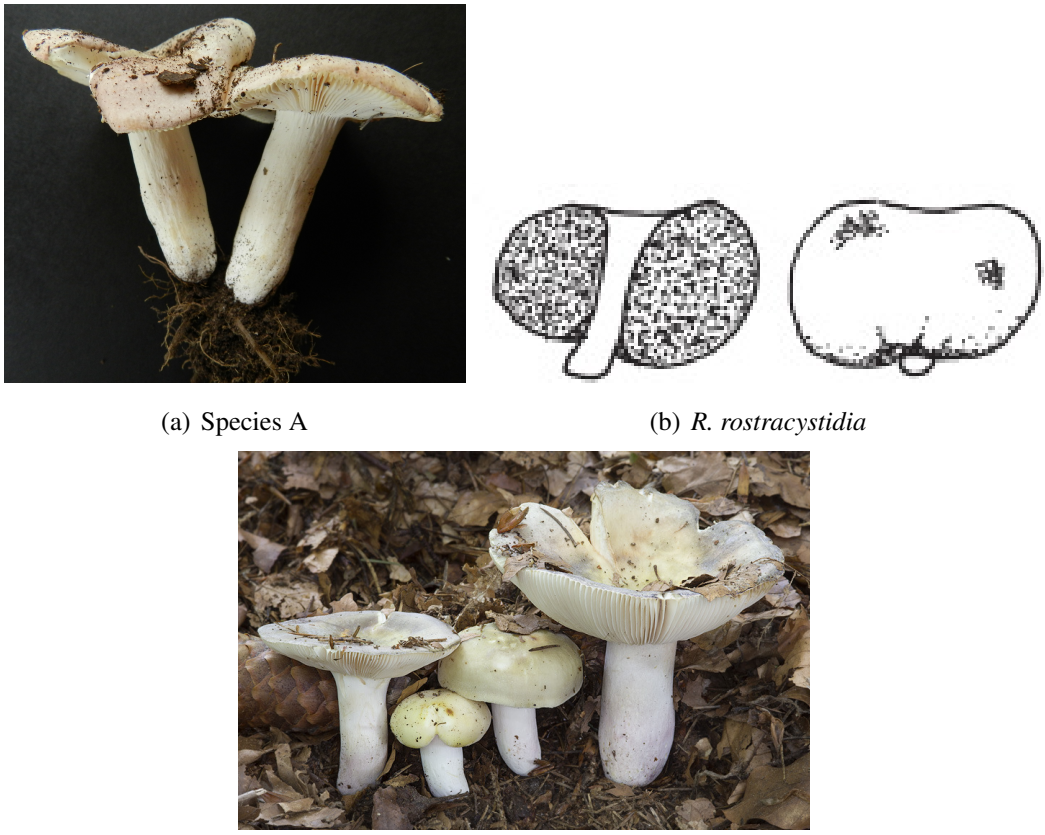
3.3.5 Discussion

Members of the Russulaceae can usually be identified to genus within the field, but identifying to species can be difficult. In this study, 83 specimens were divided into 10 groups by comparing their ITS sequences. One group contained 24 unique sequences and are most likely new species, though it would be preferable if additional specimens are examined before they can be adequately described and named. The remaining 9 groups, with sufficient evidence, may be classed as new species to be described.

The combination of molecular and morphological techniques greatly simplified species separation. By grouping the specimens based on their ITS sequences rather than on subjective, environmentally-sensitive or obscure morphological characters, the risk of misgrouping was reduced, if not eliminated. In addition, as long as a clean sample is obtained for DNA analysis, any specimen can be included regardless of its age or condition. Whilst fresh specimens provide the best opportunity for character description, photographs and preserved specimens were essential for continued reference.

Whilst molecular taxonomy identified these groups as new species, morphological taxonomy can reinforce that outcome. Therefore it is beneficial to compare the morphological features of each new species with their closest matches. (References are made to the ITS-based phylogenetic trees, Figure 3.17 and 3.18).

Species A vs *R. violeipes* vs *R. rostraticystidia* (Figure 3.20)



(a) Species A

(b) *R. rostracystidia*

(c) *R. violeipes*

Figure 3.20: Species A and its closest matches. Specimen MB87 from Species A, drawing of *R. rostraticystidia* taken from its description (Lebel & Tonkin 2007), and photograph of *R. violeipes* taken in the field in the Czech Republic (Zibrova 2016).

The closest match for Species A at 91% identity was *R. violeipes* Quél, a species commonly found in the Northern hemisphere, but not recorded in Australia or New Zealand. The original description, whilst detailed, highlights some of the difficulties in describing Russulaceae fruiting bodies. For example, the colour of the cap lists 21 colour codes for shades of yellows, greens and purples; the odour is described as that of cooking crayfish with a faint hint of Jerusalem artichokes (Quélet 1898). Unless in possession of the appropriate colour code reference and similar light source, and familiar with those scents, the description makes comparisons with other specimens quite difficult.

The closest Australian sequence was that of *R. rostraticystidia* a sequestrate species, resembling a “truffle” (Figure 3.20). This seems to be an unusual correlation, but it may

explain the growth habit of Species A whereby it does not break the soil surface. Interestingly, Figure 3.18 shows *R. variispora* also sits within the Species A/*R. rostraticystidia* clade. It is also a sequestrate species of Australian Russulaceae.

The species grouped together on the trees, with Species C nearby (Figures 3.17 and 3.18).

Species B vs Species D vs *R. tawai* vs *R. clelandii*

Species B and D have similar morphological characteristics (Figure 3.22), with their main difference being the species of their host. This is also the case with the species to which they matched. *R. tawai* is found in New Zealand, and associates with *Northofagus* sp (McNabb 1973). *R. clelandii* has been recorded in most states of Australia, and associates with a number of *Eucalyptus* species including *E. marginata* and *E. diversicolor* (Miller & Hilton 1987).

(a) Species B

(b) Species D

Figure 3.21: Comparison of Species B and D. Specimens of these two species are similarly coloured and share many of the physical characteristics. However, they appear to differ in their host specialisation: Species B was only found in association with the iron bark *E. crebra*, whilst specimens of Species D were in the proximity of the half bark *Eucalyptus tereticornis*. Scale bar = 10mm

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(a) Species B (b) Species D

(c) *R. tawai* (d) *R. clelandii*

Figure 3.22: Photographs of Species B and D and their closest matches. Specimen MB69 (Species B), specimen MB73 (Species D), *R. tawai* taken after collection from Hinewai Reserve, Akaroa NZ (Cooper 2011) and *R. clelandii* taken at Spicer's Gap, QLD (Ryan 2015). Scale bars (a) and (b) = 10mm

The most striking difference between the species is the colouration of the gills. Specimens of *Russula tawai*, possess gills that are a definite yellow, whilst the others are creamy coloured to white. The gills of Species B are bifurcated and have rare lamellulae; Species D specimens have fewer bifurcations which tend to occur near the stipe, and rare lamellulae; the gills of *R. clelandii* have no bifurcations but more lamellulae than Species B and D.

The phylogenetic tree (Figure 3.17) shows these four species and a fifth similarly-coloured species found in Toowoomba (Species G) form a well supported group.

Species C vs *R. virescens* vs *R. iterika*

(a) Species C (b) *R. virescens*

(c) *R. iterika*

Figure 3.23: Photographs of Specimen MB116 (Species C), *R. virescens* (Kuo 2009) and *R. iterika* (QMS 2011)

The caps of *R. virescens* and *R. iterika* contain a substantial amount of green colouration, a colour Species C lacks. However, *R. virescens* and Species C possess aereolate patches on the cap surface, a feature shared by Species J, which is located in the same area of the phylogenetic tree (Figure 3.17). Species J also shares the green colouration of *R. virescens* and *R. iterika* (Figure 3.24).

R. virescens is a European species; *R. iterika* has been recorded in a number of Australian states, including Queensland, NSW and Victoria.

Figure 3.24: Details of Species J. Specimen MB117 shares the green colouration and aereolate cap surface characters with related species. Scale bar = 10mm

Determining which morphological characters are important for *Russula* species identification is a more complex task than first expected. Colour descriptions are limited due to their subjectivity, variability and susceptibility to environmental conditions. However, the persistence and intensity of colouration *may* be useful. Referring again to Figure 3.17, the red colouration of the B, D, G clade is obvious as opposed to the pale, colouration of the species A, *R. violeipes* clade. However objective characters can also have limitations. The presence/absence of bifurcations and lamellulae was not definitive in separating members of the B, D, G clade, though it was useful in the Species E and F clades. This aspect of *Russula* morphology requires further study.

The ITS-sequence based trees were valuable in confirming the relationships between the four new species described. The two trees showed similarity in their placement of Species A, B and D, but there was a discrepancy in the placement of Species C. However, this appeared to be resolved by the LSU tree which grouped Species C with *R. iterika*, which was its closest BLAST match. This demonstrates the usefulness in employing multiple DNA regions for making phylogenetic inferences. Unfortunately, limited success was acheived with the LSU sequences for species A, B and D. This was possibly due to poor sequence quality (species B and D) or poor alignment during analysis (species A). Generally, the amplification of the LSU region has not been problematic in *Russula* phylogenetic analysis (Buyck et al. 2018). However some authors have reported difficulties in obtaining LSU sequences from fungal DNA (Schoch et al. 2012). Others are also starting to question its usefulness in Russulaceae phylogenetics due to a lack of variability between species Li et al. (2018).

In light of the results obtained in this Chapter, it would be worth continuing to characterise the other new species highlighted here, as well as seeking other suitable habitats in SEQ in order to locate and describe additional new species.

Chapter 4

Russulaceae fungi in *Dipodium roseum* roots

4.1 Introduction

Mycorrhizas are symbiotic associations between fungi and the roots of vascular plants which results in an exchange of nutrients - up to 90% of phosphorous and nitrogen is supplied to the plant by the associated fungi while photosynthetically derived carbon is returned (Wadud & Rahman 2010, Kennedy et al. 2011, Okayama et al. 2012). This provides both sides with significant advantages over non-mycorrhizal organisms including allowing plant life to succeed in nutritionally poor soils (Bougher 1994, Wadud & Rahman 2010), improved resistance of plants to biotic and abiotic stresses, and providing fungi with a more consistent environment, facilitating its development of sporocarps and production of spores (Wadud & Rahman 2010, Okayama et al. 2012). Host plants may also enhance the germination rate of the spores of their associated fungi (Ishida et al. 2008).

Mycorrhizas are generally termed ecto- or endo- mycorrhizas dependent on the level of penetration of the fungus in relation to the plant cell wall. They can be further subdivided based on the structures formed by the fungi and/or the plant host specificity. The features of the main associations are outlined in Table 4.1. This functional similarity is a result of convergent evolution across disparate genera, and has evolved over numerous events (Leake 1994, Taylor et al. 2002, Girlanda et al. 2006, Roy et al. 2009, Selosse et al. 2010). Arbuscular mycorrizas are the oldest and most common type of association (Leake 1994,

Brundrett et al. 1996).

Whilst mycorrhizal associations are mostly beneficial to seedlings and adult plants, approximately 10% of vascular plants rely soley on the nutrition provided by fungi for their initial growth (Leake 2005, Kottke et al. 2009). These plants tend to produce numerous small, simple seeds adapted for wind or water (rain drop) dispersal (Leake 1994, Taylor et al. 2002) and lack the energy reserves required to establish seedlings (Kennedy et al. 2011, Okayama et al. 2012, Liu et al. 2015). This level of association is referred to as mycoheterotrophy (Leake 1994). At least 400 plant species are termed *obligate* mycoheterotrophs, relying on fungal organic nutrients throughout their life cycle.

A number of characteristics are shared by obligate mycoheterotrophs, including their preferred habitat (growth in areas of reduced light and dense leaf litter, such as forests), poorly developed root structure, simplified embryos, reduced or absence of chlorophyll (Leake 1994), and the fungi with which they associate tend to form mycorrhizas on the roots of proximate autotrophic species (Girlanda et al. 2006, Riofrio et al. 2013). These plants do not go on to develop chloroplasts, are unable to generate sufficient nutrition, and so are completely dependent on their associated fungi for their entire life (Taylor et al. 2002, Leake 2005, Roy et al. 2009, Kennedy et al. 2011). Approximately 35% of these obligate mycoheterotrophs are from the family Orchidaceae (Taylor et al. 2002), making it the largest family of obligate mycoheterotrophic plants (Leake 2005).

All orchids in natural habitats require the presence of a symbiotic fungal partner. It has been found that the obligate mycoheterotrophic orchids differ in their associated fungi when compared with those that subsequently become autotrophic (Kennedy et al. 2011, Riofrio et al. 2013). The latter tend to associate with a range of Basidiomycota including the families of Tulasnellaceae, Sebacinaceae and Ceratobasidiaceae (Taylor & Bruns 1997, Leake 2005, Huynh et al. 2009, Kennedy et al. 2011, Riofrio et al. 2013). In order to ensure a consistent nutritional supply, the obligate mycoheterotrophs need to associate with the higher ectomycorrhizal fungi of neighbouring autotrophic trees and shrubs (Leake 2005, Girlanda et al. 2006, Kennedy et al. 2011). This results in a tripartite relationship formed between orchid, fungi and tree species (Bougoure & Dearnaley 2005, Leake 2005, Dearnaley 2006, Girlanda et al. 2006, Okayama et al. 2012) enabling organic and inorganic nutrients from the tree to flow to the orchid via the fungal mycelium (Leake 2005, Girlanda et al. 2006, Okayama et al. 2012). This transfer of photosynthetically derived carbon has been successfully demonstrated through radioisotopic studies (McKendrick et al. 2002).

Table 4.1: Distinguishing features of the major mycorrhizal association types. (Modified from Leake (1994))

Mycorrhiza	Type	Fungal Structure	Plant Root Structure	Plant	Fungi
Ectomycorrhizas[1]	Ecto-	Mantle surrounding roots, and Hartig net between root cells	Heterorhizy root system. Consists of distinctive short roots supported by long lateral roots	Gymnosperms and Angiosperms	Basidiomycetes; Ascomycetes
Arbutoid[2]	Ecto-	Hyphal mantle, Hartig net and hyphae penetrating epidermal cells.	The lateral roots branch in a pinnate-cruciate pattern	Subfamily Arbutoideae	Ascomycetes
Ericoid[3]	Endo-	Fungal coils formed in swollen epidermal cells	Fibrous system of delicate roots with reduced vascular and cortical tissue. No root hairs	Family Ericaceae	Ascomycetes
Monotropoid[4]	Endo-	Individual hyphal "pegs" penetrating the epidermal cells	Roots are enclosed in a dense fungal sheath	Subfamily Monotropaceae	Basidiomycetes
Arbuscular	Endo-	Intracellular vesicles or arbuscules formed in the cortical cells	Colonised and non-colonised roots show limited differences[1]	Numerous	Glomeromycetes
Orchidaceous	Endo-	Hyphal coils in the cortical cells (pelotons)	Roots are vermiform to absent and replaced by rhizomes	Family Orchidaceae	Basidiomycetes[4]; Ascomycetes[5]

[1] Brundrett et al. (1996)
[2] Kühdorf et al. (2014)
[3] Read (1996)
[4] Taylor et al. (2002)
[5] Riofrio et al. (2013)

Research has suggested the range of mycorrhizal fungi found within orchids is reasonably narrow, with each species of orchid having unique fungal associates (Dearnaley & Le Brocque 2006, Girlanda et al. 2006, Kennedy et al. 2011), mycoheterotrophic orchid species in particular being restricted to a single fungal genus or a sub-clade within (Taylor et al. 2002, Girlanda et al. 2006, Roy et al. 2009, Kennedy et al. 2011). All of the fungal ITS sequences obtained by Girlanda et al. (2006) from *Limodorum* spp. roots were from the Russulaceae family and eighty percent of these were *Russula* spp. They also found very little overlap of the individual fungal species; Of the nine species of *Hexalectris* studied by Kennedy et al. (2011), four demonstrated highly specific fungal associations, including two predominantly involving different members of the Russulaceae family.

These limited associations do not appear to be as a result of an absence of suitable alternatives (Taylor et al. 2002), nor is it related to soil fungal populations or diversity (Taylor & Bruns 1997, Liu et al. 2015) or species of localised macrofungi (Gardes & Bruns 1993). For example, Liu et al. (2015) found a highly specific relationship between *Mycena* cf. *quiniaultensis* with *Gastrodia flavilabella*. There was an abundance of the fungus present in the orchids' tubers, and very little in the surrounding soil; Taylor et al. (2002) reported in an area of greater than 100m^2, all *Corallorhiza maculata* plants associated with a single species of *Russula* - a species which had never been found fruiting on the plot. It appears the habitat, range and distribution of the plants is dictated by the fungal symbiont (Leake 2005).

The predominant structure formed by the fungus in the orchid root is the peloton, a coil of hyphae within the cortical cells (Leake 1994, Kottke et al. 2009, Kennedy et al. 2011, Riofrio et al. 2013, Pylro et al. 2013). The pelotons are digested by the plant, thus providing it with nutrition (Kristiansen et al. 2001). Two digestive strategies have been identified - tolypophagy, where the whole peloton is broken down and absorbed, and ptyophagy, where the hyphal tips are broken down so the hyphal contents can be released and made available to the orchid (Leake 1994, Pylro et al. 2013). Recent studies have also demonstrated that prior to peloton degradation, there is active transfer of fungal carbon from the live hyphae to the host (Kuga et al. 2014).

Difficulties have been encountered with the identification of the mycorrhizal species associated with obligate mycoheterotrophic orchids as many of the fungi are virtually impossible to culture artificially (Kristiansen et al. 2001, Taylor et al. 2002, Bougoure & Dearnaley 2005, Dearnaley & Le Brocque 2006, Girlanda et al. 2006, Selosse et al. 2010, Kennedy et al. 2011). However, the use of molecular techniques has greatly improved

the ability to clarify the fungi involved (Girlanda et al. 2006, Selosse et al. 2010). DNA is extracted from root samples of the orchid in question, and PCR with fungal-specific primers used to amplify the fungal ITS region. Following electrophoresis and visualisation of PCR products on an agarose gel, a number of procedures can be employed to obtain the final material for sequencing. Earlier research, such as that reported in Dearnaley & Le Brocque (2006), Girlanda et al. (2006), Okayama et al. (2012), tended to rely on cloning, whilst PCR products of later studies were often excised from the gel, purified and sequenced directly (Girlanda et al. 2006, Kottke et al. 2009, Kennedy et al. 2011, Okayama et al. 2012).

Over 1000 orchid species have been recorded in Australia, with approximately 20 of these being obligately mycoheterotrophic. *Dipodium* spp. or hyacinth orchids are large, fleshy, terrestrial orchids commonly seen in summer in Australian woodlands. Most species have limited chlorophyll, appear to be non-photosynthetic and rely on mycorrhizal fungi for their nutrition (Dearnaley & Le Brocque 2006). The flowers are required for identification of the species in the field - descriptions can be found in Table 4.2. Eleven species are currently known in Australia, some of which are considered rare or threatened.

Species of the Russulaceae family are ectomycorrhizal with a large range of plant families including Myrtaceae (Dearnaley & Le Brocque 2006), Dipterocarpaceae (Wadud & Rahman 2010), and Fagaceae (Lamus et al. 2012), particularly in well established forests (Nara 2009). However, Taylor & Bruns (1997) found they also formed endomycorrhizal associations with the Orchidaceae - they have been found in a number of orchid genera including *Corallorhiza* (Taylor et al. 2002, Girlanda et al. 2006), *Dipodium* (Bougoure & Dearnaley 2005, Dearnaley 2006, Dearnaley & Le Brocque 2006), *Limodorum* (Girlanda et al. 2006), *Hexalectris* (Kennedy et al. 2011) and *Lecanorchis* (Okayama et al. 2012).

DNA analysis has demonstrated the presence of *Russula* species within the roots of *Dipodium variegatum* (Bougoure & Dearnaley 2005, Dearnaley 2006) and *D. hamiltonianum* (Dearnaley 2006, Dearnaley & Le Brocque 2006). The fact that these plants consistently grew close to the base of *Eucalyptus* and *Callitris* trees suggested that the orchids acquire carbon and minerals from the tree host via an ectomycorrhizal connection. None of the sequences obtained in these studies matched to known Australian Russulaceae.

Table 4.2: Basic identification of achlorophyllous *Dipodium* spp. found in eastern Australia

	Base Flower	Labellum	Labellum		Petal markings		Other
Dipodium	Colour	Colour	Pattern	Hairs	Colour	Pattern	features
roseum	Pink	Pale pink	Dark stripes	Mauve	Dark pink	Diffuse	
pulchellum	Pink	Dark red	Darker stripes	Mauve	Dark red	Large, diffuse	Straight petals
variegatum	Cream to pale pink to pale green	Maroon	Darker stripes	Mauve	Maroon	Distinct	Spotted pedicel and ovaries
punctatum	Pink	Dark pink	Solid colour	Mauve	Purple-red	Dense, distinct spots	
elegantulum	Pink	Dark pink	Solid colour	White	Dark pink	Sparse, small, distinct	
atropurpureum	Dark reddish purple	Dark reddish purple	Solid colour	Mauve	Darker	Diffuse blotches	
pardalinum	Pale pink to white	White	Solid colour	White	Dark red	Small, distinct spots	
hamiltonianum	Greenish yellow	White and pink	Gradient colour	White	Deep red	Small, distinct spots	

In this project the fungal endophytes of the Australian orchid *Dipodium roseum* were investigated. As this orchid species and its fungal associates have not been examined previously, and given the results of previous research into mycorrhizas of other *Dipodium* species, it is highly likely that new species of SEQ Russulaceae will be found.

Flower spikes of *D. roseum* generally emerge from the soil in early November and had to be observed so as to confirm identification. They are readily identifiable as one of the few *Dipodium* sp. to have distinctive stripes on the labellum. DNA was extracted from colonised roots and pelotons isolated from the tissue. PCR amplification was conducted using fungal specific primers and following DNA purification, appropriate products were sent to the Australian Genome Research Facility (AGRF) for Sanger or Next Generation sequencing (NGS) utilising Illumina MiSeq technology. BLASTn searches of returned sequences against the GenBank database revealed a variety of fungi colonising the roots of *D. roseum*. This included a number of fungi not previously found as root endophytes of Australian Orchidaceae species.

4.2 Materials and Methods

4.2.1 Collection

Several healthy flower spikes of *D. roseum* were located, and the soil gently removed from the base of the plant to expose roots. Root samples were collected over several seasons, predominantly from the site at Stanthorpe, which was used due to its comparative ease of access and previous observations of the orchid's presence. The area was also known to host a high population and diversity of epigeous Russulaceae species, predominantly associating with species of *Eucalyptus*. Fresh scalpel blades were used to remove a small portion of the root and the sections briefly examined to ensure the cortex contained brown colouration, suggesting the presence of pelotons. Suitable sections were placed into labelled plastic bags or 1.5ml microfuge tubes and placed onto ice for transport back to the laboratory where they were subsequently placed at -20^{o}C until the DNA could be extracted.

4.2.2 Photomicroscopy

Frozen roots were allowed to thaw before being hand-sectioned as finely as possible with a fresh scalpel blade. Suitable sections were transferred to the well of a single concave microscopy slide, covered with a small amount of water and coverslip. The sections were initially observed without stain under 400x magnification on an Olympus CKX53 inverted microscope. Whilst pelotons were clearly visible, a small amount of 0.5% Trypan blue in lactoglycerol (1:1:1 lactic acid:glycerol:water) was added to enhance their presence. For viewing at 1000x magnification, a small amount of the stained tissue was transferred to another drop of water on a microscope slide and covered with a coverslip. Pressure was carefully applied to the coverslip, rupturing the plant cells and allowing their contents to spread out. An OMAX 9.0MP USB Digital Camera inserted in an eyepiece tube of an Olympus microscope and ToupView software were used for obtaining photographs of the isolated pelotons.

4.2.3 DNA extraction and PCR

DNA extraction

Frozen roots were rinsed in sterile water to remove excess soil and allowed to thaw before being hand-sectioned with a fresh scalpel blade. Sections were transferred to a sterile petri dish and examined under a microscope at up to 100x magnification. Sections containing pelotons were identified and aseptically transferred to sterile 1.5ml microcentrifuge tubes.

Initially, DNA extraction was performed on the complete tissue by direct addition of the appropriate volume of extraction buffer, maceration of the material with a plastic micropestle and the remaining procedure followed to completion.

Later extractions were performed on a crude peloton concentrate. Approximately 500μl of sterile water was added to the tube and the tissue macerated with a micropestle to release the pelotons. Up to 400μl of the liquid mixture was transferred to a fresh tube, leaving heavier debris behind, and centrifuged at 33,000g for 5 minutes. The supernatant was discarded and the pellet resuspended in 200μl of extraction buffer.

In both cases, DNA was extracted with either a DNeasy Plant Mini Kit, or the Extract-

N-Amp Plant PCR kit following the manufacturer's instructions. For the Qiagen kit, this involved tissue maceration or resuspension of the pellet in 400μl of Buffer AP1 and 4μl RNase A. After a brief vortex, the samples were incubated for 10 min at 65^{o}C with an occasional mix. After the addition of 130μl of buffer, the tubes were placed on ice for at least 5 minutes. The lysate was centrifuged at 15,000g for 5 minutes, an optional step recommended by the manufacturer. The supernatant was transferred into a QIAshredder spin column which was placed into a 2ml collection tube. After 2 minutes of centrifugation at 20,000g, the flow-through was transferred to a fresh 1.5ml microcentrifuge tube. The volume was estimated and a 1.5 x volume of Buffer AW1 added (note this buffer and Buffer AW2 required prior dilution with ethanol). After mixing, approximately half of the volume was transferred into a DNeasy Mini spin column placed in to a 2ml collection tube. The sample was centrifuged for 1 minute at 6,000g, and the flow-through discarded. This step was repeated with the remaining volume. The spin column was transferred to a fresh 2ml collection tube, and 500μl of Buffer AW2 added. The sample was centrifuged at 20,000g for 2 minutes, the flow-through discarded and the wash repeated. Care was taken to ensure the column did not come into contact with the discarded liquid. The spin column was finally transferred to a fresh 1.5ml microcentrifuge tube, and the sample incubated in 100μl of Buffer AE at room temperature for 5 minutes. After centrifugation for 1 minute at 6,000g, this step was repeated, producing 200μl of DNA extract.

When using the Extract-N-Amp kit, 100μl of extraction buffer was used for tissue maceration or peloton pellet resuspension. This was followed by incubation for 10 minutes at 95^{o}C, after which 100μl of diluent was added.

DNA extraction success was confirmed by gel electrophoresis. 3μl of each extract was combined with 2μl of 10x loading dye (ThermoFisher) and electrophoresed on a gel containing 1% agarose in TAE buffer and 5μl/100ml Red Safe dye (Scientifix, South Yarra, VIC, Australia). The gel was run at 250V for 15 minutes, before being viewed and photographed on a Quantum ST4 1100 Gel Doc system using Quantum-Capt software Version 15.18 (Fisher Biotech).

All extracts were stored at 4^{o}C until being used for PCR or Next Generation Sequencing (NGS).

PCR

A PCR master mix was prepared with reagents from either the Extract-N-Amp Plant PCR kit or KAPA2G Robust HotStart PCR Kit (Sigma-Aldrich, Castle Hill, NSW, Australia). The Extract-N-Amp kit contained a 2x PCR Ready Mix and required the addition of 1μl of each 10μM primer (from the combinations listed below), 1μl of each PCR additive (DMSO and 8mg/ml BSA) and 5μl of DNase-free water to 10μl of Ready Mix per 20μl reaction.

The KAPA2G kit contained separate reagents. Thus, the master mix was prepared by combining 13.4μl DNase-free water, 5μl of 5x Buffer B, 0.5μl of 10mM dNTP mix, 1.25μl of each 10μM primer, 1.25μl of each PCR additive (DMSO and BSA) and 0.1μl of 5U/μl HotStart DNA Polymerase per 25μl reaction.

Primer pairs used were either ITS1-F/ITS4 or ITS1-F/ITS4-B (White et al. 1990, Gardes & Bruns 1993). After dispensing either 19μl (Extract-N-Amp) or 24μl (KAPA2G) volumes of master mix in to thin-walled 0.2ml tubes, 1μl of extracted DNA was added.

Cycling reactions consisted of one cycle at 94^{o}C for 3 minutes, 35 cycles of the temperature sequence 95^{o}C, 50^{o}C (ITS4 set) or 55^{o}C (ITS4B set) and 72^{o}C, 1 minute for each temperature and final extension of 10 minutes at 72^{o}C. A touchdown cycle was also used for some runs which replaced the 35 cycle temperature sequence with 10 cycles of 95^{o}C, a 1^{o}C reduction from x to y, and 72^{o}C, followed by 25 cycles of the initial temperature sequence.

Following amplification, 3μl of each sample was combined with 2μl of 10x loading dye and electrophoresed on a gel containing 2% agarose in TAE buffer and 5μl/100ml Red Safe dye. Either one or threeμl volumes of MassRuler Express LR Forward DNA Ladder (ThermoFisher) were included in each well flanking the samples. The gel was run at 250V for 15 minutes, before being viewed and photographed on the GelDoc system.

PCR products of sufficient concentration and expected size (approximately 600-800 bp) were purified using either the QIAquick (Qiagen) or Monarch PCR and DNA Cleanup Kit (GeneSearch, Arundel, QLD, Australia). Both kits follow the same process – ethanol was added to the DNA Wash Buffer as directed by the manufacturers. 20μl of PCR product was combined with 100μl of DNA binding buffer before being transferred to a spin column sitting in a collection tube. Following centrifugation at 15,000g for one minute

the flow through was discarded and DNA wash buffer added, either in two lots of 200μl (GeneSearch) or a single application of 750μl (Qiagen). Following centrifugation, the spin column was transferred to a fresh microcentrifuge tube and the DNA eluted in 8-30μl of either elution buffer or nuclease-free water pre-warmed to 55^{o}C. The solvent was allowed to sit on the filter surface for up to three minutes, before centrifugation to elute the DNA.

Following visualisation and confirmation of product presence, concentration and purity, 10μl volumes of suitable products were sent to AGRF at a concentration of approximately 30ng/μl, along with 2μl of either ITS1-F or ITS4-B (10μM), depending on the original primer pair used.

Sequence chromatograms were manually checked for accuracy and corrected if necessary, using the software FinchTV version 1.3.1 (Geospiza Inc). Corrected sequences greater than 300 bases were combined into a single text file, formatted and saved in Fasta format.

Initially the file was used as data input to perform BLASTn searches on GenBank. A number of sequences were obtained from these results for inclusion in phylogenetic analysis. Sequence selection was based on a high percent identity, an E Value of 0 and match to a fully named species, preferably from vouchered specimens.

Next Generation Sequencing

Prior to submission of the DNA for diversity profiling by NGS, the Monarch PCR and DNA Cleanup Kit was used to purify the DNA extract. Apart from a change in the ratio of binding buffer to sample (2:1 rather than the 5:1 as used for purification of the PCR products above) the method was identical to that as previously described. DNA was eluted in 30μl pre-warmed nuclease-free water. Following electrophoresis to estimate DNA concentration, 20μl volumes containing a DNA concentration of 1 to 50ng/μl were submitted to AGRF.

The ITS region was amplified using primers ITS1-F and ITS2 with overhang adapters. Illumina's Nextera XT Index Kit was subsequently used to attach indices and sequencing adapters, and the Illumina MiSeq platform utilising Paired End sequencing chemistry used for sequencing.

4.2.4 Phylogenetic Analysis

Russulaceae sequences obtained from the pelotons and their two closest matches were combined with the Australian ITS sequences described in chapter 2 and outgroup sequences described in chapter 3 and aligned in MEGA X using MUSCLE with the default settings. A phylogenetic tree was created by using the Maximum Likelihood method based on the Kimura 2-parameter model and bootstrapping with 500 replications. Gaps of 5% or less were deleted.

4.3 Results

4.3.1 Collection details

A number of plants were successfully located and confirmed as being *D. roseum*, an example of which is shown in Figure 4.1.

A total number of 15 root samples were collected in November or December over the course of five years, all of which appeared to contain pelotons. All habitats were dry eucalypt woodlands, with sparse grasses. Details of samples collected are provided in Table 4.3.

4.3.2 Photomicroscopy

Microscopy confirmed the presence of pelotons in all root samples, though the density was quite variable. Staining was not required to visualise the structures, but was performed in order to enhance detail. As can be seen in Figure 4.2, the pelotons have absorbed more stain (trypan blue) than the surrounding plant tissue, and at 400x magnification, hyphae can be seen.

(a) Several plants on location

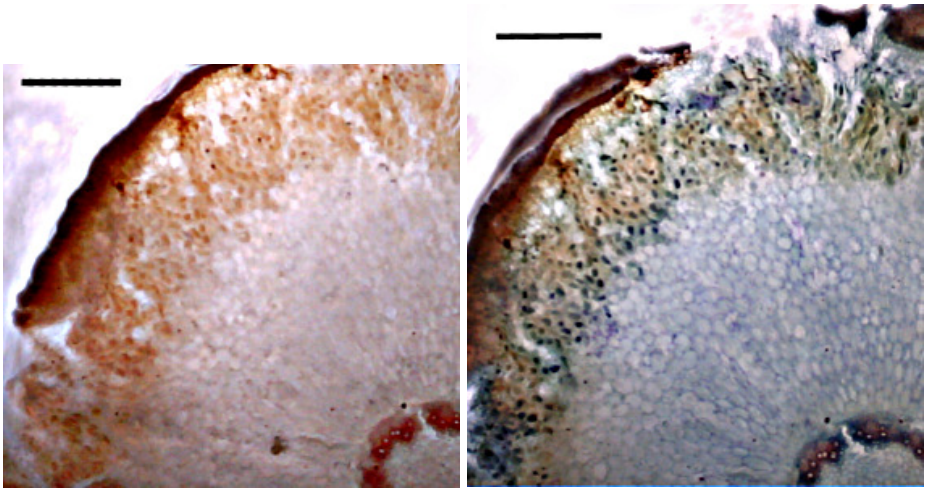
(b) Inflorescence of *D. roseum*

(c) Single flower of *D. roseum*

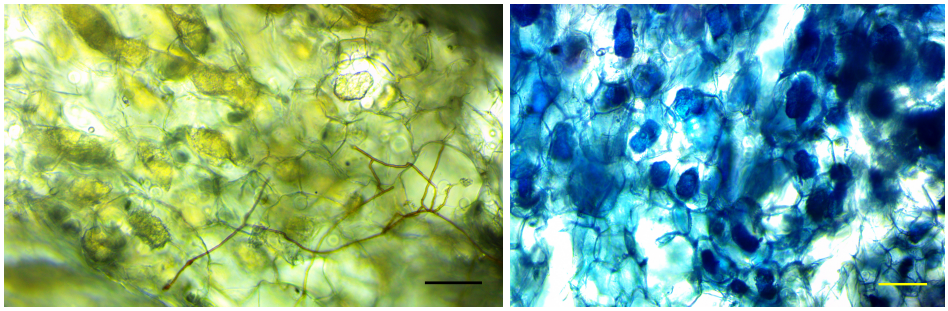
Figure 4.1: Flower stalks of *Dipodium roseum* and close up of a flower. Note the striped labellum.

Table 4.3: Date and collection site of *D. roseum* root samples

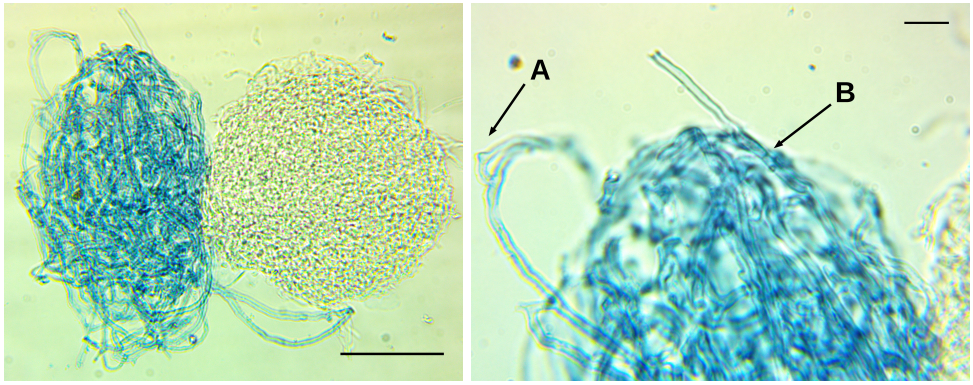
Specimen	Date	Location	Nearest tree species
OR01	08/12/13	Hell Hole Creek	*E. crebra*
OR02	27/11/14	Tully Park	*E. youmanii*
OR03	27/11/14	Tully Park	*E. banksii*
OR04	27/11/14	Tully Park	*E. andrewsii*
ORS1	08/12/15	Tully Park	*E. andrewsii*
ORS2	08/12/15	Tully Park	*E. andrewsii and E. youmanii*
ORS3	08/12/15	Tully Park	*E. youmanii*
ORP1	04/01/17	Tully Park	*E. andrewsii and E. youmanii*
ORP2	04/01/17	Tully Park	*E. andrewsii and E. youmanii*
ORD1	09/11/17	Tully Park	*E. andrewsii*
ORD2	09/11/17	Tully Park	*E. andrewsii*
ORD3	09/11/17	Tully Park	*E. andrewsii and E. youmanii*
ORD4	09/11/17	Tully Park	*E. andrewsii*



(a) Stain-free section (b) Same section stained with Trypan blue



(c) Unstained root section (d) Stained



(e) Individual pelotons (f) Important features of a peloton

Figure 4.2: Sections of the root of *D. roseum* showing pelotons within the plant cells. Photographs (a) and (b) show the placement of the pelotons within the orchid root. As can be seen, staining is not necessary for visualisation. A hyphal strand can be seen in the lower part of photograph (c). This is unlikely to be related to the pelotons, but highlights the presence of other fungi in the vicinity. The pelotons can be clearly seen within the plant cells in photograph (d). Photographs (e) and (f) show individual pelotons where clamp-like structures (A) and septate hyphae (B) can be seen. Scale bars (a) and (b) = 1mm, (c) and (d) = 100μm, (e) = 50μm, (f) = 10μm.

4.3.3 PCR, Sanger and next generation sequencing

PCR

A representative example of a gel photograph can be seen in Figure 4.3. Most PCR reactions produced multiple bands for each sample. A summary of the PCR results is shown in Table 4.4.

