UNIVERSITY OF SOUTHERN QUEENSLAND

DIVERSITY OF FUNGAL ENDOPHYTES IN THE SEMI EVERGREEN VINE THICKETS OF THE SOUTHERN BRIGALOW BELT BIOREGION AND THEIR PRODUCTION OF ANTIMICROBIAL SECONDARY METABOLITES

A dissertation submitted by

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Abstract

Endophytes are thought to make up at least half of the diversity within the fungal kingdom and yet they remain one of the least explored functional groups. What research that has been conducted has focused on tropical rainforests or grasses with comparatively little research examining diversity within other ecosystems. Semi-evergreen vine thickets (SEVT) are remnant dry rainforests which form part of the Brigalow Belt along the eastern coast of Australia. Due to the fertile soil on which they grow, SEVT are frequently cleared for agricultural use, currently no information exists regarding fungi within this ecosystem.

Leaves from 23 plants at 3 sites of SEVT were sampled and fungal endophytes were isolated and identified. Fungal specificity was examined by collecting leaves from 22 *Geijera salicifolia* plants from 5 sites of SEVT. In total, 228 and 187 fungal endophytes were isolated from the two studies. Multi-gene phylogenetic analysis was further conducted on *Nigrospora, Preussia, Guignardia* and Pezizales - four of the most commonly occurring taxa. Endophytes obtained from the diversity study were screened for antimicrobial capabilities and HPLC analysis was conducted on crude extracts obtained from endophytes showing bioactivity. Pure compounds were retested for their ability to inhibit the growth of microbial pathogens.

A wealth of novel fungal endophytes was observed within SEVT. Four new taxa within the predominately saprotrophic order Pezizales, were observed. This finding may represent an example of ecosystem specificity. A large number of *Preussia, Nigrospora* and *Guignardia* species were also observed. Fungal specificity was found

to be occurring between several species of *Guignardia* and *G. salicifolia.* 6 novel pure compounds were isolated from a *Preussia* sp. Three of these showed significant antimicrobial activity against MRSA and *C. albicans*. The results of this study indicate that SEVT harbour a vast storehouse of novel and medicinally significant endophytic fungi.

Publications

List of publications arising from this project:

Graham, R and Dearnaley J.D.W (2011) 'Biodiversity of fungal endophytes in semi-evergreen vine thickets'. XVI Congress of European Mycologists, Thessaloniki, Greece, 19-23 September 2011. Conference presentation.

Mapperson, R and Dearnaley J.D.W (2012) 'Biodiversity of fungal endophytes in semi-evergreen vine thickets'. Australasian Mycology Society, Cairns, Australia, 24-26 September 2012. Conference presentation

Mapperson, R, Kotiw, M, Davis, R.A and Dearnaley J.D.W (2013) 'The diversity and antimicrobial activity of *Preussia* sp. endophytes isolated from Australian dry rainforests.' Current Microbiology 68:30-37

Sutour, S, Mapperson, R, Dearnaley J.D.W, Kotiw, M, Kalaitzis, J, and Davis, R A (2013) 'Africanarones A-F: Antimicrobial α-Pyrone Polyketides from the Australian Endophytic Fungus *Preussia aff. Africana'*. Manuscript in preparation.

Certification of Dissertation

I certify that the ideas, experimental work, results, analyses, software and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

_____ _____

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ENDORSEMENT

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Associate Supervisor, Professor Mike Kotiw Date

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"For from Him and through Him and to Him are all things. To Him be glory forever. Amen."

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List of Abbreviations

AGRF- Australian Genome Research Facility

C-endophytes- Clavicipitaceous Endophytes

CLSI- Clinical Laboratory Standards Institute

CR-Colonisation Rates

DERM- Department of Environment and Resource Management

EPBC- Environmental Protection and Biodiversity Conservation

ICN-International Code of Nomenclature

IR- Isolation Rates

ITS- Internal Transcribed Spacer

MBC- Minimum Bactericidal Concentration

MFC- Minimum Fungicidal Concentration

MIC-Minimum Inhibitory Concentration

NC-endophytes – Non Clavicipitaceous Endophytes

OTU- Operational Taxonomic Unit

PDA- Potato Dextrose Agar

SEVT- Semi Evergreen Vine Thickets

Problem statement

Fungal endophytes are fungi which inhabit the leaves, stems, bark and roots of plants asymptomatically (Gao *et al.* 2005; Hyde and Soytong 2008; Tan and Zou 2001). Rainforests, with their high moisture environments and host plant richness are thought to harbour the greatest diversity of endophytic fungi (Arnold and Lutzoni 2007; Hyde 1996). As the Australian continent has dried out, much of the country's original rainforest vegetation has disappeared with major patches existing along the north eastern coast but only patches remaining west of the Great Dividing Range. It is possible that the ability of some of these remnant rainforests to survive harsher environmental conditions is due in part to their endophytic fungal associations (Arnold *et al.* 2002). Endophytic fungi have been shown to confer survival attributes, such as drought tolerance, through the production of secondary metabolites (Strobel and Daisy 2003).

Since the observation by Alexander Fleming that *Penicillium notatum* inhibited the growth of *Staphylococcus aureus* (Fleming 1929), the secondary metabolites of fungi have proven to be important sources of antibiotics (Guo *et al.* 2008). In recent years, the dramatic increase in antibiotic resistant bacteria has given rise to the urgent need for the development of novel antibiotics (Singh and Barrett 2006; Strobel and Daisy 2003). Despite this, over the last 20 years the number of new antibiotics coming into use each year has declined by more than 50% (Coates and Hu 2006). This combination of events has led to the speculation that we may be about to enter a period similar to the pre-antibiotic era (Fernandes 2006). The need to discover novel antibiotics has never been more important, and as such it would seem prudent to examine fungi from

previously unexplored niches. This study aims to examine the fungal diversity and antibiotic potential of endophytes of the previously unexplored dry rainforest; the semi evergreen vine thickets (SEVT) of the Southern Brigalow Belt Bioregion, Queensland, Australia.

SEVT are considered to be extreme forms of subtropical dry rainforests with high levels of flora diversity (McDonald 1996; Department of the Environment, Water, Heritage and the Arts (DEWHA), 2010). They occur patchily from Townsville in the central coast of Queensland to the northern parts of NSW and usually have high to medium fertile soils (DEWHA 2010). According to the Queensland Department of Environment and Resource Management (DERM), SEVT once covered an area of 868,200 ha (Ecosystem_Conservation_Branch 2007). Soil fertility has resulted in large areas of SEVT being cleared for agriculture and grazing (Seabrook *et al.* 2006). Indeed, as little as 17% of the original covering of vine thickets remains, resulting in this ecosystem type being classed as nationally endangered under the Environmental Protection and Biodiversity Conservation (EPBC) Act 1999 (Ecosystem_Conservation_Branch 2007). Given that habitats with high levels of host plant richness are hotspots for endophytes (Arnold and Lutzoni 2007) and therefore potential for drug discovery, it is vital that the endophytes of the SEVT are catalogued and screened before they are lost forever.

Research questions:

Ecology of fungal endophytes

1. Do the SEVT harbour a high diversity of fungal endophytes?
2. Which SEVT plant taxa harbour the greatest diversity of fungal endophytes?

3. Are there any novel fungal taxa in SEVT?
4. Does *Geijera salicifolia* exhibit fungal specificity?
5. Are there differences in overall diversity between endophytes from *Geijera salicifolia* plants at different locations of SEVT?
6. Does the species assemblage of *Geijera salicifolia* differ to those found in other plant species within SEVT?

Screening of fungal endophytes for antimicrobial compounds

7. Which fungal endophytes produce antimicrobials?
8. Is there a higher proportion of fungal endophytes producing antimicrobials in SEVT compared to other studies?
9. Are the active compounds fungicidal, bacteristatic or bactericidal?
10. What are the compounds involved?
11. What is the minimum inhibitory concentration of these compounds?

Objectives

The principal objective of this study is to examine the diversity of fungal endophytes within SEVT. Fungal specificity will also be examined by focusing on one key plant species within SEVT. Those isolates obtained from the assessment of diversity will be further tested for antimicrobial capabilities to discover novel compounds of potential use to society.

1 Literature review and Scope of project

1.1 Introduction

The kingdom fungi forms one of the five major lineages of life. Members of this kingdom may be defined as eukaryotic, heterotrophic organisms which reproduce by

way of spores and the presence of cylindrical cells which exhibit apical growth (Cooke 1986; Seifert 2009). Historically, all fungi were placed within the plant kingdom, however several important structural differences exist which warrant their separation (Shenoy *et al.* 2007). Most significantly, fungal cell walls are comprised of chitin, a major structural variation to that of the cellulose-based structure of plant cell walls (Carroll 1988; Seifert 2009). Fungi also lack chlorophyll, resulting in their heterotrophic nature (Carroll 1988). These differences are significant and it is now widely recognised that fungi are more closely related to the Kingdom Animalia than they are to the Plantae (Blackwell 2011; Carroll 1988; Seifert 2009; Shenoy *et al.* 2007).

Fungi often have complicated life cycles. Most fungi are thought to exhibit an anamorphic or asexually reproducing phase and a teleomorphic or sexually reproducing phase (Carroll 1988; Shenoy *et al.* 2007). Together, both life phases are known as the holomorph (Shenoy *et al.* 2007). Many fungi are only known by their asexual mode of reproduction (Seifert *et al.* 2000) and may exhibit multiple asexual forms under differing conditions (Seifert *et al.* 2000; Subramanian 1962; Subramanian 1983; Sutton 1980). This ability to switch between reproductive modes led to the development of a dual fungal classification system which has remained in place until 2011 (Shenoy *et al.* 2007). This system caused considerable taxonomic complications once the teleomorphic phase for a given species was described, particularly if the anamorphic name was in common usage and has resulted in a dual name for many species (Taylor 2011). In July 2011, a landmark decision was made at the International Botanical Congress in Melbourne, Australia to convert to a holomorph name for all species (Hawksworth 2011). This

decision has become known as the ‘one fungus- one name’ decision and is set to revolutionise fungal taxonomy (Hawksworth 2011; Taylor 2011).

Within the fungal kingdom, fungi may be divided into seven major divisions, or phyla, based largely on sexual reproduction, biochemical characteristics and ultrastructure (Carroll 1988; Seifert 2009; Shenoy *et al.* 2007). Of the so called ‘true fungi’ only five phyla exist (Seifert 2009; Tanabe *et al.* 2004). By far the most dominant phyla are the Ascomycota; which include many commercially and medicinally important fungi such as *Saccharomyces* and *Candida* (Schoch *et al.* 2009). Probably the most easily recognisable group due to their prominent sexual structures are the Basidiomycota, to which most macrofungi belong and include most of the edible fungi such as *Agaricus* and *Lentinula* (Carroll 1988). Previously considered Zygomycetes (Schüβler *et al.* 2001), the ecologically and economically significant arbuscular mycorrhiza are now considered to belong to a separate phylum, the Glomeromycetes (Schüβler *et al.* 2001; Stürmer 2012). The remaining two phyla are the Chytrids, which largely reproduce asexually through the production of zoospores (Gleason *et al.* 2008) and Zygomycetes, a relatively understudied phylum identified by the production of zygospores (Tanabe *et al.*). Myxomycetes (plasmodial molds) have previously been classed as fungi but are now considered protists and Oomycetes (water molds) are now placed within the Kingdom Straminopila (Seifert 2009; Shenoy *et al.* 2007). Also worth mentioning are the Deuteromycetes, this group has been given pseudo phylum status and are typified by the lack of sexual reproduction and frequent occurrence of dark septate hyphae. However, with the increase in the use of molecular identification techniques, this group

of fungi have mostly been reclassified as Ascomycetes and Basidiomycetes (Seifert 2009).