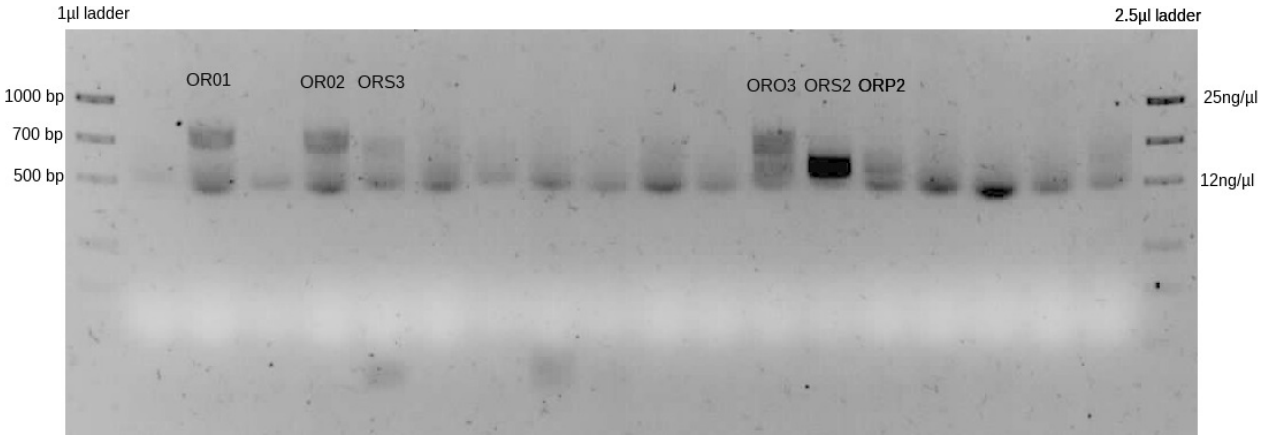


Figure 4.3: Peloton PCR gel photograph. Primers used for this PCR were ITS1-F and ITS4. Samples of extracted DNA were sent to the AGRF for sequencing, the results of which are shown in Table 4.5.

Sanger sequencing

Of the 29 products submitted for Sanger sequencing, 2 produced usable sequences. A summary of the results obtained is available in Table 4.5. The sequences obtained for specimens OR01 and OR03 can be found in Appendix D.4.

Table 4.4: Basic summary of PCR products obtained from various runs

Set	Number of samples	Tissue type	Extraction method	Primer Pair	Program	Products obtained	Sequence results
1	4	Root sections	Qiagen	ITS1-F+ITS4	Standard	0	n/a
2	9	Root sections	Sigma + EtOH	ITS1-F+ITS4-B	Standard	1	No result
3	16	Peloton positive root sections	Sigma + EtOH	ITS1-F+ITS4	Touchdown	1	No result
4	6	Peloton positive root sections	Sigma + EtOH	ITS1-F+ITS4-B	Touchdown	0	n/a
5	3	Pelotons	Qiagen and Sigma	ITS1-F+ITS4	Standard	1	No result
6	6	Pelotons	Sigma	ITS1-F+ITS4-B	Standard	6	No result
7	6	Pelotons	EdNa	ITS1+ITS4-B	Standard	0	n/a
8	17	Pelotons	Sigma	ITS1-F+ITS4	Standard	7	2x Russulaceae, 2x Ascomycetes, 3x no result

Table 4.5: Sanger sequencing and subsequent BLASTn search results

Specimen	Accession Numbers	Short Description	Percent Identity	% Query Coverage
OR01	EU019941.1	*Russula pilosella*	98.98	89
	KY697597.1	*Macowanites sp.*	96.82	93
OR02	Failed to sequence			
OR03	Failed to sequence			
OR04	Failed to sequence			
ORS1	Failed to sequence			
ORS2	Ascomycetes sequences returned			
ORS3	JN388955.1	*Lactarius bicolor*	94.57	95
ORP1	Failed to sequence			
ORP2	Ascomycetes sequences returned			
ORD1	Failed to sequence			
ORD2	Failed to sequence			
ORD3	Failed to sequence			
ORD4	Failed to sequence			

Next Generation Sequencing

A total of 339 Operational Taxonomic Units (OTU's) were obtained though over half were of unidentified fungi. Ascomycetes represented almost 30% and Basidiomycetes another 12% of OTU's. Tabular and graphical summaries of the phyla identified are shown in Table 4.6 and Figure 4.4.

Nine OTU's matched to Russulaceae (Table 4.7), as determined by a BLASTn search on GenBank. However, it should be noted that as the Next Generation sequences are comparatively short, the E-values were quite high indicating there was a chance of unrelated matches occuring.

Table 4.6: Percent of OTU classifications found in each DNA sample

Phylum	OR01	OR02	OR03	OR04	ORS1	ORS2	ORS3	ORP1	ORP2	ORD1	ORD2	ORD3	ORD4
No blast hit	0.8	16.3	1.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ascomycota	45.8	82.9	43.0	11.7	11.0	0.4	69.8	100.0	97.5	100.0	5.9	3.5	9.6
Basidiomycota	49.0	0.8	54.7	88.0	87.3	99.6	30.1	0.0	2.5	0.0	94.0	93.0	90.4
Calcarisporiellomycota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chytridiomycota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glomeromycota	2.5	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mortierellomycota	1.3	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mucoromycota	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Olpidiomycota	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Rozellomycota	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0
Unidentified fungi	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cercozoa (Protista)	0.6	0.0	0.0	0.2	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0

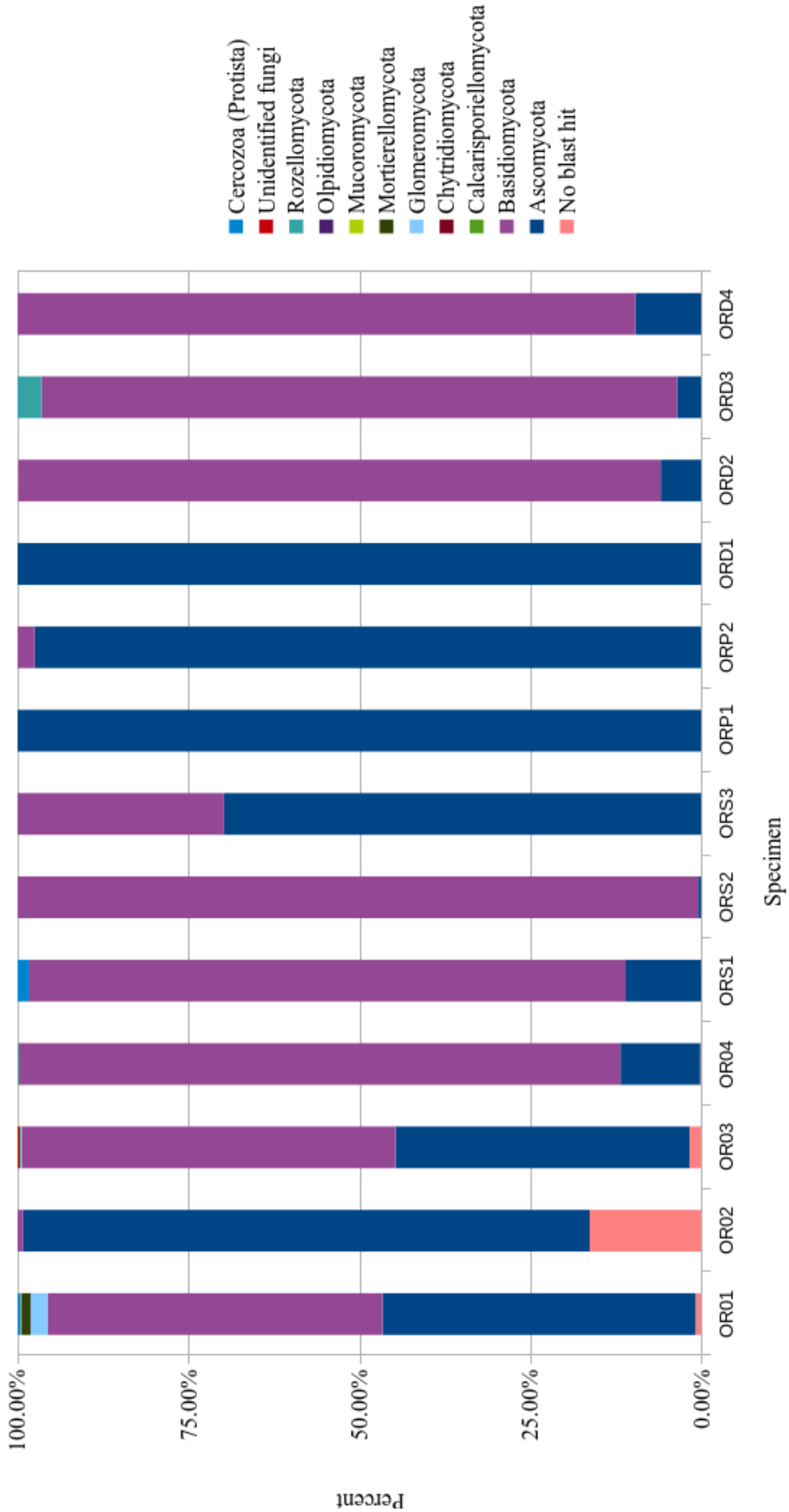


Figure 4.4: Summary of the OTU phyla obtained through NGS

Table 4.7: Number of Russulaceae-OTU reads for each sample

OTU	ID	OR01	OR02	OR03	OR04	ORS1	ORS2	ORS3	ORP1	ORP2	ORD1	ORD2	ORD3	ORD4
5	*Russula cyanoxantha*	–	–	–	227	1165	30901	–	–	1	–	1	66	–
9	*R. shingbaensis*	–	–	–	4324	33937	2	–	–	1	–	648	–	–
10	*Lactifluus oedematopus*	–	–	–	–	–	1	732	–	–	–	–	8223	–
24	*R. cascadensis*	–	–	–	–	–	–	1759	–	–	–	–	–	–
49	*R. vinaceocuticulata*	–	–	–	–	–	–	–	–	–	–	–	418	–
58	*R. tawai*	–	–	–	–	–	–	–	–	–	–	–	816	–
179	*R. shingbaensis*	–	–	–	–	29	–	–	–	–	–	–	–	–
40	*Lactarius piperatus*	–	–	749	–	–	–	–	–	–	–	–	–	–
42	*R. shingbaensis*	410	–	812	–	–	–	–	–	128	–	–	–	–

4.3.4 Phylogenetic analysis

The two closest Russulaceae matches of the two peloton sequences obtained through Sanger sequencing were included in a 44-sequence phylogenetic tree (Figure 4.5). ORO1 clustered with *R. pilosella* and *R. galbana*, two hypogeous species. ORS3 grouped with *L. bicolor* and *L. genevievae*.

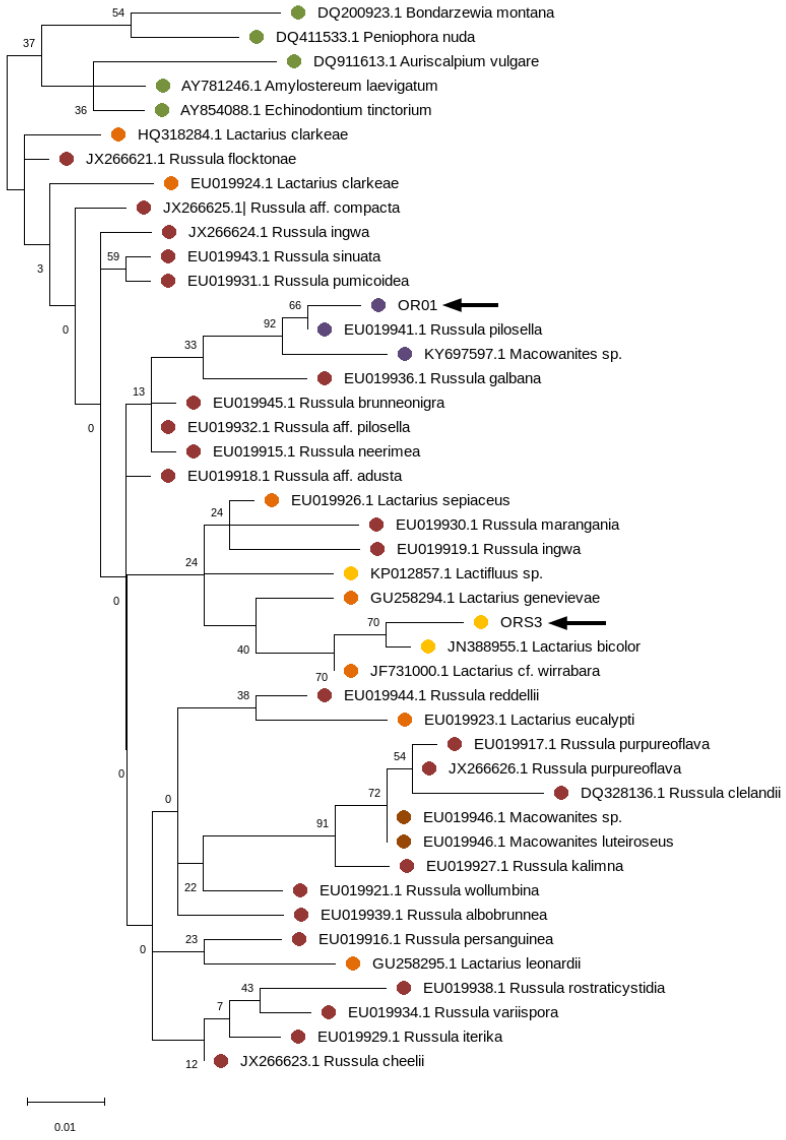


Figure 4.5: Phylogenetic tree incorporating peloton-sourced Russulaceae-ITS sequences. Sequences combined with, their closest matched sequences from GenBank, and Australian ITS sequences. A total of 304 nucleotide positions were included in the final dataset. The tree with the highest log likelihood (-1336.63) is shown. Arrows indicate the two ITS sequences obtained from *D. roseum*.

4.4 Discussion

4.4.1 DNA Extraction and PCR

Initially, thin sections of root tissue were made, transferred to a microcentrifuge tube and the DNA extraction performed without further processing of the tissue. PCR of the resulting mixture was unsuccessful. A number of reasons for this result were suggested, including insufficient fungal material, despite the use of fungal-specific primers DNA extracted from the orchid itself inhibited the reaction and cellular contents such as starch inhibited the PCR reaction.

Subsequently, thin sections of root tissue were prepared and then microscopically examined to confirm the presence of multiple pelotons. Several sections contained areas of dense pelotons, allowing excess root material to be removed. DNA extraction and PCR were performed as before, and again, the results obtained were inconsistant. Finally, effort was applied to separating the pelotons from the root material, which appeared to lead to a significant improvement in results.

Previous research has generally found that individual obligate mycoheterotrophic orchid roots are colonised by a single fungal species (Kristiansen et al. 2001, Girlanda et al. 2006) whilst autotrophic orchids can associate with multiple species simultaneously (Kennedy et al. 2011). There are exceptions, such as in comparing the mycorrhizal associations of tropical species of the orchid family Neottieae with those species found in temperate regions, Roy et al. (2009) found multiple associations in *Aphyllorchis* spp., and could not identify a consistent fungal associate.

A number of extraction and PCR methods were trialled, including the use of kits obtained from Qiagen and Sigma and the use of components prepared in the laboratory. Qiagen's Plant DNAeasy Mini Kit (Bougoure & Dearnaley 2005, Dearnaley & Le Brocque 2006, Girlanda et al. 2006, Huynh et al. 2009, Kottke et al. 2009, Kennedy et al. 2011, Okayama et al. 2012, Riofrio et al. 2013) has been predominantly used for DNA extraction of orchid root material, though other methods have been used. This results in a mixture of orchid and fungal DNA, with the orchid material generally contributing the bulk of the DNA (Gardes & Bruns 1993). The use of fungal-specific primers, such as ITS1-F and ITS4-B, were designed to preferentially amplify the ITS region of fungi (ITS1-F) and basidiomycetes (ITS4-B). Most research into fungi of orchids combined ITS1-F with the universal primer

ITS4 (Bougoure & Dearnaley 2005, Girlanda et al. 2006, Dearnaley & Le Brocque 2006, Kottke et al. 2009, Kennedy et al. 2011, Okayama et al. 2012). Whilst this can amplify any fungal DNA present in the reaction, products of potential contaminants should produce a weaker band on a gel than those amplified from the pelotons. However, unless absolutely confident a product is from a contaminant, it should not be discounted or considered as "wrong" (Selosse et al. 2010).

This combination of ITS1-F and ITS4 primers may still result in the formation of plant PCR products (Dearnaley & Le Brocque 2006), but as shown in Table 4.8 it is more selective towards fungi than the other combinations. However, if the risk of amplification of unwanted ITS products is negligible (for example, by using pure cultures (Huynh et al. 2009)) the universal primer pair ITS1 and ITS4 is quite acceptable.

Table 4.8: ITS primer combinations and their amplification preferences (Gardes & Bruns 1993)

	ITS1 and ITS4	ITS1 and ITS4-B	ITS1-F and ITS4	ITS1-F and ITS4-B
Plants	++	+	+	+
Ascomycetes	+++	-	+++	-
Basidiomycetes	+++	+++	+++	+++

- = No amplification
+ = Positive amplification

Based on these results, it appears the most successful combination of methods for amplification of the ITS region of orchid mycorrhizas from the roots of *D. roseum* include:

1. Scrutinise the tissue to ensure pelotons are present
2. Macerate the tissue in liquid to release the pelotons; collect the liquid, centrifuge to pellet the pelotons and discard the supernatant
3. Extract the DNA using the Extract-N-Amp Plant PCR kit – the peloton pellet can be resuspended in the extraction buffer

4. Amplify the ITS region of the DNA using the KAPA2G Robust HotStart PCR kit (Buffer B)

5. Utilise a Touchdown cycling program

6. Use a column-based purification method, available in a variety of commercial DNA cleanup kits, to purify the final PCR products for sequencing, but elute the DNA with pre-warmed DNase-free water

Efforts should also be made to limit the risk of contamination from organisms located on the root surface. At a minimum, surface soil should be removed as thoroughly as possible during root collection, and the root section washed in sterile water prior to peloton extraction. A more thorough de-contamination process would also require the removal of the root epidermis, as this would ensure only the DNA of endophytic fungi would be present for extraction.

4.4.2 Sequencing and Phylogenetic Analysis

Despite the effort in obtaining suitable PCR products for Sanger sequencing, this process did not appear to produce consistent results. It was therefore decided to utilise Next Generation Sequencing (NGS). Whilst this also relies on the production of PCR amplicons, they tend to be smaller with Illumina and more readily obtained. These products are subsequently ordered and matched using a reference genome in order to construct the most likely sequences.

Results of the BLASTn search of the NGS sequences revealed E-values substantially higher than those of the Sanger sequencing. The E-value (Expectation value) provides an indication of how likely a match (of a similar quality) could occur by chance alone. The calculation is dependent on the quality of the alignment (based on the number of identities, gaps and mismatched nucleotides), the length of the query sequence and the size of the database searched - generally, the smaller the query sequence, the greater the opportunity for a random hit. In a database the size of GenBank, a sequence size trial showed approximately 400 base pairs were required to achieve high quality matches with an E-value displayed as 0 (data not provided).

The sequences generated by NGS were less than 300 base pairs, so could not be used to

identify with certainty to a species. However, they were sufficient to identify fungal genera. Even so, it was interesting to note two of the NGS-GenBank matches, *R. shingbaensis* Accession number KM386692, and *R. tawai* Accession number JX178491, also appeared as matches in Chapter 2. However, they are not Australian species. It was also interesting to note the presence of *Russula* and *Lactarius* species in the NGS results, which adds support for the results of Sanger sequencing.

Two root samples yielded Russulaceae sequences from the Sanger sequencing. The first sample, OR01, matched to *R. pilosella* (99% identity) and a species of *Macowanites* (97% identity), both of which are Australian sequestrate species. These three sequences formed a clear group on the phylogenetic tree along with *R. galbana* which has a secotioid habit (Figure 4.5).

The second sample, ORS3, closely matched *Lactarius bicolor*, an agaricoid species found in Malaysia and Singapore (Stubbe et al. 2012). Other studies into the mycorrhizal partners of green orchids have recorded the presence of *Lactarius* spp. but it is not clear if they are orchid-mycorrhizas or ectomycorrhizas of nearby trees contaminating the sample (Liebel et al. 2014). In this study, however, as pelotons from within the root provided the majority source of DNA, this would suggest a species of *Lactarius* had formed an association with a *D. roseum* orchid plant, and the NGS results (Table 4.7) also reinforce this interpretation. This marks the first record of an association between *Lactarius* and an Australian orchid species.

Studies of this type are important, as the level of specificity between mycoheterotrophic orchids and associated mycorrhizal fungi needs to be taken into consideration in the conservation and management approvals (Leake 2005, Girlanda et al. 2006, Dearnaley & Le Brocque 2006). This requires determining the identity of the fungi involved (Riofrio et al. 2013). It should also be noted, a decline in ectomycorrhizal fungi populations may lead to a general reduction in plant resilience and competition (Okayama et al. 2012).

Chapter 5

Bioactive compounds from SEQ Russulaceae

5.1 Introduction

The development of antimicrobial resistance is of world-wide concern, having the potential to lead to an era where the causative agents of once treatable infections return to their pre-antimicrobial state (World Health Organisation 2014). Bacterial resistance also potentially reduces both the range of antibiotics available and the efficiency of prophylactic antibiotics administered prior to major surgery or chemotherapy, increasing the risk of serious infections and mortality (World Health Organisation 2018*a*).

Cancer is currently responsible for approximately 17% of deaths worldwide and is the second highest cause of death (World Health Organisation 2018*b*). Bioactive compounds are not only used for treatment (chemotherapy), but also for supportive (De Silva et al. 2012) and potentially preventative therapies (Cragg & Pezzuto 2015).

The economic impact of these health issues is significant and rising (World Health Organisation 2014), underscoring the desire to continue the search for additional or more effective drugs. The highest potential for discovery of these new compounds is through the examination of the bioactive metabolites produced in nature (Dias et al. 2012, De Silva et al. 2013). In plants and fungi, these compounds are frequently part of a chemical defense system, providing protection from micro-organisms, insects, animals (Liu 2007) and

other environmental stressors. Natural compounds are a large, ever changing resource as plants and fungi adapt continuously to changes within their environment (Spiteller 2015). Biologically-derived compounds often possess unique structures and when utilised medically, can have unique modes of action (Cragg & Pezzuto 2015, Stadler & Hoffmeister 2015). Natural products are either directly or indirectly responsible for over 60% of anticancer drugs (Cragg & Pezzuto 2015) and 90% of antibiotics (Wohlleben et al. 2016) currently in use.

Fungi have been shown to contain a range of biologically active compounds including antimicrobial, anticancer, antioxidant, antiviral, cholesterol-lowering and immunosuppressive agents (Türkoğlu et al. 2007, Zhang et al. 2010, Beattie et al. 2011, Cragg & Pezzuto 2015). Important examples of fungal-derived products include the antibiotics penicillin and cephalosporin, the immunosuppresent cyclosporin (De Silva et al. 2013) and anticancer compounds doxorubicin and torreyanic acid (Dias et al. 2012). However, the metabolites of only a small number of fungal species have been therapeutically examined to date (Cragg & Pezzuto 2015).

Members of the Russulaceae have been shown to contain bioactive compounds including chemicals with antimicrobial (Shittu et al. 2005, 2006, Türkoğlu et al. 2007), anticancer (Zhao et al. 2010, Zhang et al. 2010) and antioxidant (Türkoğlu et al. 2007) properties. While they do not have a major reputation for their bioactive properties, research in this area has been spasmodic and inconsistent, employing a wide variety of extraction methods and testing protocols (Shittu et al. 2005, 2006, Türkoğlu et al. 2007, Zhao et al. 2010, Beattie et al. 2011). The research has also included very few Australian species. Many species of *Lactarius* have already been examined in this context, but *Russula* spp. have been studied less frequently (Liu 2007, Malagòn et al. 2014).

Given the potentially small percentage of fungi even discovered, it would suggest they provide a greatly underutilised resource. As new species are located and characterised, the opportunity should also be taken to understand the function and properties of their metabolites. This may lead to discovery of novel and useful bioactives (Cragg & Pezzuto 2015).

Through the taxonomic and ecological investigation of the Russulaceae of SEQ, new species have been uncovered. Whilst there were limited supplies of some specimens, there was the opportunity to examine the metabolic properties of other species where multiple fruiting bodies were available. Extracts were produced from some of these species and

examined for antimicrobial and anticancer activities.

5.2 Materials and Methods

5.2.1 Specimen material

Culturing Approach

Fresh fruiting bodies of *Russula* spp. (as listed in the methods below) were obtained and used as a source of spores for culturing. Several variations of media and spore application were attempted including:

Method 1. Application of spores to standard laboratory media Malt Extract (MEA) and Sabouraud dextrose (SDA) agars (Amyl Media, Dandenong South, Victoria, Australia) and plates were prepared as per the manufacturer's instructions and stored at 4^{o}C overnight. A flamed agar cutter was carefully run along the gills of the fruiting body to collect spores or a small amount of hyphal material, and then drawn across the middle of the plate. One of each plate type was inoculated for each of the two fruit bodies sampled (MB01 and MB09). Plates were incubated at 25^{o}C.

Method 2. Alternative application of spores to standard laboratory media The cap of specimen MB03 was removed from the stipe and placed on to the surface of a MEA plate for approximately 15 minutes, so as to catch spores. The cap was subsequently transferred onto a 70% ethanol washed slide and left for approximately an hour. The spores were transferred from the slide and deposited on the surface of a second MEA plate. Both plates were incubated at 27^{o}C. This method was subsequently repeated with SDA.

Method 3. Media combined with primed water Water was primed by watering a potted *Eucalyptus leucoxylon* seedling and collecting the run-off. This was filtered through a 0.8μm syringe-driven filter to remove large particles, then through a 0.2μm filter to sterilise. The sterile filtrate was added at a ratio of 1:1 either to sterile double-strength Malt Extract broth (MEB) (Amyl Media) or sterile molten 1.2% agar. The broth was poured over sterile soil which had been collected from the base of three known *Russula*-host trees (one each of *E. tereticornis, E. melliodora* and *E. crebra*) and sterilised by autoclaving at

121^{o}C for 15 minutes; the agar was poured into 55mm petri dishes, with approximately 10ml per dish. Spores were transferred to the surface of each medium with a sterile toothpick, and in the case of the plates, the efficacy of the transfer was confirmed by microscopic observation at x40. Cultures were incubated at 18^{o}C. Spores were obtained from specimens MB04-MB07, MB09-MB15, MB17-MB20, MB23 and MB24.

Method 4. Media combined with wood shavings of *Eucalyptus* spp. Wood shavings were collected from three trees (*E. tereticornis, E. melliodora* and *E. crebra*) and 0.2g added to each of 20ml volumes of MEA, SDA, Potate dextrose agar (PDA) (Amyl Media), water agar and water agar with M9 salts. After autoclaving at 121^{0}C for 15 minutes, two 55mm plates were prepared from each mixture. A sterile toothpick was used to transfer spores to the surface of each plate, which were then incubated at 15^{o}C. A freshly collected specimen, MB69, was used as the spore source.

All plates were regularly observed over several months for signs of growth. It was anticipated this would appear as slow growing hyphae emanating from the spores.

Tissue collection

Several fruiting bodies of *Russula* were collected from a dry eucalypt forest at Preston in South-East Queensland. Following collection of approximately 100mg of material for DNA analysis, the fruiting bodies were placed into press-seal plastic bags and stored at -20^{o}C until used for extraction of potential bioactives.

5.2.2 Extraction of fungal material

Fractions of fungal extracts

Fungal tissue from specimens MB73, MB79 and MB83 was weighed, briefly rinsed in sterile water, homogenised in a small blender then transferred to a 250ml Erlenmeyer flask. One hundred percent methanol was added at a rate of 10ml methanol per gram of material, and the flask sealed with parafilm. The mixtures were subsequently shaken on an orbital shaker at 110rpm at 25^{o}C for approximately 24 hours. The mixture was filtered through a No. 54 Whatman filter paper by vacuum filtration, and the methanolic extract

stored at 4^{o}C until preparation for preparative HPLC. Solid material was discarded.

The extracts were dried using a rotary evaporator set at 50^{o}C and 240rpm, and the weight of the crude sample obtained. One gram of each sample was absorbed on to 1.5g of C18 silica gel and mixed well to ensure a homogeneous and free-flowing mixture. This mixture was used to prepare two 1g HPLC columns for each fungal sample. Where insufficient material was available to pack the second column, the space was made up with washed sand.

A Shimadzu instrument driven by Class-VP software was used for preparative HPLC. Gradient elution at a flow rate of 4ml/minute was run over 30 minutes with the compounds being progressively eluted in 10 to 95% aqueous methanol. Seven 20ml fractions were collected for each sample into pre-weighed glass vials. The fractions were subsequently dried using a nitrogen blower with heat at 50^{o}C and airflow only. After weighing, the fractions were resuspended in 100% DMSO (di-methyl sulfoxide) at a concentration of 20mg/ml.

Crude fungal extracts

Crude extracts of the same specimens (above) were also prepared in order to extend bioactive testing. In this case, frozen material was thawed, macerated and extracted in approximately 60ml dichloromethane. 20ml was removed after 5 minutes, and the remainder allowed to extract overnight. The extractions were transferred into pre-weighed tubes and dried using the nitrogen blower as done previously. After weighing, the crude extracts were resuspended in DMSO at a concentration of 10mg/ml. Two additional crude extracts were similarly prepared from a freshly collected fruiting body, MB162.

5.2.3 Microbiological testing

Sixty 90mm Sensitest and Tryptic soy agar (TSA) (Amyl Media) plates were prepared from commercially available powders as per the manufacturers' instructions, and subsequently stored at 4^{o}C until required. Sterile saline was prepared by dissolving 0.9g sodium chloride per 100ml water and dividing into 10ml volumes. After sterilisation at 121^{o}C for 15 minutes, the bottles were stored at ambient temperature until required.

Ampicillin was used as a positive control. A stock solution was prepared by dissolving 15mg of the sodium salt in 1ml of sterile water. A working solution of 100μg/ml was prepared by combining 110μl of the stock with 1.5ml of sterile water or DMSO. The stock was stored at -20^{o}C and the working solutions at 4^{o}C until required.

Due to the small volumes of test extract available, a microbe applicator was designed and made to apply discrete lines of test microbes around a central well containing the test solution. The applicator consisted of eight plastic arms arranged as regularly-spaced spokes around a central clearing, as shown in Figure 5.1. The upper edge of each arm was lined with an absorbant pad. As autoclave-safe materials were used in its manufacture, it was autoclaved before use.

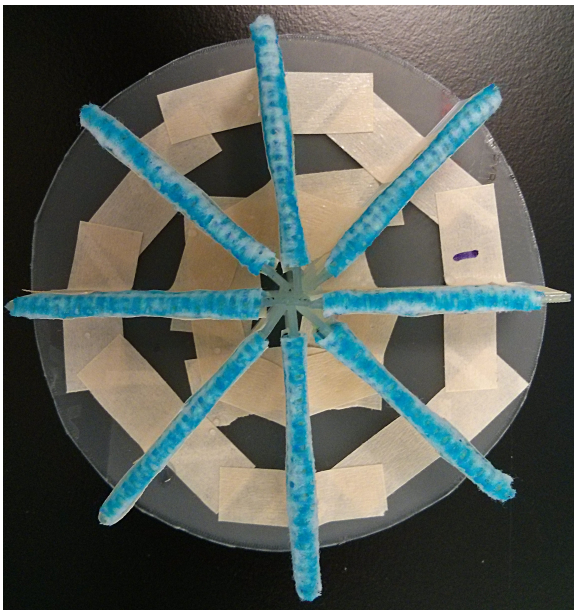


Figure 5.1: The applicator used to apply discrete lines of micro-organisms to anti-microbial test plates. The blue "lines" are the absorbant material on the spokes.

A brief trial was initially conducted in order to determine optimal response with respect to application methods, diffusion time and solvent. Suspensions of *Escherichia coli* and *Staphylococcus aureus* (details below) were used as test organisms and ampicillin as the test agent. A control plate was prepared by swabbing each bacterium on to a Sensitest agar plate and applying a commercial ampicillin (10μg) antibacterial disk in the centre of each. Subsequent test plates utilised the 100μg/ml ampicillin solutions as prepared above, as well as water and DMSO for solvent control. A sterile 5mm punch was used to create wells in the plates, and 10μl test solution added. Three conditions were trialled, relating to diffusion of the test solutions – inoculation and incubation without pre-diffusion, diffusion

overnight at 4^{o}C and diffusion for 30 minutes at room temperature. The two latter plates were inoculated and incubated after the diffusion process. The plates were incubated overnight at 37^{o}C, before being examined for zones of inhibition.

The opportunity was also taken to trial the use of the microbial applicator with all the test organisms and with the ampicillin and solvents to ensure that the procedure was feasible. A single Sensitest agar plate was used for each test, and a single central well inserted as previously described. Saline suspensions of the microbes were prepared and applied to the applicator with a sterile transfer pipette. The plates were incubated overnight at 37^{o}C, before being examined for zones of inhibition. Details of micro-organisms selected for testing are listed in Table 5.1.

Table 5.1: Micro-organisms selected for anti-microbial testing of the fungal extracts

Organism	ATCC Number
Candida albicans	60193
Staphylococcus aureus	25923
S. aureus MRSA	43300
Serratia marcescens	14756
Pseudomonas aeruginosa	10145
Escherichia coli	25922
Enterococcus faecalis	19433
Staphylococcus epidermidis	14990

The organisms were revived from their -80^{o}C storage on TSA and subcultured twice before use in the testing protocol.

For the actual procedure, Sensitest agar plates were removed from cold storage and allowed to warm to room temperature. A single well was punched in the centre of the agar, using a sterile 5mm punch, and 10μl of control or test solution added. The plates remained at room temperature and the solution was allowed to absorb into the agar for 30 minutes.

Saline suspensions of the test organisms were prepared by harvesting the growth from overnight cultures grown on TSA; the suspension was diluted to obtain an absorbance at

625nm of between 0.08 and 0.13, which is equivalent to a 0.5 McFarland standard. In the case of *E. coli*, this is approximately 1-2x10^8 CFU/ml. A micropipette was used to gradually dispense 100μl of each suspension along the length of one of the applicator arms. Once fully inoculated, the applicator was gently pressed onto the surface of each test plate. A maximum of 3 plates were inoculated at a time before the suspensions were reapplied to the applicator. Plates were incubated overnight at 35^{o}C +/- 2^{o}C before being examined for zones of inhibition.

5.2.4 Cell Viability Assay

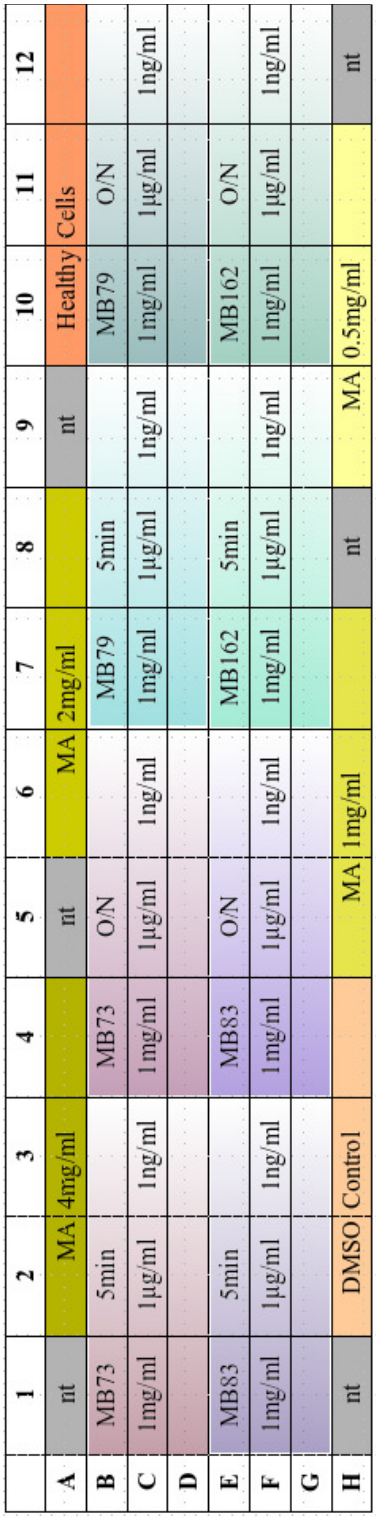
Dulbeco's modified Eagles medium (DMEM) was prepared as per the manufacturer's instructions, and supplemented with 10% foetal calf serum (FCS). Stock 10x phosphate buffered saline (PBS) was prepared by dissolving 80g sodium chloride, 2g potassium chloride, 14.4g di-sodium hydrogen orthophosphate and 2.4g potassium di-hydrogen orthophosphate in approximately 750ml water. The pH was adjusted to 7.4, and the solution made up to 1000ml. Working solution was prepared by diluting the stock 1:10 in water. Both solutions were autoclaved at 121^{o}C for 15 minutes and stored at 4^{o}C until required.

A 1mg/ml stock of each test solution was prepared by adding 400μl of the 10mg/ml crude extract to 3.6ml DMEM. As this dilution also resulted in the solution containing 10% DMSO, the DMEM required for the remaining test solutions was prepared with 10% DMSO. A final volume of 2ml was prepared for the remaining dilutions of 1μg/ml, 1ng/ml and 1pg/ml. This provided sufficient solution to perform each test with each cell line in triplicate.

Malic acid was selected as a negative control. From a stock of 4mg/ml malic acid in DMEM/DMSO, dilutions of 2mg/ml, 1mg/ml and 0.5mg/ml in DMEM/DMSO were prepared.

A plan was prepared for the 96 well layout, containing areas for test dilutions, negative control dilutions, DMEM/DMSO control and healthy cells (no intervention). The layout followed can be seen in Figure 5.2. Descriptions of the cell lines tested can be found in Table 5.2.

All incubations were conducted in a humid chamber maintained at 37^{o}C and 5% carbon dioxide.



	1	2	3	4	5	6	7	8	9	10	11	12
A	nt	MA	4mg/ml		nt	MA	2mg/ml		nt	Healthy	Cells	
B	MB73	5min		MB73	O/N		MB79	5min		MB79	O/N	
C	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml
D												
E	MB83	5min		MB83	O/N		MB162	5min		MB162	O/N	
F	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml	1mg/ml	1µg/ml	1ng/ml
G												
H	nt	DMSO	Control		MA	1mg/ml		nt	MA	0.5mg/ml		nt

Figure 5.2: Layout of the 96 well plates for the crystal violet assay. MA = Malic acid, nt = not tested.

Table 5.2: Cell lines used for the cell viability assay

ID	Initial passage number	Description
Hek-293	7	Human embryonic kidney cells
HeLa	25	Human cervical adenocarcinoma
SW620	84	Human colon adenocarcinoma

Cell lines were revived from liquid nitrogen storage and passaged twice before each being used to seed one 96 flat-well plate. After 24 hours incubation, the media was removed and replaced with 100μl test solution. For ease of rapid replacement, these had been pre-dispensed into a 96 V-well plate as per the layout described above. The plates were incubated for approximately 48 hours.

A crystal violet assay was used to assess the effect of the test solutions on cellular biomass as an indicator of cell viability. Crystal violet reagent was prepared by dissolving 2g crystal violet in 20ml methanol and making up to 100ml with water. A solution of 1% sodium dodecyl sulphate (SDS) was also prepared by carefully stirring 1g SDS into 100ml water.

Following the final incubation, the media was removed from the wells and 100μl methanol added in order to fix the remaining cell layer. After 30 seconds, the methanol was discarded and 100μl of crystal violet reagent added. The plates were incubated at room temperature with gentle mixing on a platform shaker for 30 minutes, before being emptied, carefully washed under running water and then allowed to dry. Subsequently, 100μl of 1% sodium dodecyl sulfate (SDS) was added to each well and the plates incubated for 30 minutes at room temperature on a platform shaker set to 100rpm. Absorbance of each well was measured in a plate reader at 600nm.

Triplicate results were averaged and corrected for DMSO impact by subtracting the averaged DMSO/DMEM result. The variance was calculated and possible outliers noted, but not immediately excluded. Test results were compared with those of the normal control (cells with no intervention) and determined to meet one of the conditions described in Table 5.3.

Table 5.3: Possible results of the cell viability assay, and brief description

Condition	Definition
Biomass decrease	Absorbance measured was *less* than that of the untreated control. Suggests test solution leads to cell decline.
Biomass increase	Absorbance measured was *greater* than that of the untreated control. Suggests test solution leads to increased growth.
Neutral	Measured absorbance was similar to that of the untreated control. Suggests test solution has no effect.

5.3 Results

5.3.1 Specimen material

Culturing Approach

A number of culturing methods were attempted in order to procure a sustainable amount of starting material for future extractions. After at least three months of incubation, the following results were obtained:

Method 1 No growth observed, though after seven days of incubation an ascomycete contaminant was noted and aseptically removed.

Method 2 No growth observed

Method 3 No growth observed

Method 4 No growth observed

As the culturing of the *Russula* specimens proved to be unsuccessful, optimisation of a culture method was not possible. Therefore the remaining procedures relied upon the finding of a sufficient number of fruiting bodies for characterisation and extraction.

5.3.2 Extraction of fungal material

Fractions of fungal extracts

Weights of the dried extracted material obtained from the methanolic extraction of the three fruiting body samples were as shown in Table 5.4.

Table 5.4: Dried weight of the methanolic extracts

Specimen	Starting Weight (g)	Extract Weight (g)
MB73	30.0	1.4
MB79	45.0	5.5
MB83	37.5	2.0

As the total weight of the extract from MB73 was less than 2.0g, one column was prepared with 1g of the material while the second column contained the remaining extract and 0.6g of sand. It was expected a dry powder would eventuate following the completed drying process. However, the extracts appeared to have a high fat content as indicated by a tackiness of the material obtained (Figure 5.3).

Figure 5.3: Example of one of the dried extracts, that of MB83. Note the granulated material adheres to itself due to its fatty nature.

Seven fractions of approximately 20ml were collected for each column (A and B for each fungal sample). After drying, the vials were weighed and the weight of each fraction calculated (Table 5.5).

Chromatograms generated at the time showed similar profiles for each fungal specimen, suggesting the presence of similar compounds (Figures 5.4, 5.5 and 5.6).

Crude fungal extracts

Crude, dichloromethane extracts of the four fruiting bodies resulted in the weights of extracted material as shown in Table 5.6.

Table 5.5: Fraction weights obtained for each of the 7 fractions collected following HPLC

Fraction	MB73		MB79		MB83	
	A	B	A	B	A	B
	Calculated fraction weight in mg					
1	0.7	1.2	0.2	0.3	1.2	0.3
2	269.7	172.3	218.4	93.1	200.6	173.6
3	218.8	128.7	358.8	99.8	352.1	214.3
4	15.7	16.2	26.2	17.3	34.8	15.1
5	8.9	8.7	10.4	9.7	10.3	6.1
6	12.5	9.9	7.5	5.7	6.0	4.9
7	4.7	3.9	8.3	2.9	3.9	2.6

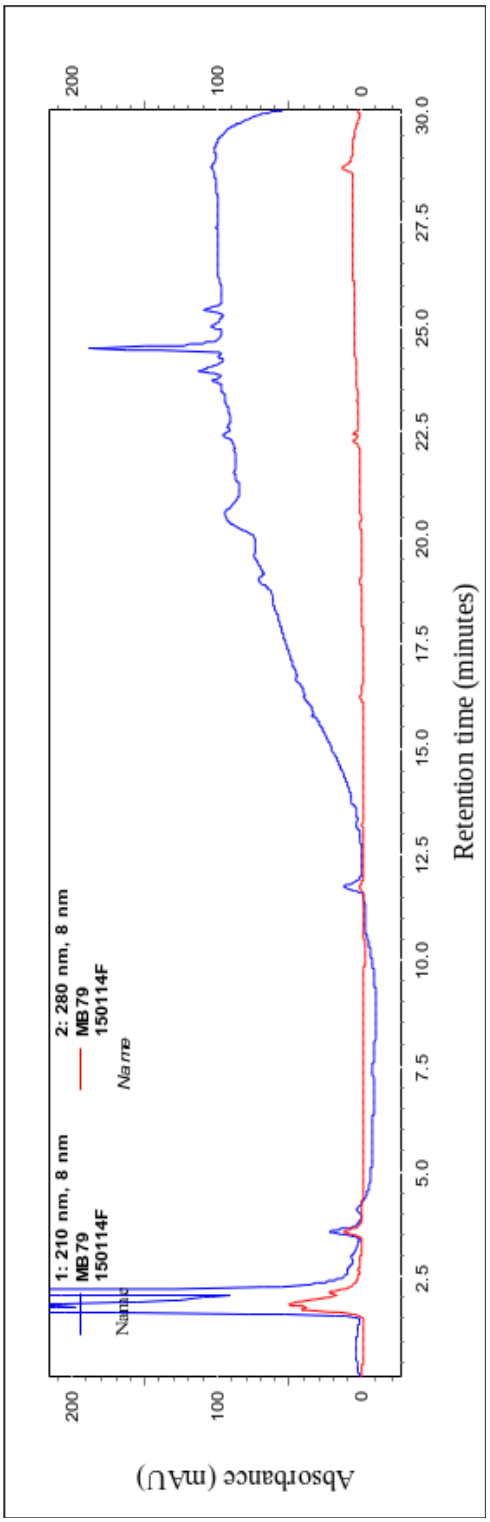


Figure 5.4: Extraction chromatogram obtained during preparative-HPLC for specimen MB73.

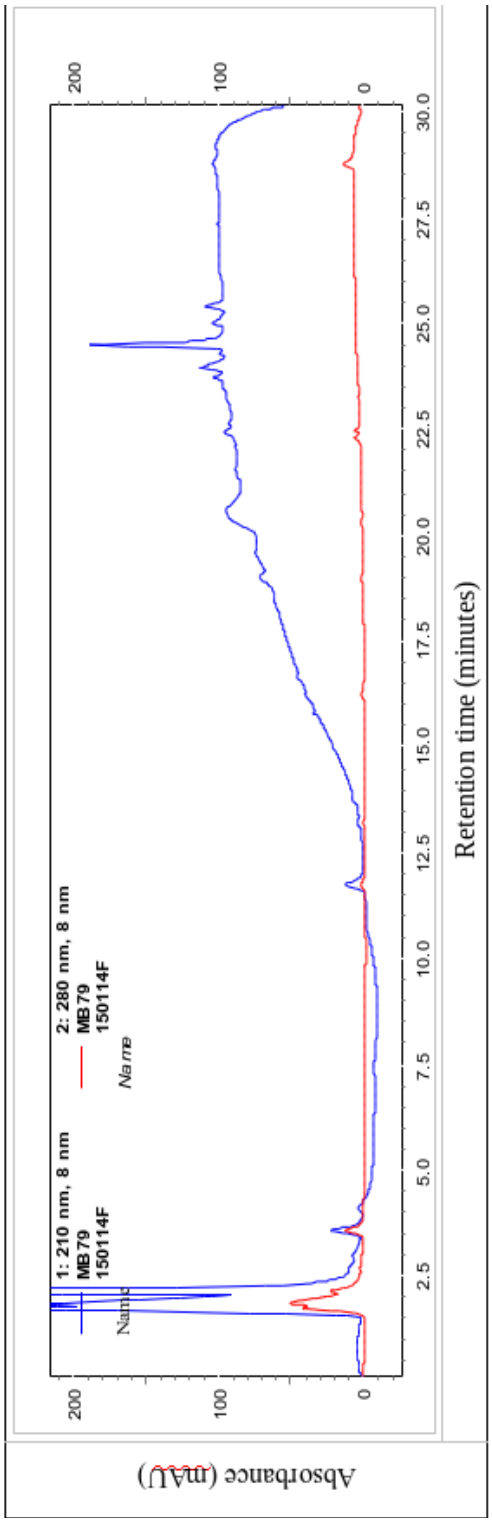


Figure 5.5: Extraction chromatograms obtained during preparative-HPLC for specimen MB79.

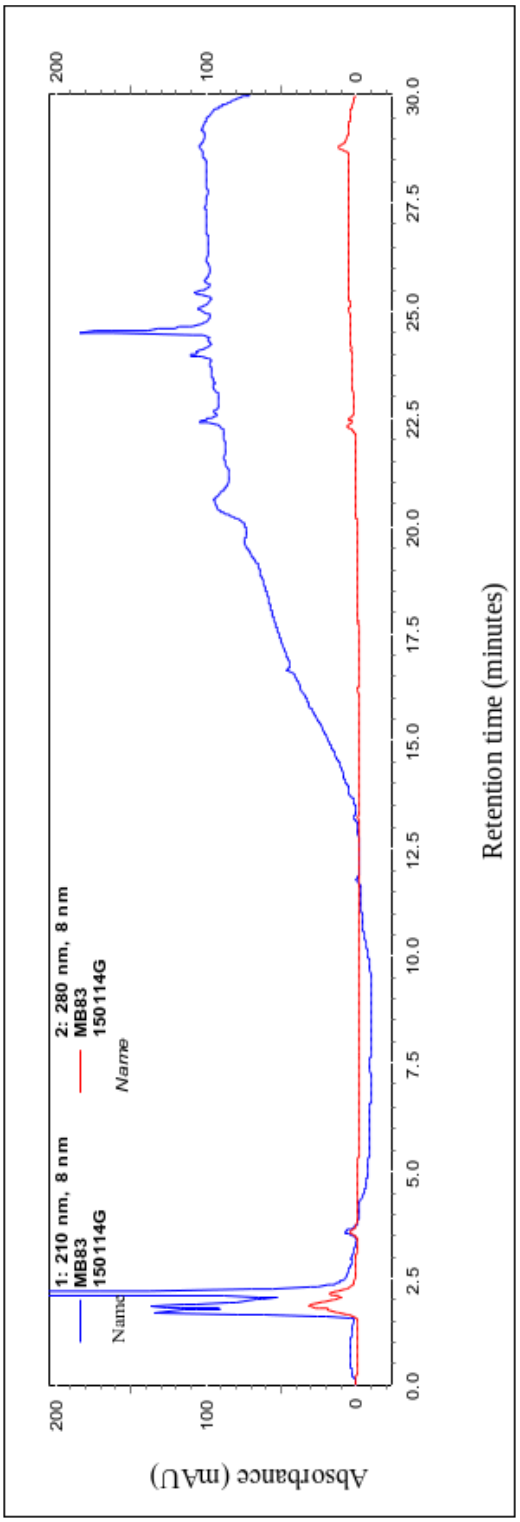


Figure 5.6: Extraction chromatograms obtained during preparative-HPLC for specimen MB83.

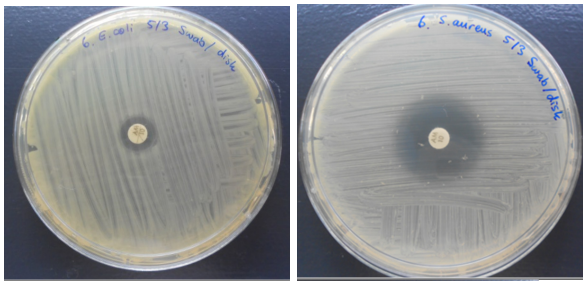
Table 5.6: Weights obtained following the 5 minute and overnight extractions of the specimens.

Specimen	Starting weight (g)	Extraction	Dry Weight (mg)
MB73	1.62	5 min	22
		Overnight	5
MB79	9.9	5 min	135
		Overnight	111
MB83	4.17	5 min	63
		Overnight	20
MB162	2.8	5 min	10
		Overnight	32

5.3.3 Microbiological testing

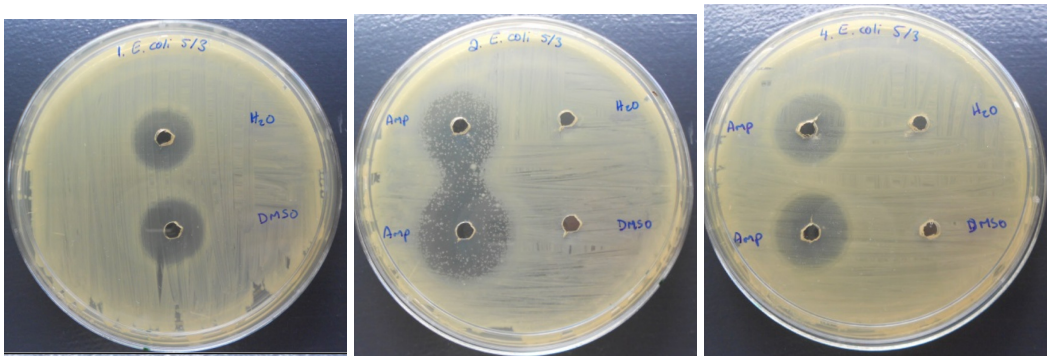
The control plates utilising the 10μg ampicillin disks, showed a zone of inhibition surrounding the disk of approximately 15mm against *E. coli* and 30mm against *S. aureus*. According to the CLSI (2014), this was within the expected results. This confirmed the test organisms were useful for testing and provided a baseline for the remaining trials (Figure 5.7).

The second set of trial plates showed the 100μl/ml ampicillin worked as expected, regardless of the solvent used. Pre-diffusion of the test substances resulted in larger zones of inhibition, though the zones produced after diffusion for 30 minutes at room temperature were cleaner and easier to interpret than those from the cold, overnight diffusion. This trial also confirmed that the solvents, water and DMSO, did not contribute to bacterial inhibition, at least in the case of these two micro-organisms (Figure 5.8).

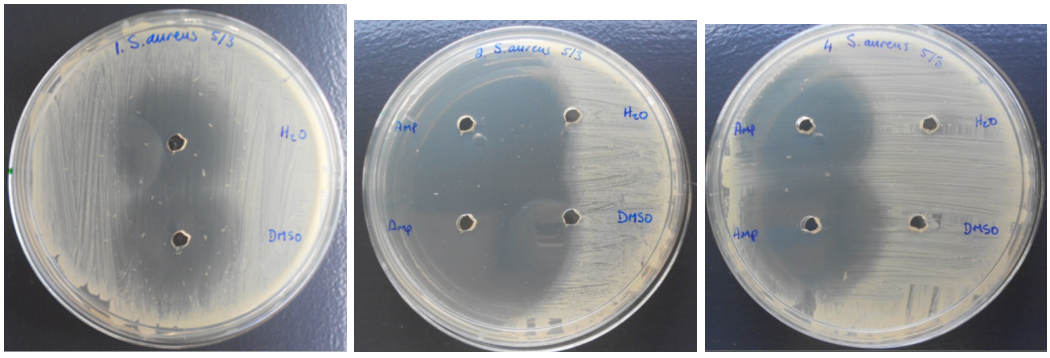


(a) *E. coli* (b) *S. aureus*

Figure 5.7: Initial control plates using the commercial AMP10 disk.



(a) No pre-diffusion (b) Pre-diffusion, 4^{o}C overnight (c) Pre-diffusion, 30 minutes at room temperature

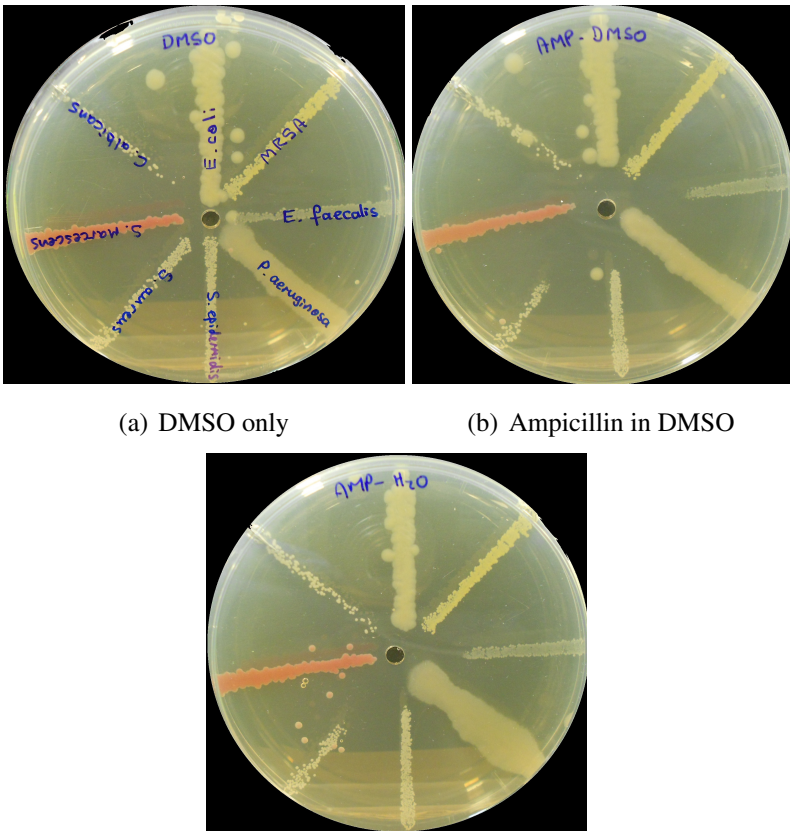


(d) No pre-diffusion (e) Pre-diffusion, 4^{o}C overnight (f) Pre-diffusion, 30 minutes at room temperature

Figure 5.8: Control plates of *E. coli* (Figures a, b and c) and *S. aureus* (Figures d, e and f) against the ampicillin solution in water or DMSO. Pre-diffusion occurred prior to inoculation and incubation of the plates.

The final trial provided a test of the applicator, a test of the solvent on the micro-organisms, and final run of the procedure. It showed the applicator worked, though care was required

to avoid cross-contamination between adjacent inocula. It also showed the remaining test organisms were not adversely effected by the solvents (Figure 5.9).



(a) DMSO only (b) Ampicillin in DMSO

(c) Ampicillin in water

Figure 5.9: Test organisms as applied to the plate surface with the applicator. Description indicates contents of the central well.

Summarised results of the anti-microbial assay can be found in Table 5.7. As can be seen, the DMSO had a small effect on *Candida albicans*, but not on the remaining test organisms; Ampicillin dissolved in DMSO was effective against the organisms expected, and not those known to be resistant; none of the three *Russula* extracts had an impact on any of the test micro-organisms. A representative image of a fraction test plate can be seen in Figure 5.10.

Table 5.7: Summarised results of the anti-microbial testing of the fractions on test micro-organisms.

Well Contents	*E. coli*	MRSA	*E. faecalis*	*P. aeruginosa*	*S. epidermidis*	*S. aureus*	*S. marcescens*	*C. albicans*
DMSO	neg	neg	neg	neg	neg	neg	neg	+
AMP-DMSO	+	+	++	neg	++	+++	neg	+[1]
AMP-H_2O	neg	neg	+	neg	+	++	neg	neg
MB73 Fractions	neg	neg	neg	neg	neg	neg	neg	neg
MB79 Fractions	neg	neg	neg	neg	neg	neg	neg	neg
MB83 Fractions	neg	neg	neg	neg	neg	neg	neg	neg

[1] A small zone was visible, but was attributed to the DMSO rather than the antibiotic
+ = Growth of the organism had been reduced
neg = Organism growth had not been affected

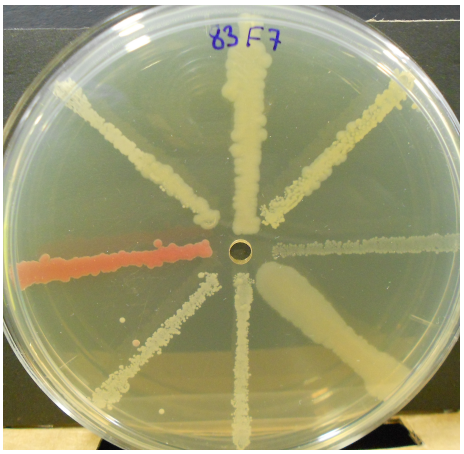


Figure 5.10: Representative image of the anti-microbial testing of the fractions. Bacterial growth extended the full length of its application, suggesting there was no anti-microbial effect.

5.3.4 Cell Viability Assay

Triplicate results of all tests and controls containing DMSO were averaged, and that of the DMSO control subtracted from those results. Triplicate results of the healthy cells (no intervention) were averaged. Averaged data was graphed for ease of interpretation, which can be found in Figures 5.11, 5.12 and 5.13.

From the figures, it can be seen that when compared with the healthy, untreated cells, the biomass of human embryonic kidney cells (Hek) was reduced by all of the crude extracts at most of the dilutions, with only the 5 minute MB83 extract increasing the cellular mass. However, in the cancerous cell lines, the majority of the crude extract dilutions appeared to result in an increase in the cell mass when compared with their untreated counterparts. In all cell types, all concentrations of the malic acid reduced the biomass when compared with the healthy cells. These results have been summarised in Table 5.8

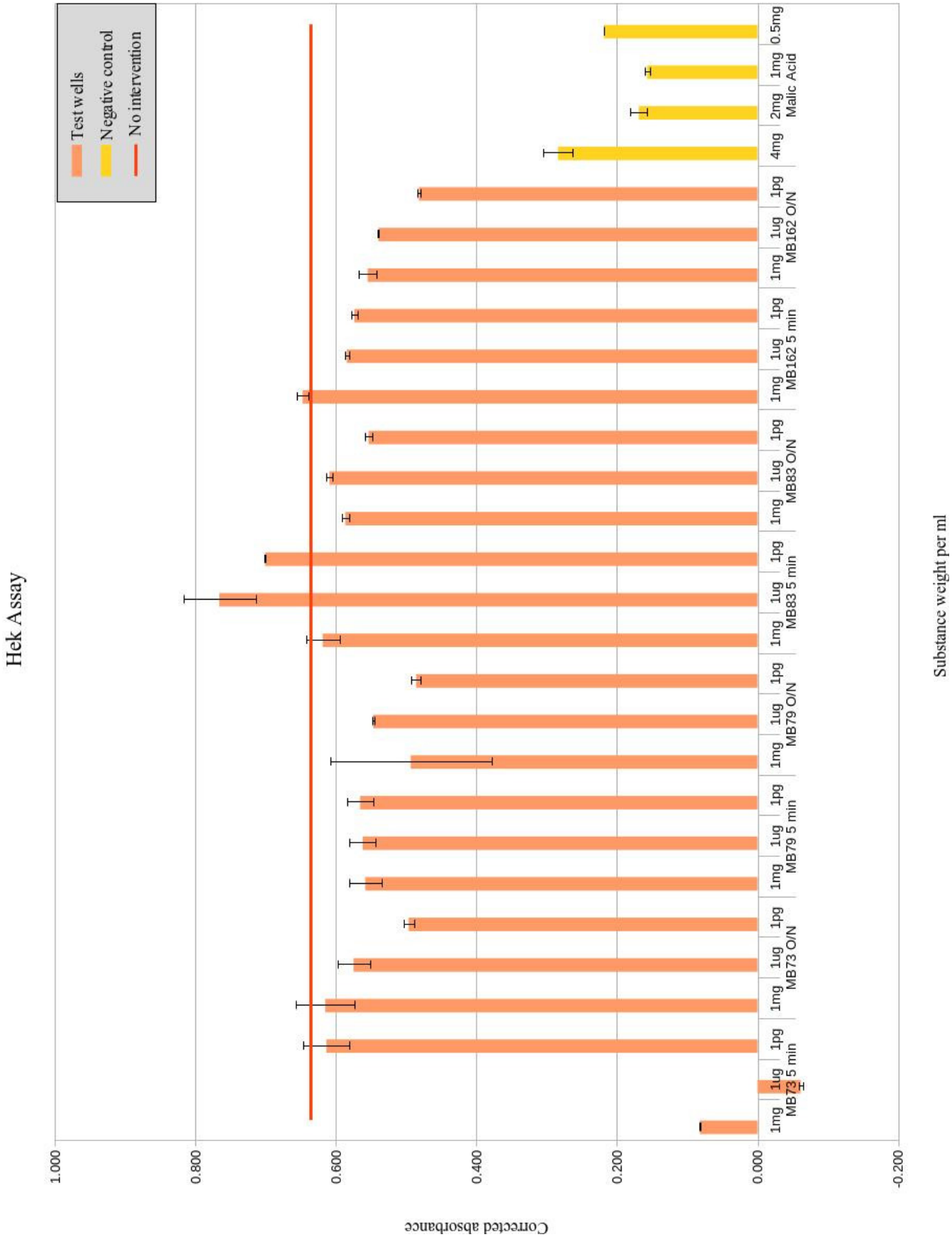


Figure 5.11: Cell assay results. Data was corrected against the blank; Error bars reflect data variance; the horizontal line shows the value obtained for the healthy, untreated cells, which was used to score the results for each test solution.

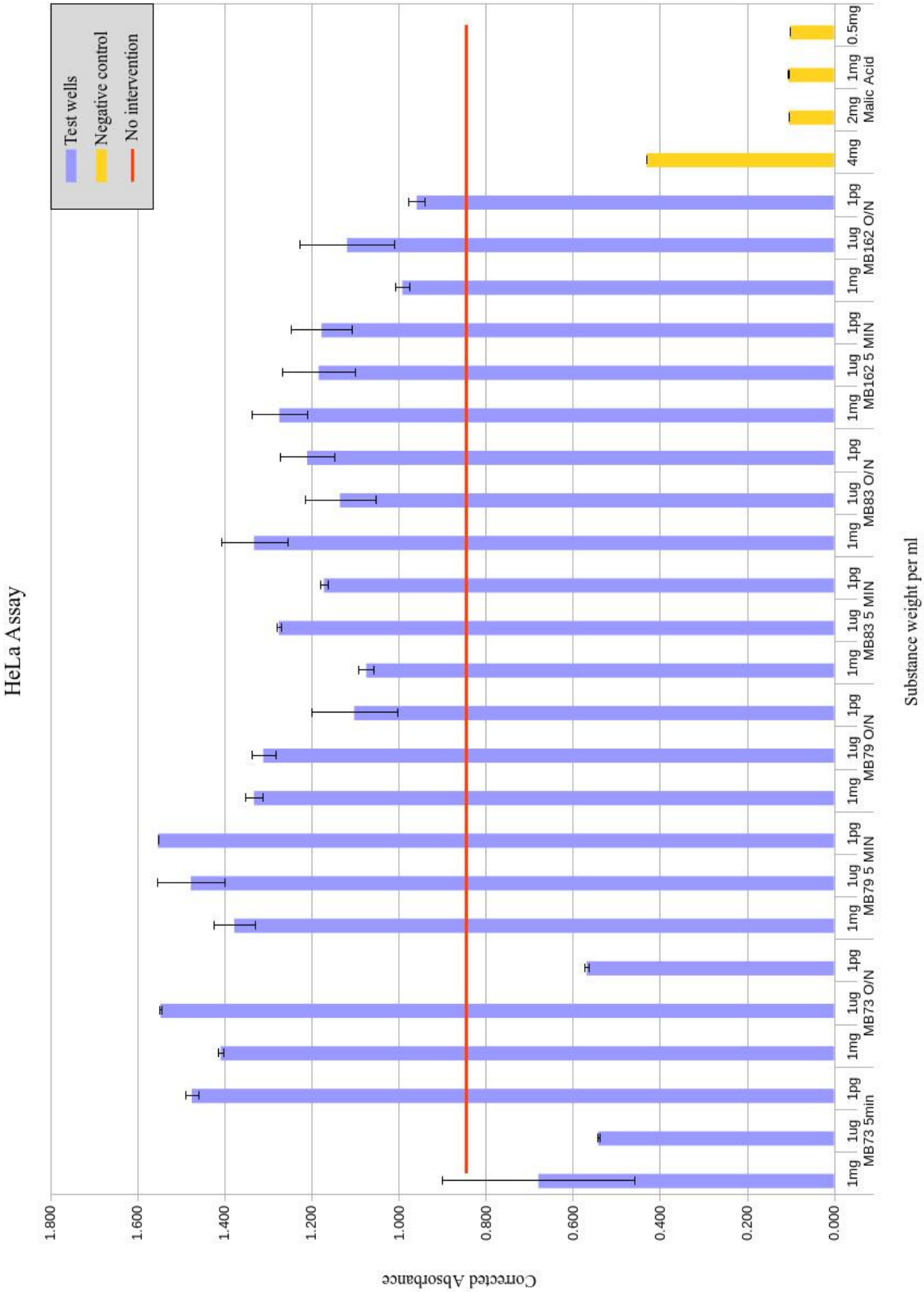


Figure 5.12: Cell assay results. Data was corrected against the blank; Error bars reflect data variance; the horizontal line shows the value obtained for the healthy, untreated cells, which was used to score the results for each test solution.

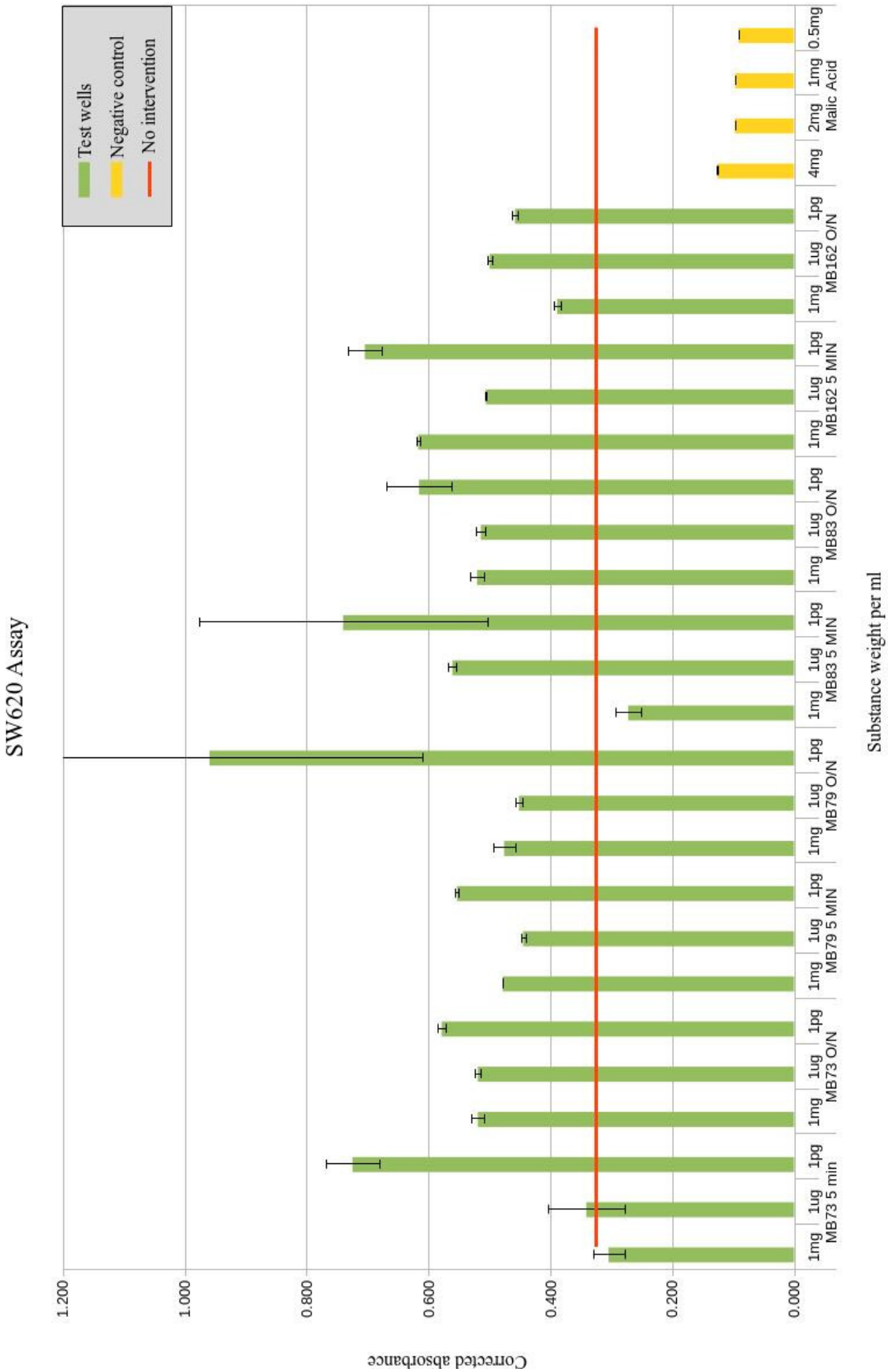


Figure 5.13: Cell assay results. Data was corrected against the blank; Error bars reflect data variance; the horizontal line shows the value obtained for the healthy, untreated cells, which was used to score the results for each test solution.

Table 5.8: Summarised results of the cell viability assay

		Hek			HeLa			SW620		
Specimen	Extraction	1mg/ml	1μg/ml	1ng/ml	1mg/ml	1μg/ml	1ng/ml	1mg/ml	1μg/ml	1ng/ml
MB73	5 min	D	D	N	D	D	I	N	N	I
	Overnight	N	D	D	I	I	D	I	I	I
MB79	5 min	D	D	D	I	I	I	I	I	I
	Overnight	D	D	D	I	I	I	I	I	I
MB83	5 min	N	I	I	I	I	I	D	I	I
	Overnight	D	D	D	I	I	I	I	I	I
MB162	5 min	N	D	D	I	I	I	I	I	I
	Overnight	D	D	D	I	I	I	I	I	I
Malic Acid		Decrease across all concentrations								

As per the definitions provided on page 130, D = Biomass decrease; I = Biomass increase; N = no effect

5.4 Discussion

A number of methods were undertaken in an attempt to culture *Russula* fungal tissue, as the extraction process ideally requires a large amount of pure starting material. However, this was attempted prior to learning of the difficulties experienced in artificially culturing ectomycorrhizal fungi. The Russulaceae, in particular, are difficult to culture (Brundrett et al. 1996, Nara 2009), possibly due to their obligate symbiotic nature (Stadler & Hoffmeister 2015).

Initial attempts used standard laboratory media used for culturing of fungal material, including Sabouraud dextrose agar. However, as there was no evidence of spore germination, it was thought substances produced by the host may have been required. This was the reasoning behind the addition of primed water or *Eucalyptus* wood shavings into the media. Ishida et al. (2008) found spore germination of some EMF was significantly improved when in the presence of host roots. However, this was not the case for the *Russula* species trialled here.

Shittu et al. (2005, 2006) reported they were able to culture a species of *Russula* and were able to obtain a sufficient amount of material for biochemical extraction. Their initial culture was obtained by inoculating a Sabouraud dextrose agar slant with spores, followed by subculturing and maintenance on additional slants (Shittu et al. 2005). Sections of mycelium from the slants were used to inoculate the working cultures which were shaken in Sabouraud dextrose broth. This was sufficient to obtain a crude extract which subsequently showed some antimicrobial activity. The following year, the same research group performed a similar experiment, but included a solid state culture using sterile rice. After 14 days of incubation they were able to obtain a greater weight of crude extract than from the broth cultures (Shittu et al. 2006). However, the identity of the species grown was not confirmed, and its reportedly rapid growth seems counterindicative with what is known about the Russulaceae. It was therefore possible the final culture was not actually a species of *Russula*.

Sangtiean & Schmidt (2002) also had some success culturing ectomycorrhizal fungi, including three species of *Russula* and one of *Lactarius*, on a modified solid medium. Initial cultures were created from freshly collected fruiting bodies by aseptically removing a small portion of the stipe and placing it on an agar surface. The medium was a complex combination of vitamins, organic nitrogen and buffer. It did not contain antibiotics, but fre-

quent subculturing kept contamination to a minimum. These cultures were subsequently used to inoculate a floating culture – a 4mm diameter disk of agar containing hyphae was transferred to a sterile nylon mesh supported by wax in each corner, which was floated on the liquid medium. Ultimately, these cultures produced a greater amount of biomass than those grown on the equivalent solid medium. After 3 months of axenic culture *in vitro* ECM associations were assessed. This was confirmed by formation of a hyphal mantle, observed under magnification.

Whilst the use of cultured hyphae as the bioactive source was not pursued, it would appear a substantial amount of attention and time would be required to achieve this, with no guarantee of success. Therefore, it was decided to perform extractions on fruit bodies rather than cultured material. The Russulaceae continue to maintain their reputation for being difficult to culture.

Numerous procedures have been used in the extraction of biochemical compounds from fungi, but they all followed a similar, basic process. The majority of extractions were prepared directly from the fungal fruiting bodies (Türkoğlu et al. 2007, Zhao et al. 2010, Beattie et al. 2011), which were generally either frozen or dried prior to homogenisation. Solvents have been added to homogenates and extractions carried out at various temperatures, times and conditions. Finally, the starting material and solvent have been separated and the solvent evaporated from the extracted material. After weighing, the extracts are typically suspended to a known concentration, usually in DMSO or water.

A variety of solvents have been employed in fungal extractions, including ethyl acetate (Stadler & Sterner 1998, Shittu et al. 2005, 2006), ethanol (Türkoğlu et al. 2007, Beattie et al. 2011), dichloromethane (Keller et al. 2002, Malagòn et al. 2014), and water (Zhao et al. 2010, Bala et al. 2011, Beattie et al. 2011), which have been used individually or as part of a sequential extraction process. Extractions have been conducted over 40 minutes (Malagòn et al. 2014) to 24 hours (Türkoğlu et al. 2007, Zhang et al. 2010) and at temperatures ranging from 4^{o}C (Zhang et al. 2010) to 95^{o}C . However, little information was provided regarding the rationale for specific solvent, temperature or extraction time and the process followed would have a major impact on the components of the extracts obtained (Valgas et al. 2007). The use of frozen or dried material could result in a finer homogenate than using fresh tissue, which would have the advantage of improved extraction effeciency due to the increased surface area. Dried material would also have the advantage of not diluting the solvent. Solvents have different properties and will preferentially extract different compounds; volatile solvents would also be easier to remove from

the extract than non-volatile solvents, reducing the risk of solvent contamination. Finally, variances in temperature and extraction times may have impact on the quality and quantity of the extracted compounds.

It has also been found, particularly in members of the Russulaceae family, that changes in chemical composition can occur when fruiting bodies are damaged (Stadler & Sterner 1998, Spiteller 2008, Malagòn et al. 2014). The mushrooms contain fatty acid esters of sesquiterpenoid alcohols which are converted through injury-induced hydrolysis into pungent, acrid or bitter compounds (Malagòn et al. 2014, Spiteller 2015). These changes can occur within seconds and continue over several hours. It has also been found that the original metabolites can form artefacts due to degradation caused by some solvents, including ethanol and methanol (Malagòn et al. 2014). Therefore, it was decided to include the fourth mushroom specimen, MB162, use dichloromethane as the extraction solvent rather than methanol and to obtain short-term (5 minute) and long-term (overnight) extractions.