Fungi have been observed forming an array of interactions in every known ecological niche (Blackwell 2011), many of which are critical for ecosystem function. Mycorrhizal fungi form close associations within the roots of plants and provide nutritional benefits to their host (Den Bakker *et al.* 2004; Molina *et al.* 1997; Taylor *et al.* 2003). Saprotrophic fungi play an important role in the breakdown and recycling of wood and leaf litter (Posada *et al.* 2012). Other symbiotic associations such as those that occur within lichens and pathogenic interactions are just further examples of the diverse list of interactions that fungi carry out within the environment (Tedersoo *et al.* 2013). The most understudied and yet potentially largest functional group are the endophyte Endophytes are potentially the largest known life mode of the fungal kingdom and yet represent one of the most understudied groups to date (Gamboa and Bayman 2001).

1.2 Fungal Endophytes

1.2.1 Definition and role of fungal endophytes in plants

Fungal endophytes are defined as fungi which inhabit the leaves, stems, barks and roots of plants without causing disease symptoms (Gao *et al.* 2005; Hyde and Soytong 2008; Tan and Zou 2001). Some debate over their precise definition still exists and centres around two main issues. Firstly the lack of conclusive evidence regarding the function of endophytes within plants (Douanla-Meli *et al.* 2013), and secondly, the observation that some fungal endophytes may 'switch' to a parasitic or pathogenic relationship under certain environmental conditions (Carroll 1988; Saikkonen *et al.* 1998).

Despite this lack of conclusive evidence, the consensus is that endophytes are largely beneficial to the host (Arnold *et al.* 2003; Douanla-Meli *et al.* 2013; Huang *et al.* 2008). Research has unearthed an array of functions of endophytes in plants which further vary depending on the host plant identity (Aly *et al.* 2011; Arnold *et al.* 2003; Dearnaley *et al.* 2012; Dingle and Mcgee 2003; Gimenez *et al.* 2007; Redman *et al.* 2011; Rodriguez *et al.* 2004a; Rodriguez *et al.* 2009). Observed plant benefits of endophyte colonisation include increased plant resistance to drought and fungal diseases (Dingle and McgGee 2003), increased tolerance to heavy metals (Malinowski and Belesky 2000; Tan and Zou 2001) and improved resistance to herbivores (Wagner and Lewis 2000). Other studies have found increased vegetative production in plants highly colonised with some fungal endophytes (Clay and Schardl 2002) and improved salt tolerance in some plant species (Waller *et al.* 2005). Redman *et al*. (2002) found that plants had an improved ability to withstand extreme temperature fluctuations when heavily colonised with fungal endophytes. The increased fitness afforded by fungal endophytes has also been shown to improve the colonising ability of plants in more extreme environments (Rodriguez *et al.* 2004a). Of the 17 studies which have compared grasses with and without endophytes, all but one found an increase in plant productivity (Sumarah *et al.* 2010). Under stressful conditions, the difference between plants was found to be even more marked, giving further support to the theory that endophytes are offering some level of protection or benefit to their host. It has also been reported that environmental pressures may influence the function of endophytes within their host (Arnold *et al.* 2003; Baldani *et al.* 1986; Barraquio *et al.* 1997; Dingle and Mcgee 2003; Gasoni and De Gurfinkel 1997).

The second area of debate surrounding the definition of fungal endophytes involves the observation that in some cases, they may be latent pathogens. For example, under certain environmental conditions such as when the host plant has become stressed, some endophytes have been observed to switch to a pathogenic life mode (Photita *et al.* 2004). Furthermore, some smuts are known to lie dormant for several years before symptoms appear (Clay and Schardl 2002). This has led to debate over whether some endophytes are really just latent pathogens (Carroll 1988; Saikkonen *et al.* 1998). However, most researchers would agree, that the correct definition of a fungal endophyte would include those who are not causing any visible signs of disease but recognise that this may include latent pathogens (Gao *et al.* 2005; Hyde and Soytong 2008; Photita *et al.* 2004; Tan and Zou 2001).

Rodriguez *et*. *al* (2009) further separated endophytes into four major classes. The first class, covering endophytes in grasses, is by far the most widely researched to date (Baldani *et al.* 1986; Barraquio *et al.* 1997; Gasoni and De Gurfinkel 1997; Hyde and Soytong 2008). Clavicipitaceous endophytes (C- endophytes), described by Rodriguez *et al*. (2009) as Class 1 endophytes, are well described and their largely beneficial role in plants has been well documented (Sumarah *et al.* 2010). Class 2 endophytes, predominately ascomycota and occasional basidiomycota, are described as those which are found in leaves stems and roots of plants. The distinguishing feature of Class 2 endophytes is that they may be found in above and below ground tissue. They are also capable of extensively colonising their host tissues. By contrast, Class 3 and 4 endophytes occur only in above ground tissues. Class 3 may be distinguished from Class 4 endophytes in that they form only highly localised infections (Rodriguez *et al*. 2009).

1.2.2 Method of colonisation

As has been previously alluded to, the method with which fungal endophytes colonise their host varies depending on the class of fungi. The method of fungal colonisation may then also affect sampling technique and the observed diversity. Generations of C-endophytes, or Class 1 as referred to be Rodriquez *et al*. (2009), are transmitted vertically through seeds being infected from the parent plant (Clay 1988; Johnston *et al.* 2006; Rodriguez *et al.* 2009). This results in very little variation in endophyte diversity within the host and in some cases may even result in a single species being observed (Johnston *et al.* 2006). By contrast, Non Clavicipitaceous fungi (NC-endophytes) colonise via horizontal transmission resulting in highly diverse, mostly ascomycetous, assemblages of endophytes (Rodriguez *et al.* 2009). It should be noted however, that Class 2 NC-endophytes may also use vertical transmission as a means of populating succeeding generations (Redman *et al.* 2002). Given that fungal endophytes colonise their host through horizontal transmission, leaf age is an important factor in the diversity of NC-endophytes which may be observed during sampling (Arnold and Herre 2003). Given this, only mature leaves of NC-endophytes will be sampled in this study.

Tissue colonisation appears to be similar regardless of host colonisation method and has been observed to occur in two main ways. Entry is either gained directly through the epidermal layer or through stomata (Johnston *et al.* 2006). This may be through the use of appressoria or via the direct penetration of hyphae (Ernst *et al.* 2003). Importantly, hyphae do not penetrate the interior of plant cells but rather, grow alongside the tissue (Rodriguez *et al.* 2009). Many of the benefits afforded to the host are thought to occur

as a result of the fungal endophytes' production of secondary metabolites (Sumarah *et al.* 2010; Yu *et al.* 2010).

1.2.3 Diversity

Fungal endophytes are a large but poorly studied group (Arnold *et al.* 2007). Based on conservative assumptions of a 6:1 fungal endophyte species: plant ratio, recent estimates suggest that there may be 1.5 million different species of fungi (Hawksworth 1991; Hawksworth 2002). Recent findings that plants harbour much higher numbers of fungal endophyte species suggests that current worldwide fungal estimates are inadequate (O'Brien *et al.* 2005). The observation that some endophytes may switch lifestyles adds further complications to the attempts to assess overall endophyte diversity in plants.

In recent years many studies have examined the diversity of fungal endophytes in particular plant species. Most of these studies have focused on agricultural grass species or plants from tropical rainforests where very high levels of fungal diversity have been commonly observed (Lodge *et al.* 1996; Phongpaichit and Rukachaisirikul 2006; Raviraja *et al.* 2006). For example Gamboa and Bayman (2001) identified 63 endophyte species taken from 268 leaf fragments of 14 plants of *Guarea quidonia* and suggested that this was still an underestimate of the level of fungal diversity in the plant. Other plant species such as *Picea rubens* have been found to contain up to 150 different endophyte species (Sumarah *et al.* 2010). These findings suggest that when endophyte diversity is taken into account, the estimate of a global 1.5 million fungal species is conservative. Recently, it has been argued that based on current results from molecular investigations of plant-endophyte diversity, global fungal diversity may be somewhere between 3.5

million to 5.5 million (O'Brien *et al.* 2005). Whatever the true number, only 0.1 million of all fungi have been described (Strobel 2003) leaving the vast majority of species and their associated plant benefits yet to be discovered. Given that fungal endophytes appear to be such a highly diverse functional group containing many novel species , it seems logical that these would present great potential for antibiotic discovery (Gamboa and Bayman 2001; Lodge *et al.* 1996; Schulz and Boyle 2005).

Ascomycetes are the most frequently isolated endophytes. Of these, the most commonly reported species worldwide are *Colletotrichum* spp, *Phomopsis* spp, *Alternaria* spp. *Phoma* spp, *Pestalotiopsis* spp and *Xylariales* spp. (Yu *et al.* 2010). However, it should be noted that this is largely a method dependent observation. Standard methods of endophyte isolation typically involve plating of surface sterilised leaf, bark or stem material, commonly on mediums such as Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) or Potato Carrot Agar (PCA) (Huang *et al.* 2009). This tends to favour faster growing isolates over their slower growing counterparts and is dependent on an endophyte's ability to grow in culture. Identification is then based on morphological characteristics and this also may produce inaccuracies since a considerable number of isolates do not produce spores in culture and are deemed mycelia sterilia. Relying on morphological characteristics to identify species can result in an underestimation of the endophytic species present. This was noted by Arnold et al, (2007) when diversity of endophytes was assessed in loblolly pines. Endophytes that failed to produce spores in culture were grouped together based on morphological characteristics. These isolates were then classed as separate morphological groups when they differed in two or more characteristics, a common method used for mycelia

sterilia in the past (Lacap *et al.* 2003). The use of a species area curve initially suggested that adequate samples had been collected to indicate endophyte diversity within the loblolly pine. However, when these fungal isolates were then analysed using molecular methods (Arnold and Lutzoni 2007; Arnold 2007), the diversity was found to be considerably higher and that the sampling strategy was actually insufficient (Lacap *et al.* 2003).

There are several factors which may affect observed diversity within a sampled population which warrant discussion. These factors may be grouped into two broad categories: ecological factors and sampling methodology. Environmental/or ecological factors such as disturbance, geographical location and replanting of old growth forests with nursery grown plants has been shown to dramatically affect the diversity of fungal endophytes observed (Arnold 2007; Arnold and Herre 2003; Helander *et al.* 1993; Suryanarayanan *et al.* 2011; Suryanarayanan *et al.* 2002). Another factor which may affect endophyte diversity is the genetics of the host. Given that factors such as light, pH and temperature are critical in determining endophyte species composition, plants growing in extreme environmental conditions may contain lower endophyte diversity (Suryanarayanan *et al.* 2011). When comparing host genetics with environmental conditions, Suryanarayanan *et al*. (2011) found that while host selection was a factor, the overall environmental conditions were far more important. Higher rainfall levels (Suryanarayanan *et al.* 2002) and proximity to the equator (Arnold *et al.* 2002) are also significant factors which may increase endophyte diversity. These factors need to be taken into consideration when analysing endophyte diversity at any given location.

The method used also has an impact on the ability to accurately determine the diversity within a given host. Leaf age, fragment size, location of fragment collection (high, mid or low in the canopy; stem, bark, root or leaf), agar type when plating methods are used and whether molecular-based methods were used independently or in conjunction with culture-based methods are just a few of the factors which may affect the diversity of fungal endophytes observed (Arnold 2007; Gamboa and Bayman 2001; Unterseher and Schnittler 2009). Given that most endophytes are transferred horizontally, typically through wind or rain, mature leaf material should harbour greater numbers of endophytes (Arnold and Herre 2003). According to Gamboa and Bayman (2002) sampling using large numbers of small tissue fragments increases the number of fungal endophytes observed. This may also help in combating the problem of faster growing fungi out-competing slower growing endophytes. As mentioned earlier, various types of agar may also be used during fungal isolation. Due to the different nutritional requirements of individual endophytes, using different agar types may allow a broader selection of fungi to be obtained (Hughes *et al.* 2009). Since it is well known that culture-based methods fail to capture the complete diversity in a given sample (Arnold *et al.* 2007; Lacap *et al.* 2003), many researchers use a combined approach. These predominantly molecular-based methods are discussed in detail in section 1.2.4. By using traditional plating methods in conjunction with culture independent methods, a much more accurate picture of endophyte diversity may be obtained.

1.2.4 Molecular methods for identifying diversity

The use of molecular methods to assess endophyte diversity has recently been developed to include techniques which do not require *in vitro* cultivation of endophytes

(Crozier *et al.* 2008; Duong *et al.* 2006; Nikolcheva and Bärlocher 2004a, b). By using PCR and fungal specific primers on DNA extracted from surface sterilised leaves or other plant tissue, endophytes can be identified regardless of their ability to grow in culture. Interestingly, when DNA-based identification methods were utilised, some studies found that the most common endophytes isolated were actually basidiomycetes (Crozier *et al.* 2008). This may suggest that the importance of ascomycetes as endophytes may be overestimated relative to basidiomycetes when only culture-based methods are used. It would seem prudent for diversity assays to include a combination of both molecular and morphological assessments to gain a more accurate understanding of fungal endophytes *in situ*.

The use of molecular techniques has long been used to assess soil fungal diversity (Anderson *et al.* 2003; Borneman and Hartin 2000). Common methods of identification involve the PCR amplification of the ITS region and then comparing sequences with entries in databases such as GenBank. Sequences obtained through solely molecular methods are termed molecular operational taxonomic units (MOTU). An operational taxonomic unit simply refers to the taxonomic level of sampling within a study. MOTU are frequently equated as individuals and likely species. Most commonly a cut off of 97% similarity is used to identify individual species (Hibbett *et al.* 2011). There has been much comment about the problems associated with assessing diversity in this way. Particularly since sequences in GenBank may be inaccurately labelled leading to incorrect identification of new samples (Brock *et al.* 2009; Vilgalys 2003). Furthermore, PCR bias has been raised as an issue in assessing fungal diversity. As the PCR reaction comes to completion, larger amounts of DNA may be preferentially amplified over

smaller, less common sequences potentially leading to PCR bias (Anderson *et al.* 2003). This can lead to fungal species which only occur in low levels being missed entirely. Primer bias may also be an issue. Some of the commonly used primers such as the ITS1F and ITS4 primer pair may preferentially select for one fungal group over another (Larena *et al.* 1999; Manter and Vivanco 2007). For example, ITS1F is thought to favour ascomycetes over certain groups of basidiomycetes, which may or may not be amplifiable (Toju *et al.* 2012). Furthermore, it has been shown that when pooled samples are used such as is required for environmental samples, rarely is the full diversity observed (Avis *et al.* 2010). In their study, Avis *et al*. (2010) found that, while cloning was the only method successful in identifying the diversity within a sample, it failed to give an accurate indication of richness. Problems such as this are yet to be overcome.

One relatively new technology which may assist in overcoming the above problems is 454 pyrosequencing. Thousands of sequences may be identified very quickly from very small initial sample material. Very high levels of diversity have been observed when using this method, suggesting that fungal endophytes may be even more hyperdiverse than was previously thought (Jumpponen and Jones 2009; Manter *et al.* 2010; Unterseher *et al.* 2011). However, more recent research is discovering that 454 pyrosequencing is susceptible to many errors and may therefore result in an overestimation of MOTU (Lücking *et al.* 2013). During phylogenetic analysis, such errors can be minimised by only using sequences of high quality (longer reads), by not including singletons and through replication of the original PCR and sequencing procedure for comparison (Lücking *et al.* 2013). On a less technical note, 454

pyrosequencing requires specialised equipment which may not always be accessible. Companies such as the Australian Genome Research Facility (AGRF) routinely provide 454 sequencing services but at great cost in comparison to regular sequencing. In well funded labs such costs would not be an issue, however for many small grant projects, it may become cost prohibitive. Despite the issues associated with 454 pyrosequencing, this technology is continuously improving and will likely provide many insights into species richness and diversity in the future.

Despite these problems which have been discussed, genomic approaches of assessing the diversity of fungal endophytes is increasing in popularity and the overall number of newly described endophyte species appears to be rapidly growing (Hibbett *et al.* 2011). Indeed, in recent years, one would be hard pressed to find research which has not at least combined molecular based approaches with the more traditional methods. While these methods are certainly not without their faults, with careful examination of the results obtained, a clearer picture of the great wealth of endophyte diversity is being obtained.

1.2.5 Secondary metabolites in fungal endophytes

Secondary metabolites are generally low molecular weight chemical compounds produced in varying quantities by fungal endophytes (Debbab *et al.* 2009). Secondary metabolites have also been shown to be produced in response to environmental factors such as the presence of insects or other endophytes. Accordingly, it has been suggested, that selecting plants subject to environmental stress may provide endophytes which produce more commercially important secondary metabolites (Strobel and Daisy 2003; Yu *et al.* 2010). In a recent paper, Sumarah *et al*. (2010) found that several of the

secondary metabolites isolated from endophytes of the pine *Picea rubens* were toxic to the forest pest *Choristoneura fumiferana*. Findings such as this add further support to the hypothesis that endophytes produce secondary metabolites which act in the defence of their host.

Secondary metabolites commonly produced by endophytes include an unusually diverse array of chemical compounds. Most commonly these are aliphatic compounds, alkaloids, flavinoids, phenols, quinones, steroids and terpenoids (Schulz *et al.* 2002; Tan and Zou 2001; Yu *et al.* 2010). Research conducted by Schulz *et al.* (2002) found that 51% of the secondary metabolites isolated from endophytes were previously unidentified compounds. This is due in part to the lack of research which has focussed on the secondary metabolites of fungal endophytes (Schulz *et al.* 2002), and would suggest that fungal endophytes may provide a useful source of novel antibiotics.

1.3 Antibiotics

Natural products have proven to be valuable sources of useful drugs for thousands of years. Traditionally this was through the utilisation of plants for medicinal purposes (Singh and Barrett 2006; Strobel and Daisy 2003). In fact some of the most common known drugs in today's society have originated from plant-based compounds, for example, salicylic acid (aspirin), initially isolated from the bark of the weeping willow (Strobel *et al.* 2004). Comparatively new to medicine is the discovery of the therapeutics from fungal and bacterial sources.

As previously noted, fungi are thought to be more closely related to animals than any other kingdom (Baldauf and Palmer 1993). This is a useful attribute since many of the microbes which affect humans are also problematic for fungi (Baldauf and Palmer

1993). In defence against microbial attacks, many fungi produce secondary metabolites which inhibit or kill these pathogens and may therefore be useful for medicinal purposes (Dingle and Mcgee 2003; Firáková *et al.* 2007; Gimenez *et al.* 2007). It should therefore come as no great surprise the discovery by Alexander Fleming that the secondary metabolites produced by the fungus *Penicillium notatum* inhibited the growth of *Staphylococcus aureus* (Fleming 1929). Since then, many different fungi have been found to have useful antimicrobial properties (Strobel and Daisy 2003; Yu *et al.*).

Considerable recent research has examined the medicinal properties of the endophytic and mycorrhizal? partners of plants (Bills *et al.* 2002; Boddington 2009; Debbab *et al.* 2009; Firáková *et al.* 2007; Raviraja *et al.* 2006). Research has found that many medicinally important plant species harbour fungi producing similar chemicals to their host (Raviraja *et al.* 2006). An example of this was observed in the production of taxol, an important anticancer drug. Originally taxol was found to be produced in the bark of trees from the genus *Taxus*. The endophytic *Taxomyces andreanae* was then found to also produce this compound (Stierle *et al.* 1995). Since then, several different taxol producing fungal endophytes have been isolated from a variety of host plants, such as the recently discovered Wollemi pine (Kumaran *et al.* 2008; Strobel *et al.* 1997).

1.3.1 Historical context of antibiotic development

The development of antibiotics began with the accidental discovery of penicillin by Alexander Fleming in 1929 (Fleming 1929). The development of penicillin, which had its first real test during World War II, led to the 'golden age' (1940-1962) of antibiotic usage and discovery (Singh and Barrett 2006). During this time, a wealth of antibiotics derived from natural sources became available. Many of these still in common usage

today include chloramphenicol, tetracycline, streptomycin, erythromycin, vancomycin and quinolines (Singh and Barrett, 2006). Such was the level of discovery during this 'golden age' that it was speculated that by the year 2000 the world would be bacteria free (Time Magazine, February 1966). Interestingly, just six months later, the Time magazine reported concerns regarding the emergence of antibiotic resistant bacteria (Time Magazine, August 1966). In fact, antibiotic resistance was first noted in 1947 (Singh and Barrett, 2006). By the late 1960's, antibiotic discovery declined and resistant bacteria began to gain more prominence (Singh and Barrett 2006). Eventual resistance is now considered inevitable for all drugs (Coates and Hu 2006; Li 2005; Walsh 2000). Bacterial control methods now rely on remaining one step ahead of resistance through novel drug discovery.

In the last 25 years, 70% of all new drug discoveries in the area of anticancer and infectious diseases have come from natural sources (Newman and Cragg 2007). Despite this, many drug companies are pulling back from screening natural products (Lam 2007) with the focus shifting to methods based solely on chemical synthesis. This has largely been due to the fact that screening of plants or microbials for potential drug sources is often time consuming (Strobel and Daisy 2003). However, in the last ten years, many of the new drugs on the market were sourced from natural products (Lam 2007), suggesting that natural products remain an important supply for future drug discovery.