The microbe-applicator worked appropriately in the application of the micro-organisms to the plate surface. A small amount of inadvertent inoculation occurred, possibly due to overloading the material with the microbial suspensions. However, it allowed the simultaneous application of numerous organisms to the agar surface without contamination of the central well. It was felt this method provided a suitable means to maximise the test organisms with such a limited amount of test material.

No anti-microbial activity was detected in the methanolic extracts of the *Russula* specimens examined, a result that was inconsistent with work conducted elsewhere. For example, Türkoğlu et al. (2007) found an ethanolic extract of *R. delica* which had significant antioxidation properties was also mildly effective in inhibiting the growth of *S. aureus, B. cereus* and *Micrococcus flavus*; Bala et al. (2011) found a highly concentrated water-based sonicated extract of *R. erumpens* showed activity against *S. aureus*; and Chen et al. (2008) showed that a fermentation broth from *Russula vinosa* had activity against *S. aureus* and *E. coli*.

It was initially intended to use the crude dichloromethane extracts for the cell viability assay, saving the fractions for targeted assays following a positive result. However, the results obtained could not be so easily defined, and an increase in the cancer-cell populations was quite unexpected.

Compound effects on non-cancerous cells was evaluated using the human embryonic cell

line (Hek-293). In general, cell viability decreased when exposed to the test extracts, though only by approximately 25%. This would suggest there is an adverse effect on the cells - ideally, healthy cells should not be compromised.

Compound effects on cancerous cells was evaluated using human cervical (HeLa) and colon (SW620) adenocarcinoma cell lines. In general, cell viability *increased* when exposed to the test extracts, suggesting the compounds either directly aided the cell growth and/or allowed the cells to overcome the toxicity of the well contents. It was also possible the fats present in the extracts played a major role here. It has long been assumed that proliferating cells of cancerous cell lines in culture synthesise lipids for membrane growth *de novo* from the glucose or glutamine supplied in the medium. However, when the medium contains the lipid palmitate, Yao et al. (2016) have shown that some lines, including HeLa, preferentially utilise that.

Closer examination of the results (Table 5.9) shows the two highest concentrations (1mg/ml and 1μg/ml) of the 5 minute MB73 extract decreased the Hek and HeLa cellular biomass and had no obvious effect on the SW620. However if, for example, the fatty acids in the extracts did provide a food source for the cells, it could be suggested an additional component of those two extracts was sufficiently strong enough to inhibit the growth response of these tested cell lines and it wasn't until the lowest extract concentration (1ng/ml) that it was no longer effective. This may also explain the result obtained for the 5 minute MB83 extract on the SW620 cell line (Table 5.8).

The overnight extraction of MB73 may have resulted in the extraction of additional or an increased concentration of bioactive compounds. The lowest concentration of this extract had no effect on the Hek cell line in the 5 minute extract when compared with the overnight extract. Likewise, the SW620 cell line was not affected by the 5 minute extract at 1μg/ml, but cell growth was increased when incubated with the overnight extract.

Table 5.9: From Table 5.8, results of the MB73 extracts on the cell viability assay.

	Hek			HeLa			SW620		
Extraction	1mg/ml	1μg/ml	1ng/ml	1mg/ml	1μg/ml	1ng/ml	1mg/ml	1μg/ml	1ng/ml
5 min	D	D	N	D	D	I	N	N	I
Overnight	N	D	D	I	I	D	I	I	I

D = Biomass decrease I = Biomass increase N = No effect

It is interesting to note, most cases of Russulaceae bioactivity success occurred with the use of purified products. For example, Liu (2007) isolated the compound Rufuslactone from *Lactarius rufus*, which demonstrated antifungal properties; (Zhao et al. 2010) isolated a lectin from *R. delica* which demonstrated antiproliferative activity against breast adenocarcinoma and hepatoma cells, and inhibited HIV-1 reverse transcriptase, while (Zhang et al. 2010) isolated a lectin with similar properties from the fruiting bodies of *R. lepida*. On the contrary, a crude ethanolic extract from *R. clelandii* showed low to no cytotoxic activity in research conducted by Beattie et al. (2011) and crude methanolic extracts from a variety of Russulacaea species showed no effect on murine cancer cell lines (Tomasi et al. 2004).

Crude fungal extracts can contain a variety of compounds in varying proportions. For example, Yaltirak et al. (2009) were able to identify 19 components in an ethanolic extract prepared from *R. delica*, whilst Mohamed & Farghaly (2014) detected a total of 107 metabolites in the ethanolic extract of the agaric mushroom *Pleurotus ostreatus* (Agaricales). Of greater interest, however, is the latter study which involved a comparison of the extracts sourced from either fresh or dried fruiting bodies. Of the 107 metabolites identified, only 14 were found in both extracts. Another 56 were only found in the extract of fresh material and the remaining 37 were only found in the extract of dried fruiting bodies. Combined with the knowledge discussed previously of metabolite variation due to injury, this clearly demonstrates the impact of material treatment prior to extraction.

The isolation of individual products potentially requires a targeted approach based on chemical knowledge, laboratory capabilities (such as requirements for specific instrumentation) and supply of starting material. As an example, in order to obtain 44mg of a single compound, Tan et al. (2004) began with 12kg of starting material (*R. nigricans*). The use of such large amounts of starting material, and volumes of solvents required to extract it,

is not efficient or necessary for an initial screening of natural product extracts. However, if bioactives are present in a crude extract, their concentration may be too low to exhibit an effect, or other compounds within the extract may interfere. The use of bioautographic methods may therefore be beneficial (Valgas et al. 2007). Essentially, the compounds of a crude extract are separated through chromatography before testing against biological agents. For example, Masoko & Eloff (2006) utilised thin layer chromatography (TLC) in this regard. After separation, a suspension containing medium and one of 5 pathogenic fungi was sprayed onto the surface of the TLC plate and allowed to grow. Subsequently, a colourimetric assay was utilised to determine the presence or absence of growth. Results from this initial procedure may assist in the refinement of subsequent compound isolation and experimentation.

Whilst bioactive extracts were obtained from the *Russula* species sampled, improvements could be made to the extraction process in order to increase the yield. In particular, the material used in the preparation of these extracts was not finely processed, due to the use of either fresh or thawed fruiting bodies and a small domestic blender. Where fresh material is being used it should be frozen (snap frozen if possible) and homogenised while frozen. A sequential extraction process, where a number of different solvents are progressively used, may also be beneficial in order to maximise the range of metabolites extracted. As there tends to be minimal starting material in this type of research, efforts must be made to maximise their use.

The cell viability assay needs to be repeated not only with the fractionated extracts, but also the crude extracts if the fats can be removed. It would also be interesting to try a bioautographic method for antimicrobial testing. Rather than provide a definitive answer, it might indicate an area of interest so efforts could be made to isolate and concentrate a portion of the extract. Other assays may also be worthwhile - as mentioned previously, products isolated from the Russulaceae have included antifungals, antivirals and antioxidants. However, they may also demonstrate bioactivity against other agents such as protozoa, nematodes and plant pathogens, or be a source of specific enzymes and pigments.

In chapter 2 it was noted taste was one of the useful characteristics for field identification of Russulaceae. However it was not appreciated how valuable this approach is. Sesquiterpenes are a class of chemical found in the Russulaceae, which contribute to a chemical defense system aimed at discouraging damage to the fruiting body caused by opportunistic animals, insects or micro-organisms (Liu 2007). These chemicals are responsible for changes to colour, smell and taste of the fruiting bodies (Liu 2007, Velisek & Cejpek

2011, Malagòn et al. 2014). There are significant differences in the chemical composition of intact, or non-injured fruiting bodies, and those which have incurred some amount of damage (Stadler & Sterner 1998, Malagòn et al. 2014). Physical damage of the fruiting body initiates changes to the biologically inactive sesquiterpene-precursor, resulting in the production of secondary metabolites, evidenced by changes in colour and/or taste. Malagòn et al. (2014) has found little difference between species in the composition of the precursor components. However, the composition of the secondary metabolites varies considerably, to the point they refer to it as being chemotaxonomic. Thus there is importance of noting changes to colour and taste which commence the moment a fruiting body is collected, or a small portion is broken off to taste. Taste remains highly subjective, but this may be an area where further study may result in improved identification of Russulaceae species in the field, as well as the discovery of useful bioactive compounds.

As highlighted in the earlier chapters of this thesis, there are still a number of SEQ Russulaceae species to be fully described. Examination of the bioactives of these species may yield important new drugs and be crucial to the improved identification of species. Exploration of how does environment effect metabolite composition may be worthy. Does time of collection have an impact? Is there a metabolic difference based on structure (cap, stipe, hyphae)? There are many questions in this area of research, and not many answers as yet. The hunt for novel fungal-derived natural products will continue.

Chapter 6

Conclusion

The Kingdom Fungi contains a large number of undocumented species. With the drastic rates of land clearing occurring in many parts of the world, there is a serious concern that many fungi will never be formally described or studied. This is unacceptable from an ethical point of view but also may mean that important human food materials, agriculturally useful taxa and sources of novel bioactive compounds may be permanently lost.

SEQ contains a great diversity of habitats and an associated fungal biodiversity, but there are few mycologists working in northern Australia. One main fungal society, the Queensland Mycological Society, currently lists on their website, a substantial number of undescribed taxa of macrofungi particularly in the Agaricomycete families, Agaricaeae, Boletelaceae and the Russulaceae. Therefore, this study and others like it, are essential in order to contribute to the knowledge of the diversity of Australian fungi, and the fungi in SEQ in particular.

The Russulaceae is an ectomycorrhizal macrofungal family of 4 genera: *Russula, Lactarius, Lactifluus* and *Multifurca*. They can be found in a range of environments, but are most commonly found in temperate forests and in Australia, in wet and dry eucalypt forests. Members of the group form typical mantle structures around the roots of *Eucalyptus* and *Casuarina* species and provide mineral nutrients for their hosts in return for plant carbon.

The first part of the study required the location and collection of Russulaceae specimens from a range of environments in SEQ. This was performed over the course of 6 years, yielding over 300 specimens. Russulaceae species are notoriously difficult to identify

using morphological characteristics alone, so molecular techniques were the focus of the study. DNA was successfully extracted from all of the specimens collected, but the results of the subsequent amplification of the ITS region was variable. It was thought this was most likely due to the presence of PCR inhibitors, or particularly in the case of the dried specimens, degraded or damaged DNA. Extensive experimentation was subsequently aimed at improving the success rate of the PCR. Different DNA extraction protocols were tried in an attempt to either remove or decrease the impact of potential inhibitors and changes were made to the PCR master mix or thermalcycling parameters in order to increase sensitivity or reduce the risk of failure. There was some success in this regard, but PCR optimisation is an area of that requires further improvement in studies of this type.

DNA sequencing of collected specimens confirmed the presence of some common Russulaceae species found in the southern states, such as *Russula cheelii* and *Lactarius eucalyptii*, but also highlighted the presence of a number of new taxa. Both molecular and morphological taxonomic approaches were used to describe four of these new taxa, currently designated as species A, B, C and D. There is still a substantial amount of work that could be carried out in describing the other new taxa highlighted by this study. Exploration of additional areas in Queensland would also likely yield further novel Russulaceae taxa. For example, Dearnaley (unpublished) has recently conducted field work in the Cape York region of North Queensland and suggested the presence of a number of potential new species, unusually, for members of the Russulaceae, present in tropical rainforest habitats.

Most orchids form mycorrhizal associations with members of the fungal families, Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae. These saprotrophic fungi provide water, phosphorus and micronutrients to their adult plant partners and receive photosynthate in return for their services. A minority of orchids are unable to photosynthesise as adults and rely on fungal carbon in addition to inorganic nutrients. These obligate mycoheterotrophic orchids are usually found in heavily shaded habitats such as forests. Obligate mycoheterotrophic orchids typically obtain their carbon indirectly from neighbouring tree species via an ectomycorrhizal conduit, commonly members of the Russulaceae. Australia has a number of genera of obligate mycoheterotrophic orchids including *Erythrorchis, Gastrodia, Epipogium* and *Dipodium*. Recent studies of several *Dipodium* orchid species have highlighted the presence of *Russula* mutualists Bougoure & Dearnaley 2005, Dearnaley & Le Brocque 2006). This study confirmed this via both Sanger and Next Generation sequencing of root and peloton DNA. In addition to mycorrhizal Russula fungi in the target species, *D. roseum, Lactarius* fungi were detected for the first time in Aus-

tralia. This observation has implications for habitat management of threatened *Dipodium* species such as *D. pictum* in northern Australia. The presence of both *Russula* and *Lactarius* species and their tree hosts may be important for monitoring and maintaining the habitat health of the orchids.

Fungi have provided a source of many important medical compounds over the past century. Most prominent have been antibiotics such as penicillins and cephalosporins and the immunosuppressant, cyclosporin which has allowed organ transplants to occur in humans without the threat of tissue rejection. Fungi have also provided a source of antiviral, anticancer and cholesterol lowering drugs. In this study, compounds were extracted from tissues of newly documented *Russula* species. Initial attempts to grow these fungi in pure culture were unsuccessful and thus the compounds were sourced from fruiting body collections of individual *Russula* species. Although these compounds had little antimicrobial activity, they did have impacts on the growth of a number of cancer cell lines. Further investigation of purified fractions of these extracts might isolate the responsible molecule and possibly uncover aspects of the biology of these cells that might be therapeutically significant. Further study of the antiviral and cholesterol lowering capabilities of these compounds may also be warranted. So too might be additional studies on the other novel species identified in this study. This appears particularly worthy given the diversity of bioactivity found in the family.

In summary, this study has highlighted a number of new Russulaceae species present in SEQ. It has demonstrated the utility of both molecular and morphological approaches in the taxonomy of the group. Investigation of the ecology of these fungal taxa has uncovered some novel aspects of orchid mycorrhizal biology in Australia. While not specifically isolating key bioactive compounds from these fungi, this project has suggested that *Russula* fungi should continue to be investigated for their medical value.

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Appendix A

Data collection forms

Forms used for data collection. Figure A.1 shows the initial form used to record location and collection details for each specimen. As there were multiple forms on each page, it allowed rapid collection of information and ease in the allocation of unique identifiers to each specimen collected.

Figure A.2 illustrates the second form used to collect information regarding macromorphology of the specimens, while Figure A.3 was used for recording the microscopic characteristics. The forms were not only useful for recording the information in an orderly fasion. They also acted as a checklist, ensuring the appropriate information was collected.

Lab. # _____ **SPECIES:** _____

<table>
<tr><td>DATE</td><td>LOCALITY</td><td>GPS</td><td rowspan="2">SPECIMEN AGE</td><td>SUBSTRATE</td><td>ASSOCIATION</td></tr>
<tr><td>/ /</td><td></td><td></td><td></td><td></td></tr>
<tr><td>COLLECTOR</td><td></td><td></td><td>Immature</td><td></td><td></td></tr>
<tr><td></td><td rowspan="2">SPECIMEN NUMBER</td><td>ALT</td><td>Mature</td><td colspan="2">GROWTH HABIT</td></tr>
<tr><td>CHARACTER</td><td>ACC</td><td>Old</td><td colspan="2">Single / Scattered / Caespitose / Grouped</td></tr>
<tr><td></td><td></td><td colspan="4">SPECIMEN NOTES:</td></tr>
<tr><td colspan="6"></td></tr>
</table>

Figure A.1: Short form for the assignment of specimen numbers and recording of collection details

Lab. # _____ **SPECIES:** _____

SEQUENCED Group -

PILEUS (CAP)

SHAPE – SIDE	**SHAPE – TOP**	**COLOUR & REFERENCE**
SIZE	**TEXTURE**	
PEELING N / Y x	**SURFACE MARGIN** Smooth / Striate /	
CAP MARGIN Plane /	**KOH**	**NOTES**

FLESH

COLOUR & REFERENCE	**TEXTURE**	**SIZE**	**NOTES**

STIPE

SHAPE	**COLOUR & REF**	**DIMENSIONS:**	**ATTACHMENT**
		WIDEST	**TEXTURE**
		NARROW	**INTERIOR**
		LENGTH	**$FeSO_4$**
NOTES			

GILL

COLOUR & REF	**ATTACHMENT** Adnate / Subdecurrent /	**SPACING**
		LAMELLULAE
	MARGIN Smooth /	**ARRANGEMENT**
GILLS / ½ CAP	**THICKNESS**	**NOTES**

EXUDATES/BRUISING

AREA Cap / Gills / Flesh / Stipe	**COLOUR, REFERENCE & DESCRIPTION**

OTHER

ILLUSTRATIONS

Figure A.2: Form used for recording a specimen's macroscopic characteristics

Lab. # _____ DATE: _____

CHARACTERISTICS – MICROSCOPIC

SPORES

MAGNIFICATION: x _____

DIMENSIONS (Not including ornamentation or apiculus) AVG STD

L:												
W:												
Q:												

SHAPE: _____ COLOUR (KOH): _____

SPORE WALL: LAYERS: _____ THICKNESS: _____

ORNAMENTATION (In Meltzer's): _____

OTHER: _____

ILLUSTRATIONS:

PELLIS

MAG: x _____ STAIN: _____

Pilei- _____

Supra- _____

Stipiti- _____

HYMENIUM

BASIDIA

MAGNIFICATION: x _____ STAIN: _____

DIMENSIONS (Not including sterigmata) AVG STD

L:												
W:												

SHAPE & CONTENTS: _____

STRUCTURAL NOTES: _____

STERIGMATA: NUMBER: _____ LENGTH: _____

OTHER: _____

Figure A.3: The form used for recording a specimen's microscopic details

CYSTIDIA

	STAIN		MAG.	DESCRIPTION (Shape, abundance, colour, distribution etc)								
CHEILO-												
L:												
W:												
PLEURO-												
L:												
W:												
DERMATO-												
L:												
W:												
CAULO-												
L:												
W:												
GLOEO-												
L:												
W:												
MACRO-												
L:												
W:												
PSEUDO-												
L:												
W:												

SUBHYMENIUM (HYPHAE)

MAG: x _____ STAIN: _____

DESCRIPTION: _____

HYMENOPHORAL TRAMA

MAG: x _____ STAIN: _____

SPHAEROCYSTS: (Position, arrangement, abundance) _____

_____

_____

LACTIFERS: _____

ILLUSTRATIONS:

Appendix B

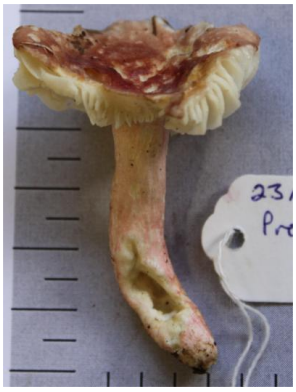
Colour plates of fresh specimens

No photo MB01 Lodbroke 12/02/12 96% ID *R. violepes* Acc# KF361797.1	No photo MB02 Lodbroke 12/02/12 91% ID *R. tawai* Acc# JX178491.1	 MB03 Lodbroke 26/02/12 90% ID *R. tawai* Acc# JX178491.1
No photo – squashed during transport MB04 Crows Nest 17/03/12 90% ID *R. crustosa* Acc# EU598193.1 Sp. C	 MB05 Lodbroke 18/03/12 91% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB06 Lodbroke 18/03/12 92% ID *R. tawai* Acc# JX178491.1 Sp. D.
 MB07 Lodbroke 18/03/12 Failed PCR	 MB08 Lodbroke 12/03/12 92% ID *R. delica* Acc# KX812842.1 Sp. E.	 MB09 Duggan Park 23/03/12 91% ID *R. tawai* Acc# JX178491.1

MB10
Lodbroke 23/03/12
91% ID *R. tawai*
Acc# JX178491.1

MB11
Lodbroke 23/03/12
90% ID *R. tawai*
Acc# JX178491.1

MB12
Lodbroke 23/03/12
91% ID *R. tawai*
Acc# JX178491.1



MB13
Lodbroke 23/03/12
91% ID *R. tawai*
Acc# JX178491.1

MB14
Lodbroke 23/03/12
91% ID *R. tawai*
Acc# JX178491.1

MB15
Lodbroke 23/03/12
91% ID *R. tawai*
Acc# JX178491.1

MB16
Lodbroke 24/03/12
Short sequence

MB17
Lodbroke 24/03/12
92% ID *R. tawai*
Acc# JX178491.1
Sp. D.

MB18
Lodbroke 24/03/12
92% ID *R. tawai*
Acc# JX178491.1
Sp. D.

 MB19 Lodbroke 04/05/12 Not Russulaceae	 MB20 Lodbroke 04/05/12 Not Russulaceae	 MB21 Lodbroke 09/06/12 Not Russulaceae
 MB22 Ravensbourne 17/06/12 Not Russulaceae	 MB23 Ravensbourne 17/06/12 90% ID *R. tawai* Acc# JX178491.1	 MB24 Ravensbourne 17/06/12 Unique sequence ~ *R. acrolamellata*
 MB25 Lodbroke 02/03/13 90% ID *R. tawai* Acc# JX178491.1	 MB26 Tully Park 04/03/13 Not Russulaceae	 MB27 Tully Park 04/03/13 Failed to amplify

MB28
Tully Park 04/03/13
Failed to amplify

MB29
Tully Park 04/03/13
Failed to amplify

MB30
Tully Park 04/03/13
Failed to amplify

MB31
Tully Park 04/03/13
Failed to sequence

MB32
Tully Park 04/03/2013
Unique sequence
~ *R. archaeosuberis*

MB33
Tully Park 04/03/13
Failed to amplify

MB34
Tully Park 04/03/13
Failed to amplify

MB35
Tully Park 04/03/2013
Unique sequence
~ *R. subfoetens*

MB36
Tully Park 04/03/13
Failed to amplify

 MB37 Tully Park 04/03/2013 88% ID *R. tawai* Acc# JX178491.1	 MB38 Tully Park 04/03/2013 87% ID R. foetens Acc# KX095018.1	 MB39 Crows Nest 09/03/2013 Unique sequence ~ *R. subfoetens*
 MB40 Crows Nest 09/03/13 80% ID R. kanadii Acc# NR_153247	 MB41 Crows Nest 09/03/13 Unique sequence ~ *R. tawai*	 MB42 Crows Nest 09/03/13 Failed to amplify
 MB43 Crows Nest 09/03/13 Sequence too short	 MB44 Crows Nest 09/03/13 Unique sequence ~ *R. subfoetens*	 MB45 Crows Nest 09/03/13 93% ID *R. delica* Acc# KX812842.1 Sp. F

MB46
Crows Nest 09/03/13
94% ID *R. delica*
Acc# KX812842.1
Sp. F.

MB47
Crows Nest 09/03/13
80% ID *R. acrolamellata*
Acc# MF461612.1

MB48
ANWR 10/03/13
Unique sequence
~ *R. tawai*

MB49
ANWR 10/03/13
95% ID *R. tawai*
Jacc# JX178491.1

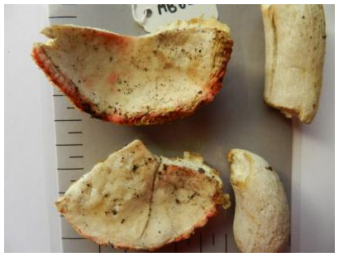
MB50
ANWR 12/03/13
96% ID *R. tawai*
Acc# JX178491.1

MB51
Incorrect sequence
obtained

MB52
Lodbroke 14/04/13
92% ID *R. tawai*
Acc# JX178491.1
Sp. D.

MB53
Lodbroke 14/04/13
Sequence too short

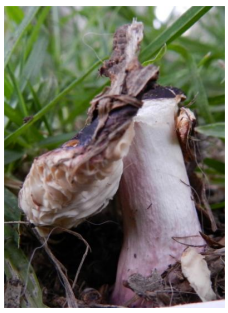
MB54
21/04/13
Sequence too short

 MB55 Ravensbourne 20/04/13 85% ID *R. purpureoflava* Acc# EU019914	 MB56 Crows Nest 20/04/13 Failed to amplify	 MB57 Crows Nest 20/04/13 Failed to amplify
 MB58 Crows Nest 20/04/13 Failed to amplify	 MB59 21/04/13 Unique sequence ~ *R. redolens*	 MB60 21/04/13 Unique sequence ~ *R. foetens*
 MB61 Crows Nest 21/04/13 92% ID *R. virescens* Acc# MG680184 Sp. C	 MB62 Crows Nest 21/04/13 92% ID *R. kanadii* Acc# KM275230 Sp. C	 MB63 Ravensbourne 19/04/13 96% ID *R. atroviridis* Acc# JX178493.1 Sp. H

 MB64 Crows Nest 21/04/13 88% ID R. virescens Acc# LC008519	 MB65 Ravensbourne 21/04/13 Failed to amplify	 MB66 Ravensbourne 21/04/13 Failed to amplify
MB67 Lodbroke 14/07/13 Failed to amplify	 MB68 21/04/13 Sequence too short	 MB69 Lodbroke 14/07/13 91% ID *R. tawai* Acc# JX178491.1
 MB70 Lodbroke 29/07/13 92% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB71 15/04/14 92% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB72 Lodbroke 17/04/14 92% ID *R. tawai* Acc# JX178491.1 Sp. D.

 MB73 Lodbroke 17/04/14 89% ID *R. tawai* Acc# GU222263.1 Sp. I? (Now D)	 MB74 Lodbroke 18/04/14 Failed to amplify	 MB75 Lodbroke 18/04/14 94% ID *R. delica* Acc# KX812842 Sp. E.
 MB76 ANWR 24/04/14	 MB77 Brisbane 24/04/14 90% ID *R. crustosa* Acc# EU598193 Sp. J	 MB78 Brisbane 24/04/14 93% ID *R. delica* Acc# KX812842 Sp. E.
 MB79 Lodbroke 26/04/14 95% ID *R. violeipes* Acc# AY061726.1 Sp. A.	 MB80 Lodbroke 26/04/14 95% ID *R. violeipes* Acc# AY061726.1 Sp. A	 MB81 Lodbroke 26/04/14

 MB82 Lodbroke 26/04/14 No significant similarity found	 MB83 Lodbroke 27/04/14 Incorrect band size obtained	 MB84 Lodbroke 27/04/14 89% ID *R. tawai* Acc# GU222263.1 Sp. D.
 MB85 Lodbroke 27/04/14	 MB86 Lodbroke 27/04/14	 MB87 Lodbroke 27/04/14 93% ID *R. violeipes* Acc# AY061726.1 Sp. A.
 MB88 Lodbroke 03/05/14 92% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB89 Lodbroke 03/05/14 92% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB90 Lodbroke 03/05/14 95% ID *R. tawai* Acc# JX178491.1 Sp. D.

 MB91 Lodbroke 03/05/14 95% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB92 Lodbroke 04/05/14 93% ID *R. tawai* Acc# JX178491.1 Sp. D.	 MB93 Lodbroke 04/05/14 Failed to amplify
 MB94 Lodbroke 04/05/14 No significant similarity found	 MB95 Lodbroke 27/05/14 Failed to sequence	 MB96 Lodbroke 27/05/14 92% ID *R. tawai* Acc# JX178491.1 Sp. D.
 MB97 Lodbroke 01/06/14 Sequence too short	 MB98 Lodbroke 18/06/14	 MB99 Lodbroke 12/02/15 94% ID *R. violeipes* Acc# AY061726.1 Sp. A

MB100
Lodbroke 13/02/15
Failed to amplify

MB101
Lodbroke 14/02/15
Incorrect sequence

MB102
Lodbroke 21/02/15
Failed to amplify

MB103
Lodbroke 21/02/15
Failed to sequence

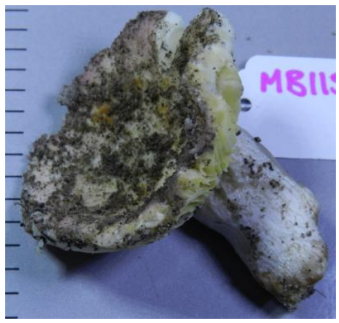
MB104
Lodbroke 21/02/15
Failed to amplify

MB105
Lodbroke 21/02/15
Incorrect sequence

MB106
Lodbroke 22/02/15
Multiple traces

MB107
Lodbroke 22/02/15
Multiple traces

MB108
Rockmount 27/03/15
Not Russulaceae

 MB109 Lodbroke 09/04/15 Not Russulaceae	 MB110 Tully Park 10/04/15 Not Russulaceae	 MB111 Tully Park 09/04/15 Sequence too short
 MB112 Tully Park 09/04/15 Unique sequence ~ *Lactifluus allardii*	 MB113 Tully Park 09/04/15 Failed to amplify	 MB114 Tully Park 09/04/15 Failed to amplify
 MB115 Tully Park 09/04/15 Failed to amplify	 MB116 Wilson's Downfall 12/04/15 92% ID *R. kanadii* Acc# KJ866936 Sp. C	 MB117 Wilson's Downfall 12/04/15 90% ID *R. crustosa* Acc# EU598193 Sp. J.

 MB118 Wilson's Downfall 12/04/15 Failed to amplify	 MB119 Wilson's Downfall 12/04/15 89% ID *R. tawai* Acc# JX178491.1	 MB120 Wilson's Downfall 12/04/15 Failed to amplify
 MB121 Wilson's Downfall 12/04/15 92% ID *R. kanadii* Acc# KJ866936 Sp. C	 MB122 Wilson's Downfall 12/04/15 Failed to amplify	 MB123 Wilson's Downfall 12/04/15 Failed to amplify
 MB124 Wilson's Downfall 12/04/15 Failed to sequence	 MB125 Wilson's Downfall 12/04/15 Failed to amplify	 MB126 Wilson's Downfall 12/04/15 Failed to amplify

MB127
Lodbroke 22/04/15
94% ID *R. violeipes*
Acc# JF908655.1
Sp. A

MB128
Crows Nest 04/05/15

MB129
Tully Park 02/06/15

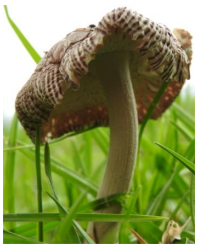
MB130
Tully Park 02/06/15
95% ID *R. marangania*
Acc# EU019930
Sp. H

MB131
Tully Park 02/06/15
Sequence too short
(Photo I. Milinovitch)

MB132
Tully Park 02/06/15
97% ID R. wollumbina
Acc# GU222260
(Sp. H)

MB133
Tully Park 3/6/15

MB134
Tully Park 3/6/15
98% ID *R. wollumbina*
Acc# EU019921



MB135
Lodbroke 27/12/15
Not Russulaceae

 MB136 Lodbroke 28/12/15	 MB137 Lodbroke 28/12/15	 MB138 Lodbroke 29/12/15 Incorrect sequence returned
 MB139 Lodbroke 29/12/15	 MB140 Lodbroke 29/12/15 Not Russulaceae	 MB141 Lodbroke 29/12/15
 MB142 Lodbroke 28/12/15 Incorrect sequence returned	 MB143 Lodbroke 29/12/15 Not Russulaceae	 MB144 Lodbroke 29/12/15
 MB145 Lodbroke 29/12/15	 MB146 Lodbroke 31/12/15 No significant similarity found	 MB147 Lodbroke 31/12/15

MB148
Lodbroke 03/01/16

MB149
Lodbroke 04/01/15

MB150
Lodbroke 05/01/16

MB151
Lodbroke 05/01/16

MB152
Lodbroke 05/01/16

MB153
Lodbroke 05/01/16

MB154
Lodbroke 05/01/16

MB155
Lodbroke 05/01/16
96% ID *R. violeipes*
Acc# KY693648.1
Sp. A

MB156
Lodbroke 05/01/16

MB157
Lodbroke 07/01/16
No significant similarity found

MB158
Lodbroke 07/01/16

MB159
Lodbroke 07/01/16

MB160
Lodbroke 16/01/16

MB161
Lodbroke 16/01/16
Not Russulaceae
(Spores not amyloid)

MB162
Lodbroke 16/01/16

MB163
Lodbroke 16/01/16

MB164
Lodbroke 20/03/16

MB165
Lodbroke 20/03/16

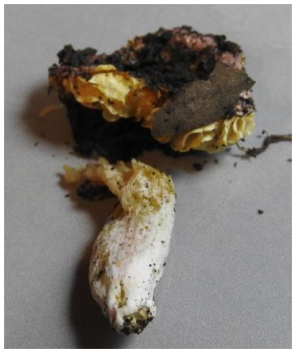
MB166
Lodbroke 20/03/16

MB167
Lodbroke 20/03/16

MB168
Lodbroke 20/03/16

MB169
Lodbroke 20/03/16 ?

MB170
Lodbroke 27/03/16



MB171
Lodbroke 27/03/16

MB172
Toowoomba 27/03/16

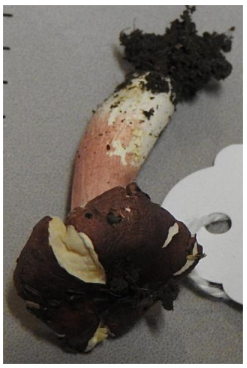
MB173
Toowoomba 27/03/16

MB174
Toowoomba 27/03/16

MB175
Toowoomba 27/03/16

MB176
Toowoomba 27/03/16

MB177
Lodbroke 21/08/16

 MB178 Lodbroke 10/09/16	 MB179 Lodbroke 16/09/16	 MB180 Lodbroke 17/09/16
 MB181 Lodbroke 19/09/16 Failed to amplify	 MB182 Lodbroke 19/09/16	 MB183 Lodbroke 19/09/16
 MB184 Lodbroke 22/09/16	 MB185 Lodbroke 22/09/16	 MB186 Lodbroke 23/09/16
 MB187 Lodbroke 23/09/16	 MB188 Lodbroke 27/09/16	 MB189 Lodbroke 27/09/16

MB190
Lodbroke 27/09/16

MB191
Lodbroke 30/03/17

MB192
Lodbroke 30/03/17
Not Russulaceae

MB193
Lodbroke 30/03/17
Not Russulaceae
(Spores not amyloid)

MB194
Lodbroke 30/03/17
Not Russulaceae
(Spores not amyloid)

MB195
Lodbroke 31/03/17

MB196
Lodbroke 02/04/17

MB197
Lodbroke 02/04/17

MB198
Lodbroke 02/04/17

MB199
Lodbroke 09/04/17

MB200
Lodbroke 09/04/17

MB201
Lodbroke 09/04/17

MB202
Lodbroke 09/04/17

MB203
Lodbroke 14/04/17

MB204
Lodbroke 14/04/17

MB205
Lodbroke 14/04/2017

MB206
Lodbroke 14/04/2017

MB207
Lodbroke 14/04/2017

MB208
Lodbroke 14/04/17

MB209
Lodbroke 15/04/2017

MB210
Lodbroke 15/04/2017

MB211
Lodbroke 15/04/2017

MB212
Lodbroke 16/04/2017

MB213
Lodbroke 22/04/2017

 MB214 Lodbroke 24/04/17	 MB215 Lodbroke 27/04/17 Not Russulaceae	 MB216 Lodbroke 14/12/17
 MB217 Lodbroke 17/12/17	 MB218 Lodbroke 20/12/17	 MB219 Lodbroke 24/03/18
 MB220 Lodbroke 24/03/18		

Appendix C

Specimen DNA and sequencing summary

C.1 Fresh specimens

Table C.1: Sample collection - Fresh specimens

Collection			Closest match			Closest Australian match	
Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB01	12/02/12	Lodbroke	*R. violeipes*	KF361797.1	96.0	*R. rostraticystidia*	94.6
MB02	12/02/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.0
MB03	26/02/12	Lodbroke	*R. tawai*	JX178491.1	90.0	*R. clelandii*	88.0
MB04	17/03/12	Crows Nest NP	*R. kanadii*	KJ866936	91.3	*R. iterika*	86[2]
MB05	18/03/12	Lodbroke	*R. tawai*	JX178491.1	91[1]		
MB06	18/03/12	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.2
MB07	18/03/12	Lodbroke	Failed to amplify				
MB08	12/03/12	Lodbroke	*R. delica*	KX812842.1	92.0	*R. pumicoidea*	93.1
MB09	23/03/12	Duggan Park	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.1
MB10	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.1

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB11	23/03/12	Lodbroke	*R. tawai*	JX178491.1	90.0	*R. clelandii*	89.0
MB12	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.7
MB13	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.0
MB14	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.7
MB15	23/03/12	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.7
MB16	24/03/12	Lodbroke	*R. tawai*	JX178491.1	86[1]		
MB17	24/03/12	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	90.9
MB18	24/03/12	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.4
MB19	04/05/12	Lodbroke	*Melanoleuca strictipes*	JF908359.1	94.0		
MB20	04/05/12	Lodbroke	*Melanoleuca strictipes*	JF908359.1	94.0		
MB21	09/06/12	Lodbroke	Taxonomically not Russulaceae				
MB22	17/06/12	Ravensbourne	Taxonomically not Russulaceae				
MB23	17/06/12	Ravensbourne	*R. tawai*	JX178491.1	90.0	*R. clelandii*	88.6
MB24	17/06/12	Ravensbourne	*R. acrolamellata*	MF461612	94.8	*R. neerimea*	96.2
MB25	02/03/13	Lodbroke	*R. tawai*	JX178491.1	90.0	*R. clelandii*	88.7
MB26	04/03/13	Tully Park	Taxonomically not Russulaceae				
MB27	04/03/13	Tully Park	Failed to amplify				
MB28	04/03/13	Tully Park	Failed to amplify				
MB29	04/03/13	Tully Park	Failed to amplify				
MB30	04/03/13	Tully Park	Failed to amplify				
MB31	04/03/13	Tully Park	Failed to sequence				
MB32	04/03/13	Tully Park	*R. archaea*	EU598165.1	87.0	*R. galbana*	84[2]
MB33	04/03/13	Tully Park	Failed to amplify				
MB34	04/03/13	Tully Park	Failed to amplify				
MB35	04/03/13	Tully Park	*R. subfoetens*	KF002757.1	89.7	*R. neerimea*	90.6
MB36	04/03/13	Tully Park	Failed to amplify				
MB37	04/03/13	Tully Park	*R. tawai*	JX178491.1	88[1]		
MB38	04/03/13	Tully Park	*R. foetens*	KX095018.1	87[1]		
MB39	09/03/13	Crows Nest NP	*R. acrolamellata*	MF461612	79[1]		