1.3.2 Methods of antibiotic function

Broadly speaking, currently available antibiotics may be categorised as either bacteriostatic or bactericidal (Kohanski *et al.* 2007). That is, the bacteria are either inhibited and further growth prevented (bacteristatic) or the bacteria are killed

(bactericidal) (Hancock 2005; Walsh 2000). Antibiotics have been shown to inhibit or kill bacteria through three main methods: (i) Disruption of cell membrane function and cell wall synthesis, (ii) inhibition of bacterial protein synthesis, or (iii) Inhibition of DNA or RNA synthesis (Pankey and Sabath 2004; Walsh 2000).

1.3.3 Mechanisms of resistance

Singh and Barrett (2006) suggest six main methods by which bacteria display resistance to antibiotics:

- inactivation of antibiotics by enzymatic reactions
- efflux pumps which pump antibiotic out of cells
- target mutation to decrease binding efficiency of antibiotic peptide
- overproduction of the target molecule
- bypassing of the affected metabolic pathway and/or
- decreased uptake of antibiotics.

Resistance has been further shown to be conveyed from one bacterial species to another through horizontal gene transfer through transposons or plasmid exchange (Mazel and Davies 1999).

1.4 Semi-evergreen vine thickets of the Southern Brigalow Belt

SEVT, also referred to as bottle scrub, softwood scrub or vine scrub, are remnant dry rainforests which form part of the Brigalow Belt (DERM 2007). Well known for their rich diversity of plant species, SEVT are thought to be extreme forms of subtropical dry rainforests (McDonald 1996). Characteristic emergent trees include the bottle tree *Brachychiton rupestris* (McDonald 2010), brush wilga *Geijera salicifolia* and Kurrajong *Brachychiton populneus* (DERM 2007; Sattler and Williams 1999). The high nutrient soils

characteristic of this habitat type have made SEVT particularly targeted for clearing for agriculture. Only 17% of the original coverage of SEVT is thought to remain (DERM 2007; McDonald 2010), resulting in SEVT being listed as a nationally endangered ecosystem under the Environmental Protection and Biodiversity Conservation (EPBC) Act of 1999. The Darling Downs forms part of the Southern Brigalow Belt bioregion. Many of the remnant SEVT have not been mapped for this region, and are therefore at greater risk of clearing (McDonald 2010).

2 Fungal endophyte diversity from plants within Semi Evergreen vine thickets

2.1 Introduction

Fungi are highly diverse organisms found in virtually all ecosystems (Tejesvi *et al.* 2011). Even areas thought to be largely inhospitable to life such as deep marine sediments have been found to contain a range of fungal taxa (Bhimba *et al.* 2012; Biddle *et al.* 2005). From soil to mosses, to large trees, fungi may be found growing on, within or beside all living and even non living things (Aly *et al.* 2011; Anderson and Cairney 2004; Arnold and Herre 2003; Chakraborty *et al.* 2000; Cuomo *et al.* 1995; Ernst *et al.* 2003). Such habitat flexibility has led to considerable discussion over the global diversity of the fungal kingdom (Hawksworth 1991; Hawksworth 2001; O'Brien *et al.* 2005).

The most universally accepted estimate of global fungal diversity is 1.5 million fungal species (Hawksworth 2001) although this approximation is probably conservative, based on the low estimation given to endophytic fungi (Blackwell 2011; O'Brien *et al.* 2005). Currently, just under 100,000 species have been formally described (Blackwell 2011).

Furthermore if Hawksworth's estimate is correct, at the current rate of discovery, it is predicted that it will take over 1000 years to identify and describe all existing fungal diversity (Hibbett *et al.* 2011).

Endophytes are thought to make up at least half of the fungal kingdom (Gamboa and Bayman 2001), with potentially more than 1 million species yet to be identified (Clay 1988). Endophytes can be defined as fungi which inhabit the leaves, stems, barks and roots of plants without causing any visible sign of disease (Gao *et al.* 2005; Hyde and Soytong 2008; Tan and Zou 2001). Rainforests, with their high moisture environments and host plant richness are thought to harbour the greatest diversity of endophytic fungi (Arnold and Lutzoni 2007; Hyde 1996). The level of diversity for endophytic fungal has been well described in tropical and subtropical rainforests around the world (Arnold and Lutzoni 2007; Arnold *et al.* 2002; Arnold *et al.* 2001; Gamboa and Bayman 2001; Hawksworth 2001; Hyde 1996; Schulz and Boyle 2005; Suryanarayanan and Johnson 2005), but to date, comparatively little research has examined levels of diversity within other ecosystems. Given the lack of research to date, it would therefore seem logical that these ecosystems contain high numbers of undescribed fungal endophytes.

The precise role that fungal endophytes serve within their host plant is still a matter of conjecture although it is thought to be largely beneficial (Arnold *et al.* 2003; Carroll 1988; Hyde and Soytong 2008; Martín *et al.* 2013; Schulz *et al.* 1999; Sieber 2007). Various benefits have been proposed, such as increased resistance to pathogens (Arnold *et al.* 2003; Sieber 2007), improved drought tolerance (Dingle and Mcgee 2003) and increased tolerance to salinity (Waller *et al.* 2005). These benefits would then

enable host plants to persist in areas outside their optimum environment (Rodriguez *et al.* 2004a; Sumarah *et al.* 2010).

Well known for their rich diversity of plant species, SEVT are thought to be extreme forms of subtropical dry rainforests which form part of the Brigalow Belt along the eastern coast of Australia (DERM 2007). Due to the high nutrient soils characteristic of this habitat type only 17% of the original coverage of SEVT is thought to remain (DERM 2007; McDonald 2010) with less than 25% of this thought to occur in protected areas such as national parks (Department of Environment, Water, Heritage and the Arts (DEWHA) 2010). As such, SEVT have been listed as a nationally endangered ecosystem under the (EPBC) Act of 1999. The Darling Downs forms part of the Southern Brigalow Belt bioregion where many SEVT occur where many of the remnant SEVT have not been mapped, making them at greater risk of loss (McDonald 2010).

2.2 Aims

This study examined the diversity of fungal endophytes within the Darling Downs region of the previously unexplored SEVT of the Southern Brigalow Belt Bioregion. The following questions were addressed in this chapter:

1. Do the SEVT harbour a high diversity of fungal endophytes?
2. Which SEVT plant taxa harbour the greatest diversity of fungal endophytes?
3. Are there any novel fungal taxa in SEVT?

These questions were addressed by examining the fungal endophyte diversity in multiple host species across three sites. Locations within plants and between site

variation were also examined. A measure of the mean endophytic diversity was also obtained for the three sites.

2.3 Methods

Leaf samples were collected from three SEVT sites in the Darling Downs; a nature reserve at Highfields, Mt Kingsthorpe park and a private property at Boodua (Figure 1). These three sites were all listed as regional ecosystem type 11.8.13; Semi evergreen vine thicket on Cainozoic igneous rock at a latitude of -27^{0}N (http://www.environment.gov.au). The purpose of this site selection was to minimise any variation that may be due to habitat type.

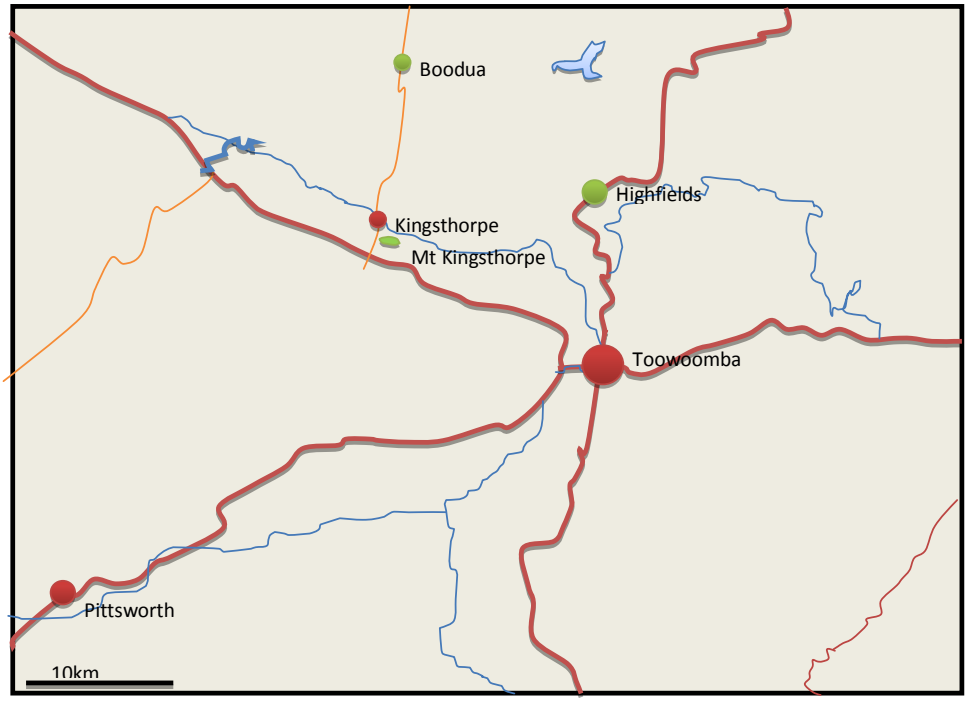

Figure 1 Sites of SEVT included in this study. Sampling occurred at locations marked in green

2.3.1 Sample collection and isolation

A total of 25 individual plants from 22 species (Table 1) were sampled across the three different SEVT sites. Plants were identified based on Harden *et al.* (2009). In the case of the trees and shrubs, small branches were taken from each of high, mid and low

regions of the canopy. The same number of leaves were collected for the vines and ground cover plants but from three locations within the plant. All samples were washed and plated within 10 hours of collection.

Table 1 Location and growth form of plants sampled

Location	Plant sampled	Family	Growth form
Highfields	*Citriobatus lancifolius*	Pittosporaceae	Tree
Highfields	*Croton insularis*	Euphorbiaceae	Tree
Highfields	*Elattostachys xylocarpa*	Sapindaceae	Tree
Highfields	*Eleaocarpus obovatus*	Eleaocarpaceae	Tree
Highfields	*Geijera salicifolia*	Rutaceae	Tree
Mt Kingsthorpe	*Alphitonia excelsa*	Rhamnaceae	Tree
Mt Kingsthorpe	*Brachychiton populneus*	Sterculiaceae	Tree
Mt Kingsthorpe	*Eustrephus latifolius*	Philesiaceae	Vine
Mt Kingsthorpe	*Geijera salicifolia*	Rutaceae	Tree
Mt Kingsthorpe	*Pandorea pandorana*	Bignoniaceae	Vine
Mt Kingsthorpe	*Pittosporum rhombifolium*	Pittosporaceae	Tree
Mt Kingsthorpe	*Sophora fraseri*	Fabaceae	Shrub
Boodua	*Alectryon diversifolius*	Sapindaceae	Tree
Boodua	*Alphitonia excelsa*	Rhamnaceae	Tree
Boodua	*Brachychiton populneus*	Sterculiaceae	Tree
Boodua	*Bursaria spinosa*	Pittosporaceae	Shrub
Boodua	*Capparis mitchelli*	Capparaceae	Tree
Boodua	*Cassine australis*	Celastraceae	Tree
Boodua	*Ehretia membranifolia*	Boraginaceae	Shrub
Boodua	*Erythroxylum australe*	Erythroxylaceae	Tree
Boodua	*Geijera salicifolia*	Rutaceae	Tree
Boodua	*Myoporum debile*	Myoporaceae	Ground cover
Boodua	*Notelaea venosa*	Oleaceae	Shrub
Boodua	*Pittosporum angustifolium*	Pittosporaceae	Tree
Boodua	*Santalum lanceolatum*	Santalaceae	Tree

Endophytes were isolated by using a variation of the method developed by Boddington (2009). Depending on size, two to three leaves per location (high mid or low) were selected for processing. Only those which appeared mature and had no visible signs of disease were selected for sterilising. Leaves were surface sterilised to remove any fungal epiphytes by first washing vigorously in tap water three times and then by soaking in sterile milli-q water for a minimum of three minutes. Leaves were then immersed in 95% ethanol for 90 seconds, before being rapidly passed through a flame and then pressed onto a control plate prior to sampling. This procedure ensured that the surface sterilisation procedure had been successful. A sterile 5mm hole punch was next used to remove eight tissue segments from two-three leaves (depending on leaf size) per plate onto Potato Dextrose Agar (PDA; Sigma, Castle Hill, NSW, Australia) containing 1.5mg/ml sterile streptomycin and tetracycline. In the case of *Myoporum debile* and *Eustrephus latifolius,* which had leaf widths smaller than could be hole punched, leaves were cut to approximate the same amount of leaf area per plate as the other samples and the number of leaves used was recorded. Plates were then incubated in the dark at 23^{o}C and checked regularly for growth. When hyphae were observed to grow from the leaf material, they were transferred to a fresh PDA plate without antibiotics.

Colonisation rates (CR) were determined based on %= N_i/N_s X 100 and isolation rates (IR) were based on N_e/N_s where N_i=number of samples with at least one isolate, N_e=number of endophytes collected, N_s= total number of samples investigated where 1 sample=1 leaf (Douanla-Meli *et al.* 2013; Fróhlich *et al.* 2000).

2.3.2 Fungal identification

Endophytes were identified using morphological characteristics as well as through the use of molecular methods. Sporulating fungi were identified using the following guides: (Bhimba *et al.* 2012; Carvalho *et al.* 2012; Ellis 1960). In some cases, identification was only to the genus or family level (Huang *et al.* 2009). For those fungal isolates that did not produce spores in culture, several attempts were made to induce sporulation. Initially, colonies were moved into a 4^{o}C refrigerator to encourage the production of spores. For those that still did not sporulate, colonies were moved to PDA plates containing sterilised leaf tissue from the original host plant. Since this second method was found to have no effect, it was discontinued for later samples. Colonies were also left under UV light for a month. If colonies still did not sporulate, isolates were classed as mycelia sterilia and were identified using molecular methods (see below). As each fungal isolate was collected, they were given a unique code depending on which plant and which location in the canopy the leaf tissue was isolated from. For example, isolates from *Alphitonia excelsa* collected from midway in the canopy from the Boodua (site 2) location were given the code AEM2.x where x reflects the number collected from that plant. So if two samples had previously been collected, the next sample would be AEM2.3.

2.3.3 DNA extraction

Fungal DNA was extracted from all endophytes that did not sporulate and/or those that showed antimicrobial activity using the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, Castle Hill, NSW, Australia) following the manufacturer's instructions. Once the DNA had been extracted, PCR with fungal specific primers were used to amplify endophytic fungal DNA using the Extract-N-Amp™ Plant PCR ready mix (Sigma-Aldrich, Castle Hill, NSW, Australia) together with 1µl of each primer, and 1µl of the previously extracted

DNA. The fungal specific primers included the ITS1F forward primer (Gardes and Bruns 1993) and ITS4 reverse primer (White *et al.* 1990).

All initial PCR reactions were performed in duplicate and a control tube containing 0.5µl of sterile H_2O instead of DNA was also included. Samples were amplified with 35 cycles of 95°C for one minute, 50°C for one minute and 72°C for one minute, with a final elongation step of ten minutes at 72°C. 2µl of the PCR product was then run on a 2% agarose gel and viewed under UV light using the Quantum ST4 gel documentation system (Vilber Lourmat, Fisher Biotec) and photographed using the Quantum capture ST4® image acquisition and analysis software package.

PCR products were purified in preparation for sequencing using a DNA purification kit (Macherey Nagel, Cheltenham, Australia) as per the manufacturer's instructions. 2µl of the resulting purified DNA was then run on a 2% w/v agarose gel to check the concentration of the purified sample.

2.3.4 Sequencing of isolated DNA

Samples containing between 17ng and 30ng of DNA were sent to the Brisbane laboratory of the Australian Genome Research Facility (AGRF) for sequencing. Upon receipt of the returned sequences, samples were viewed using the Chromas® 2.0 program to check for contamination. A BLAST search using the National Centre for Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov/> was used to determine closest species matches.

2.3.5 Statistical analysis

A one way ANOVA was used to determine differences in isolation rates of endophytes between sites and within sites using SPSS (IBM_Corp. Released 2012). The homogeneity of variance was tested using Levene's statistic and a Tukey post hoc test was used to confirm the suitability of the data. Data was checked for normalcy prior to analysis and those which did not display a normal distribution were then log transformed.

2.4 Results

A total of 228 fungal cultures from 88 different fungal genera were isolated from 286 leaves of 23 plants (2 plants failed to produce any endophytes and were not included in the analysis; Appendices Table A.14). For leaves where growth was observed, 26.6% of the leaf fragments contained one or more endophytes and 36.7% (SE = 0.11) of the leaves collected contained endophytes which were able to grow on agar (CR). The mean IR was 0.8 fungal endophytes per leaf. Several plants had a low individual IR. The lowest IR rate of 0.16 observed was from *Sophora fraseri,* a small shrub (Table 2). One of the *Geijera salicifolia* trees sampled at Mt Kingsthorpe had a low IR of 0.2. The small tree, *Ehretia membranifolia* and the ground cover *Myoporum debile*, were next lowest with IR of 0.3 and 0.4 respectively. *Alphitonia excelsa* was collected from two sites, and had an IR of 0.3 and 0.5. In most cases, surface sterilisation was found to be adequate in removing epiphytic fungi. Control plates for *Pittosporum angustifolium, Santalum lanceolatum, Elattostachys xylocarpus* and *Geijera salicifolia* (Boodua) all had epiphytic contamination from *Nigrospora* sp. and *Notelaea venosa* had one contaminating epiphytic colony of *Xylaria* sp. (Table 2). These fungal taxa are ubiquitous and were also isolated as endophytes from plates with no epiphyte contamination.

Table 2 Colonisation and isolation rates of endophytes collected from three sites.

Location	Plant sampled	Family	Colonisation rate	Isolation rate	Total no. fungi	Control plate
Highfields	*Citriobatus lancifolius*	Pittosporaceae	50.0%	2.0	12	ng
Highfields	*Croton insularis*	Euphorbiaceae	33.3%	0.6	7	ng
Highfields	*Croton insularis*	Euphorbiaceae	33.3%	0.5	6	ng
Highfields	*Elattostachys xylocarpa*	Sapindaceae	25.0%	1.0	12	ng
Highfields	*Elattostachys xylocarpa*	Sapindaceae	41.7%	1.0	11	ng
Highfields	*Eleaocarpus obovatus*	Eleaocarpaceae	33.3%	0.6	7	ng
Highfields	*Geijera salicifolia*	Rutaceae	33.3%	0.8	8	ng
Mt Kingsthorpe	*Alphitonia excelsa*	Rhamnaceae	16.7%	0.3	3	ng
Mt Kingsthorpe	*Eustrephus latifolius*	Philesiaceae	41.7%	1.8	19	ng
Mt Kingsthorpe	*Geijera salicifolia*	Rutaceae	16.7%	0.2	2	ng
Mt Kingsthorpe	*Pandorea pandorana*	Bignoniaceae	33.3%	0.6	7	ng
Mt Kingsthorpe	*Sophora fraseri*	Fabaceae	16.7%	0.16	2	ng
Boodua	*Alectryon diversifolius*	Sapindaceae	50.0%	1.4	17	*Nigrospora* sp.
Boodua	*Alphitonia excelsa*	Rhamnaceae	50.0%	0.5	2	ng

Location	Plant sampled	Family	Colonisation rate	Isolation rate	Total no. fungi	Control plate
Boodua	*Brachychiton populneus*	Sterculiaceae	33.3%	0.4	5	ng
Boodua	*Bursaria spinosa*	Pittosporaceae	50.0%	1.1	13	ng
Boodua	*Capparis mitchelli*	Capparaceae	50.0%	1.3	15	ng
Boodua	*Cassine australis*	Celastraceae	50.0%	1.2	14	ng
Boodua	*Ehretia membranifolia*	Boraginaceae	16.7%	0.3	3	*Nigrospora* sp.
Boodua	*Erythroxylum australe*	Erythroxylaceae	41.7%	1.1	13	ng
Boodua	*Geijera salicifolia*	Rutaceae	33.3%	0.7	8	*Xylaria* sp.
Boodua	*Myoporum debile*	Myoporaceae	41.7%	0.4	5	ng
Boodua	*Notelaea venosa*	Oleaceae	41.7%	1.1	13	*Nigrospora* sp.
Boodua	*Pittosporum angustifolium*	Pittosporaceae	33.3%	0.6	7	*Nigrospora* sp.
Boodua	*Santalum lanceolatum*	Santalaceae	50.0%	1.4	17	ng
Mean			36.7%	0.8	9.9	

Ng= no growth of epiphytic fungi

Note: Two plants failed to grow any fungi at all. It was decided this was most likely as a result of over sterilising and so these trees were not included in any of the analysis.

Two plants collected from Mt Kingsthorpe failed to grow any fungi at all. When these samples were included, a statistically significant difference in endophyte totals was observed, as determined by one-way ANOVA, between Mt Kingsthorpe and Boodua (F (2,20) = 4.896, p = 0.024). However, when they were excluded from analysis no statistical difference was observed between the three sites (F (2,20) = 1.042, p = 0.371; Appendices Table A. 15 and Table A.16). The lack of growth from these two samples was thought to be due to over sterilisation and so these two plants were not included in any other analysis. On average, 9.9(std 5.4) fungal endophytes were isolated per plant, with no significant differences found between the number of endophyte species isolated at each location within the canopy (Figure 2), F (2, 47)= 1.807, p =0.175 (see appendices Table A.17).