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB40	09/03/13	Crows Nest NP	*R. virescens*	MK782587	80[1]		
MB41	09/03/13	Crows Nest NP	*R. atroviridis*	GU222285.1	79[1]		
MB42	09/03/13	Crows Nest NP	Failed to amplify				
MB43	09/03/13	Crows Nest NP	*R. roseostipitata*	GU222324.1	94.0		
MB44	09/03/13	Crows Nest NP	*R. subfoetens*	KF002757.1	93.0	*R. neerimea*	94.0
MB45	09/03/13	Crows Nest NP	*R. delica*	KX812842.1	93.0	*R. sinuata*	91.8
MB46	09/03/13	Crows Nest NP	*R. delica*	KX812842.1	94.0	*R. sinuata*	93.4
MB47	09/03/13	Crows Nest NP	*R. acrolamellata*	MF461612	80[1]		
MB48	10/03/13	ANWR	*R. tawai*	JX178491.1	94.0		
MB49	10/03/13	ANWR	*R. tawai*	JX178491.1	95.0		
MB50	12/03/13	ANWR	*R. tawai*	JX178491.1	96.0		
MB51	14/03/13		Incorrect sequence obtained				
MB52	14/04/13	Lodbroke	*R. tawai*	JX178491.1	92.1	*R. clelandii*	91.2
MB53	14/04/13	Lodbroke	*Insufficient sequence*				
MB54	21/04/13	Crows Nest NP	*R. redolens*	KT933950.1	75[1]		
MB55	20/04/13	Ravensbourne	*R. purpureoflava*	EU019914	85[1]		
MB56	20/04/13	Crows Nest NP	Failed to amplify				
MB57	20/04/13	Crows Nest NP	Failed to amplify				
MB58	21/04/13	Crows Nest NP	Failed to amplify				
MB59	21/04/13	Crows Nest NP	*R. redolens*	KT933950.1	94.0	*R. galbana*	87[1]
MB60	21/04/13	Crows Nest NP	*R. foetens*	KF245487.1	94.0	*R. galbana*	91.6
MB61	21/04/13	Crows Nest NP	*R. virescens*	MG680184	92.0	*R. iterika*	85[2]
MB62	21/04/13	Crows Nest NP	*R. kanadii*	KM275230.1	92.0	*R. iterika*	88[2]
MB63	19/04/13	Ravensbourne	*R. atroviridis*	JX178493.1	96.0	*R. wollumbina*	95.5
MB64	21/04/13	Crows Nest NP	*R. virescens*	LC008519	88[1]		
MB65	21/04/13	Ravensbourne	Failed to amplify				
MB66	21/04/13	Ravensbourne	Failed to amplify				
MB67	14/07/13	Lodbroke	Failed to amplify				
MB68	21/04/13	Crows Nest NP	*R. virescens*	KX267657	82[1]		

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB69	14/07/13	Lodbroke	*R. tawai*	JX178491.1	91.0	*R. clelandii*	89.1
MB70	29/07/13	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.4
MB71	15/04/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.2
MB72	17/04/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.1
MB73	17/04/14	Lodbroke	*R. tawai*	GU222263.1	89.0	*R. clelandii*	88.5
MB74	18/04/14	Lodbroke	Failed to amplify				
MB75	18/04/14	Lodbroke	*R. delica*	KX812842.1	94.0	*R. sinuata*	93.7
MB76	24/04/14	ANWR	*Not sequenced*				
MB77	24/04/14	Brisbane	*R. crustosa*	EU598193.1	90.0	*R. marangania*	86[2]
MB78	24/04/14	Brisbane	*R. delica*	KX812842.1	93.0	*R. sinuata*	91.8
MB79	26/04/14	Lodbroke	*R. violeipes*	AY061726.1	95[1]		
MB80	26/04/14	Lodbroke	*R. violeipes*	AY061726.1	95[1]		
MB81	26/04/14	Lodbroke	DNA extracted only				
MB82	26/04/14	Lodbroke	No significant similarity found				
MB83	27/04/14	Lodbroke	*Incorrect band size obtained*				
MB84	27/04/14	Lodbroke	*R. tawai*	GU222263.1	93.0	*R. clelandii*	91.4
MB85	27/04/14	Lodbroke	DNA extracted only				
MB86	27/04/14	Lodbroke	DNA extracted only				
MB87	27/04/14	Lodbroke	*R. violeipes*	AY061726.1	93.0	*R. rostraticystidia*	94.0
MB88	03/05/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.1
MB89	03/05/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.2
MB90	03/05/14	Lodbroke	*R. tawai*	JX178491.1	95.0	*R. clelandii*	93.4
MB91	03/05/14	Lodbroke	*R. tawai*	JX178491.1	95.0	*R. clelandii*	93.3
MB92	04/05/14	Lodbroke	*R. tawai*	JX178491.1	93.0	*R. clelandii*	91.7
MB93	04/05/14	Lodbroke	Failed to amplify				
MB94	04/05/14	Lodbroke	No significant similarity found				
MB95	27/05/14	Lodbroke	Failed to sequence				
MB96	27/05/14	Lodbroke	*R. tawai*	JX178491.1	92.0	*R. clelandii*	91.4
MB97	01/06/14	Lodbroke	*R. tapawera*	EU019942	80[1]		

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB98	18/06/14	Lodbroke	*Possibly Species D*				
MB99	12/02/15	Lodbroke	*R. violeipes*	AY061726.1	94.0	*R. rostraticystidia*	94.4
MB100	13/02/15	Lodbroke	Failed to amplify				
MB101	14/02/15	Lodbroke	Incorrect sequence obtained				
MB102	21/02/15	Lodbroke	Failed to amplify				
MB103	21/02/15	Lodbroke	Failed to sequence				
MB104	21/02/15	Lodbroke	Failed to amplify				
MB105	21/02/15	Lodbroke	Incorrect sequence obtained				
MB106	22/02/15	Lodbroke	Multiple traces				
MB107	22/02/15	Lodbroke	Multiple traces				
MB108	27/03/15	Rockmount	Taxonomically not Russulaceae				
MB109	09/04/15	Tully Park	Taxonomically not Russulaceae				
MB110	09/04/15	Tully Park	Taxonomically not Russulaceae				
MB111	09/04/15	Tully Park	*R. purpureoflava*	JX266626	92[1]		
MB112	09/04/15	Tully Park	*Lactifluus volemus*	HQ318222	79[1]		
MB113	09/04/15	Tully Park	Failed to amplify				
MB114	09/04/15	Tully Park	Failed to amplify				
MB115	09/04/15	Tully Park	Failed to amplify				
MB116	12/04/15	Wilson's Downfall Site A	*R. kanadii*	KJ866936.1	92.0	*R. iterika*	85[2]
MB117	12/04/15	Wilson's Downfall Site B	*R. crustosa*	EU598193.1	90.0	*R. marangania*	86[2]
MB118	12/04/15	Wilson's Downfall Site B	Failed to amplify				
MB119	12/04/15	Wilson's Downfall Site	*R. tawai*	JX178491.1	89.0	*R. clelandii*	88.1
MB120	12/04/15	Wilson's Downfall Site	Failed to amplify				
MB121	12/04/15	Wilson's Downfall Site	*R. kanadii*	KJ866936.1	92.0	*R. iterika*	85[2]

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB122	12/04/15	Wilson's Downfall Site	Failed to amplify				
MB123	12/04/15	Wilson's Downfall Site	Failed to amplify				
MB124	12/04/15	Wilson's Downfall Site D	Failed to sequence				
MB125	12/04/15	Wilson's Downfall Site E	Failed to amplify				
MB126	12/04/15	Wilson's Downfall Site E	Failed to amplify				
MB127	22/04/15	Lodbroke	*R. violeipes*	JF908655.1—	94.0	*R. rostraticystidia*	94.4
MB128		Crows Nest NP	DNA extracted only				
MB129	03/06/15	Tully Park	DNA extracted only				
MB130	03/06/15	Tully Park	*R. marangania*	EU019930	95.0	*R. marangania*	98.6
MB131	03/06/15	Tully Park	*R. tapawera*	EU019935.1	94[1]		
MB132	03/06/15	Tully Park	*R. atrovirens*	GU222260.1	97.0	*R. wollumbina*	98.9
MB133	03/06/15	Tully Park	DNA extracted only				
MB134	03/06/15	Tully Park	*R. wollumbina*	EU019921.1	97.7	*R. wollumbina*	97.7
MB135	27/12/15	Lodbroke	Taxonomically not Russulaceae				
MB136	28/12/15	Lodbroke	DNA extracted only				
MB137	28/12/15	Lodbroke	DNA extracted only				
MB138	29/12/15	Lodbroke	Incorrect sequence obtained				
MB139	29/12/15	Lodbroke	DNA extracted only				
MB140	29/12/15	Lodbroke	Taxonomically not Russulaceae				
MB141	29/12/15	Lodbroke	DNA extracted only				
MB142	28/12/15	Lodbroke	Incorrect sequence obtained				
MB143	29/12/15	Lodbroke	Taxonomically not Russulaceae				
MB144	29/12/15	Lodbroke	DNA extracted only				
MB145	29/12/15	Lodbroke	DNA extracted only				
MB146	31/12/15	Lodbroke	No significant similarity found				
MB147	31/12/15	Lodbroke	DNA extracted only				

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB148	03/01/16	Lodbroke	DNA extracted only				
MB149	04/01/16	Lodbroke	DNA extracted only				
MB150	05/01/16	Lodbroke	DNA extracted only				
MB151	05/01/16	Lodbroke	*R. tawai*	JX178491.1	83[1]		
MB152	05/01/16	Lodbroke	DNA extracted only				
MB153	05/01/16	Lodbroke	DNA extracted only				
MB154	05/01/16	Lodbroke	DNA extracted only				
MB155	05/01/16	Lodbroke	*R. violeipes*	KY693648.1	96.0	*R. rostraticystidia*	94.7
MB156	05/01/16	Lodbroke	DNA extracted only				
MB157	07/01/16	Lodbroke	No significant similarity found				
MB158	07/01/16	Lodbroke	DNA extracted only				
MB159	07/01/16	Lodbroke	DNA extracted only				
MB160	16/01/16	Lodbroke	DNA extracted only				
MB161	17/01/16	Lodbroke	Taxonomically not Russulaceae				
MB162	16/01/16	Lodbroke	DNA extracted only				
MB163		Lodbroke	DNA extracted only				
MB164	20/03/16	Lodbroke	DNA extracted only				
MB165	20/03/16	Lodbroke	DNA extracted only				
MB166	20/03/16	Lodbroke	DNA extracted only				
MB167	20/03/16	Lodbroke	DNA extracted only				
MB168	20/03/16	Lodbroke	DNA extracted only				
MB169	20/03/16	Lodbroke	DNA extracted only				
MB170	27/03/16	Lodbroke	DNA extracted only				
MB171	27/03/16	Lodbroke	DNA extracted only				
MB172	27/03/16	Toowoomba	DNA extracted only				
MB173	27/03/16	Toowoomba	DNA extracted only				
MB174	27/03/16	Toowoomba	DNA extracted only				
MB175	27/03/16	Toowoomba	DNA extracted only				
MB176	27/03/16	Toowoomba	DNA extracted only				

...continued on next page

Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB177	21/08/16	Lodbroke	DNA extracted only				
MB178	10/09/16	Lodbroke	DNA extracted only				
MB179	16/09/16	Lodbroke	DNA extracted only				
MB180	17/09/16	Lodbroke	DNA extracted only				
MB181	19/09/16	Lodbroke	Failed to amplify				
MB182	19/09/16	Lodbroke	DNA extracted only				
MB183	19/09/16	Lodbroke	DNA extracted only				
MB184	22/09/16	Lodbroke	DNA extracted only				
MB185	22/09/16	Lodbroke	DNA extracted only				
MB186	23/09/16	Lodbroke	DNA extracted only				
MB187	23/09/16	Lodbroke	DNA extracted only				
MB188	27/09/16	Lodbroke	DNA extracted only				
MB189	27/09/16	Lodbroke	DNA extracted only				
MB190	27/09/16	Lodbroke	DNA extracted only				
MB191	30/03/17	Lodbroke	DNA extracted only				
MB192	30/03/17	Lodbroke	Taxonomically not Russulaceae				
MB193	30/03/17	Lodbroke	Taxonomically not Russulaceae				
MB194	30/03/17	Lodbroke	Taxonomically not Russulaceae				
MB195	31/03/17	Lodbroke	DNA extracted only				
MB196	02/04/17	Lodbroke	DNA extracted only				
MB197	02/04/17	Lodbroke	DNA extracted only				
MB198	02/04/17	Lodbroke	DNA extracted only				
MB199	09/04/17	Lodbroke	DNA extracted only				
MB200	09/04/17	Lodbroke	DNA extracted only				
MB201	09/04/17	Lodbroke	DNA extracted only				
MB202	09/04/17	Lodbroke	DNA extracted only				
MB203	14/04/17	Lodbroke	DNA extracted only				
MB204	14/04/17	Lodbroke	DNA extracted only				
MB205	14/04/17	Lodbroke	DNA extracted only				
MB206	14/04/17	Lodbroke	DNA extracted only				

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Spec. ID	Date	Location	Species	Accession number	Ident (%)	Species	Ident (%)
MB207	14/04/17	Lodbroke	DNA extracted only				
MB208	14/04/17	Lodbroke	DNA extracted only				
MB209	15/04/17	Lodbroke	DNA extracted only				
MB210	15/04/17	Lodbroke	DNA extracted only				
MB211	15/04/17	Lodbroke	DNA extracted only				
MB212	16/04/17	Lodbroke	DNA extracted only				
MB214	24/04/17	Lodbroke	DNA extracted only				
MB215	27/04/17	Lodbroke	Taxonomically not Russulaceae				
MB216	14/12/17	Lodbroke	DNA extracted only				
MB217	17/12/17	Lodbroke	DNA extracted only				
MB218	20/12/17	Lodbroke	DNA extracted only				
MB219	24/03/18	Lodbroke	DNA extracted only				
CW1	08/02/16	Coonabarabran	*R. delica*	KX812842.1	75[1]		
CP2	08/02/16	Coonabarabran	Failed to amplify				
CP1	08/02/16	Coonabarabran	Failed to amplify				
CW2	08/02/16	Coonabarabran	*R. delica*	KX812842.1	90.0		

[1] Short specimen sequence, so percent identity provides an indicator of the genus and clade only

[2] Matched sequence contains large numbers of gaps and/or mismatches

C.2 Dried specimens

Table C.2: Sample collection - Dried specimens

	Collection			Closest match		
Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS01	PL19708	17/07/12	Maroochy BBG	*R. tricholomopsis*	JX178492.1	89.9
DS02	PL1712			*R. tawai*	JX178491.1	94
DS03	PL4712	17/07/12	Maroochy BBG	*R. tricholomopsis*	GU222261.1	94.4

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Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS04	PL18412	21/04/12	Obi Obi	*R. virescens*	KU552087	89.8
DS05	FG2100107P009	07/01/10	Dilkusha, Maleny	*R. violeipes*	KR673669.1	91[1]
DS06	FG8712	13/07/12	Dilkusha, Maleny	*R. tawai*	JX178491.1	90
DS07	FG2012023	20/02/12	Dilkusha, Maleny	*R. cerolens*	KX095042	92
DS08	PL4412	07/04/12	Mt Tamborine	*R. foetens*	KF245487.1	94
DS09	PL15412	16/04/12	Linda Garrett	*R. shingbaensis*	KM386692.1	97
DS10	PL16412	17/04/12	Linda Garrett	*R. tapawera*	EU019942.1	82[1]
DS11	PL14412	16/04/12	Linda Garrett	*R. tapawera*	EU019942.1	90[1]
DS12	CH022	31/03/12	Chermside Hills	*R. senecis*	KX574699.1	95
DS13	PL16509	24/05/09	Lithgow, NSW	Matched to Ascomycete		
DS14	PL41312	24/03/12	Mt Norman Track, Girraween NP	*R. brevipes*	FJ845429.1	93
DS15	BP21412	21/04/12	Obi Obi	*R. senecis*	KX574699.1	93
DS16	FE6 25412	21/04/12	Obi Obi	*Incorrect sequence*		
DS17	PL79313	2013	Linda Garrett	Failed to amplify		
DS18	PL90313	16/03/13	Linda Garrett	Failed to amplify		
DS19	PL44411	2011	Girraween	Failed to amplify		
DS20	PL51313	20/03/13	Girraween	Failed to amplify		
DS21	KS2566	11/04/11	Cootharaba	Failed to amplify		
DS22	PL76313	16/03/13	Linda Garrett	Failed to amplify		
DS23	PL47313	09/03/13	Mapleton Falls NP	Failed to amplify		
DS24	F2012/024	22/02/13	Dilkusha, Maleny	Failed to amplify		
DS25	PL77313	16/03/13	Linda Garrett	Failed to amplify		
DS26	PL45313	09/03/13	Mapleton Falls NP	Failed to amplify		
DS27	PL72313	16/03/13	Linda Garrett	*R. ingwa*	EU019919.1	94

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Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS28	PL31411	17/04/11	Tully Park, Girraween	*R. griseobrunnea*	MF461604.1	98.1
DS29	PL71313	16/03/13	Linda Garrett	Failed to amplify		
DS30	PL471107	29/11/07	Maroochy BBG	Failed to amplify		
DS31	QMS GIR 64	16/04/11	Underground Ck., Girraween	Failed to amplify		
DS32	PL35410	10/04/10	Linda Garrett	Failed to amplify		
DS33	SP11-68	12/03/11	Ankida, Springbrook	Failed to amplify		
DS34	PL3712	2012	Maroochy BBG	Failed to amplify		
DS35	PL52408	13/04/08	Harry's Hut	Failed to amplify		
DS36	PL103313	2013	Bald Rock NP NSW	*Lactarius volemus*	LC176757.1	89
DS37	PL104313	20/03/13	Bald Rock NP NSW	Failed to amplify		
DS38	PL14411	03/04/11	Yidney Scrub, Fraser Island	Failed to amplify		
DS39	PL30313	07/03/13	Maroochy BBG	Failed to amplify		
DS40	PL40312	24/03/12	Mt Norman Track,Girraween NP	Failed to amplify		
DS41	PL108313	20/03/13	Bald Rock NP NSW	Failed to amplify		
DS42	S. Nelles	26/02/13	Alexandra Hills	No significant similarity found		
DS43	RB31	19/04/13	Ravensbourne	DNA extracted only		
DS44	RB37/38	19/04/13	Ravensbourne	DNA extracted only		
DS45	GW	08/05/13	Bellthorpe	DNA extracted only		
DS46	KS2845	2013	Denmark WA	DNA extracted only		
DS47	CH16	2013	Chermside Hills	DNA extracted only		
DS48	KS2871	2013	Denmark WA	DNA extracted only		
DS49	PL82313	2013	Linda Garrett	DNA extracted only		
DS50	PL20708	2008	Linda Garrett	DNA extracted only		
DS51	PL271107	2007	Mapleton	DNA extracted only		

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Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS52	PL13409	2009	Noosa NP	DNA extracted only		
DS53	CN63	20/04/13	Crows Nest	DNA extracted only		
DS54	PL131608			DNA extracted only		
DS55	PL19312	2012	Obi Obi	DNA extracted only		
DS56	PL2308	2008	Maroochydore	DNA extracted only		
DS57	PL16411	2011	Fraser Island	DNA extracted only		
DS58	PL45411	2011	Girraween	DNA extracted only		
DS59	PL41413	2013	Cooloola	Failed to amplify		
DS60	PL1310	2010	Maroochydore	DNA extracted only		
DS61	KS2566	2011	Cooloola	DNA extracted only		
DS62	KS2855	2013	Walpole WA	DNA extracted only		
DS63	PL471107	2011	Manjimup WA	*R. subfoetens*	KY681430.1	94
DS64	PL6711	2011	Walpole WA	DNA extracted only		
DS65	PL17708	2008	Maroochydore	DNA extracted only		
DS66	PL69413	2013	Maroochydore	DNA extracted only		
DS67	RB18	2013	Ravensbourne	*Lactifluus luteolus*	KR364016	91
DS68	PL11010	2010	Obi Obi	DNA extracted only		
DS69	PL102313	2013	Bald Rock NP NSW	*Lactarius piperatus*	DQ422035.1	94
DS71	PL161208	2008	Obi Obi	*Lactarius clarkeae*	HQ318283.1	99
DS72	CN62	2013	Crows Nest	Failed to sequence		
DS73	PL17412	2012	Obi Obi	*Lactarius sepiaceus*	EU019926.1	99
DS74	PL43413	2013	Cooloola	*Lactaris leonardii*	GU258288.1	97
DS75	PL51413	2013	Cooloola	*Lactarius cf. Wirrabara*	JF731001	99
DS76	RB145	2013	Ravensbourne	*L. longipilus*	HQ318256	80[1]
DS77	RR245	2011	Manjimup WA	*R. flavida*	KX267651.1	87[1]
DS78	PL15411	2011	Fraser Island	Matched to Ascomycete		

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Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS79	PL59408	2008	Maroochy BBG	Matched to Ascomycete		
DS80	SP11-74	2011	Springbrook	Failed to amplify		
DS81	NDR22	11/04/15	North Dr Roberts	*R. acrolamellata*	MF461612.1	94
DS82	NDR15	11/04/15	North Dr Roberts	*Lactarius decipiens*	KT165312.1	97
DS83	WD2-15	12/04/15	Wilson's Downfall	*Lactifluus luteolus*	KR364016	87[1]
DS84	PL630415	10/04/15	Bald Rock NP NSW	*Lactarius glaucescens*	AB509515	93[1]
DS85	PL640415	10/04/15	Bald Rock NP NSW	*Lactarius volemus*	HQ318273	73[1]
DS86	PL710415	10/04/15	Bald Rock NP NSW	*Lactarius crocatus*	JN388985.1	94
DS87	PL720415	10/04/15	Bald Rock NP NSW	*L. volemus*	HQ318273	78[1]
DS88	PL730415	10/04/15	Bald Rock NP NSW	*R. cheelii*	JX266623.1	97.9
DS89	PL870415	16/04/15	Mt Norman Track,Girraween NP	*R. psedoareolata*	JX178488.1	97
DS90	PL25614	14/06/14	Linda Garrett	*Multifurca stenophylla*	MH063867	99.9
DS91	MEL2297389	25/03/05	Yarra State Forest	Matched to Ascomycete		
DS92	PL700415	10/04/15	Bald Rock NP NSW	*R. vesca*	HM189956	99
DS93	Bellthorpe 17c	29/03/11	Bellthorpe	*R. crustosa*	EU598193.1	90
DS94	DCFC 005.	20/04/15	Davies Creek Falls Circuit	No significant similarity found		
DS95	DFR2	20/04/15	Dinden State Forest	DNA extracted only		
DS96	DCFC - 15	20/04/15	Davies Creek	No significant similarity found		
DS97	2DCFC - 1	21/04/15	Davies Creek	*R. chloroides*	KF432954.1	86[1]
DS98	2DCFC - 2	21/04/15	Davies Creek	*R. aeruginea*	HQ604837.1	92

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Spec. ID	QMS ID	Date	Location	Species	Accession number	Ident (%)
DS99	2DCFC-10	21/04/15	Davies Creek	No significant similarity found		
DS100	2DCFC-12	21/04/15	Davies Creek Falls Circuit	DNA extracted only		
DS101	TDS2B - 6	22/04/15	Tinaroo Forest Reserve	DNA extracted only		
DS102	TDS-SS-1	24/04/15	Tinaroo Forest Reserve	*R. virescens*	DQ422014.1	88
DS103	WD4 45	10/04/15	Wilson's Downfall	DNA extracted only		
DS104	PL370415	06/03/15	Mapleton Forest	DNA extracted only		
DS105	LG64	13/06/15	Linda Garrett	DNA extracted only		
DS106	LG62	13/06/15	Linda Garrett	DNA extracted only		
DS107	DCFC 6	20/04/15	Davies Creek	DNA extracted only		
DS108	PL1420316	31/03/16	Lake Cootharaba	DNA extracted only		
DS109				DNA extracted only		
DS110	PL1380312	31/03/16	Lake Cootharaba	DNA extracted only		
DS111	PL1920416			DNA extracted only		
DS112	JOR12	09/06/16	John Oxley Reserve	DNA extracted only		
DS113	Q16RR-12	06/08/16	Ravensbourne	DNA extracted only		
DS114	Q16RC02	05/08/16	Ravensbourne	DNA extracted only		
DS115	SN18414	14/04/14	Alexandra Hills	DNA extracted only		
DS116	SN18414	14/04/14	Alexandra Hills	DNA extracted only		
DS117	SN17414	14/04/14	Alexandra Hills	DNA extracted only		
DS118	SN16414	14/04/14	Alexandra Hills	DNA extracted only		

[1] Short specimen sequence, so percent identity provides an indicator of the genus and clade only

Appendix D

Sequences generated in this study

In the following lists of sequences, lower-case letters are corrections made to the sequence during examination of the chromatogram, and may indicate a nucleotide insertion or correction of a miscall. A lower-case "n" indicates a multi-coloured peak and inability to discern the most likely nucleotide in that position.

D.1 Fresh specimens

>MB01

ACA CAC ACA CAC ACC TTT ATG TAT AGA ATG TCT TAC TTT TTG CGG TGA TAC GCA ATA AAT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ACA TCC TCA ACC TTC TTT TGG GTT TTT GAC CGG GGG AAG GCT TGG ACT TTG GAG GCC CTT TCA TTG CTG GTA TGC TTT TAA AGC CAG CTC CTC CTA AAT GAA TCA GTG GGA TCC GCT TCG GTG ATC CTT GAC GTG ATA AGT ATT ATT CTA CGT CTT GGG TTT TCA CAG CAC CTG CTT CCA ACC GTC TTT AAA CAA AGA CAA TGT TCG GGT TTC GAC CTG AAT CAC AAA ACC TGA CCT CA

>MB02

AGG TGA CCT GCG GAA GGA TCA TTA TTG TAT AAC AGG GGT GTA AGG GCT GTT GCT AAC CTT TAA AAG GGT TGT GCA CGC CTG AGC ACT CTT GAA TGT CCA TCT TAC CCA TTT GTG CAC CTC TGC GTA GGC CTC TTT GCA AAA AGA GGG CCT GCG TTT TTT TAT ATT AAA CTC TGT GTG TAG AAT GTT ATA TTT TGC TGT TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAT GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGT TTG ATT GTT TCT AAT CAG TAA AAG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TGT CTT AAA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGA TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC ATT TTG GAT TTG GTA TTG TCT CTT AGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG TTT CTG GTG TCT GCT ATT ATA TTA GCA GTT AGC TTA GAC CCA TGA AAT AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA A

>MB03

TGC GGA AGG ATC ATT TCT TGT ATA ACA GGG GTC GTT AAG GGC TGT TGC TAA CCT TTA AAA GGG TTG TGC ATG CCT GAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CCT CTT TGC AAA AAG AGG GCC TGC GTT TTT TTA TAT TAA ACT CTG TGT GTA GAA TGT TAT ATT TTG CTG TTA TGC AAT TAA TAC AAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA TGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGG ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TAG ACC CAT GAA ATA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT TAA

>MB04

CCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GTG CTT CCC CTG TTG GGC TTG TTC CGA GGG GGG GTT CAC GTT TTT ATA CAA ACA CCA TTT ATG TAT AAA AAG GCC TTA CTT TTT GCG ATC ATG CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ACC CTC AAC CTT TTT GGT TTC TTG ACC AAG AAG GCT TGG ACT TTT GGA GGT TTT TCC TTG CTG GCC TCC CTT TGA AGC CAG CTC CTC CTA AAT GAA TTA GTG GGG TTC GCA TTG CTG ATC CTT GAC GTG ATA AGT ATG CTT CTA CGT CTT GGA TTT TGC ACC TTT GCC TTG GAA CCC GCT TCC AAC TGT CTT TGA ATG AAG ACA ATG TTT TGA GTT CCA TCG TGA CTC AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA G

>MB05

GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGC CAT TCC GAG GGG CAC ACC TCT TTG AGT GTC GTG AAA TTC TCA AAA ACC CAA TTG CTT GAT TGT TTC TGA TCA GAA AAG GGA TTT TGT ACT TGG AGG GGA GAA TGC ATG CTT TTA TCT TGG AAG CAA GCA CCC CCC CCC TAA ATC AGT CGG GTC TGC TTT GCT

>MB06

GGT GAC CTG CGG AGG ATC ATT ATC TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AGC ATA TCA ATA

>MB08

TGC ATC ACC GCG TGG GTC CCC CCT TTC TTC GGA GTA GGG GGT GCT CAC GTT TTT AAC ATC GAA CAC CCA TTA GAA CGT AGT GTA GAA TGT TCT TTG CGC AAT CGC GCG CGA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA CAT CCT CAA CCT GCT TGG TTT TAC TTG AAA CCA AGT AGG CTT GGA ATT TGG AGG TTT TCT GCT GGC CGC CTT TGA TGC CAG CTC CTC TTA AAT GTA TTA GTG GGA TCC GCT TTG CTA GAT CCT TGA CGT TGA TAA GAT GTT TCT ACG TCT TGG GTT TCG CTC AGG AAT GAC CCG CTT CTA ATG CGT CCC ATC AGG GAC AAT GTT CGA GAG CCG ATC GCC CTT CAT GGG GTG GGA AGC TTT TTG ACC TTT CAT GCC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AAG CGG GAG GAA AAG GAA ACT AAC AAG GGA TTC CCC CTA AGT AAC TGC GAA TTG AAA GCG GGG AAA AAG CTT CAA AAT TTT AAA AAT CTG GGG GGG TCC TTT TTG GGC CCA TCC CGA AGT TTG GTA AAT TTT T

>MB09

AGG TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GGG GTG TAA GGG CTG TTG CTA ACC TTT AAA AGG GTT GTG CAT GCC TGA GCA CTC TTG AAT GTC CAT CTT ACC CAT TTG TGC ACC TCT GCG TAG GCC TCT TTG CAA AAA GAG GGC CTG CGT TTT TTT ATA TTA AAC TCT GTG TGT AGA ATG TTA TAT TTT GCT GTT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGA ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TAG ACC CAT GAA ATA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT T

>MB10

GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG GGG TGT AAG GGC TGT TGC TAA CCT TTA AAA GGG TTG TGC ACG CCT GAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CCT CTT TGC AAA AAG AGG GCC TGC ATT TTT TTA TAT TAA ACT CTG TGT GTA GAA TGT TAT ATT TTG CTG TTA TGC AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA TGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG TTT GAT TGT TTC TAA TCA GTA AAA GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTG TCT TAA AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG ATC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT CTA CAT TTT GAA TTT GGT ATT GTC TCT TAG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GTT TCT GGT GTC TGC TAT TAT ATT AGC AGT TAG CTT AGA CCC ATG AAA TAA ATC TTG ACC TCA AAT CAG GTG AGA CTA CCT GCT GAA CT

>MB11

GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG GGG TGT AAG GGC TGT TGC TAA CCT TTA AAA GGG TTG TGC ACG CCT GAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CCT CTT TGC AAA AAG AGG GCC TGC GTT TTT TTA TAT TAA ACT CTG TGT GTA GAA TGT TAT ATT TTG CTG TTA TGC AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA TGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG TTT GAT TGT TTC TAA TCA GTA AAA GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTG TCT TAA AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG ATC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT CTA CAT TTT GGA TTT GGT ATT GTC TCT TAG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GTT TCT GGT GTC TGC TAT TAT ATT AGC AGT TAG CTT TAG ACC CAT GAA ATA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT T

>MB12

TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GGG GTG TAA GGG CTG TTG CTA ACC TTT AAA AGG GTT GTG CAC GCC TGA GCA CTC TTG AAT GTC CAT CTT ACC CAT TTG TGC ACC TCT GCG TAG GCC TCT TTG CAA AAA GAG GGC CTG CGT TTT TTT ATA TTA AAC TCT GTG TGT AGA ATG TTA TAT TTT GCT GTT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGT GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGA ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TA

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GTG ACC TGC GGA AGG ATC ATT ATT GTA TAA CAG GGG TGT AAG GGC TGT TGC TAA CCT TTA AAA GGG TTG TGC ACG CCT GAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CCT CTT TGC AAA AAG AGG GCC TGC GTT TTT TTA TAT TAA ACT CTG TGT GTA GAA TGT TAT ATT TTG CTG TTA TGC AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GTG ATA TGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG TTT GAT TGT TTC TAA TCA GTA AAA GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTG TCT TAA AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG ATC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT CTA CAT TTT GAA TTT GGT ATT GTC TCT TAG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GTT TCT GGT GTC TGC TAT TAT ATT AGC AGT TAG CTT AGA CCC ATG AAA TAA ATC TTG ACC TCA AAT CAG GTG AGA CTA CCT GCT GAA CTT AA

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GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG GGG TGT AAG GGC TGT TGC TAA CCT TTA AAA GGG TTG TGC ACG CCT GAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CCT CTT TGC AAA AAG AGG GCC TGC GTT TTT TTA TAT TAA ACT CTG TGT GTA GAA TGT TAT ATT TTG CTG TTA TGC AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GTG ATA TGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG TTT GAT TGT TTC TAA TCA GTA AAA GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTG TCT TAA AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG ATC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT CTA CAT TTT GAA TTT GGT ATT GTC TCT TAG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GTT TCT GGT GTC TGC TAT TAT ATT AGC AGT TAG CTT A

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AGG TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GGG GTG TAA GGG CTG TTG CTA ACC TTT AAA AGG GTT GTG CAC GCC TGA GCA CTC TTG AAT GTC CAT CTT ACC CAT TTG TGC ACC TCT GCG TAG GCC TCT TTG CAA AAA GAG GGC CTG CGT TTT TTT ATA TTA AAC TCT GTG TGT AGA ATG TTA TAT TTT GCT GTT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGG ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TA

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TGC GGA GGA TCA TTA TTG TAT AAC AGA GGG TGT AAG GGC TGT TGC TAA CCC TTA AAG GGT TGT GCA CGC CTT AGC ACT CTT AAA CAT CCA TCT CAC CCA TTT GTG CAC CTC TGC GTG GGC CCC CTT TGC GAA GAG GGC CTG CGT TTT TTT AAT ATA TAT AAA CCC CAC CTG GAT AAA GGA TTA TAT TTT GCT GGT ATA TGC AAT TAA ATA CCA CTT TCA ACA ACG GAT CCC TTG GCT CCC GCA TCC ATG AAG AAC GCA GCG AAA TGG GAT ACC TAA TGG GAA TTG CAG AAT TCC GTG AAT CCT CGA ATC CTT GAA CGC CAC TTG GGC CCC TTG GCA TTC CGA GGG GCA CAC CTG GTT GAA TGG CGT GAA ATT CCC AAA AAC CCT TTT GCT TGA TTG GTT CCG AAC CGA AAA GGG GTT TTG GAC TTT GAA GTT TAA TGC TTG GCT TT

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TGC GGA AGG ATC ATT ATT GTA TAA CAG AGG GTG TAA GGG CTG TTG CTA ACC CTT AAA GGG TTG TGC ACG CCT TAG CAC TCT TAA ACA TCC ATC TCA CCC ATT TGT GCA CCT CTG CGT GGG CCC CCT TTG CGA AGA GGG CCT GCG TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AGT ATT ATA TTT TGC TGT TAT ATG CAA TTA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGC TTG ATT GTT TCT GAT CAG AAA AGG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTG TAC ATT TTG AAT TTG GCA TTG TCT CTT GGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG CTT CTG ATT ACG TCT ATT TTT ATA TTA GGC AGA CA

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TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AGC ATA

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AGG TGA CCT GCG GAG GAT CAT TAA TGA ATA AAC TCG GTG GAT TGT TGC TGG TTC TTA GGA GCA TGT GCA CAT CTA CCA TTG TTT CAT TCT TTC TCC ACC TGT GCA CCT TTT GTA GGC TTG AAT AAC TCT CAA GGA ATG AAT TAC AAT CAT TCC TTG GAT TGA AGG ATT GGT TTT AAA TAA AAC TCT CCT TTA TAT TTC CAG TCT ATG TTT TTA TAA TTT ACA CCC TAT TAG TAT GGC TTA GAA TGT TTA TTA TTT GGC CTT TCT TTT GAT AGG CTT TAA AAC TTA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA AGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCT CCT TGG TAT TCC GAG GAG CAT GCC TGT TTG AGT GTC ATT AAA TTC TCA ATC CTT TCT GAG TTT ATT CTT AGC TAG GGT TGG ATA TGG GAG TTA TAT GCG GGC TTT GCA AAG TCA AAG TCA GCT CTC CTG AAA GTC ATT AGC AAG ACG CTT GTT GCA ACC TTC TAT CTG GTG TGA TAA TTA TCT ACA TCA TAG ATT GTA TGC AGT TTA TTA TGT CTT GCT TCT AAT AGT CCA TTA AAT CGG ACA AAA CTC TGA CAA TTT GAC CTC AAA TCA GGT AGG ACT ACC CGC TGA ACT TA

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CTG CGG AGG ATC ATT AAT GAA TAA ACT CGG TGG ATT GTT GCT GGT TCT TAG GAG CAT GTG CAC ATC TAC CAT TGT TTC ATT CTT TCT CCA CCT GTG CAC CTT TTG TAG GCT TGA ATA ACT CTC AAG GAA TGA ATT ACA ATC ATT CCT TGG ATT GAA GGA TTG GTT TTA AAT AAA ACT CTC CTT TAT ATT TCC AGT CTA TGT TTT TAT AAT TTA CAC CCT ATT AGT ATG GCT TAG AAT GTT TAT TAT TTG GCC TTT CTT TTG ATA GGC TTT AAA ACT TAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAA GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CTC CTT GGT ATT CCG AGG AGC ATG CCT GTT TGA GTG TCA TTA AAT TCT CAA TCC TTT CTG AGT TTA TTC TTA GCT AGG GTT GGA TAT GGG AGT TAT ATG CGG GCT TTG CAA AGT CAA AGT CAG CTC TCC TGA AAG TCA TTA GCA AGA CGC TTG TTG CAA CCT TCT ATC TGG TGT GAT AAT TAT CTA CAT CAT AGA TTG TAT GCA GTT TAT TAT GTC TTG CTT CTA ATA GTC CAT TAA ATC GGA CAA AAC TCT GAC AAT TTG ACC TCA AAT CAG GTA GGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AAG CGG AGG A

>MB23

AGG GCT GTT GCT AAC CTT TAA AGG GTT GTG CAT GCC TGA GCA CTC TTG AAT GTC CAT CTT ACC CAT TTG TGC ACC TCT GCG TAG GCC TCT TTG CAA AAA GAG GGC CTG CGT TTT TTT ATA TTA AAC TCT GTG TGT AGA ATG TTA TAT TTT GCT GTT ATG CAA TTA ATA CAA CTT TTA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGA ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TAG ACC CAT GAA ATA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT TAA GCA TAT CAA TAA GCA GAG GAA AAG AAA CTA ACA AGG ATT CCC CTA GTA ACT GCG AGT GAA GCG GGA AAA GCT CAA ATT TAA AAT CTG GTG GTC TTT AGC CAT CCG AGT TGT AAT TTA GAG AAG CAT CTT CCG