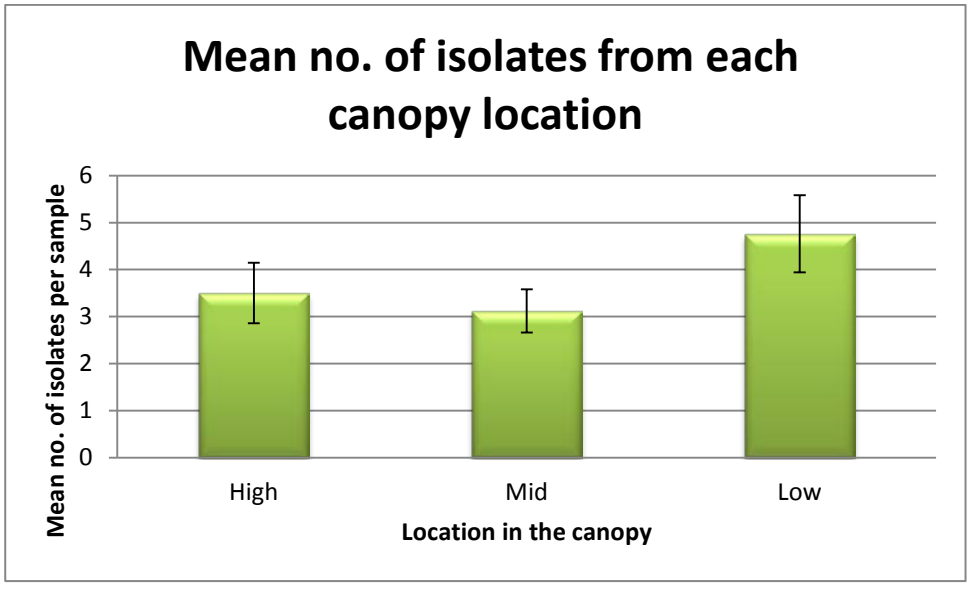

Figure 2. No significant differences were recorded for the number of fungal endophytes occurring in different canopy locations SE+/-

The most common endophytic genera were *Nigrospora* (66 isolates), *Preussia* (19 isolates), *Cladosporium* (10 isolates), *Guignardia* (9 isolates), *Phomopsis* (8 isolates) and Sarcosomataceae (20). Despite *Nigrospora* occurring commonly across the three sites,

only one species was identified (Table 3). In the case of the *Preussia* isolates, 11 different species were present in 20 isolates (Table 3). More than half of the genera isolated were observed less than ten times. However approximately 13% were isolated between 10 and 20 times and the remaining third all belonged to a single genus, *Nigrospora* (Table 4).

Table 3 Frequency of fungal genera observed compared with the number of species present

Genera	No. of times isolated	No. of species
Acremonium spp.	4	2
Cladosporium spp.	10	9
Epicoccum spp.	4	4
Guignardia spp.	9	7
Nigrospora spp.	66	1
Pestalotiopsis spp.	4	4
Pezizales spp.	25	11
Phomopsis spp.	8	6
Preussia spp.	19	11
Sordaria spp.	4	4
Xylaria spp.	10	4
Mycelia sterilia	21	20
other	44	40
Total	228	123

Table 4 Percentage of fungal genera isolated less than ten times compared to those more frequently isolated

Frequency	Number of isolates	Number of genera	%
<10	133	82	58.33%
10-20	29	5	12.72%
20 >	66	1	28.95%
Total	228	88	100%

Of the 228 isolates, 137 (60%) failed to produce any identifiable features in culture. Of these, a further 21 could not be successfully sequenced and were classed as mycelia

sterilia. From 116 isolates where DNA sequences were successfully obtained, 62 (53.4%) returned ITS sequence similarities of less than 97% to known species in GenBank (Table 5). A further nine had sequence similarities of 90% or less while 15 were between 91% and 93%. One sequence had a closest match of 96% however this was only over 86% of the sequence. Isolate PAH2.7 was very slow growing and forming a bright pink glossy ridged colony (Figure 3). When it was analysed using the BLAST option in GenBank, it returned a closest match of 80% with an unknown environmental sequence. 15 sequenced isolates were found to belong to the order Pezizales, 13 of these had a less than 97% sequence similarity to other known sequences in GenBank (Table 5). Further analysis of the 13 sequences found that they belonged to four families within the order Pezizales. These were Sarcoscyphaceae (3), Sarcosomataceae (8), Pyronemataceae (1) and one *incertae sedis*.

Table 5 Closest GenBank match of the ITS region for all fungi successfully sequenced

Code	Closest Match	Identity	%	GenBank Code
ADH2.15	*Nigrospora oryzae* JN662419.1	382/395	97%	
ADL2.2	*Nigrospora* sp. HQ889723.1	447/447	100%	KF227798
ADL2.4	*Preussia* sp. FJ210518.1	422/426	99%	JN418766
ADL2.5	*Nigrospora* sp. JQ246358.1	493/494	99%	KF227799
ADL2.6	*Sporormiella isomera* EU551184.1	493/498	98%	JN418765
ADM2.11	*Preussia isomera* GQ203763.1	335/352	95%	JN418767
AE2.1	*Pyronema domesticum* HM016895.1	431/440	98%	KF227800
AE2.2	Pezizomycetes sp. JQ760765.1	407/426	93%	KF227801
AEH3.2	*Oedocephalum adhaerens* FJ695215.1	398/415	96%	KF227802

Code	Closest Match	Identity	%	GenBank Code
AEL3.1	*Pyronema* sp. HQ829058.1	473/474	99%	KF227803
BPL2.1	*Nigrospora* sp. HQ608030.1	512/513	99%	KF227804
BPL2.2	*Phoma* sp. HQ630999.1	531/535	99%	KF227805
BPM2.4	*Nigrospora* sp. HQ608063.1	507/507	100%	KF227806
BPM2.5	*Conoplea fusca* EU552114.1	470/510	92%	KF227807
BSH2.13	*Nigrospora* sp. JN903534.1	464/466	99%	KF227808
BSH2.9	*Preussia Africana* EU551195.1	504/507	99%	JN418768
BSL2.4	*Nigrospora* sp. GQ999518.1	406/473	86%	
BSL2.5	*Nigrospora* sp. GU073125.1	471/476	98%	KF227809
BSM2.6	*Nigrospora* sp. HQ607936.1	515/515	100%	KF227810
CAH2.11	*Preussia africana* JQ031265.1	467/476	98%	JN566153
CAH2.12	Fungal sp. ARIZ AZ0908 HM123589.1	500/529	95%	KF227811
CAH2.13	*Pezizomycetes* sp. JQ760750.1	511/550	93%	KF227812
CAH2.14	*Guignardia mangiferae* HQ874762.1	383/396	97%	KF227813
CAH2.9	*Sporormiella isomera* EU551184.1	497/503	98%	JN418769
CAL2.1	*Sporormiella isomera* EU551184.2	448/450	99%	JN418770
CAL2.2	*Pestalotiopsis* sp. HQ288238.1	498/504	99%	KF227814
CAL2.3	*Nigrospora* sp. JF817271.1	461/472	97%	KF227815
CAL2.5	*Hypoxylon fragiforme* EU715619.1	446/458	97%	KF227816
CIL1.1	*Colletotrichum* sp. FJ904824.1	497/500	99%	KF227817
CIL1.2	*Guignardia mangiferae* FJ538333.1	592/625	95%	KF227818
CIM1.14	*Fimetariella* sp. HQ406808.1	542/544	99%	KF227819

Code	Closest Match	Identity	%	GenBank Code
CIM1.15	*Hypoxylon* sp. JN979430.1	516/522	99%	KF227820
CIM1.16	*Hypoxylon* sp. JN979435.1	526/531	99%	KF227821
CIM1.17	*Cercophora* sp. HQ631039.1	472/515	92%	KF227822
CIM1.3	*Biscogniauxia anceps* EF026132.1	570/664	86%	KF227823
CIM1.4	*Pithya cupressina* PCU66009	524/572	92%	KF227824
CIM1.6	*Pithya cupressina* PCU66009	530/580	91%	KF227825
CLH1.10	*Phomopsis theicola* GQ281809.1	522/538	97%	KF227826
CLH1.11	*Phomopsis* sp. FN868477.1	562/578	97%	KF227827
CLH1.12	*Biscogniauxia* sp. JQ327868.1	550/589	92%	KF227828
CLM1.6	*Nigrospora* sp. HQ248210.1	472/481	98%	KF227829
CLM1.8	*Nigrospora* sp. HM565952.1	503/511	98%	KF227830
CMH2.13	*Nigrospora* sp. HQ608063.1	487/488	99%	KF227831
CMH2.14	*Nigrospora* sp. HM565952.1	464/484	95%	KF227832
CMH2.15	*Nigrospora sp.* GU934549.1	422/423	99%	KF227833
CML2.4	*Acremonium sp*. HQ829107.1|	560/569	98%	KF227834
CML2.7	*Neurospora*sp. AY681187.1|	541/547	99%	KF227835
CMM2.11	*Nigrospora* sp. HQ608030.1	483/485	99%	KF227836
EAH2.13	*Guignardia* sp. FJ538333.1	595/597	99%	KF227837
EAL2.4	*Preussia pilosella* HQ637314.1	498/517	96%	JN566152
EAL2.5	*Pezizomycotina* sp. GU212409.1	419/451	92%	JN418771
EAL2.7	*Preussia pilosella* DQ468033.1	454/474	96%	JN418772
EAM2.10	*Nigrospora* sp. JF819161.1	515/525	98%	KF227838

Code	Closest Match	Identity	%	GenBank Code
ELV3.1	Xylariaceae sp. HQ130668.1	521/547	95%	KF227839
ELV3.10	HM123589.1			
ELV3.11	*Preussia* sp. HQ602666.1	422/471	89%	JN418774
ELV3.12	*Coniochaeta prunicola* GQ154541.1	357/372	96%	KF227840
ELV3.13	*Chaetomium globosum* FJ904822.1	472/478	99%	KF227841
ELV3.14	*Nigrospora* sp. HQ607936.1	509/509	100%	KF227842
ELV3.15	*Coniochaeta prunicola* GQ154541.1	398/424	94%	KF227843
ELV3.16	*Conoplea fusca* EU552114.1	531/590	90%	KF227844
ELV3.18	*Guignardia mangiferae* EU677818.1	563/592	95%	KF227845
ELV3.19	*Guignardia mangiferae* FJ538333.1	592/620	95%	KF227846
ELV3.2	*Preussia* sp. HQ602666.2	382/426	89%	JN418773
ELV3.3	Xylariaceae sp. FJ487924.1	519/548	95%	KF227849
ELV3.4	Dothidiomycete JQ760353.1	434/468	93%	KF227850
ELV3.6	*Guignardia mangiferae* EU677811.1	569/596	95%	KF227851
ELV3.7	*Xylaria sp.* FJ205450.1	385/395	97%	KF227852
EMH.2.3	Pezizomycetes sp. JQ760747.1	468/497	93%	KF227853
EOL.1.2	*Microdiplodia hawaiiensis* EU715661.1	548/572	96%	KF227854
EXH1.14	*Conoplea fusca* EU552114.1	506/564	90%	KF227855
EXH1.21	Pezizomycetes sp. JQ761977.1	506/509	92%	KF227856
EXH1.22	*Muscodor yucatanensis* FJ917287.1	494/512	96%	KF227857
EXH1.23	*Guignardia* sp. FJ538333.1	591/592	99%	KF227858
EXL1.16	*Phomopsis* sp. HQ829058.1	564/565	99%	KF227859