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AGG TGA CCT GCG GAG GAT CAT TAT TAT ACA ATG GAG GTG CTA AGG TTG TCG CTG ACT TTT GAA AGG GTC GTG CAC ACC TCG GTG CTC TCA CAT ATA ATC CAT CTC ACC CCT TTT GTG CAT TAC CGC GTG GGG ACC CCT TTT AGC TAG TTC TAA GAG GGG TCT TCA TGT TTT TAC CAA TTT TAA CGC AAT GTG TAG AAT GTG TTA CTT TTT GCG ATC ATA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CTT TGG CAT TCC GAG GGG CAC ACC CGT TTG AGT GTC ATG ACA TTC TCA AAC CTT CTT GGT TTC TTG ACC AGG AAG GCT TTG GAC TTG GAG GCT TTT GCT GGT GTT CCT TTG TTG GAG CCA GCT CCT CTG AAA TGG ATT AGT GGG GTC TGC TTT GCC TAT CCT CGA CGT GAT AAG ATG TTT TCT ACG TCT TGG GTT TTG CAC TGT TTC CTG CTT CTA ACT GTC TTA CAG AAG ACA ATA GTC AAG TGA TTG CCA CTT GAC CCA TGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AAG CGG A

>MB25

GAC CTG CGG AGG TCA TTA TTG TAT AAC AGG GGT GTA AGG GCT GTT GCT AAC CTT TAA AAG GGT TGT GCA TGC CTG AGC ACT CTT GAA TGT CCA TCT TAC CCA TTT GTG CAC CTC TGC GTA GGC CTC TTT GCA AAA AGA GGG CCT GCG TTT TTT TAT ATT AAA CTC TGT GTG TAG AAT GTT ATA TTT TGC TGT TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAT GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ATG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGT TTG ATT GTT TCT AAT CAG TAA AAG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TGT CTT AAA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGA TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC ATT TTG GAT TTG GTA TTG TCT CTT AGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG TTT CTG GTG TCT GCT ATT ATA TTA GCA GTT AGC TTA GAC CCA TGA AAT AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AG

>MB32

GGT GAC CTG CGG AGG ATC ATT ATC GTA CCT CAG AGG CGT AAT GGC TGT CGC TGA CCA TAT TCA AAG GTC GTG CAC GCC AGA GCG CAC TCA CAC ATT ATC CAC TTC ACC CTT GTG CAT CAC TGC GTG GGG AGC CCC CTT TGG CTT GCT TCA AGG GGG GTC CAC GTC TTT TAC ACA CAC CCC TTT AAC AAA GTT TAG AAT GTC CTT TGC GAT CAC ACG CAA TTA ATA CAA CTT TCA ACA ATG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAC ATT CTC AAA CCT TTC TTG GTT TCT TAA TCA GAA AGG CTT TGG ACT TTG GAG GCA TTT TGC TGG GTC CCT TTG AAA CCC AGC TCC TCC CAA ATG TAT TAG TGG GGT CAC CTT TGC CAC TCC CCT GAT GTG ATA AGA TGT TTC TGC ATC TTG GGT TTG GCT CTG TAA CCT GCT TCT AAC CGT CTC ATC AGA GAC TAT GTT TGG GTT TGC CAC CTT TCC CGG GTG GTA AGC TTG ACC CTA GAA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA C

>MB35

AGG TGA CCT GCG GAA GGA TCA TTA CCG TAT AAT GGA GGT GCT GGG GTT GTC GCT GAC TTT CTG AAT GGG GGA GGT CGT GCA CGC CTC GGT GCT CCC ACA CAC AAT CCA CAT CAC CCC TTT TGT GCA TCA TCG CGT GGG GGA CCC TCT CTG AGG AGG GGG GGG GCC TTT CAC GTT TTT ACA AAT TTG AAC GCA ATG TGT AGA ATG TCT TTA TTA CTT TTT GCG ATC ACA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AAA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CTT TGG CAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG CTA TCT TCA AAC CTT CTT GGT TTC TTG ACC GGG GAA GGC TTT GGA CTT TGG AGG CTT TTG CTG GCT TTC ATT TGT TGG AAC CAG CTC CTC TGA AAT GGA TTA GTG GGT CTG CTT TGC TTA TCC TCG ACG TGA TAA GAT GTT TTC TAC GTC TTG GGT TTT GCA CCG TCT CCT GCT TCT AAC CAG TCT CAC AGA AGA CAA TGG TCA AGT GAT TGC CAC TTG ACC GCA TGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAC

>MB37

TGC ACG TCT AAG CAC TCT TAA ACA TCC ATC TCA CCC CAT TGT GCA TCA CTG CGT GAG CCT GCT TTG TAA AGA GGG CTT GCG TTT TTT TTA AAC ATA ATA CCT GAT ACA GTA TAG AAA GGT TTT TTT TGC TAT TAT ATG CAA TTA ATA CAA CTT TCC ACC ACG GAT CCC TTG GCT CCC GCA TCC ATG AAG AAC TCA GCC AAA TGC GAT ATG GAA GGG GAT TGC AGA AAT CAG TGG ATC ATC GAA TCT TTG AAC CCA CTT GCG CCC CTT GGA TTT CGA GGG GCA CAC CTG TTT AGT GTC GGA AAT TCC CAA ACC TTT TCT TTG AAA AGG CTT TGG ATT TGG AGA ATC ATG CTT GCT TTT

>MB38

TAC CGT ATA ATG GAG GTG CTG GGG TTG TCG CTG ACT TTC TGA ATG GGG GAG GTC GTG CAC GCC TCG GTG CTC CCA CAC ACA ATC CAC ATC ACC CCT TTT GTG CAT CAT CGC GTG GGG GAC CCT CTC TGA GGA GGG GGG GGG GGG GGG GGG TTT TCC CAA TTT TAA ACC CAT GGG GCA AAG GGG TTT AAT TCT TTT TTC CAT TTT

>MB39

TCA TTA TCG TAC AAT GGA GGT GCT GGG GCT GTC GCT GAC TTT TGA AAG GGT CGT GCA CGC CTC GGT GCT CTC ACA CAT GAA TCC CTT CTC CCC CCT TTT GTG CAC TAC CGC ACG GGG ACC CCT TTT ATT TAG GGG TCT TCA TGT TTT TAC AAA TTT TAA CGC AAT GTG CAG AAT GTC TTA CTT TTT GCA ATC ACA CCC ATC CAT AAC ACT TTT CAC CAA CGA ATC TTT GGC CCC CCC CAC CAA TAA AAA ACC CAC CAA ATG GCA AAC CTA ATG GGA ATT GGC AAA ATT CAT GGA ACC TCC AAT CCT TTA ACC CCA CCT GGC CCC CTT GGC ATT TCC AAG GGG CAC ACC CGT TGA ATG GCC GGG ACT CCT CCA ACC CTC CTG GTT TCC TGG ACC AGA AAG GCT TGG AAC TTG GAA GCT TTT GGC GGC CTT CCC TTG GTG GAA CCC ACT TCT CCG GAA TGG AAT AAT GGG TCC GCC TTG GCT AAT CTT CAA CTT ATT AAA GGT TTT

>MB40

GCG CAC GCC CAG ATG CGC TCT CAT ATC TCC TAT CTC ACC CCT TTG TGC ATC ACC GCG TGC GCT TCC GTG GTG GGT TTG CTC AGA GGG GGG CTC ACG TTT TTA TAC AAA CAC TTT TTA TGT ATA AAA TGT TTT ATT TTT TGA GAT CGT GCG CAC TCT ATA CCT TTT TCA ACA ACG GAT CTC GTG GCT CTC GCA TCT AAG AAA AAC GCA GCA AAA TGA GAT ACG TAG TGA GAA TTG CAA AAT TCA GTG AAT CCT CAA CTC TTT GAA CGC CCC GTG CGC CCT GTG ATA TTC AGA GGG GCA CCC CCG TTA GAG TGT CGA GAA CAC CCT CCC CCT TTT TTG TTT TCT TGA CCA AGA AGG GTT GTA TTT AGG AGT TTT TCG TTG CGG GCC TCC TTA TGA AGC CAC CTC CTC CAA AAA GAA TTG GGG GGG TTC TTA TTG ATG ATC GTT GAC GTA ATA AGG ATG CTT CTA CGT CGT GTA T

>MB41

GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG GGG GTG TAA GGG CTG TTG CTG ACC ATT AAA GGG TTG TGC ACG CCT AAG CAC TCT TGA ATA TCC ATT TCA CCC ATT TGT GCA CCT CTG CGT AGG CCC CTT TGC AAA AAA AGG GCC GCG CTT TTT TAA TAA TAT ATA ACA CTC TGT GTG TAA AAT AAT ATT TTT TGC TAT TAT ATG CTT TTA ACA CCT TTT TCA ACC GGG GAT CTT GTG TCT CTC GCA TCT AAA AAA AAC GCC GCG AAG CGA GAT ACA TAG TGA AAT TTG CAG AAT TCT GAG ACT CCT CAA CTC TGA GAA CGC CTT TTG CCC CTC GTG ATT TTC CGA GGC GCA CAT GTG TTA GAG TGT CGA GAT CTC CTC AAA CAC TTT TTG TGT GAT TAT TTA TTC TCA AAA AAT GTT TGG ACA CGT GGA TTT TTG CGC GTG CTT TAT CGG GAC GCG CTC TCT CTG AAT ATA CAA TAG GCT GCG CTG CGC AGA CTC ATG TGT ATA T

>MB43

AGA ACG CAG CGA AGT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG ATA TCC TCA AAA ACC CTT TGG TTT GAT TAT TTT TAA TCA GAA AAA GGT TTT TGG ACT GGG AGG TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AGA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC AT

>MB44

GTG ACC TGC GGA GGA TCA TTA TCG TAC AAT GGA GGT GCT GGG GCT GTC GCT GAC TTT TGA AAG GGT CGT GCA CGC CTC GGT GCT CTC ACA CAT AAT CCA TTC CAC CCC CTT TTG TGC ATT ACC GCG CGG GGA CCC CTT TTA TTT AGG GGT CTT CAC GTT TTT ACA AAA TTT AAC GCA ATG TGC AGA ATG TCT TAC TTT TGC GAT CAT AAG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCT TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAC ATC TTC AAA CCT TCT TGG TTT CTT GAC CAG GAA GGC TTT GGA CTT TGG AGG CTT TTG CTG GCG TTC CTT TGT TGG AGC CAG CTC CTC TGA AAT GGA TTA GTG GGG TCT GCT TTG CCT ATC CTC GAC GTG ATA AGA TGT TTT CTA CGT CTT GGG TTT TGC ACT GTT TCC TGC TTC TAA CCG TCT CAC AGA AGA CAA TGG TCA AGT GAT TGC CAC TTG ACC GCA CGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG C

>MB45

ATC TCA CCC CTT TGT GCA TCC CCG CGT GTG TCC CCC CCC TTC CCG GGG GGT GCT CAC GTT TTT AAC ATT GAA CAC CCA TTC GAA CGT AGT GTA GAA TGT TCT TTG CGC GAC CAC GCG CGA CCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GCA TTC CGA GGG GCA CGC CCG TTT GAG TGT CGT GAA CAT CCT CAA CCT ACT TGG TTT TCT CCA AAC CAA GTA GGC TTG GAA TTT GGA GGT TTT CTG CTG GCC TCC TTT GAA GCC AGC TCC TCT TAA ATG TAT TAG TGG GAT CCG CTT TGC TAG ATC CTC GAC GTT GAT ATA ATA TGT TTC TAC GTC TTG GGT TTC GCT TAG GAA TGA CCT GCT TCT AAC AGT CCC GTC GTG GAC AAC GTT CGA GAG GCC GAT CAC CCT TTC ACG GGG TGG GAA GCT TTT CGA CCT TTC ATG CCC TGA CCT C

>MB46

GGG GGG TGC TCA CGT TTG TAA CAT CGA ACA CCC ATT CGA ACG TAG TGT AGA ATG TTC TTT GCG CGA CCA CGC GCG ACC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACG CCC GTT TGA GTG TCG TGA ACA TCC TCA ACC TAC TTG GTT TTC TCC AAA CCA AGT AGG CTT GGA ATT TGG AGG TTT TCT GCT GGC CTC CTT TGA AGC CAG CTC CTC TTA AAT GTA TTA GTG GGA TCC GCT TTG CTA GAT CCT CGA CGT TGA TAT AAT ATG TTT CTA CGT CTT GGG TTT CGC TTA GGA ATG ACC TGC TTC TAA CAG TCC CGT CGT GGA CAA CGT TCG AGA GGC CGA TCA CCC TTT CAC GGG GTG GGA AGC TTT TCG ACC TTT CAT GCC CTG ACC TCA

>MB47

ACC TGC GGA GGA TCA TTA TCG TAC AAT AGG AGG TGC TGG GGC TGT CGC TGA CTT TTG AAA GGG TCG TGC ACG CCT CGG TGC TCT CGC ACA TGT ACT CTT CTT CCC CCT TTT TGG GCT CAA CCG CAG GGG AAC CCC TTT TAT CTA GGG TCC TTC ATG TTT TTA CAA ATT TTA ACG CAT GGG GAA AAA GGT CTA ATT TTT TGA TAT CAC ACG CAA TCA ATA CAT TTT TCA ACA AGG GAT CTC GTG TCT CTC GCA TCT AAA AAA AAC GCA GCA AAG TGA GAT ACA AAG TGA GAT TTG CAA ATT TCA GAG AAT CCT CAA CTC TTT GAA CGC CCC TTG CCC CTT TTG GTT TTC AGA GGG GCA CAC CCT TTA GAG TGT CGA GAC ATC TTC AAC CCT TCG TGT TCT CTT GAC CAA AAG GGT TTT GGA CTT TGG AGG CTT TTG CTG GCT TTC TTG TGT TAG ACC GAG CTC TCT GAT ATG TAT TAG TGG TCT GTG CTT TGC ATA CCC

>MB48

CAT TAT TGT TTA ACA GGG GGT GTA AGG GTT GTT GCT GAC CTT TAA ATG GTT GTG CAT GCC TAA GCA CTC TTA AAC ATC CAT CTC ACC CAT TGT GCA CCA CCG CGT GGG CCC CTT TGC AAA GAG GGC CTG CGT TTT TTT AAT ACA TAA AAA CTT GAT GTG GTA TAG AAT ATT ATA TTT TGC TAT TAT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATC CTC AAA AAT CTT TTA CTT TAA TTG TTT CTG ATC AGG AAA AGG TTT TTG GAC TTG GAG GTT TAA TGC TTG CTC TTG TCT TAG AAG CAA GCT CCT CTG AAA TAA ATT AGT AGG GTC TGC TTT GCT GGT TCT TAA TGT AAT AAG TTG CTT CTA CAT TTT GAA TTT GGC ATT GTC TCA TAG GAT GCC TGC TTC TAA CTG TCT TAT GGA CAA TGA TGG TGC TTT TGG TTA CTG CTA CTT ATA TTA GCA GAC AGC TAG ACC CAT ATA AAA TAA AAT AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC

>MB49

TGC GGA GGA TCA TTA TTG TTT AAC AGG GGG TGT AAG GGT TGT TGC TGA CCT TTA AAT GGT TGT GCA TGC CTA AGC ACT CTT AAA CAT CCA TCT CAC CCA TTG TGC ACC ACC GCG TGG GCC CCT TTG CAA AGA GGG CCT GCG TTT TTT TAA TAC ATA AAA ACT TGA TGT GGT ATA GAA TAT TAT ATT TTG CTA TTA TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT CCT CAA AAA TCT TTT ACT TTA ATT GTT TCT GAT CAG GAA AAG GTT TTT GGA CTT GGA GGT TTA ATG CTT GCT CTT GTC TTA GAA GCA AGC TCC TCT GAA ATA AAT TAG TAG GGT CTG CTT TGC TGG TTC TTA ATG TAA TAA GTT GCT TCT ACA TTT TGA ATT TGG CAT TGT CTC ATA GGA TGC CTG CTT CTA ACT GTC TTA TGG ACA ATG ATG GTG CTT TTG GTT ACT GCT ACT TAT ATT AGC AGA CAG CTA GAC CCA TAT AAA ATA AAA TAA ATC TTG ACC TCA AAT CAG GTG AGA CTA CCT GCT GAA CT

>MB50

AGG TGA CCT GCG GAG GAT CAT TAT TGT TTA ACA GGG GGT GTA AGG GTT GTT GCT GAC CTT TAA ATG GTT GTG CAT GCC TAA GCA CTC TTA AAC ATC CAT CTC ACC CAT TGT GCA CCA CCG CGT GGG CCC CTT TGC AAA GAG GGC CTG CGT TTT TTT AAT ACA TAA AAA CTT GAT GTG GTA TAG AAT ATT ATA TTT TGC TAT TAT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATC CTC AAA AAT CTT TTA CTT TAA TTG TTT CTG ATC AGG AAA AGG TTT TTG GAC TTG GAG GTT TAA TGC TTG CTC TTG TCT TAG AAG CAA GCT CCT CTG AAA TAA ATT AGT AGG GTC TGC TTT GCT GGT TCT TAA TGT AAT AAG TTG CTT CTA CAT TTT GAA TTT GGC ATT GTC TCA TAG GAT GCC TGC TTC TAA CTG TCT TAT GGA CAA TGA TGG TGC TTT TGG TTA CTG CTA CTT ATA TTA GCA GAC AGC TAG ACC CAT ATA AAA TAA AAT AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AGC A

>MB52

GCG GAG GAT CAT TAT TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC ACC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA A

>MB54

GGT GAC CTG CGG AGG ATC ATT ATC GTA TAA CCA GAG GTG CTG GGG CTG TCG CTG GCC TCC CCC TTA AAA ATG GGG GGT CGT GCA CGC CTC AAG TAT CCT CAA CAA ACC CAA TAC ACC CCC CCT CTT GTG CAT CAC CGC GTG GGC TCT CTT AAA AAA GGG GGC TTG CGT TTT TCA TAC AAA CGA TTC AGT GGT TGT AGA ATG TCT TTT TTT GTT TTG GGG CTA GAA CAC TTT CAT ATA TAT AAA ACC TTT CAA CAA CGA ACC TCT GGG CCC CCG CAT CGA TAA AAA ACG CAG CGA AAT GCA AAA CGA AAG GGA AAT TGC AAA ATT CAG GGA ATC ATC AAA CCT TTG AAC GCA TCT GGC CCC CCT GGG AAT CCC AAG GGG AAC ACC CGT TTG AGT GTC GGG AAA CCT TCA ACC TTC ACC CTT TCT GGA AAT CGG GGA AAG GCT GGA ACT TGG AAG GCA ATG GCG GAC CCC AAT CAA TAA AGC CAG CTC CCC TAA AAT AAA TAA TGA AGG CCG CCC TTT TGC CAA TCT CTG GAA GGT GAT AGG TGG CTT TTT T

>MB55

GGT GAC CTG CGG AGG ATC ATT ATT GTA TAA CAT GTA GGG TGT AAG GGC TGT TGC TAA CCC TTA AAG GGT TGT GCA CGC CTT AGC ACT CTT AAA CAT CCA TCT CAC CCA TTT GTG CAC CTC TGC GTG GGC CCC CTT TGC GAA GAG GGC CCG CGT TTT TTT TAT ATA TAT AAA CTC TCC ATG TGT AAA GAA TAA TAT TTT GCT GTT ATA TGG GAT TAA ATA CGA ATT TAA CCC CGG ATC TCT TGT GCC TCG CGG TGA TAA CAA GAC CCG CCA CAA CTC ATA CGG AAC GTG CCC TGG GGA AGG TCG TGC CCT TTT GAA ACT TTG TGC GCA ACC CCC GCC CCG GGG GGC AAA AAG GGG GAC GGC CTG GGG AGA GTC GCG CAG GAC CCC GAA

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GAA CCT GCG GAA GGA TCA TTT ATC GTA TAA TCG AGG CAC CGG GGC TGT CGC TGA CCT TTG AAA GGG TCG TGC ACG CCC CTG TGC ACT CAC ACA TCC ATC TCA CAC CTT TGT GCA TCA CCG CGT GGG CCC TCT TTG GCT TGT TCC GAG GGG GTT CAC GTT TTT ACA CAC ATA CAC GAC GCA TGT ATC GAA TGT CTT TAC TTA TTT TTG CGA TCA CAC GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA CAT CCT CAA CCT TTC TTG GTT TCT CTT GAC CAA GGG AGG CTT GGA CTT GGG GGG TTT TCT TTT TT

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TGA GGT TGT CGC TGA CTT TTG AAG GGT CGT GCA CGC CTC GGT GCT CTC ACA CAC AAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG AGG GCC CCC TTT TGG CTA GTT CTG AGG GGG GTT TTC ACG TTT TTA CAC AGA CAC CCG TTT TAA TGC AAT GTG TAG AAT GTC TTA GTT TTT TGC GAT CAC GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA CAT CCT CAA ACC TTC TTG GTT TCT TGA CCG GGA AGG CTT TGG ACT TTG GAG GCT TTT GCT GGC CTT CCC TTT TTG AAG CCA GCT CCT CTT AAA TGA ATT AGT GGG GTC TGC TTT GCT GAT CCT TGA CGT GAT AAG ATA TTT CTA CGT CTT GGG TTT GGC ACT GTT TGC TTT AGA TCC TGC TTC TAA ATG TCT TAT AGA AGA CAA TGG TCG AGT GAT TGC CAC TTG ACC CAT CGA CCT TGA CCT CAG ATC GGG TGA GAC TAC CCG CTG AAC TTA AGC ATA TCA ATA AGC GGA GGA AAA GAA ACT AAC AAG GAT TCC CCT AGT AAC TGC GAG TGA AGC GGG AAA AGC TCA AAT TTA AAA TCT GGT GGT CTT TGG CCA TCC GAG TTG TAA TTT AGA GAA GCG TCT TCC GCG C

>MB61

AGG GCT GTC GCT GAC CTT CAC AGG TTG TGC ACG CCC AAA GGT GCT CTC TTA TAT CCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GTG CTT CCC CTG TTG GGC TTG TTC CGA GGG GGG GTT CAC GTT TTT ATA CAA ACA CCA TTT ATG TAT AGA ATG CCT TAC TTT TTG CGA TCA TGC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA ACA CCC TCA ACC TTT TTG GTT TCT TGA CCA AGA AGG CTT GGA CTT TGG AGG TTT TCC TTG CTG GCC TCC CTT TGA AGC CAG CTC CTC CTA AAT GAA TTA GTG GGG TTC GCA TTG CTG ATC CTT GAC GTG ATA AGT ATG CTT CTA CGT CTT GGA TTT TGC ACC TTT GCC TTG GAA CCC GCT TCC AAC TGT CTT TGA ATG AAG ACA ATG TTT TGA GTT CCA TCG TGA CTC AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA GCA TAT CAA TAA GCG GAG GAA AAG AAA CTA ACA AGG ATT CCC CTA GTA ACT GCG AGT GAA GCG GGA AAA GCT CAA ATT TAA AAT CTG GTG GCC TTT GGC CGT CCG AGT TGT AAT TTA GAG AAG CAT CTT CCG CGC TGG AC

>MB62

CCC TGT TGG GCT TGT TCC GAG GGG GGG TTC ACG TTT TTA TAC AAA CAC CAT TTA TGT ATA GAA TGC CTT ACT TTT TGC GAT CAT GCG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GTA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA CAC CCT CAA CCT TTT TGG TTT CTT GAC CAA GAA GGC TTG GAC TTT GGA GGT TTT CCT TGC TGG CCT CCC TTT GAA GCC AGC TCC TCC TAA ATG AAT TAG TGG GGT TCG CAT TGC TGA TCC TTG ACG TGA TAA GTA TGC TTC TAC GTC TTG GAT TTT GCA CCT TTG CCT TGG AAC CCG CTT CCA ACA GTC TTT GAA TGA AGA CGA ATG TTT TGA GTT CCA TCG TGA CTC AAA CTC AGC GAC CTG

>MB63

GTG ACC TGC GGA GGA TCA TTA TCA TAC AAT GGA GGT GCG AGG GCT GTC GCT GAC CTT TAA AAG TCG TGC ACG CCC GAG TGC TCT CAT ACA TCC ATT TCA CCC CTT TGT GCA TCA CCG CGT GGG GGT CCC TTG AAA GGG GGA GGC CCA CGT TTT TTT TTT CTT TCA CAC AAG CCT CAA AGT TGT GTA GAA TGT ACT TTT ATT TTA TTT TTG CGG TGA TAC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA AAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA AAT CCT CAA AAC CCC CCC TTC CCT TGA ACA TTT TGA TTG AAA AAA AGG TTG GGT TTT TGG

>MB64

GAG GAT CAT TAT CGT ATA ACA GAG ATG CCC TAG GGC TGT CGC TGA CCT TCA CAG GTT GTG CAC GCC CAA AGG TGC TCT CTT ATA TCC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGT GCT TCC CCT GTT GGG CTT GTT CCG AGG GGG GTT CAC GTT TTT ATA CAA ACA CCA TTT ATG TAT AAA AAG CCT TAC TTT TTG CGA ACA TGC CCA ATC AAT AAA ACT TTC AAC AAC GGA ACT CTT GGC TCT CGC ATC GAT GAA AAA CGC CGC GAA ATG CGA AAC GTA ATG GGA ATT GGA GAA TTC AGT GAA TCA TCC AAT CTT TTA ACG CAC CTT GGG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA ATG GCG TGA ACA CCC TCA ACC TTT TTG GTT TCC TGA CCA AGA AGG CTT GGA ATT TGG AGG TTT TCC TTG GTG GCC TCC CTT TGA AGC CAG CTC CTC CTA AAT GAA TTA ATG GGG TTC GCA TTG CTG AAC CTT GAC GTG ATA AGT AAG CCT CCA CGT CCT GGA ATT TGC ACC TTT GCC TTG GAA CCC GCT TCC AAC TGG CTT TGA ATG AAG ACC ATG TTT TGA GTT CCA TCG TGA CTC AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT G

>MB68

GAT CAT TAT CGT ATA ACG GAG GTG CCT AGG GCT GTC GCT GAC CTT TCT GGG TCG TGC ACG CCC GAA AGT GCT CTC TCG TGT CCC ATT ATT ATC CCT CAC CCC TTT TGT GCA TCA CCG CGT GGG CTT CCC CCC TAT TGG GAT TGT TCT GTT CCG AAG GGG GGG GGT TCA CGT TTT TTT TTT TTT TTA CAT AAA CCC CTT TTC TTT TAA GGC AGG GGT AAA AGG TAT CAC CTT TTG CGA TGA GGC CCA ATT AAA ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA AAA CGC ACC GAA ATG CGA TAC GTA ATG GGA ATT GCA AAA TTC AGG GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGA ATC CCG AGG GGC ACC CCG TT

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AGG TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GGG GTG TAA GGG CTG TTG CTA ACC TTT AAA AGG GTT GTG CAC GCC TGA GCA CTC TTG AAT GTC CAT CTT ACC CAT TTG TGC ACC TCT GCG TAG GCC TCT TTG CAA AAA GAG GGC CTG CGT TTT TTT ATA TTA AAC TCT GTG TGT AGA ATG TTA TAT TTT GCT GTT ATG CAA TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ATG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ATC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TCT ACA TTT TGG ATT TGG TAT TGT CTC TTA GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGT TTC TGG TGT CTG CTA TTA TAT TAG CAG TTA GCT TAG ACC CAT GAA ATA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT TAA GCA TA

>MB70

TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AGC

>MB71

CTG CGG AGG ATC ATT ATT GTA TAA CAG AGG GTG TAA GGG CTG TTG CTA ACC CTT AAA GGG TTG TGC ACA CCT TAG CAC TCT TAA ACA TCC ATC TCA CCC ATT TGT GCA CCT CTG CGT GGG CCC CCT TTG CGA AGA GGG CCT GCG TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AGT ATT ATA TTT TGC TGT TAT ATG CAA TTA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGC TTG ATT GTT TCT GAT CAG AAA AGG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTG TAC ATT TTG AAT TTG GCA TTG TCT CTT GGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG CTT CTG GTT ACT GCT ATT TTT ATA TTA GCA GAC AGC TAG ACC CAT AAA AAA AAT CTT GAC CTC AAA TCA GGT GAG ACT ACC TGC TGA ACT T

>MB72

TGC GGA GGA TCA TTA TTG TAT AAC AGA GGG TGT AAG GGC TGT TGC TAA CCC TTA AAG GGT TGT GCA CAC CTT AGC ACT CTT AAA CAT CCA TCT CAC CCA TTT GTG CAC CTC TGC GTG GGC CCC CTT TGC GAA GAG GGC CTG CGT TTT TTT AAT ATA TAT AAA CTC TAC ATG TAT AAA GTA TTA TAT TTT GCT GTT ATA TGC AAT TAA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GCT TGA TTG TTT CTG ATC AGA AAA GGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT ATC TTG GAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GGT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TGT ACA TTT TGA ATT TGG CAT TGT CTC TTG GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGC TTC TGG TTA CTG CTA TTT TTA TAT TAG CAG ACA GCT AGA CCC ATA AAA AAA ATC TTG ACC TCA AAT CAG GGT GAA GAC TAC CTT GCT GAA CTT TAA CCA TAT C

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AAG TAA AAG TCG ATA ACA AGG GTT CCG GTA GGG GGA CCC TGC GGG AAG GGT TCA TTT TTT GTA TAA CCG GGG GGT GTT AAG GGC TGT TTG CTA ACC CCT TAA AGG GGT TGT GCA CCC CCT TAG CAC TCT TAA AAC ATC CAT CTC ACC CCA TTT TGT GCA CCT CTT GCG TGG GCC CCC TTT GCG AAG AGG GCC CTG CGT TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AAG TAT TAT ATT TTT GCT GTT ATA TGC AAT TAA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAA CGC AGC GAA ATT GCG ATA CGT AAT TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT A

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CCC CCC TTT TTT GGA GTT GGG GGG GCT CAC GTT TTT TTA ACA TCG AAC ACC CAT TAG AAC GTA GTG TAG AAT GTT CTT TGC GCG ATC GTG CGC GAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ATC CTC AAC CTG CTT GGT TTT TCT TGA AAC CAA GTA GGC TTG GAA TTT GGA GGT TTT CTG CTG GCC TCC TTT GAA GCC AGC TCC TCT TAA ATG TAT TAG TGG GAT CCG CTT TGC TAG ATC CTC GAC GTT GAT AAG ATG TTT CTA CGT CTT GGG TTT CGC TCA GGA ATG ACC CGC TTC TAA CGG TCC CAT CAG GGG ACA ACG TTC GAG AGC CGA TCG CCC TTC ACG GGG TGG GAA GCT TTT CGA CCT TTC ATG CCT TGA CCT CAA ATC

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TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG ATG CCC ATG GCT GTC GCT GAC CTA CAC AGG TCG TGC ACG CCT GAA GGT GCT CTC TCA CAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG TGC TCC CCT ATT GGG CTT GTT TCA AAG GAG GGT TCA CGT TTT TAT ATG GAC ACC AAG CAT GTG TAG AAT GTC TTA CTT TTT GTG ATT ATG CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG TAT TCC GAG GGG TAC ACC CGT TTG AGT GTC GTG AAC ACC CTC AAC CTT CTT GGT TTC TTA ATC AAG GAG GCT TGG ACT TTG GAG GTT TTT CTT GCT GGC CTT TCC TTT AAA GCC AGC TCC TCC TAA ACG CAT TAG TGG TGT TTG CTT TGC CGA TCC TTG ACA TGA TAA GTA TGC TTC TAT GTC TTG GGT TTG CAC TGT TGC CAC TTC CAA TGG TCT TCA AGT GAA GAC AAT GTT TGA GTT GCA CTG CGA CTG AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA

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TGC GGA GGA TCA TTA TCG TAT ACA ATG GGG GTA CGA GGG CTG TCG CTG ACT CTT TGT CGT GCA CGC CCG AGT GCT CTT CAT CCA TCT CCC CCC TTT GTG CAT CAC CGC GTG GGT CCC CCT TTC TTC GGA GTA GGG GGT GCT CAC GTT TTT AAC ATC GAA CAC CCA TTA AAA CGT AGT GTA GAA TGT TCT TTG CGC GAT CGT GCG CGA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACC TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA CAT CCT CAA CCT GCT TGG TTT TAC TTG AAA CCA AGT AGG CTT GGA ATT TGG AGG TTT TCT GCT GGC CTC CTT CGA AGC CAG CTC CTC TTA AAT GTA TTA GTG GGA TCC GCT TTG CTA GAT CCT CGA CGT TGA TAA GAT GTT TCT ACG TCT TGG GTT TCG CTC AGG AAT GAC CTG CTT CTA ATC GTC CCA TCA GGG ACA ACG TTC GAG AGC CGA TCG CCC CGT GAA GGG GTG GGA AGC TTT TCG ACC TTT CAT GCC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AA

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GTG ACC TGC GGA AGG ATC ATT ATC ATA TAA CCG AGG CAC TAG GGC TGT CGC TGA CCC GTC GAA GGG TAG TGC ACG CCC AAG TGC TCT CTC TCA CAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG GGC CAC CTT TTT GGC TTG TTT CAA AGA GGT TGG TTC GCG TTT TTA CAC ACA CAC ACA CCT TTA TGT ATA GAA TGT CTT ACT TTT TGC GGT GAT ACG CAA TAA ATA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA CAA CGC ATC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC

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GCG GAG GAT CAT TAT CAT ATA ACC GAG GCA CTA GGG CTG TCG CTG ACC CGT CGA AGG GTA GTG CAC GCC CAA GTG CTC TCT CTC ACA TCC ATC TCA CCC CTT TGT GCA TCA CCG CGG GGG CCA CCT TTT TGG CTT GTT TCA AAG AGG TTG GTT CGC GTT TTT ACA CAC ACA CAC ACC TTT ATG TAT AAA ATG TCT TAC TTT TTG CGG GGA TAC GCA ATA AAT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC

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TAT AAC CGA GGC CTA GGG CTG TCG CTG ACC CGT CGA AGG GGG GGG ACG CCC AAG TGC TCT CTC CCA CAT CCC TTC CAC CCC CTT GTG TGT CAC CGC ATG GGC TGC TTT TTG GCT TGT GTC AAA GGA AAA TGG TTC GCG GTT TTA CCG ACA CAC ACG CCT TTA TTT ATA GCT TCT TTG TAT TTT TTG CGG TGA TGC GGC ATA AAT ATA TAC AAA TTT CTT CAT TAG GAC CTC TGG GCT CTT ACA TTG ATG AGT AAC GCA GAA TTA ATG CGA ACC GTC TAT TTC TAT TGC AGA ATT CAT GGA ATG ATT GAA TCT TTG AAC GTA CCT CGT GCC CCT TAG TAT TCC GAG GGA CAC CCC ACT GTT GAA ATG TCG CGA ACA AAT TAC AAT GCC TTC TTT TGG GTT TTT GAT TG

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TAG GGG AAG TAA AAG TCG TAA CAA GGT TTC CGT AGG TGA ACC TGC GGA AGG ATC ATT ATT GTA TAA CAG AGG GTG TAA GGG CTG TTG CTA ACC CTT AAA GGG TTG TGC ACA CCT TAG CAC TCT TAA ACA TCC ATC TCA CCC ATT TGT GCA CCT CTG CGT GGG CCC CCT TTG CGA AGA GGG CCT GCG TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AGT ATT ATA TTT TGC TGT TAT ATG CAA TTA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGC TTG ATT GTT TCT GAT CAG AAA AGG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTG TAC ATT TTG AAT TTG GCA TTG TCT CTT GGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG CTT CTG GTT ACT GCT ATT TTT ATA TTA GCA GAC AGC TAG ACC CAT AAA AAA AAT

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TAT CAT ATA ACC GAG GCA CTA GGG CTG TCG CTG ACC CGT CGA AGG GTA GTG CAC GCC CAA GTG CTC TCT CTC ACA TCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GGG CCA CCT TTT TGG CTT GTT TCA AAG AGG TTG GTT CGC GTT TTT ACA CAC ACA CAC ACC TTT ATG TAT AGA ATG TCT TAC TTT TTG CGG TGA TAC GCA ATA AAT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ACA TCC TCA ACC TTC TTT TGG GTT TTT GAC CGG GGG AAG GCT TGG ACT TTG GAG GCC CTT TCA TTG CTG GTA TGC TTT TAA AGC CAG CTC CTC CTA AAT GAA TCA GTG GGA TCC GCT TCG GTG ATC CTT GAC GTG ATA AGT ATT ATT CTA CGT CTT GGG TTT TCA CAG CAC CTG CTT CCA ACC GTC TTT TAA CCA AGA CAA TGT TCG GGT TTC GAC CTG AAT CAA AAA ACC TTG ACC TCA AAT CGG GTG AAA CTA CCC CCT GAA CTT AAG CCT ATT ATT AAG CGG AGG AAA AGA AAC TAA CAG GGA TTC CCC CAG GAA CTG CCA ATG AAG CGG GAA AAG CTC AAA TTT AAA AAC CGG GGG GCC TTT TGG

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GTG ACC TGC GGA AGG ATC ATT ATC TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG

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AGG TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAA GAT GCT TGT ACA TTT TGA ATT TGG CAT TGT CTC TTG GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGC TTC TGG TTA CTG CTA TTT TTA TAT TAG CAG ACA GCT AGA CCC ATA AAA AAA ATC TTG ACC TCA AAT CAG GTG AGA CTA CCT GCT GAA TTA

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TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AGT ATT ATA TTT TGC TGT TAT ATG CAA TTA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGC TTG ATT GTT TCT GAT CAG AAA AGG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTG TAC ATT TTG AAT TTG GCA TTG TCT CTT GGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG CTT CTG GTT ACT GCT ATT TTT ATA TTA GCA GAC AGC TAG ACC CAT AAA AAA AAT CTT GAC CTC AA

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TTT TTT TAA TAT ATA TAA ACT CTA CAT GTA TAA AGT ATT ATA TTT TGC TGT TAT ATG CAA TTA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGC TTG ATT GTT TCT GAT CAG AAA AGG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TAT CTT GGA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTG TAC ATT TTG AAT TTG GCA TTG TCT CTT GGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG CTT CTG GTT ACT GCT ATT TTT ATA TTA GCA GAC AGC TAG ACC CAT AAA AAA AAT CTT GAC CTC

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TGC GGA GGA TCA TTA TTG TAT AAC AGA GGG TGT AAG GGC TGT TGC TAA CCC TTA AAG GGT TGT GCA CGC CTT AGC ACT CTT AAA CAT CCA TCT CAC CCA TTT GTG CAC CTC TGC GTG GGC CCC CTT TGC GAA GAG GGC CTG CGT TTT TTT AAT ATA TAT AAA CTC TAC ATG TAT AAA GTA TTA TAT TTT GCT GTT ATA TGC AAT TAA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC AAC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CTG TTT GAG TGT CGT GAA ATT CTC AAA AAC CCT TTT GCT TGA TTG TTT CTG ATC AGA AAA GGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT ATC TTG GAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GGT CTG CTT TGC TGA TTC TTA ATG TAA TAA GAT GCT TGT ACA TTT TGA ATT TGG CAT TGT CTC TTG GAT GCC TGC TTC TAA CTG TCT TAC GGA CAA TAA TGG TGC TTC TGG TTA CTG CTA TTT TTA TAT TAG CAG ACA GCT AGA CCC ATA AAA AAA A

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TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG GGT GTA AGG GCT GTT GCT AAC CCT TAA AGG GTT GTG CAC GCC TTA GCA CTC TTA AAC ATC CAT CTC ACC CAT TTG TGC ACC TCT GCG TGG GCC CCC TTT GCG AAG AGG GCC TGC GTT TTT TTA ATA TAT ATA AAC TCT ACA TGT ATA AAG TAT TAT ATT TTG CTG TTA TAT GCA ATT AAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG ACT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCT CCT CTG AAA TAA ATC AGT AGG GTC TGC TTT GCT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CT

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TGC GGA GGA TCA TTA TCA TAT AAC CGA GGC ACT AGG GCT GTC GCT GAC CCG TCG AAG GGT AGT GCA CGC CCA AGT GCT CTC TCT CAC ATC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGG GCC ACC TTT TTG GCT TGT TTC AAA GAG GTT GGT TCG CGT TTT TAC ACA CAC ACA CAC CTT TAT GTA TAG AAT GTC TTA CTT TTT GCG GTG ATA CGC AAT AAA TAA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GTA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA CAC ATC CTC AAC CTT CTT TTG GGT TTT TGA CCG GGG GAA GGC TTG GAC TTT GGA GGC CCT TTC ATT GCT GGT ATG CTT TTA AAG CCA GCT CCT CCT AAA TGA ATC AGT GGG ATC CGC TTC GGT GAT CCT TGA CGT GAT AAG TAT TAT TCT ACG TCT TGG GTT TTC ACA GCA CCT GCT TCC AAC CGT CTT TAA ACA AAG ACA ATG TTC GGG TTT CGA CCT GAA TCA CAA AAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA GCA T

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TCA TTA TTG TAT AAC AGG AGG TGC CGA GGT TGT CGC TGA TCC CCC CTT AGG TCG TGC ACG CCT CGA GCA TAT TCT TAC TCC TCC TCA TCC ACC TCT CCA CCC CTT GTG CAT CCC CGC GTG GGC TTT CTC TTC CAC CAC GAG GGA GAG AGA GAG AGC CGG CTT CCT TTA AAC AAA CAC AAA GCG GAG AAG GAT GTT TTT TTG TAT GTT GTG ATG CAA ATA ATA CTT TTT TCA ACA ACG GAT CTG TTG CTT CTC GCA TCG ATG AAG GAC GCA ACG TGC TGC AAT ACA TGA TGT GAG TTG AAG AAT TCG ATG CAT CAT CTC ATC TAT GAA CGC TCC TTG CCC CCG TTG TTA TTA CGA AGG CAA CCC CCG GAT GAG TGT GGA GAA GTT CTC CCC CCT TCA TCT TTT GAA GAA GAG GGT AGT ATT TTG GAG GTT TTT GCA GTA CTT CTT CTT GAC GGA GAA CAT CGC CTC TTC TTT AAT GTA GTA GGA AGA CCC TGC GGT GCT CGT CGT CGA GGT GAT GCT GCT TTC

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GCG GAG GAT CAT TAT TGT ATA ACG GAG ATG CCC TAG GGC TGT CGC TGA CCT TCA CAG GTT GTG CAC GCC CAA AGG TGC TCT CTC ATA TCC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGT GCT TCC CTG TTG GGC TTG TTC CGA GGG GGG GTT CAC GTT TTT ATA CAA ACA CCA TTT ATG TAT AGA ATG CCT TAC TTT TTG CGA TCA TGC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA ACA CCC TCA ACC TTT TTG GTT TTT TGA CCA AGA AGG CTT GGA CTT TGG AGG TTT TCC TTG CTG GCC TCC CTT TGA AGC CAG CTC CTC CTA AAT GAA TTA GTG GGG TTC GCA TTG CTG ATC CTT GAC GTG ATA AGT ATG CTT CTA CGT CTT GGA TTT TGC ACC TTT GCC TTG GAA CCC GCT TCC AAC TGT CTT TGA ATG AAG ACA ATG TTT TGA GTT CCA TCG TGA CTC AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA

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GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG AGA TGC CCA TGG CTG TCG CTG ACC TAC ACA GGT CGT GCA CGC CTG AAG GTG CTC TCT CAC ATC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGT GCT CCC CTA TTG GGC TTG TTT CAA AGG AGG GTT CAC GTT TTT ATA TGG ACA CCA AGC ATG TGT AGA ATG TCT TAC TTT TTG TGA TTA TGC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCA CCC CTT GGT ATT CCG AGG GGT ACA CCC GTT TGA GTG TCG TGA ACA CCC TCA ACC TTC TTG GTT TCT TAA TCA AGG AGG CTT GGA CTT TGG AGG TTT TTC TTG CTG GCC TTT CCT TTA AAG CCA GCT CCT CCT AAA CGC ATT AGT GGT GTT TGC TTT GCC GAT CCT TGA CAT GAT AAG TAT GCT TCT ATG TCT TGG GTT TGC ACT GTT GCC ACT TCC AAT GGT CTT CAA GTG AAG ACA ATG TTT GAG TTG CAC TGC GAC TGA AAC TCA GCG ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT A

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TGC GGA GGA TCA TTA TTG TAT AAC AGG GGT GTA AGG GCT GTT GCT AAC CTT TAA AAG GGT TGT GCA CGC CTG AGC ACT CTT GAA TGT CCA TCT TAC CCA TTT GTG CAC CTC TGC GTA GGC CTC TTT GCA AAA AGA GGG CCT GCG TTT TTT TAT ATT AAA CTC TGT GTG TAG AAT GTT ATA TTT TGC TGT TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAT GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGT TTG ATT GTT TCT AAT CAG TAA AAG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TGT CTT AAA AGC AAG CTC CTC TGA AAT AAA TCA GTA GGA TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC ATT TTG AAT TTG GTA TTG TCT CTA AGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GGG TTT TCC GGG TGT CTG CCA ATT ATA TTA AGC AGT TAG CTT TAG ACC CAT GAA ATA AAT CTT GGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAT TAA AAA ACT CAC ATA AAG GAA AAA AAA AAA AAA A

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GCG GAG GAT CAT TAT CTG TAT AAC GGA GAT GCC CTA GGG CTG TCG CTG ACC TTC ACA GGT TGT GCA CGC CCA AAG GTG CTC TCT CAT ATC CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG TGC TTC CCT GTT GGG CTT GTT CCG AGG GGG GGT TCA CGT TTT TAT ACA AAC ACC ATT TAT GTA TAG AAT GCC TTA CTT TTT GCG ATC ATG CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ACC CTC AAC CTT TTT GGT TTC TTG ACC AAG AAG GCT TGG ACT TTG GAG GTT TTC CTT GCT GGC CTC CCT CTG AAG CCA GCT CCT CCT AAA TGA ATT AGT GGG GTT CGC ATT GCT GAT CCT TGA CGT GAT AAG TAT GCT TCT ACG TCT TGG ATT TTG CAC CTT TGC CTT GGA ACC CGC TTC CAA CTG TCT TTG AAT GAA GAC AAT GTT TTG AGT TCC ATC GTG ACT CAA ACT CAG CGA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA A

>MB127

GAC CTG CGG AGG ATC ATT ATC ATA TAA CCG AGG CAC TAG GGC TGT CGC TGA CCC GTC GAA GGG TAG TGC ACG CCC AAG TGC TCT CTC TCA CAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG GGC CAC CTT TTT GGC TTG TTT CAA AGA GGT TGG TTC GCG TTT TTA CAC ACA CAC ACA CCT TTA TGT ATA GAA TGT CTT ACT TTT TGC GGT GAT ACG CAA TAA ATA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA ACA CAT CCT CAA CCT TCT TTT GGG TTT TTG ACC GGG GGA AGG CTT GGA CTT TGG AGG CCC TTT CAT TGC TGG TAT GCT TTT AAA GCC AGC TCC TCC TAA ATG AAT CAG TGG GAT CCG CTT CGG TGA TCC TTG ACG TGA TAA GTA TTA TTC TAC GTC TTG GGT TTT CAC AGC ACC TGC TTC CAA CCG TCT TTA AAC AAA GAC AAT GTT CGG GTT TCG ACC TGA ATC ACA AAA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AA

>MB130

GAT CAT TAT CAT ATG ATG GAg GCC CAG GgT TGT TGC TGA CCC tag gaG GGn ACG CCC gAG TGC TCT caC aCa CaT CCA TCC CTC aCC CCC TTT GTG CAT CTC CGC TTT GgG CCC CTT TCG GCT CGT TCC GAA GGC CGC TCG GTT TTT AAT ACA GAC CAT TAT AAT GCA GTG TAG AAT GTC TTT TAT CCg ATG ATA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG TAT TCC GAG GGG TAC ACC CGT TTG AGT GTC GTG ACA TAC TCA ACC TTC TTG GTT CTT GAT CGG GAA GGC TTG GAT TTG GAG TTT CTG CTG GCC TCT CTC GAA GTC AGC TCC TCT AAA TGG ATT AGT nGG GTC TGC TTG CCG ATC CTT GAC GTG ATA GGA TCC TnT CTA CGT CTC GTG

>MB131

CTC CGC AGG cTA GTn TnG CCT GGa TTn Gnn GTC AAa aTT TTA TTT NTA TAT GGG TAT AGC CGC GTC TnT TAT TAT TTn ATA GCA nTA ACC nnA AnC ACC ATT ATT GTC CAT AAG ACA GTT AGA AGC nGG CnT CTA AGA GAC AAT GCC AAA TTC AAA ATG TAG AAG CAT CTT ATT ACA TTA AGA ATC AGC AAA GCA GAC CCT ACT AAT TTA TTT CAG AGG AGC TTG CTT TCA ACA CAA AAG CAA Gnn TTG GAC CTC CAA GTC CAA AAA CCT TTT CnT GnC Cnn AAA AAC ATT CtA ACA AAA AAG GTT TTT GAg aTT TCT CnG CAC TCA AAC AGG TGT GCC CCT nTT nAT ACC AaG GGG CCC nAG TTG CnT TTG AAG ATT CG

>MB132

GAC CTG CGG AGG ATC ATT ATC ATA CAA TGG AGG TGC GAG GGC TGT CGC TGA CCT TTA AAA GTT GTG CAC GCC CGA GTG CTC TCA TAC ATC CAT TTC ACC CCT TTG TGC ATC GCC GCG TGG GGG TCC CTT GAA AGG GGG AGG CCC ACG TTT TTC TTT CTT TCA CAC AAG CCT CAA AGT TGT GTA GAA TGT ACT TTT ATT TTA TTT TTG CGG TGA TAC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT CCT CAA AAC CCC CCT TCC CTT GAT CAT TTT GAT CGA GAA GAG GGT TTT TGG ACT TGG AGG ATG AAT GCT TTC CCT CAT TTT TGA GTG AGA GCT CCT CTT AAA TGA ATC AGC GGG GTT GGC TTT GCT GAT CCT TGA CGT GAT AAA GTG ATT CTA CGT TTT GGA TCT GGC ACT GTC CCT TTA AAT GCC TGC TTC TAA CCG TCT TAT GGA CAA TGA TGG TGT TCT GGT CAC CCG CTG CCT TGT GTT GGT GGG AGG CTA GAC CCA CAA GAA ATG ACC TTG ACC TCA AAT CAG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AA

>MB134

CCC GCG TGG GGG TCC CTT GAA AGG GGG AGG CCC ACG TTT TTC TTT CTT TCA CAC AAG CCT CAA AGT TGT GTA GAA GGT ACT TTT ATT TTA TTT TTG CGG TGA TAC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTG GCG CCC CTG GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT CCT CAA AAC CCC CCT TCC CTT GAT CAT TTT GAT CGA GAA GAG GGT TTT TGG ACT TGG AGG ATG AAT GCT TTC CCT CAT TTT TGA GTG AGA GCT CCT CTT AGA TGA ATC AGC GGG GTT GGC TTT GCT GAT CCT TGA CGG GAT AAA GTG ATT CTA CGT TTT GGA TCT GGC AGT GTC CCT TTA AAT GCC TGC GTC TAA CCG TCT TAT G

>MB146

CCC AAG TGC GCT CTC TTC ATC CGT CTC TTC CCT TGG TGC ATC ACC GCG TGT AGC CTC ACT TTG GTT TGT TTC AAG GAT TTG TGA CGA GTT TTT ACA CCC ACA CAT TCC TTT TTG TTA AAA ATG GCT GAC ATT TTG CGG TGA TAA AAC CCA AAA CAA TAA AAC TTA CTT ATC CCG A

>MB151

TGc AGG GGA ATC CTT GTT AGT TTC TTT TCC cTC TGC TTA TTG ATA TGC TTA AGT CCA GCA GGt AGC tCT CAC CCT GAT TTG AGG TCA AGA TTT TTT TTA Tnn nTC TAG CTG TCT GCT AAT AnA AAA ATA GcA GTA nCc AGA AGC ACC ATT ATT ATC CGC nAn AnA nTA AGA AGC AGC CGT CCt AGA gAn AAT GCC nnA ATC tAA AtG TAC AAG CAT CTT ATt ACA TtA AGA ATc AGC AAA GgA GAC CCn nCT nAT TTA TTT CnG AnG AGC TTG CTT CCA AGA tAA AAG CAA GCA TtA AAA CCt CCn nGT CCA AAA ACC CTT TTC TGA TCA GAA ACA ATC AAG CAA AAG GGT TTT TGA GAA TAT CAC GAC ACT CAA ACA GGT GTG CCC CTC GTA ATA CCA nGG GGn GCA AGT CGC GTn CAA AnA Ann GAT GAT TCA CTG AAT TCT GCA ATT TA

>MB155

GTG CAT CAC CGC GTG GGC CAC CTT TTT GGC TTG TTT CAA AGA GGT TGG TTC GCG TTT TTA CAC ACA CAC ACA CCT TTA TGT ATA GAA TGT CTT ACT TTT TGC GGT GAT ACG CAA TAA ATA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGT ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA ACA CAT CCT CAA CCT TCT TTT GGG TTT TTG ACC GGG GGA AGG CTT GGA CTT TGG AGG CCC TTT CAT TGC TGG TAT GCT TTT AAA GCC AGC TCC TCC TAA ATG AAT CAG TGG GAT CCG CTT CGG TGA TCC TTG ACG TGA TAA GTA TTA TTC TAC GTC TTG GGT TTT CAC AGC ACC TGC TTC CAA CCG TCT TTA AAC AAA GAC AAT GAT CGG GTT TCG ACC TGA ATC ACA AAA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAA CTT AAG CAT ATC AAT AAG C

>MB157

ATC aTA ATT TTA AAT TTG AGC TTT TGC CGC TGg Ctg gnn nnG GGT AGG GGA ATC CTT GTG CGt CTC TCT CtC CTC CGT CTC Tcn nnn TGG TGA ATC ACg GCG Gtn nCC TCT TTT TGn TTG AGG TCA GGA TTG TGA tCG AGg TTT TAA CaC ACA CAT TGC TTT TaG TTT AAA ATG GCT GAA nTT TTG CGG TGA TAn AAC CCA AAA AAA TAA AAA TTA CTT ATC CGG ATC tGG AGc CTG Ann ATC AAT CCA GAA CTT CAT TTA GGA CGA GCT GGA TTT AAA TTG CCC ATT CTG AAG GGC CTC CAT CTT tCA ACC CTT CtC GCG CCC CTA GAC CCA CAA gAg AGG CAC ACC AGT gTG AG

>CW1

TCA TTA TCG TAT ACA ATG GGG GTT ACG AGG GCT GTC GCT Gnn nnn Gnn GTC GTG CAC GCC CAA GTG CTC TTC CAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG GGT CCC CCC CCT TCC nGG GGG GGG CnC nCG TTT TTA ACT TTG AcC CCC CTT TCA AAC GTA GGG AAA AAG GTT CTT GGC nCn ACC CCn CCC AaC CAA TAC AnT TTT CAA CAA CGA ATC TCT GGG CCC TCC Ctt CAT TAA GAA CCG nAC CnA ATn GCA TTA CTA AAG GTG ATT GGC AAA ATT CGG GGA aCC TTC AAa CTT TTA ACC GCc CTT GCC GCC CCT GGG CAT tCG GGG GGc cGG CCC GTT gAG GgG TGG GAA CAT CTT CAA CTT CCT GGg TTT TCT CAA ACC

>CW2

TGC GGA AGG ATC ATT ATC GTA TAC AAT GGG GGT TAC GAG GGC TGT CGC TGA CTT TGT TGT CGT GCA CGC CCA AGT GCT CTT CCA TCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GGG TCC CCC CCT TCC CGG GGG GTG CTC ACG TTT TTA ACA TTG AAC ACC CAT TCG AAC GTA GTG TAG AAT GTT CTT TGC GCG ACC ACG CGC GAC CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG CAT TCC GAG GGG CAC GCC CGT TTG AGT GTC GTG AAC ATC CTC AAC CTA CTT GGT TTT CTC CAA ACC AAG TAG GCT TGG AAT TTG GAG GTT TTC TGC TGG CCT CCT TTG AAG CCA GCT CCT CTC AAA TGT ATT AGA GGG ATC CGC TTT GCT AGA TCC TCG ACG CTG ATA TAA TAT GTG TCT ACG CCT CGG GGT TCG CTT AGG AAT GAC CTG CGT CTA ACA GTC CCG TCG CGG ACA ACG TTC GAG AGA CCG ATC ACA CTT TCA CGG GGT GGG AAG CTT TTC GAC CTT TCA TGC GCT TGA CCT CAC ATC GGG TGA GAC TAC CCG CTG AGC TTA AGC GTA TC

D.2 Dried specimens

>DS01

AGG TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GGG GGT GTA AGG GCT GTT GCT AAC CTT TAA AAA GGG TTG TGC ACG CCT AAG CAC TCT TGA ATG TCC ATC TTA CCC ATT TGT GCA CCT CTG CGT AGG CTC CTT TGC AAA AAG AGG GCC TGC GTT TTT TAT ATT AAA CTC TGT GTG TAG AAT GTT ATA TTT TGC TGT TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT TCT CAA AAA TCC TTT TGT TTG ATT GTT TCT AAT CAG AAA AAG GTT TTT GGA GTT GGA GGT TTT AAT GCT TGC TTT TGT CTT GAA AGC AAG CTC CTC TGA AAT AAA TTA GTG GGA TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC ATT TTG AAT TTG GCA TTG TCT CTT AGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG TTT CTG TTG TCT GCT ATT ATT ATT AGT AGT CAG CTT AGA CCC ATA TGA AAG AAA TCT TGA CCT CAA ATC AGG TGG AGA CTA CCT GCT GA

>DS02

TTT TTT AAC ATA AAA CTT GAT ACT AGT ATA GAA TAT TAT ATA TAT TTG CTA TTA TGT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA AAT CCT CAA AAA CCT TTT GTT TGA TTG TTT CTA GTC AGG AAA AGG TTT TTG GAC TTG GAG GTT TAA TGC TTG CTT TTG TCT TGG AAG CAA GCT CCT CTG AAA TAA ATT AGT AGG ATC TGC TTT GCT GAT TCT TAA TGT GAT AAG ATG CTT CTA CAT TTT GAA TTT GCC ATT GTC TCT TAG ATT CCT GCT TCT AAT TGT CTT ACA GAC AAT AAT GGT GCT TCT GAC TAC TGC TAT TTA CAT TGG CAG ACG GCC AGA CCC

>DS03

AAC ATC CAT CTC ACC CCA TTG TGC ATC CCC GCG TGG GCC CCC TTT GCA AAG AGG GCC TGC GTT TTT TTA ACA TAA AAC TTG ATA CGG TAT AGA ATA TTA TAT TTT GCT ATT ATA TGC AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC TTT TTG TTT GAT TGT TTC CGA TCA GGA AAA GGT TTT TGG ACT TGG AGG TTT AAT GCT TGG TTT TAT CTT GAA AAC AAG CTC CTC TGA AAT AAA TTA GTA GGG TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC GTT TTG AAT TTG CCA TTG TCT TTT AGA TAC CTG CTC CTA ACT GTC TTA TGG ACA ATA ATG GTG CTT CTG GTT ACT GCT ATT TTT ATA TTG GCA GAC AGC TAG ACC CAT GTA AAA AAA

>DS04

TGA CCT GCG GAG GAT CAT TAT TGT ATA ACA GAG ATG CCC ATG GCT GTC GCT GAC CTA CAC AGG TCG TGC ACG CCT GAA GGT GCT CTC TCA CAT CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG TGC TCC CCT ATT GGG CTT GTT TCA AAG GAG GGT TCA CGT TTT TAT ATG GAC ACC AAG CAT GTG TAG AAT GTC TTA CTT TTT GTG ATT ATG CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG TAT TCC GAG GGG TAC ACC CGT TTG AGT GTC GTG AAC ACC CTC AAC CTT CTT GGT TTC TTA ATC AAG GAG GCT TGG ACT TTG GAG GTT TTT CTT GCT GGC CTT TCC TTT AAA GCC AGC TCC TCC TAA ACG CAT TAG TGG TGT TTG CTT TGC CGA TCC TTG ACA TGA TAA GTA TGC TTC TAT GTC TTG GGT TTG CAC TGT TGC CAC TTC CAA TGG TCT TCA AGT GAA GAC AAT GTT TGA GTT GCA CTG CGA CTG AAA CTC AGC GAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT

>DS05

AGT GCA CGC CCA AGT GCT CTC TCT CAC ATC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGG GCC ACC TTT TTG GCT TGT TTC AAA GAG GTT GGT TCG CGT TTT TAC ACA CAC ACA CCT TTA TGT GTA TAG TGT CTC ACT TTT TGC GGG GAG ACG CGC TAT ATA TAA ACA CCT TTT TAC ACC GGA TCT CTT GTC TCT CGC ATC TAT AAA AAA AAC GCC GCA ATG CGA GAT ATA ATG TGA ATT GCG CAA ATT TGT GAA TCT CCG AAT CTC TGT ACG CAC ATT GCG CGC CTT GTG ATA CTG AGA GGC ACA CAC GTG TGA GAG TCG TGA GCA CAT CCT CAA CCT TCT CTT GGG TTT TTT

>DS06

GTG TAG GGC TGT TGC TAA CCT TTA AAA AGG GTT GTG CAC GCC TAA GCA CTC TTA AAT GTC CTA TCT TAC CCA TTT GTG CAC CTC TGC GTA GGC TTC TTT GCA AAA AGA CAG CCT GCG TTT TTT TAT ATT AAA CTC TGT GTG TAG AAT GTT ATA TTT TGC TGT TAT GCA ATT AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT TGC ATC GAT GAA GAA CGC AGC GAA ATG TGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAA CTT GCG CCC CTT GGC ATT CTG AGG GGC ACA TCT GTT TGA GTG TCG TGA AAT TCT CAA AAA CCC TTT TGT TTG ATT GTT TCT AAT CAG AAA AAG GTT TTT GGA CTT GGA GGT TTT AAT GCT TGC TTT TGT CTT GAA AGC AAG CTC CTC TGA AAT AAA TCA GTG GGA TCT GCT TTG CTG ATT CTT AAT GTA ATA AGA TGC TTC TAC ATT TTG AAT TTG GCA TTG TCT CTT AGA TGC CTG CTT CTA ACT GTC TTA CGG ACA ATA ATG GTG TTT CTG GTT TCT GCT ATT ATT TTA GCA GTC AGC TTT AAA ACC ATG GTA AAA AAA

>DS07

GTG ACC TGC GGA AGG ATC ATT ATC GTA CAA TGG AGG TGC CGA GGT TGT CGC TGA CTT TTG AAA GGG TTG TGC ACA CCT CGG TGC TCT CAC ATA TAA TCC ATC TCA CCC TTT TGT GCA TTA CCG CGT GGG GAC CCC TTT TGG TTA GTT CTG AGA AGG GTT TTC ACG TTT TTA TAC AAA CAC CTT TTT AAT GCA ATG TGT AGA ATG TCT TAC TTT TTT GTG GTC ACA CAC AAT CAA TAT AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CTT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG ACA TTC TCA AAC CTT CTT GGT TTC TTG ACT AGG AAG GCT TTG GAC TTT GGA GGC TTT TGC TGG CAT TCC CTT GTT GAA GCC AGC TCC TCT GAA ATG AAT TAG TGG GGT CTA CTT TGC TGA TCC TTG ACG TGA TAA GAT GCT TCT ACT ACG TCT TGG GTT TTT GCA CTG TTT ACT CTG GAT CCT GCT TCT AAC CGT CTC ATA GAA GAC AAT AAT TGA CAA CCC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT A

>DS08

GTG CTG GGG TTG TCG CTG ACT TGT GAA GGG TCG TGC ACG CCT TGG TGC TTT CGC ACA TAA TCC ATT TCA CCC CTT TGT GCA TTA CCG GCG TGG GGA CCC CTT TTA GCT AGG GGT CTT CAC GTT TTT ACA AAT TTT AAA CCC AAT GAA TGT GTA GAA TGT CTT GCT TTT TGC GAC CAC ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCT TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAC ATC TTC AAA CCT TCT TGG TTT CTT GAC CGG GAA GGC TTT GGA CAT TGG AGG CTT TTG CTG GTG TTC CTT TGT TGG AGC CAG CTC CTC TGA AAT GGA TTA GTG GGG TCT GCT TTG CCT ATC CTC GAC GTG ATA AGA TGT TTT CTA TGT CTT GGA TTT TGC ACT GTT TCC TGC TTC TAA CCG TCT CAC AGA AGA CAA TGG TCA AGT GAT TGC CAC TTG ACC GCA CGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AAG CGG AGG AAA AGA AAC TAA CAA GGA TTC CCC TAG TAA CTG CGA GTG AAG CGG GAA AAG CTC AAA TTT AAA ATC TGG TGG TCT TTG GCC ATC CGA GTT GTA ATT TAG AGA AGC GTC TTC CGC GCT GGA

>DS09

TTT TTT TTT TTT AAG GGA GGG TTC ACG TTT TTC TTT ACA CAA ACA CCT TTT TAA TGC ATG TCT AGA ATG TCT TTA CTA TTT GCG CTC ATA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ATC CTC AAC CTT TCG TTG GTT TCT TGA CCA AGG GGA GGC TTG GAC TTT GGA GGC TTT ATT TTT GCT GGC ATC CTT GGA AGC CAG CTC CTC CTA AAT GAA TTA GTA GGG TCT GCT TTG CTG ATC CTT GAC GTG ATA AGG TTA TTT CTA CGT CTT GGA TTG TGC ACA AGT CAG TTT TGG ATT CTG CTT CTA ACC GTC TTT GCA AAA GAC AAT GTT GTA AAC CTG ACC TCA AAT

>DS10

GCC TAA GCA CTC TTA AAC ATC CAT CTC ACC CCA TTG TGC ATC ACC GCG TGG GCC CCC TTT GCA AAG AGG GCT TGC GTT TTT TTA CAT AAA ACT TGT GAC GGG ATA TAA AAT AAT ATT TTT TTA TTA TAT GCG ATT ATT ACA CCT TTT ACA AAC GGA TCT CTT GTG TCT CGC ACC GCT AAA AAA CGC ACC GCA ATG CGA GAC GCG TTG TGA ATT GTG CAA TTC TGA GAA TCT TCG CAT CTT TGA GCG CAA CTT GTG CCC CCT GTG ATA CCG AGA GGC ACA CCT GTG TTA GAG TGG CGA AAT TTT CTC AAA ACC TTT TGT GTG AGA GTG TCC GAT ATC GAA AAA TTT TTG GAC ATG TAG ATT TAT AGC GCG GGT TTA TCT TGA AAC TCG CGC CTC TCA GAT AA

>DS11

GTG ACT GCG GAG GAT CAT TAT TGT ATA ACA GGG GGT GTA AGG GCT GTT GCT GAC CTC TAA AGG GTT GTG CAC GCC TAA GCA CTC TTA AAC ATC CAT CTC ACC CCA TTG TGC ATC ACC GCG TGG GCC CCC TTT GCA AAG AGG GCT TGC GTT TTT TAA CCT AAA ACT TGA TAC CGG AAA AAA AAT TAT TTT TTG GCA ATA TTA GCC ATT AAT ACC ACT TTC AAC CAC GGA TCT CCT GGC TCC CCG CTC GAT GGA GAA CGC CAC CGA ATG CGA TAC GTA TGG GAA TTG GAG AAT TCA GTG ATC ATC GAT CTT TG

>DS12

AGG TGA CCT GCG GAG GAT CAT TAT CGT ACA ATG GAA GTG CCG GAG TTG TCG CTG ACC CTA ACA GGG TCG TGC ACG CCT CGG TGC TCT CAC ACA TAA TCC ATC TCA CCC CTT TTG TGC ATC ACC GCG TGG GGA CCC CCT TTT GGT TAG TTC CGA GGG GGG GTT TTC ACG TTT TTA CAA AGA CAC CCT CTT AAT GCA ATG TAT AGA ATG TCT CAC TTT TTG CGA TCT CAT ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCT TCG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAC ATC CTC AAA CCT TCT CGG TTT CTC GAC CGG GGA AGG CTT TGG ACT TTG GAG GCT TTT GCT GAC CTT CCT TCG TTG GGG CCA GCT CCT CTG AAA TGA ATT AGT AGG GTC TAC TTT GCC GAT CCT TGA CGT GAT AAG ATG TTT TCT ACG TCT TGG GTT TTG CAC TGT TTC GTC CTG GAT CCT GCT TCT AAC CGT CTC GCA GAA GAC AAA CGG TCA AGT GAT CGC CAC TTG ACC CAC AAA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA

>DS14

AGG TGA CCT GCG GAA GGA TCA TTA TCG TAC AAG GGG GGG TTA CGA GGG CTG TCG CTG ACG TCA AGT CGT GCA CGC CCG AGT GCT CTC CCA TAC AAA TAT CTA TCT CAC CCC TTT GTG CAT CAC CGC GTG GGG GTC CCC TTC CTC GGA GGG GGG TGC TCA CGT TTT TAA CAT CGA ACA CCC ATT CGA ACG TAG TGT AGA ATG TTC TTT GCG CGA CCA CGC GTG ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA ACA CCC TCA ACC TGC TTG GTT TTC TCC AAA CCA AGT AGG CTT GGA ATT TGG AGG TTT TCT GCT GGC CTC CTT CGA AGC CAG CTC CTC TCA AAT GTA TTA GTG GGA TCC GCT TTG CTA GAT CCT CGA CGT TGA TAA GAT GTT TCT ACG TCT TGG GTT TTG CTC AGG AAT GAC CTG CTT CTA ACA GTC CCA TCA GGG ACG ACG TTC GAG AGC CGA TCG CCC CGT GAA GGG GGT GGG AAG CTT TTC GAC CTT TCA TGC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TA

>DS15

AGG TGA CCT GCG GAG GAT CAT TAT CGT ACA ATG GAA GTG CCG GAG TTG TCG CTG ACC CTA ACA GGG TCG TGC ACG CCT CGG TGC TCT CAC ACA TAA TCC ATC TCA CCC CTT TTG TGC ATC ACC GCG TGG GGA CCC CCC TTT GGG TAA TTT CCA AGG GGG GGT TTT CAC GTT TTT ACA AAA ACA CCC CCT TAA TGC AAT GTA TAG AAA GTC TCA CTT TTT GCG ATC TCA TAC GCA ATC AAT ACA ACT TTT AAC AAC GGA TCT CTT GGG TCT CGC ATC GAT GAA AAA AGC ACC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA ATC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC TTC GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA CAT CCC CAA ACC TTC TCG GTT TCT CGA CCG GGG AAG GCT TTG GAC TTT GGA GGC TTT TGC TGA CCT TCC TTT GTT GGG GCC AGC TCC CCT GAA ATG AAT TAT AAG GGG CTA CTT TGC CGA TCC TTG ACG TGA TAA AAT GTT TTC TAC GTC TTG GGT TTT GCA CTG TTT TGT CC

>DS27

TAT CGT ACT ACA GAG GCA CGA AGG TTG TCG CTG ACC TTT CCA AAA GGT CGT GCA CGC CGG AGT GCT CTC ACA CAC AAT CCA CCT CAC CAC TTG TGC ACC ACC ATG CGG GGG CCC CTT GCT CTT GGC TCG CTT CGA GAG GGG GGA GGC TTT CGT TTT TTT TAA CAC ACA CAC ACC CAA AAC AGC CGG GAA GTA AAA AAG GTC ATT CAT TTG CAA AAA CAC GCA ATC AAA ACA ACT TTC AAC AAG GAA CCT CTG GGC TCT CGC ATC GAG AAA AAA CGC AGC GAA ATG CAA AAC GAA AGG TGA ATT GCA AAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCA CCC CTT GGC ATT CCG AGG GGT ACA CCC GTT TGA GTG TCG TGA AAT TCT CAA ACC TTC TTG GTT TCT TGA CCA AGA TGG CTT TGG ACT TTG GAG GTC TTT TGC TGG CTT TGC GAA AAG CCA GCT CCT CTT AAA TGC ATT AGT GGG GTC CTC TTT GCC GAT CTC CAG GCG TTG ATA AGA TGT TTC TAC GTC TTG GGA TTT GCT CTG TTC ATT GGG AAC CCG CTT CTA ACC GTC TCG CTA TGG GAG ACA TTG TTC GAG CTT GCT CGA CCT ACG AAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA GCA T

>DS28

TGC GCA GGA TCA TTA TCG TAC AAC AGA GGT GCG AGG GCT GTC GCT GAC CTT TCC AAA GGT CGT GCA CGC CCG AGT GCT CTC GAC TAC AAT CCA CCT CAC CCC TTG TGC ACA ACC GCG TGA GGT CTC CCC TTT GGC TCG TTC CGA GGG AGG CTC ACG TTT TTC ATA CAA ACA CCC TTT TAG TTT GGA ATG TCA TTC ATT TGC GAT CAC ACA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAA TTC TCA AAC CCA TTT GGT TTC TTG ACC AAG AAG GGT TTG GAT TTT GGA GGG ATT TTG CTG ACT TCA AGA CAA GTC AGC TCC TCT TAA ATG CAT TAG TGG AGT CCT CTT TGC GGA TCC TCG GAC GTG ATA AGA TGC TTC TAC GTC TTG GGA TTT GTA CTG TTC TTT AGG AAC CTG CTT ACA ACC GTC TCC CAT GAG ACA TCG TTC GAG TTT GCT CGA CCC ACG AAC CTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA GCA T

>DS36

GTG ACC TGC GGA AGG ATC ATT ATC GTA TAA CAA GAG AGG TGC CCG GGG GCT GTC GCT GAC CTC CCC TTC CAG GTC GTG CAC GCC CCC GAA GCA TTC TCT CAC TCG CAT CCA CCT CAC CTT TTG TGC ATC ACC GCG TGG GCT TCC GAG TAA AAA CCG GGG GCC TGC GTT TAT TAC ACA AAC GTC CTT CCA AGT GTA GAA TGG TCT TGT GCA TTG CAA TCG CGA TGC GAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG CAT TCC GAG GGG TAC ACC CGT TTG AGT GTC ATG AAG CTC TCA ACC TTT CTC CGT TTC TTT CTT TGA TCG GGG TTA AAG GCT TGG ACT TTG GAG GCT TTT CTG CTG GCC TTT TTC TTT TTG AAG GCC TAG CTC CTC TTA AAT GAA TTA GCA GGG TCC CTT TCG TCG ATC CTC GAT GTG ATA AGG TTT CTC CTA CAT CCG GGG TTT TGG CTT TCT AAG GCC TGC TTC TAA TCC GTC TCG TAA GTC GAG ACA GAG AAA ACC CTT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA A

>DS42

ATC ATT ATT GTA TAA TTG GGG GTG CAT AGG CTG TTG CTA ACC TTT AAA AAG GGT TGT GCA CGT CTA AGT GCT CTT GAA ATA TCC ATT TTT ACC TCA TTG TGC ATC ATT GCG TGA GCT ACT TTT ATT AAA GCA GCT TGC GTT TTT TTT TCC TCC CCA AAT TTT GAA ACC GTA AGG GAA AAT ATT TAT TTT TTT TTT GCT CTT ATA AGC TAT AAA CAA AAC AAT TTT CAA CAG ATC TTT TGG GGC TTC CAT CTT TAA AAA AAA CCG CAA AAA GCG GTA TTT TAT GTG GAT TGT TGA AAA CTT CGA AGC ATC AAA TCT TTT AAC ACC ACT TGT GCC CCC CGT GGT TCT TAG GGG GGC CCC TGT TTT ATG GGC GTA AAA AAA TCC AAA AAC TTT TTT AAA AGA TTT TTG AGG AGG AGG AGT TAT GCT GGT TTT TTT TAC AAC CTC CTC CTA AAA AAA ATA TTA AAA AAT ACT TTT TTG ATG CTT AAT GGG GTA AAA AGA TGC TAC AAT TTT

>DS63

TAC CGT ATA ATG GAG GTG CTG GGG TTG TCG CTG ACT TTC TGA ATG GGG GAG GTC GTG CAC GCC TCG GTG CTC TCA CAC ACA ATC CAC ATC ACC CCT TTT GTG CAT CAT CGC GTG GGG GAG TCC NNN CCN NTC ACG TTN TGG GGN NNN TGA ACG CAA TGT GTA TAA AGT CTT ATT ACT TTT TGC GAT CAC ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAC AAC GCA GCG AAA TGC GAT ACG AAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CAA ATC TTT GAA CGC ACC TTG CGC CCT TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAT ATC TTC AAA CCT TCT TGG TTT CTT GAC CGG GGA AGG CTT TGG ACT TTG GAG GCT TTT GCT GGC TTT CAT TTG TTG GAA CCA GCT CCT CTG AAA TGG ATT AGT GGG TCT GCT TTG CCT ATC CTC GAC GTG ATA AGA TGT TTT CTA CGT CTT GGG TTT TGC ACC GTC TCC TGC TTC TAA CCG TCT CAC AGA AGA CAA TGG TCA AGT GAT TGC CAC TTG ACC GCA TGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA

>DS67

TGC GGA GGA TCA TTA TCG TAT AAC CAG AGG TGC TGG GGC TGT CGC TGG CCT CCC CCT TAA AAA TGG GGG GTC GTG CAC GCC TCA AGT ATC CTC AAC AAA CCC AAT ACA CCC CCC CTC TTG TGC ATC ACC GCG TGG GCT CTC TTA AAA AAG GGG GCT TGC GTT TTT CAT ACA AAC GAT TCA GTG GTT GTA GAA TGT CTT TTT TGT TTT GTG GCT AGT ACA CAT TCA TAT ATA TAA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ATC TTG CGC CCC TTG GTA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA ACC TTC AAC CTT CAC CCT TTC TTG GAA TCG GGG GAA GGC TTG GAC TTT GGA GGC TAT TGC TGA TCT CTA TCT ATG AAG TCA GCT CCT CTT AAA TGA ATT AGT GAG GTC GTC CTT TTG CCG ATC TCT TGG ATG TGA TAA GTT GCT TTT TTA CGT CCT TGA GTT TTG GTC AAC ACT TTC AAG GCC TTG CTT CTA ACC ATT ATC ATT ATC ATT TTT TTT TGA CCT CAA ATC GGG TGA AAC TAC CCG CTG AAC TTA AGC A