Code	Closest Match	Identity	%	GenBank Code
EXL1.17	*Pyronema sp.* DQ780461.1	557/565	99%	KF227860
EXL1.3	*Halorosellinia* sp. JF773589.1	474/491	97%	KF227861
EXL1.4	*Biscogniauxia* sp. JQ327868.1	594/638	93%	KF227862
EXL1.6	*Diaporthe* sp. JF304628.1	400/425	93%	KF227863
EXL1.9	*Phomopsis* sp. DQ780461.1	524/539	97%	KF227864
EXM1.11	*Conoplea fusca* EU552114.1	551/616	89%	KF227865
EXM1.19	*Microdiplodia hawaiiensis* EU715661.1	561/586	96%	KF227866
GSH2.8	*Devriesia fraseriae* HQ599602.1	528/549	96%	KF128864
GSH2.9	Uncultured soil fungus clone EU826926.1	519/542	96%	KF128865
GSL1.2	*Diaporthe leucospermi* JN712460.1	510/512	99%	
GSL1.5	*Phomopsis* sp. HM595506.1	517/536	96%	KF128868
GSL2.1	*Nigrospora* sp. HQ832828.1	538/542	99%	
GSL2.2	*Lecythophora* sp. AY219880.1	501/534	94%	KF128870
GSM3.1	*Preussia africana* DQ865095.1	506/509	99%	JN418775
GSM3.2	*Neofusicoccum vitifusiforme* EF445349.1	386/387	99%	
MDC2.3	*Guignardia mangiferae* JF261459.1	534/573	93%	KF227867
MDC2.4	*Toxicocladosporium protearum* HQ599586.1	532/548	97%	KF227868
MDC2.5	*Paraphaeosphaeria* sp. HQ630993.1	438/459	95%	KF227869
NVH2.11	*Lecythophora* sp. AY219880.1	549/570	96%	KF227870
NVL2.1	*Gelasinospora* sp. JN207268.1	549/556	99%	KF227871
NVM2.4	*Fimetariella rabenhorstii* EU781677.1	510/510	100%	KF227872
NVM2.5	*Preussia Africana* EU551195.1	480/503	95%	JN418776

Code	Closest Match	Identity	%	GenBank Code
NVM2.6	*Sporormiella isomera* EU551184.1	497/499	99%	JN418777
NVM2.7	*Cladosporium tenuissimum* FR799495.1	530/530	100%	KF227873
PAH2.7	Uncultured ascomycetes AM901772.1	493/594	80%	KF227874
PAL2.1	*Sporormiella isomera* EU551184.2	507/511	98%	JN418778
PAL2.3	*Preussia* sp. HQ130664.1	416/439	95%	JN418779
PAL2.4	Xylaria sp. EF157664.1	549/560	98%	KF227875
PPV3.1	*Xylaria* sp. EF157664.1	487/530	92%	
PPV3.2	*Aureobasidium* sp. JF439462.1	531/536	99%	KF227876
PPV3.4	Pleosporales sp. HQ007235.1	438/512	86%	KF227877
PPV3.5	*Aureobasidium pullulans* HM855960.1	561/580	97%	KF227878
PPV3.6	*Preussia* sp. FJ210521.1	508/514	98%	JN418780
SFL3.1	*Periconia* sp. HQ608027.1	540/549	98%	KF227879
SLH2.12	*Nigrospora* sp. JQ936183.1	513/515	99%	KF227880
SLH2.13	*Colletotrichum boninense* EU822802.1	578/580	99%	KF227881
SLH2.16	*Phoma glomerata* AY183371.1	476/494	96%	KF227882
SLH2.17	*Pseudocercospora casuarinae* HQ599603.1	510/531	96%	KF227883
SLL2.3	*Conoplea fusca* EU552114.1	501/549	91%	KF227884
SLM2.10	*Nigrospora* sp. JN207298.1	524/534	98%	KF227885
SLM2.6	*Nigrospora* sp. GQ999518.1		100%	
SLM2.8	*Fimetariella rabenhorstii* HQ406808.1	382/385	99%	KF227886

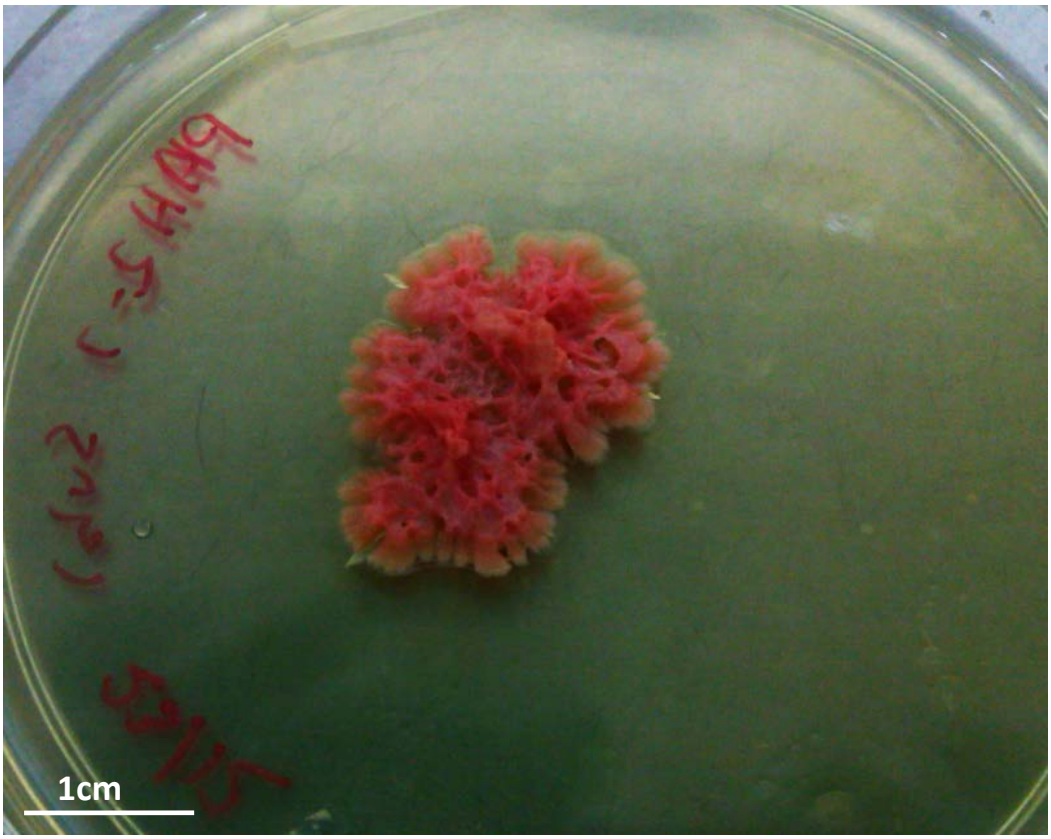

Figure 3 Isolate PAH2.7 on PDA after 6 months growth

2.5 Discussion

This study isolated 228 fungal endophytes from SEVT in south east Queensland. The average colonisation rate of 36.7% was lower than other studies of this type. Toofanee and Dulymamode (2002) found an overall leaf CR of 64% when examining fungal endophytes of the rainforest tree species, *Cordemoya integrifolia.* Similarly when the roots, stems and leaves of the orchid, *Dendrobium nobile* were investigated, a 59% endophytic CR was recorded (Yuan *et al.* 2009). Other studies examining tropical rainforests have found CR of 81% (Fróhlich *et al.* 2000), 95% (Gamboa and Bayman 2001) and as low as 54% (Douanla-Meli and Langer 2012). Low CR of between 9.2-33% have been observed before in studies examining temperate palms (Taylor *et al.* 1999) and pine needles (Guo *et al.* 2008b). CR have also been shown to vary widely between plants sampled at a particular site and also when examined before or after recent rain

periods (Krishnamurthy *et al.* 2008). Arnold and Lutzoni (2007) examined diversity across latitudinal gradients and found that endophyte diversity decreased with increased latitude. Based on this and given the latitude of the sites examined in this study (27^0N), a CR around 30% would have been expected and so the observed CR of 36.7% is not entirely surprising. Based on CR, levels of diversity observed here were more similar to those from temperate rainforests with similar latitudes (Arnold and Lutzoni 2007; Hoffman and Arnold 2008). Therefore, the low CR observed here may be a reflection of the latitudinal gradient of SEVT.

It should also be noted that a novel sterilisation technique was used in this study. Leaves were flame sterilised instead of solely using bleach or hydrogen peroxide. Flame sterilisation may potentially raise the internal temperature which may have affected endophytic viability and altered the CR rates. Further study could examine whether a different CR would be observed using more traditional sterilisation methods.

The IR observed in this study (0.8) falls within the range of those commonly reported (Douanla-Meli *et al.* 2013; Huang *et al.* 2008). Although the IR varied considerably between individual plants examined (0.1-1.8), this was not the case between sites and reflected commonly reported differences (Huang *et al.* 2008). IR have often been shown to vary widely between plants at individual sites. For example, Douanla-Meli and Langer (2012) reported an average IR of 0.57 per leaf while Fróhlich *et al*. (2000) observed an IR of 1.44. Huang *et al.* (2008) also found IR which varied between 0.45 and 1.75 in their study of 29 plant species from various sites. Given that these rates are based on culturable endophytes only, the variation may in part be explained by the ratio of fungal endophytes able to grow in culture to those that are unable to grow. While studies such

as Nikolcheva and Bärlocher (2004a) and Crozier *et al*. (2008) have compared the techniques of culture and molecular-based methods and found differing species compositions, how they relate to IR has not been specifically examined. Another possible explanation of the variation may be due to slight differences in micro environments provided by individual host plants (Huang *et al.* 2008). Huang *et al.* (2008) found that increased levels of phenolic compounds in host plants led to lower overall fungal richness. Many studies, however, found that differences in richness are more a reflection of site-based factors than host species selection (Helander *et al.* 1993; Hoffman and Arnold 2008; Suryanarayanan *et al.* 2011). Since few plants were replicated in this study it is difficult to determine whether there was any statistical variation between plants. More research is needed to determine whether the differences seen here are true reflections of the fungal richness within individual host plants. Further research could also examine whether low levels of richness are a reflection of the proportion of species able to grow in culture to those only observable using molecular-based methods or whether they are associated with host plant species.