>DS69

TGC GGA AGG ATC ATT ATT GTA ACA CAG GGG GTG CCA GGG CTG TCG CTG ACC TTC AAG GTT GTG CAC GCC CCG AGC ATT CTC TCA TTC ACA TCC ATC ACA CCT TTG TGC ATC ACC GCG TGG GCC CCC TTC TCT TTA AAA AAG AAG GGT GCT TGC GTT TTC ACA CGT ACA CAC AAT TGC ATA GAA GGT TTG CAC TTG CGA TCA CAC GCA AAT TAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG CAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAA TCT TCA ACC TTC TCT GCT TCT TTT TTT TGA TCC GGG GAA AGG CTT GGA CTT TGG AGG CTT TTG CTG GCA TCT CGT GAA GTC GGC TCC TCT TAA ATG CAT TAG CGG GGG TTT CCT TTT GTC AAT CCT CGA CGT GAT AAA GTT TTT CTA CGT CTG GGG CTT GGC TTT AAG ACC CGC TTC TAA TGG TCT CGT CGA GAC ACT TGG TTG AGT TTT GCC TCT TAG TTG GGG TGA GAC TCT GGC CCA TAA TGA ACC ATT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA AGC ATA TCA ATA AGC GGA GGA AAA GAA ACT AAC AAG GAT TCC CCT AGT AAC TGC GAG TGA AGC GGG AAA AGC TCA AAT TTA AAA TCT GGC AGT CTT TTG GCT GTC CGA GTT GTA ATT TAG AGA AGC GTC TTC CGC GCT GGA C

>DS71

TGC GGA AGG ATC ATT ATC GTA CGA CGA GAG GTG CCG GGG CTG TCG CTG ACC TTT TTA AAG TCG TGC ACG CCC CGA GCA TTC TCT CTC ACA CAT CCA TCT AAC CTT TGT GCA TCA CCG CGT GGG CTC CGT AAA GGA GCT TGC GTC TTT CAC ACA GAC GCC AGT GTA GAA TGA CCT TTT TGC GAT TAA TAC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA AAT CCT CAA CCT TCA CTG TTT CTT TCG ATC AGG GAA GGC TTG GAC TTT GGA GGC TAT TGC TGG TCT CTT ACC TGA AGC CAG CTC CTC TTA AAT GGA TTA GCA GGG TCC CCT TTG CCG ATC CTC GGA CGT GTG ATA AGC TGC TTC TAC GTC CTC GGG TTT GGT TTC TCT TGC CTT GCT CCT AAT CCT TTC GGG GAC TGT GAA CCC CCA CGA ACC CTT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA AGC ATA T

>DS73

GTT TGG GGC TGT CGC TGA CTT GTA AAT AAA GTC GTG CAC GCC CTG GGC ATT CTC ATT CGC AAA TCC ATC TCA CCC TTT GTG CAT CAC CGC GTG GGC CCC CTT TTA AAG GTG GTC TAC GTT TTA CAC AAA CGT TGC CTT AGT TTT TGA ATG TTC ATA CTC TTT GCG GTC ACA CGC AAT AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG CAT TCC GAG GGG TAC ACC CGT TTG AGT GTC GTG AAT CTC TCA ACC TTT TCA AAG TTT ATT TTT GTA AAG GCT TGG ACT TTG GAG GCT TTT TGC TGG GCG TTT CTT TCT CTG AAT GCC TCA GCT CCT CTT AAA TGG ATT AGT GGG GTC CTT TTT GCC GGT CCC TCG ACG TTG ATA AGA CTT TCT CTA CGT CCG AGG TTT GGC TCT GTC AAG GCC CGC TTC TAA CCG TCT CTA TCG GAG ACA CGT TTT TGA ACC CTT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TT

>DS74

GGT GAC CTG CGG AGG ATC ATT ATC ATA TAA CAA GAG GCA CCC AGG GCT GTC GCT GAC CCC TTA GGT CGT GCA CGC CTC GAG TGT TTT CTT TCA CAT CCA TCT CAC CCT TTG TGC ACC ACC GCG TGG GCC TTT TCT CTT TAA AGA GGG GGG CTT ACG TGT TTT TAC ACA GAC ACA TCC TTA CGT AGA ATG GGG TAC CTT TTG CGA TCA CAC GCA ATT AAA ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCC GTT TGA GTG TCG TGA AAT TCT CAA CCT CCC TAA TTT GGG GGA AGG CTT GGA TTT TGG AGG CTT TTG CTG GCC TTT TCG GAA GCC AGC TCC TCT TAA ACG GAT TAG TAG GGT CCT CTT TGC CGA TCC CTT GAC GTG ATA AGA ATT TTC TTA CGT CCG AGG TTC GCG AAG ACC TGC TTC CAA TGG TCA CGA TTG AAC TCT TTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA GCA TAT CAT A

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GTG ACC TGC GGA GGA TCA TTA TCG TAC AAC AAG GGG CGT TCG GGG GCT GTC GCT GAC CTC TAA AGT CGT GCA CGC CCT TTG ATC GTC CTC GTT TAC ACA TCC ATC TCA CCC CCT TGT GCA TCA CCG CGT GGG CCC CTC CCT TTT GAT TCG AAA GGG TGG CCT GCG TTT TAT ATT TTC ACA CAC ACA AAC CTG GTC ACA GTG AAC GTC TTT TTA CTT TTT GTA ATA ATC AGT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCA CCC CTT GGC ATT CCG AGG GGT ACA CCC GTT TGA GTG TCG TGA AAT TCT CAA CCT TCT CTG ATT TAT CGG GGA AAG GCT TGG ACT TTG GAG GCT TTT GCT GGT CCC TCT TTT CTT TTG AAG CCA GCT CCT CTT AAA CGA ATT AGT GGG GTC CCT CTT TGC CGG TCC CTC GAC GTT ATA AGA TTT CTT TAC GTC CGA GAT TTG GCT CTG CAG CCC TGC TTC TAA TGA CGG TCT CTT CAC CGA GAC ACG TTT TTT GAA CCC TTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA

>DS76

TCA TTA TCG TAT AAA CAG GAG GTG CTG AGG TTG TCG CTG ACT GGG TGA AAC CCC CCT TAG GTC GTG CAC GCC TCG AGC ATT CTT ACT CCT CAT CCA CCT CTC TCA CCC CCC TTG GGC ATC CCC CCG GGG GCT TTT CCT CTT CTT CCA CAA AAA AAA AAA AAC CGG GGT CCT TTA CAC CAA CAC CTA AAA ACA GTG GAA AAA GGT CTT TTG GAT TTT GTT ATG CAG TTA AAA CAA CTT TCC ACC ACG GAA CTC TTG GCT CTC GCA TCC ATT AAG GGC GCA ACG AAA TGG GAT ATG GAA TGG GAA TTG GAA AAT TTC GTG AAT CCT CCA ATC CTT GAA CGC CCC TTG GGC CCC TTG GTA TTC CGA AAG GCA CCC CCC TTT GAG TGG CGT GGA ATT CTC AAC CCT TCT TCA TTT TAA GAA AAG GCT TGG ACT TTG GAA GCT TTT GCT TGA CCT CTT CTT GAC GGA AAT CCG CTC CTC TTA ATG GAT TAA TAA GAA CCC

>DS77

ATC ATT TAT CGT ACG ACG AGG GGT GGC GGG GCT GTC GCT GAA CCT TTT CAG GGC GTG CAC GCC CCT GAA CAT TCT CTC TCA CAC CTC CCT CTA ACC CTT GGG CAT CAA CGC GGG GGC TCC GTA AAA GGG GCT TGG GTT TTT CAC ACC AAC CCC GGG GTA AAA AGG CCC TTT TGC GAA TAA TAC CCA ATC AAA ACC ACT TTC CAC AAC GGA ACT TTT GGC TCT TGC ATC CAT TAA AAA CGC AGC GAA ATG GGA TAC GTA ATG GGA ATT GGA AAA TTC CGT GAA TCA TCC AAT CTT TGA ACG CAC CTT GGG CCC CTT GGC ATT CCC AGG GGC ACA CCC CTT TGA ATG GCG TGA AAT CCT CAA CCT TCA CTG GTC CTT TGG TCA GGG AAG GGT TGG ACT TTG GAG GCT ATT GCC GGG CTC TAC CCC AAA CCG GGT CCC CTT AAA TGG ATT AAT AGG GTC CCC GTT GCC CAT CCT CGG ACG CGA TTA GCT GCC TCT ACG TCC CTG GGT TC

>DS79

TGA TGA GAG CCG CCC TCC GGG GGG GTG ACA CTT TCC CAT CCT TGG CTA TCT CTA CCC TGT TGC TTC CGC GGG CCC GCC TAA CCT TTG TGC ATC AGA TCC TTA GGG GGG GGC CAG GAG CTT GCG GGG GCC ACA CGC CCC CCG GCC CGT AAC GTC CTT TTA CTC CCA CTT GCA ACG TTG ATA CAA GTT TGA ACA CCG AAT CTT TTG TGG AAA AAA ATC GAT AAA AAA TTC AAC AAA GTG CCA TAC GTA ATG GGA ATC GAT GAA TTC CGC GAA TCA TCG AAT CTT TGA ACG CGA ATT GCG CAC CTC GGT GTT CCA AGG GGC CTT TGA ACG TGA GTG GCG CCC AAT GCT CTT CCT TGG GTG TTG CTT TCC ATA GCG TAA TGC TTG CCC TTT GGA GGC TAT TGC GGG TCG CTT ACC CGA CCC CGG CTC CTT TTA TGC GCA TCA GCA GGG TCC CCA TTG CCG ATC CTC GGA CGT GTG ATA GGC T

>DS81

GGT GAC CTG CGG AGG ATC ATT ATC GTA ACA ATG GAG GTG CTG GGG TTG TCG CTG ACT TTT GAA AGG GTC GTG CAC ACC TCG GTG CTC TCG CAC ACA ATC CAT CTT ACC CCT TTT GTG CAT TAC GCG TGG GGA CCC CTT TTT AGC TAG TTC TGA GAG GGG TCT TCC ACG TTT TTA CAA ATT TTA ACA CAA TGT GCA GAA TGT CAT ACT TTT TGC GAT TAC ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCT TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAC ATC TTC AAA CCT TCT TGG TTT CTT GAC CGG GAA GGC TTT GGA CGT TGG AGG CTT TTG CTG GCC TTC CTT TGT TGG AGC CAG CTC CTC TGA AAT GGA TTA GTG GGG TCT GCT TTG CCT ATC CTC GAC GTG ATA AGA TGT TTT CTA CGT CTT GGG GTT CGC ACT GTT TCC TGC TTC TAA CCG TCT CAC AGA AGA CAA TGG TCA AGT GAT TGC CAC TTG ACC CCA TGA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT G

>DS82

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>DS83

GGT GAC CTG CGG AGG ATC ATT ATC GTA TAA CCA GAG GTG CTG GGG CTG TCG CTG GCC TCC CCC TTA AAA ATG GGG GGT CGT GCA CGC CTC AAG TAT CCT CAA CAA ACC CAA TAC ACC CCC CCT CTT GTG CAT CAC CGC GTG GGC TCT CTT AAA AAA GGG GGC TTG CGT TTT TCA TAC AAA CGA TTC AGT GGT TGT AGA AAG TCT TTT TTG TTT TGT GGC TAG TAC ACA TTC ATA TAT TTT ACA ACT TTC AAC AAC GGA ACT CTT GGC TCT CGC ATC GAT TAA AAA CGC AAC GAA ATG CGA TAC GTA ATG GGA ATT

>DS84

TCA ATA GTG TCT CTA TGA GAG AAT TGG AAG CAG GTC TCA AAA AGC CAA GCC CCC AAA CGT AGA AAA TAT CTC ATC ATG TCA AGG ATT GAC CAA AAG GAA ACC TCC GCT AAT GCA TTT AAG AGG AGC TGG CTT AAA GAG AAG GCC AGC AGA AGC CTC CAA AGT CCA AGC CTT CTC TAA CAG AGA AGG TTG A

>DS85

TCA TTA TCG TAT AAC AGG AGG TGC CGA GGT TGT CGC TGA TCC CCC TTA AGG CGG GGA ACC CTC CAG GAT ATT CCT AAC CCC CCC CCT CCA CCT TTC CCC CCC TTG GGG CTC CCC CCG GGG GCC TTT CCT TCC ACC CCG AAG GAA AAA AAA CCC GGC TCC TTT ACC CCA ACC CCA AAA CCG TGG AAA AAG GTC TTT TGG CTT TTG GTA TGG CAT TTA AAA CAC TTT TAA CCA CGG AAC CTT TGG CTC TTG CCT CCA TTA AAG AAG CCG CCA AAT GGG AAA CCT AAT GGG AAT TGG CGA AAT TCA TGG ATC CAC CAA ACC TTT AAC CCC CCT TGG GCC CCT TGG TAT TCC CAA AGG GAC CCC CGG TTG AAT GGC CTG GAA TTT TCC ACC CTT CCT CCA TTG AAG AAA AAG GTT GGA ATT TGG AAG GCT TTG GCG GAA TTC CCC TTG GCC GAA GAA AAT GGC TCC CCC TAA ATG GAT TAA GAA GAA CCC

>DS86

GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG GAG GTG CCG AGG TTG TCG CTG ACC CTA GGT CGT GCA CGC CTT GAG CAC ATT CTT ACT CCA CCT CAC CCC TTG TGC ATC CCC GCG TGG GCT CTC TCT TAT CGA GAA GAG CCG GCG TCT TTA ACA CGG ACA CAA AGC AGT ATA GAA TGT TCT TTT TGC ATT TGT ATG CAG TTA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG GAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GTA TTC CGA AGG GCA CAC CCG TTT GAG TGT CGT GAA GTT CTC AAC CTT CTT CAT TCT TGG GGA ATG GCT TGG ACT TTG GAG GCT GTT TTT GCT GGA CTC TCT TGA TCG AGT ATC GGC TCC TCT TAA ATG GAT TAG TAG GGT CCC TTT TTG CTG GTC CTT GAC GTG ATA AGT CTT TTC TAC GTC TGG AGC TTA GCT TAT GAG GCC TGC TTC TAA TCG TCT CTT TAG ACA AAT CGG TTC AGA GAA TTC CCT TTC TGA CCG GTG AAC CCC TTT GAC CTC AAA TCG GGT GAG ACT ACC CGC TGA ACT TAA

>DS87

GAC TGC GGA GGA TCA TTA TCG TAT AAC AGG AGG TGC CGA GGT TGT CGC TGA TCC CCC CTT AGG TCG TGC ACG CCT CGA GCA TAT TCT TAC TCC TCC TCA TCC ACC TCT CCA CCC CTT GTG CAT CCC CGC GTG GGC TTT CTC TTC CAC CAC GAG nGA GAG AGA Gnn GGG GTC CTT Tnn ACn nAC ACA nAA GCC GGG GAA AAn GGT CnT TTG GnT TTT GgT ATG CAG GTA AAA CAA CTT TTA Ann ACG GAA CCn TTG GGT CTn GCn TCG nTT AAG Gnn GCn GCn AAA TGn nAn aCG naA TGG TGA tTG CnG GAT TCC nnG AAT CAT CnA AnC nTT GaA CCG CCC TGG GCC CCT GGG ATT cCG AaG GGA CAC CGT TTA GGG CGT GAA GTC TTA ACC CTC TTC AAT TGA AGA AAA GG

>DS88

CCA AGA CGT AGA AAT AAT CTT ATC ACG TCA AGG ATC AGC AAA GCA GAT CCT ACT AAT TCA TTT AGG AGG AGC TGG CTT CCA AGG ATG CCA GCA AAA ATA AAG CCT CCA AAG TCC AAG CCT TCC CTC AGT CAA GAA ACC AAA GAA AGG TTG AGG ATG TTC ACG ACA CTC AAA CGG GTG TGC CCC TCG GAA TAC CAA GGG GCG CAA GGT GCG TTC AAA GAT TCG ATG ATT CAC TGA ATT CTG CAA TTC AAA TTA CGT ATC GCA TTT CGC TGC GTT CTT CAT CGA TGC GAG AGC CAA GAG ATC CGT TGT TGA AAG TTG TAT TGA TTG CAT ATG AGT GCA AAT AGT AAA GAC ATT CTA GAC ATG CAT TAA AAA GGT GTT TGT GTG AGG AAA AAC GTG AAC CCT CCC TTT TTT TAA AAA AAT GGG GGG GGG CCT

>DS89

TGA CCT GCG GAG GAT CAT TAT CGT ATA ACC GAG GTG CGA GGG CTG TCG CTG ACC TTT CGG TCG TGC ACG CCC GAG TGC TCT CAC ATA CAT CCA TCT CAC CCC ATT TGT GCA TCA TCG CGT GGG CCC CGC TCT TCG AAG GGG GGC TCG CGT TTT CAT ACA AAC ACC CCT TTT TAA TGC AGT GTA GAA TGT TCA TTG CGA TGA CTC GCA ATA AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCG CCC CTT GGC ATT CCG AGG GGC ACA CCT GTT TGA GTG TCG TGA CAT TCT CAA AGA ACC ATT CTT GGT CCT TGT GAT CGA GAA AGG CTT TTT GGA CTT TGG AGG TTA TTG CTG GTT TCC CCA GCT CCT CCT AAA TGA ATT AGC AGG GAC CAC TTT GCT GAC CCT GGA CGT GAT AAG ATG TTT CTG CGT CTT GGG TTT TCG CTC TGT CCT TTG GAG ACC TGC TTC TAA CCG TCT TGT CAA AAG ACA ACG TTC GAG CCT TCA TCG AAG GCT TGA CCC ATG ACC CTT GAC CTC AAA TCA GGT GAG ACT ACC CGC TGA ACT TAA GC

>DS90

TGA CCT GCG GAG GAT CAT TAT TGT ACA ACG TGG GCG CGA GGG CTG TCG CTG ACT TTT TCT CAA AAG TTG TGC ACG CCC AAA GCG TCC TCA TCC ATC TCT CAC CCC TTG TGC ATC ACC GCG TGG GCT CTC CTC TGG ATC AGA CCG GTC CAG AGG TGG CTT GCG TTT TTC CAC ACA AAC ACA CTG TGC AGG ATG TCT CTT CTT TGC GAT CAC ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GCA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA ATT CTC AAC CTT CTC GGT TTC TTT GAC CGG GCA AGG CTT GGA CTT TGG AGG CTT ATG CTG GCA CAA CCT CTC CTG GAA GCC AGC TCC TCT CAA ATG GAT TAG TGG GGT CCT CTT TGC TGA TCT CCT TGA CGT GAT AAG ATG TTT CTA CGT CTT GGG CTT GGC TCT GTT TCT TGG GAA CCT GCT CCT AAC TGT CTC GTC GAG ACA ACG GTT CAA AGT CTC CTC CCC AGG GGA CTT TGA CCC ATG AAC CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA A

>DS92

TGC GGA GGA TCA TTA TCG TAT AAA ATG GAG GTG CTG GGG CTG TCG CTG ACC TTT GAA AAA GGT CGT GCA CGC CCG GAG CAC TCT CTC ATA TCC ATC TCA CCC CTT TGT GCA TTG CCG CGT GGG CCC CCT TTT GGC TTG TTC CAG AGG GGG TGA CCT GCG TTT TTA CAT AGA CAC CCT TTG AAT GCA TGT GTA GAA CGT CTT ACT TTT TGC GAT CAC ACG CAA TCA ATA CAA CTT TCA ACA ACG GAT CTC TTG GCT CTC GCA TCG ATG AAG AAC GCA GCG AAA TGC GAT ACG TAA TGT GAA TTG CAG AAT TCA GTG AAT CAT CGA ATC TTT GAA CGC ACC TTG CGC CCC TTG GTA TTC CGA GGG GCA CAC CCG TTT GAG TGT CGT GAA TAC TCT CAA CCT TCT TGG TTT CTT TGA CCA CGA AGG CTT GGA CTT TGG AGG TTT TTC TTG CTG GCC TCT TTT GAA GCC AGC TCC TCC TAA ATG AAT GGG TGG GGT CCG CTT TGC TGA TCC TCG ACG TGA TAA GCA TTT CTT CTA CGT CTC AGT GTC AGC TCG GAA CCC GCT TTC CAA CCG TCT TTG GAC AAA GAC AAT GTT CGA GTT GTG ACT CGA CCT TAC AAA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TT

>DS93

GAC CTG CGG AGG ATC ATT ATT GTA TAA CAG AGA TGC CCA TGG CTG TCG CTG ACC TAC ACA GGT CGT GCA CGC CTG AAG GTG CTC TCT CAC ATC CAT CTC ACC CCT TTG TGC ATC ACC GCG TGT GCT CCC CTA TTG GGC TTG TTT CAA AGG AGG GTT CAC GTT TTT ATA TGG ACA CCA AGC ATG TGT AGA ATG TCT TAC TTT TTG TGA TTA TGC GCA ATC AAT ACA ACT TTC AAC AAC GGA TCT CTT GGC TCT CGC ATC GAT GAA GAA CGC AGC GAA ATG CGA TAC GTA ATG TGA ATT GCA GAA TTC AGT GAA TCA TCG AAT CTT TGA ACG CAC CTT GCA CCC CTT GGT ATT CCG AGG GGT ACA CCC GTT TGA GTG TCG TGA ACA CCC TCA ACC TTC TTG GTT TCT TAA TCA AGG AGG CTT GGA CTT TGG AGG TTT TTC TTG CTG GCC TTT CCT TTA AAG CCA GCT CCT CCT AAA CGC ATT AGT GGT GTT TGC TTT GCC GAT CCT TGA CAT GAT AAG TAT GCT TCT ATG TCT TGG GTT TGC ACT GTT GCC ACT TCC AAT GGT CTT CAA GTG AAG ACA ATG TTT GAG TTG CAC TGC GAC TGA AAC TCA GCG ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GA

>DS94

GAA TCC TTG TTA GCT TGT TTT CCT CCG GTT ATT GAT AGG CTC AAG TTC AGC GGT GAG TTT CAC CCG ATT TTC GGT CAA AGG GTT CAT CCG GAG GCT TAA AAT GCC CCC CCT GGG GTG ACA AGC GGG TGA ACA AAA ACC AAA CCC GTG CCT TGC GCT CAC TGC CCG CTT TCC CCT CGG ATC GCC AGT CGA GCC CTC TGC TTT ATA TTT GAA AGG AGC GCG CGG GGA GAG GAG GTT TGC GAA TTG GGT GCT CTT CCG CTT CCT CCG TCG ATG AAG CGA TGC GGT CAG GCG GCC GCC TGC ACC GAT CGG TAT CAG CTC TCC CCC TGG CGA TAC TAA GGT TAT CCT CAG AGT TGA TGG ATT TCG CCG CTT CGA ACA TAT GTG TTT CTC TGA TTG GGA AAT GCT GGT AAC CTT GCC TAC CGC AGG ATG CAG ACG GGG GTT CCG AGG CGG GGG GGC CGA TGT ACC TG

>DS96

CGA TGT GTC GTC AAG GAG CAT GGT GTT CAT CCG TGA CCT GAA TAT TTT CCC TCC TCA ATG AAA TGG CAA GCG GCT CCT CGG AAA CCC TGA ATG CCA CCA TCC ACT TAT CAC CCA GGT TTT CAC CCG GGA ATT TTC CCC CGA CCT TAT TTG TCA GCT GCG GGC TCC TTA CGA CCC TTT AAT TCC AAC TTC CAA TGT CCG CCT TTC CAC TGT CCG TTG CCT GCT TTG GTT CAA CCG GCT TGA CTT CCG GTT CGA CGT CCA TCC CAA CTA TTA TGG GAG GGT TTT TGA TCC AGC CAA TGT CAT TAT TTG CCG TTT GCC TGG CTT TCC TCG GCC CCT TTG TCG AAT ACC CCT TAC TTC CTT TTG CCA T

>DS97

GAT CAT TAT CGT ACT GTG GGG GTA CGA GGG CTG TCG CTG ACA Cnn nGn CGT GCA CGC CCG AGT GCT CTT CAT CCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GGG TGT CCC CCC TTC CTC GGA GGG GGG nGG TCA CGG cTT TTA AnA TCC AAA AnC CTT CAT TGC CGA AAA ATG TTT GTT GGA TCT TGC ACG ATG ACC ATG ACT TTn TCC AAC GGA TCA CTT GTT TCT TGn nTC AAT CAA AAA AAG TTC GAA ATG TTT TAC CAA ATG tGA GGA GCT GAA TTC TTT GAT CCA TCT aAT CTT C

>DS98

TGA CCT GCG GAG GAT CAT TAT AGT ATA ACG GAG GCC CCC GGG CTG TCG CTG ACC TTT TGA GGA CGT GCA CGC CCG GAG CGC TCT CTC ACA TCC ATC TCA CCC CTT TGT GCA TCA CCG CGT GGG GCC CCT TCT CTT TTG GCT CGT TCC GGA GAG GGG GGT TTC ACG TTT TTA CTC AAA CAC CCA TTA ATG AAT GTG TAG AAT GTC TTA CTT TTT GCG GTC ACA CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA ACT TGC GCC CCT TGG TAT TCC GAG GGG TGC ACC CGT TTG AGT GTC GTG AAC ACC TTC AAC CTA CTC GGT TTA TCG ACC GGG GAA GGC TTG GAC TTT GGA GGT TTT ACT GCT GGC CTC CTT TGA AGC CAG CTC CTC CTA AAT GAA TTA GTG GGG TCA GCC GTG CCG ATC CTC GAC GTG ATA AGT TCT ACG TCT TGT GCG GTT TTC ACG CGG TCC CGC TTC CAA CCG TCT TTG AAT GAA GAC AAT GTT CGA GTC ACG ACT CGA CCT TAT AAA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA AGC ATA TCA ATA AGC GGA GGA AAA GAA ACT AAC AAG GAT TCC CCT AGT AAC TGC GAG TGA AGC GGG AAA AGC TCA AAT TTA AAA TCT GGT GGT CTT TGG CCA TCC GAG TTG TAA TTT AGA GAA GCG TCT TCC GCG CTG G

>DS99

CCG GAG CGA TCT CTC ACA TCT ATC CTC ACC CCT TTG TGC GTC ACC GCG TGG GGA CCC TTC CCT TTT GGC TTG TTC CCG ATA AGG GGG GGC TTC TTG TAT TCA AAA ATT TTC CCC TCT GTG CAT GTA TTT AAA GTC CCC TCT CTG AAA CCC TAA CTT CCT ACA TAC AAT TTT CAT CCG GTT TTC ACC CCG GGA CTT GCC CCT TAA TTT AGG TGA CAA GCC TAC ATA CTC TTT CCC ATT GAT TTT CAA AAT ACC CCT TGT GCC ATC CGA ATT AGG ACG GCT GCT ATT GGT TAT TCC CGT AGA TTC TTC TCG TAC CAC CAT CAT TAC CAT CTC TTA TGC AAC ATC TTT ACG ATG TAG ACG TTT TCG TTT TTT GCC GCT TTT GCT T

>DS102

GCG GAG GAT CAT TAT CGT ATA ACA GGA GAT GCC CTA GGG CTG TCG CTG ACC TTC ACA GGT TGT GCA CGC CCA AAG GTG CTC TCT TAT ATC CCA TCT CAC CCC TTT GTG CAT CAC CGC GTG TGC TTC CCC GGT GGG GTT TGT CCG AAG GGG GGT TCA CGT TTT TAT ACA AAC ACC ATT TAT GTA TAG AAT GCC TTA CTT TTT GCG ATC ATG CGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC GCC CCT TGG TAT TCC GAG GGG CAC ACC CGT TTG AGT GTC GTG AAC ACC CTC AAC CTT TTT GGT TTC TTG ACC AAG AAG GCT TGG ACT TTG GAG GTT TTC CTT GCT GGC CTC CCT TTG AAG CCA GCT CCT CCT AAA TGA ATT AGT AGG GTT CGC ATT GCT GAT CCT TGA CGT GAT AAG TAT GCT TCT ACG TCT TGG ATT TTG CAC CTT TGC CTT GGA ACC TGC TTC CAA CTG TCT TTG AAT GAA GAC AAT GTT TTG AGT TCC ATC GTG ACT CAA ACT CAG CGA CCT TGA CCT CAA ATC GGG TGA GAC TAC CCG CTG AAC TTA AGC ATA TCA ATA AGC GGA GGA AAA GAA ACT AAC AAG GAT TCC CCT AGT AAC TGC GAG TGA AGC GGG AAA AGC TCA AAT TTA AAA TCT GGT GGC CTT TGG CCG TCC GAG TTG TAA TTT AGA GAA GCA TCT TCC GCG CAG GAC CGT GTA CAA

D.3 LSU sequences

LSU sequences obtained from the four new species

>Species A

ATC GAA TCT TTG AAC GCA CCT TGC GCC CCA TTG GAA TTC CGA GGG GAA CCC CGT TTG AGT GTC GTG AAC ACA TCC TCA ACC TTC TTT TGG GTT TTT GAC CGG GGG AAG GCT TGG ACT TTG GAG GCC CTT TCA TTG CTG GTA TGC TTT TAA AGC CAG CTC CTC CTA AAT GAA TGA GTG GGA TCC GCT TCG GTG ATC CTT GAC GTG ATA CGT ATT ATT CTA CGT CTT GGG TTT TCA CAG CAC CTG CTT CCA ACC GTC TTT AAA CAA AGA CAA TGT TCG GGT TTC GAC CTG AAT CAC AAA ACC TTG ACC TCA AAT CGG GTG AGA CTA CCC GCT GAA CTT AAG CAT ATC AAT AAG CGG AGG AAA AGA AAC TAA CAA GGA TTC CCC TAG TAA CTG CGA GTG AAG CGG GAA AAG CTC AAA TTT AAA ATC TGG TGG CCT TTT GGT CAT CCG AGT TGT AAT TTA GAG AAG CGT CTT CCG CGC TGA ACC GTG CAC AAG TCT CCT GGA ATG GAG CGT CAT AGA GGG TGA GAA TCC CGT CTT TGG CAC GGA ACA TCA GGG CTT CTG TGA TGC GCT CTC GAC GAG TCG AGT TGT TTG GGA ATG CAG CTC AAA ATG GGT GGT GAA CTC CAT CTC AAG CTA AAT ATT GGC GAG AGA CCG ATA GCG AAC AAG TAC CGT GAG GAA AGA TGA AAA GCA CTT TGG AAA GAG AGT GAA CAG TAC GTG AAA TTG TTG AGA GGG AAC GCT TGA AGT CAG TCG CGT CGA CCG AGA CTC AGC NTG CCT NGC TTG GTG TAC TTC TCG GCT GAC GAG CAG CAT CAA TNT TGG TCT TGG ATA AGG CAA GGG AAA NNA GAA CTC TCT AGG TGG TTA NNN CCC TCT GTC GCG GAG GGG ATC CCC ATG AAG GAC TTN NNN NGA GCT GTG GNN TCG AGA CTT

> Species B

TG AAC GCA ACT TGC GCC CCT TGG ATT CCG AGG GGA TAC CTG TTT GAG TGT CGC GAA ACT CTC AAA AAC CCT TTT GTT TGA TTG TTT CTA ACC AGT AAA AGG TTT TTG GAC TTG GAG GTT TTA ATG CTT GCT TTT GTC TTA AAA GCA AGC TCC TCT GAA ATA AAT CAG TAG GAT CTG CTT TCT GAT TCT TAA TGT AAT AAG ATG CTT CTA CAT TTT GAA TTT GGT ATT GTC TCT GAG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GTT TCT GGT GTC TGC TAT CAT ATT AGC AGT TAG CTT AGA CCC ATG AAA TAA ATC TTG ACC TCA AAT CAG GTG AGA CTA CCT GCT GGA CTT AAG CAT ATT AAT CAG AAG AGG AAA AGA AAC TAA CAA GGA TTC CCC TAG TAA CTG CGA GTG AAG CGG GAA AAG CTC AAA TTT AAA ATC TGG TGG TCT TTA GCC ATC CGA GTT GTA ATT TAG AGA AGC ATC TTC CGC GCT GAA CCG TGT ACA AGT CTC CTG GAA TGG AGC GTC ATA GAG GGT GAG AAT CCC GTC TTT GGC ACG GAC ATG TCA GGG CTT TTG TGA TGT GCT CTC AAA GAG TCG AGT TGT TTG GGA ATG CAG CTC AAA ATG GGT GGT GAA CTC CAT CTA AAG CTA AAT AT

>Species C

G AGT TTC CTC TGG CTT CAC CCT ATT CAG GCA TAG TTC ACC ATC TTT CGG GTC CCA ACA TAC ACG CTC TAC CGC AGA GCG TCA CAG AAG GTC TGG TCC GGG CGT CGG TGC CCT CCA CGA CAG AGG TCC CAA CTT TTA CTT TCA TTA CGC GCA CGG GTT TTC CAC CCG AAC ACT CGC GGG TAT GTT AGA CTC CTT GGT CCG TGT TTC AAG ACG GGT CGT TTA AAG CCA TTA CGC CAG CAT CCT AAG CAC GTA ACG TGG GCA AAG ACC CCG ACA CCC GGA GGT GCG TGC TGA GTT CCT CAA TCC CAA CCC TCG CAT GCG ACA GAG GAC TAT AAC ACA CCT AAA AAG GTG CTA CCT TTC CCC TGC CAT TAT CCA AGG ATC GAA ATT GAT GCT GAC CCG TCG AGC CGA GAA GCA CAC CAA GCC GAG GCA AGG CTG AGT CTC GGT CGA CGC GAC TGA CTT CAA GCG TTT CCC TTT CAA CAA TTT CAC GTA CTG TTT CAC TCT CTT TCC AAA GTG CTT TTC ATC TTT CCC TCA CGG TAC TTG TTC GCT ATC GGT CTC TCG CCA ATA TTT AGC TTT AGA TGG AGT TCA CCA CCC ATT TTG AGC TGC ATT CCC AAA CAA CTC GAC TCT TCG AGA GCA CAT CGC AAA AGC ACC GGG AGT CCG TGC CAA AGA CGG GAT TCT CAC CCT CTA TGA CGC TCC ATT CCA GGA GAC TTG TGC ACG GTC CAG CGC GGA AGA TGC TTC TCT AAA TTA CAA CTC GGA CGG CCA AAG GCC ACC NGN ATT TTA AAT TTG AGC TTT TCC CGC TTC ACT CGC AGT TAC TAG GGG GAA TCC TTG GTT AGG TTT CTT TTT CNC CCG CTT ATT GGA TAT GCT TTA AGT TCA GCC GGG TAG TCT TCA CCC CGA TTT GGA GGT CCA AGG TCC GCT GAA GTT TGG AGT CAA CGA TTG GAC CTC AAA AAC

>Species D

CTT TGA ACG CAA CTT GCG CCC CTT GCA TTC CGA GGG GAC CCC TGT TTG AGT GTC GTG AAA TTC TCA AAA ACC CTT TTG CTT GAT TGT TTC TGA TCA GAA AAG GGT TTT TGG CCT TGG AGG TTT TAA TGC TTG CTT TTA TCT TGG AAG CAA GCG CCT CTG AAA TAA ATC AGT AGC GTC TGC TTT GTT GAT TCT TAA TGT AAT AAG ATG CTT GTA CAT TTT GAA TTT GGC ATT GTC TCT TGG ATG CCT GCT TCT AAC TGT CTT ACG GAC AAT AAT GGT GCT TCT GGT TAC TGC TAT TTT TAT ATT AGC AGA CAG CTA GAC CCA TAA AAA AAA TCT TGA CCT CAA ATC AGG TGA GAC TAC CTG CTG AAC TTA AGC ATA TCA ATA AGC AGA GGA AAA GAA ACT AAC AAG GAT TCC CCT AGT AAC TGC GAG TGA AGC GGG AAA AGC TCA AAT TTA AAA TCT GGT GGT CTT TAG CCA TCC GAG TTG TAA TTT AGA GAA GCA TCT TCC GCG CTG GAC CAT GCA CAA GTC TCC TGG AAT GGA GCG TCA TAG AGG GTG AGA ATC CCG TCT TTA GCA TGG ACA TAT CAG GGC TTT TGT GAT ATG CTC TCA AAG AGT CGA GTT GTT TGG GAA TGC AGC TCA TAA TGG GTG GTG AAC TCC ATC TAA GCT AAA TAT TGG CGA GAG ACC GAT AGC GAA CAG TCC CGT GAG GAA AGA TGA AAA GCA CTT TGG AAA GAG AGT GAA CAG TCG TGA AAT GTT GAA AGG GAA ACG CTT CGA AGT CAG TAT GTG GAC CAA GAC TCA GCC TTG CCT CCG CTT GGG TGT AAG TGT GGG TGA TAC GCC AGC ATC AAT TTT GTC TCT CGC A

D.4 Peloton Sanger sequences

Russulaceae ITS-sequences obtained from pelotons.

> OR01

TT TTT TTT TTT GCC ATT ATA TGC AAT CAA TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAT GCA TCT TGC GCC CTT TGG CAT TCC AAA GGG CAC ACC CGT TTG AGT GTC GTG AAA TTC TCA AAC CTT TTT AGT TTC ATG ACT GGG AAG GCT TTG GAC TTT GGA GGC TTT TGC TGG CTT TCC CTT GTT GGG GCC AGC TCC TCT GAA ATG AAT TAG TGG GGT CTG CTT TGC TGA TTC TTG ACG TGA TAA GAT GTT TTT ACG TCT TGG GTT TCG CAC TGT TTT GTC TGG ATC CTG CTT CTA ACC GTC TCA TAG AAG ACA ATG GTC AAG TGA TTG CCA CTT GAT TGC ACA AAC CTG ACC TCA AAT C

> ORS3

T CTT GGT CCA ATT AGA GGA AGT AAA AGT CGT AAC AAG GTT TCC GTA GGT GAA CCT GCG GAA GGA TCA TTA TCG TAC AAC AAG AGG CGC TCG GGG CTG TCG CTG ACC CTT AAA GTC GTG CAC GCC TCG ACG TTC TCG TTT ACA CAT CCA TCT CAC CCC TTG TGC ATC ACC GCG TGG GCC CTC CCT TTT GAT TCA AAA GGT GGC TTG CGT TTT ATT ATC ACA CAC AAA CCT GGT CAC AGT GAA CGT CTT TTA CTT TTT TGT AAT AAT CAG TAC AAC TTT CAA CAA CGG ATC TCT TGG CTC TCG CAT CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AAT GTG AAT TGC AGA ATT CAG TGA ATC ATC GAA TCT TTG AAC GCA CCT TGC ACC CCT TGG CAT TCC GAG GGG TAC ACC CGT TTG AGT GTC GTG AAA TTC TCA ACC TCC CCA ATT TCT TGA GGA AGG CTT GGA CTT TGG AGG CTT TTG CTG GTC TCG CTT TTG AAG CCA GCT CCT CTT AAA CGA ATT AGC GGG GTC CCT CTT TGC CGG TCC CTC GAC GTT ATA AGC TTT CTT TAC GTC CGA GGT TTG GCT CTG TAG CCC TGC TTC TAA TAG TGG TCT CTT CCT TCT GAG CCA CGT TTT TG