IR for several of the plants appeared to be low. Of particular interest was the low IR observed in *Alphitonia excelsa* across the two sampled sites. Commonly known as Red ash, or Soap bush, *A. excelsa* is easily identified due to the orang/red leaves it produces on maturity and the grey waxy underside of leaves (Harden *et al.* 2009). Leaves are also commonly observed exhibiting signs of heavy insect predation (Brisbane Rainforest Action & Information Network (BRAIN) 2007). It is therefore quite interesting that the IR observed in this study was low for this species and would support other research suggesting that fungal endophytes play a role in anti-herbivory (Saikkonen *et al.* 1998; Wagner and Lewis 2000). An insufficient sample size prevented statistical analysis from

confirming whether the differences seen here were significant. The similarity in IR observed between the two plants sampled, however, does suggest a potential explanation for the high level of insect damage observed on almost all leaves. Furthermore, it should be noted that during leaf sampling, where possible, only intact leaves were used. In the case of *A. excelsa* this was difficult at times since many leaves displayed evidence of insect herbivory. Further research is needed in order to compare the level of herbivory observed in *A. excelsa* across a broad range of sites and geographical locations to determine whether the low species richness observed in this study is statistically significant and whether it is related to the common observation of heavy predation of *A. excelsa* leaves.

While no statistical difference was observed between the three sites, several of the plants with lower IR came from trees or shrubs growing on Mt Kingsthorpe. This site is a park with a path which winds its way up the side of the mountain through the SEVT, effectively creating small patch sizes. The lower endophyte numbers could be a reflection of the general health of this particular SEVT. The effect of poor habitat health on endophytic richness has not been well studied (Chaverri and Vílchez 2006; Tsui *et al.* 1998). Early work examining diversity of fungi within palms found that palms in disturbed sites contained significantly less diversity than those in old growth areas (Taylor 1998). More recently, Douanla-Meli *et al.* (2013) found a correlation between lower endophytic diversity and poor plant health in *Citrus limon*. Similarly Hall *et al.* (2012) found decreased diversity of fungal endophytes in disturbed grasslands. Furthermore, *Geijera salicifolia* was sampled at this site and two of the others sites but exhibited lower CR and IR only at Mt Kingsthorpe, suggesting that these results are site dependent and not a reflection of the choice of plant species selected. This would

suggest that the results seen here are most likely an indicator of the poor health of this site. Interestingly, the two vines sampled had IR similar to those isolated in the rest of the study, suggesting that endophytic diversity within vines is not affected in this case. Unfortunately, not enough plants were sampled to conclude whether these results were significantly lower. Further research could examine this site over subsequent years to determine if the CR and IR are consistently lower and to ascertain whether this site is in decline.

Somewhat surprisingly, no difference in IR was observed between the three canopy locations that were sampled. Areas more exposed to light were expected to contain fewer endophytes than those found lower in the canopy (Johnson and Whitney 1989). Leaf age is thought to also be a significant contributor to endophyte density and this increases progressively (with accompanying endophytic colonisation) down through the canopy (Saikkonen 2007). Early work on fungal endophytes found varying results in this respect. While some research found higher levels of endophytic abundance towards the trunk (Johnson and Whitney 1989), others did not (Bernstein and Carroll 1977). Therefore, it seems that other factors such as moisture, temperature, pH, leaf age and distance to nearest neighbour may play a more significant role in species abundance (Saikkonen 2007). Helander and associates (2007) found a strong link between endophyte abundance and nearest neighbour, a result also recorded by Arnold and Herre (2003). Given that many endophyte propagules are thought to be dispersed via water droplets (Helander *et al.* 2007; Saikkonen 2007), such results are not surprising. SEVT are closed canopy forests with trees in close proximity to each other. Most likely as a result of a decreased access to water, trees in SEVT also tend to be shorter in height than those typically found in more tropical areas (McDonald 1996; McDonald 2010).

This would then mean that there is relatively little variation in light at the various levels. The lack of endophytic variation between canopy locations observed in this study may therefore be a result of the close proximity of neighbouring trees in SEVT and the easy transference of propagules coupled with small height of the trees sampled.

Of interest was the observation that the large herbaceous shrub, *Ehretia membranifolia,* the ground cover *Myoporum debile,* and the small Fabaceae shrub *Sophora fraseri* had relatively low IR compared to the other plants isolated in this study. While there was an insufficient sample size for statistical comparison, it would appear that these smaller plants have fewer endophytes than the larger plants sampled. A possible explanation for the ground cover and small shrub could be that since SEVT have reduced access to water (Ecosystem Conservation Branch 2007) and most endophyte propagules are thought to be dispersed by water (Helander *et al.* 2007; Saikkonen 2007; Saikkonen *et al.* 1998), these small low lying shrubs are less exposed to colonising propagules. In the case of *Ehretia membranifolia*, the leaves of this species are very thin and so may have been more affected by the surface sterilisation procedure, a factor potentially applicable to the other two plant species as well. Further research would need to be undertaken in order to confirm this.

All fungi isolated in this study were ascomycetes, a result not entirely surprising particularly when culture-based isolation methods are used (Aly *et al.* 2011; Arnold 2007; Huang *et al.* 2009; Hyde and Soytong 2008; Kumar and Hyde 2004). Even when culture independent methods are used in endophyte identification, the vast majority of endophytes observed appear to be ascomycetes (Allen *et al.* 2003) although high numbers of basidiomycetes have been recorded (Crozier *et al.* 2008). It should be also

noted that basidiomycetes have been observed forming specialised endophytic associations with orchid species, oil palms and several other plant species (Graham and Dearnaley 2012; Guo *et al.* 2001; Rungjindamai *et al.* 2008). Furthermore, some basidiomycetes are recalcitrant to amplification using standard fungal ITS primers (Toju *et al.* 2012). Given that 21 isolates did not produce any identifiable morphological features and could not be successfully sequenced, it is possible that basidiomycetes may have been present in this study but were unable to be identified.

The most common fungal taxa observed in this study differed from those frequently found in other investigations of endophytic diversity. Regularly reported endophytic species include *Colletotrichum* spp, *Phomopsis* spp, *Alternaria* spp. *Phoma* spp, *Pestalotiopsis* spp and *Xylariales* spp. (Aly *et al.* 2011; Arnold and Lutzoni 2007; Helander *et al.* 1993; Huang *et al.* 2008; Suryanarayanan *et al.* 2011). These are endophytes with broad host ranges, which is thought to be the main reason for their common occurrence (Arnold and Lutzoni 2007). While members from these genera were observed, the most common taxa recorded were *Nigrospora* spp., *Preussia* spp., *Cladosporium* spp., and *Guignardia* spp. No such endophytic community patterns have been observed before, however studies have found novel endophytic fungal species compositions in previously unexplored habitats in the past. When endophyte communities were examined in coastal redwoods, the most common species observed were *Pleuroplaconema* sp. and *Pestalotiopsis funereal* (Espinosa Garcia and Langenheim 1990). Similarly, Sakayaroj and associates (2010) found a variety of Sordariomycetes, Eurotiomycetes and Dothidiomycetes as the most abundant groupings from endophytes of tropical seagrasses(Sakayaroj *et al.* 2010). Common genera observed in that study were *Cladosporium*, *Cordyceps*, *Geosmithia, Penicillium* and *Eurotium*. SEVT are

remnant ecosystems from a time in Australia's past which saw much higher rainfall (McDonald 1996). Given that nothing is known about the fungi in SEVT, the novel assemblages observed in this study may be a reflection of the uniqueness of the habitat and the particular ecological pressures experienced within this environment.

The most common genus observed in this was *Nigrospora*, being isolated 66 (28%) times. *Nigrospora* spp. are well known aerial molds whose numbers peak in the autumn months (Das and Gupta-Bhattacharya 2008; Ellis 1960; Webster 1952). Several species of *Nigrospora* are also pathogens of agricultural importance (Palmateer *et al.* 2003). Considered to be true endophytes, they are generally described as generalists which may be found occurring abundantly within a broad range of plant hosts (Suryanarayanan *et al.* 2005). Chakraborty *et al.* (2000) found that spore counts of *Nigrospora* spp., peaked during periods of increased rain, a result also observed by Das and Gupta-Bhattacharya (2008). At the time of sampling, the region had been experiencing unseasonally high rainfall (Bureau of Meterology (BOM) 2011) and this may explain the large number of *Nigrospora* spp. observed in this study.

The second most prolific fungal taxon observed was *Preussia*. Interestingly, *Preussia* spp. are not usually considered endophytic (Kruys and Wedin 2009) but are typically coprophilous or saprotrophs of wood, soil and dead plant matter (Arenal *et al.* 2005; Kruys and Wedin 2009; Poch and Gloer 1991). Although recently, Arenal *et al*. (2007) found that plants collected from a sclerophyllous evergreen forest of the Mediterranean basin west of Spain supported a large number of *Preussia* spp. Together with the results of this study, it would suggest that the endophytic life mode may be more frequent than first thought for *Preussia* spp.

Three families belonging to the order Pezizales were isolated frequently in this study. 11/15 Pezizales isolated in this study are likely to be new species belonging to the Sarcosomataceae and Sarcoscyphaceae. Of the remaining four isolates, three are likely to be *Pyronema domesticum* (Pyrenomataceae) and one new species related to isolates that are *incerta sedis*. Members of the Sarcosomataceae and Sarcoscyphaceae are most commonly known as cup fungi due to the prominent, often brightly coloured apothecia that are produced on the ground or on decaying leaf and bark material (Jumpponen and Jones 2009), (Unterseher *et al.* 2011). On some occasions members of the order Pezizales have been shown to form ectomycorrhizal associations (Tedersoo *et al.* 2006), and on rare occasions have been observed occurring endophytically, although not in the high numbers observed here. Recently, Soca-Chafre *et al.* (2011) isolated one unidentified Sarcosomataceae species from *Taxus globosa,* while Weber *et al.* (2002) isolated one species of *Galiella rufa* from the twig of *Cistus salvifolius*. More research is needed to determine whether the results in this study are common to a wider sampling of SEVT, and what role these species may be playing within this ecosystem.

A large number of mycelia sterilia were observed in this study. Isolation frequencies of 54% have been recorded for non-sporulating fungi before and thus the present result is not unusual (Arnold and Lutzoni 2007; Lacap *et al.* 2003). Of these, a further 21 could not be successfully sequenced and so were unable to be identified. Often missed due to their slow growing nature, basidiomycetes are notoriously difficult to sporulate in culture (Hansen 1979; McLaughlin and McLaughlin 1972) and may be missed entirely if they contain ITS regions not easily amplified by conventional primers. Therefore, as mentioned earlier, some of these unidentifiable samples may potentially belong to the basidiomycota. For those samples which were able to be identified through molecular

methods, formal naming is prevented under the current conditions of the ICBN code. Consequently, isolates could only be identified to the family or genus level. Results such as this highlight the need for some provision under the ICBN code for naming such samples.

In this study, over half of the isolates from which ITS data was obtained had a less than 97% sequence similarity to taxa in GenBank, indicating that they may be potentially new species. However, the vast majority of these are mycelia sterilia and as mentioned above, naming is prohibited under the ICBN code. The ITS and related regions are now routinely used as a DNA barcode for most fungal species (Tedersoo *et al.* 2006) and it would seem logical that formal naming methods should take advantage of molecular techniques. Through the use of peer reviewed reference databases such as UNITE (http://unite.ut.ee), unknown sequences could be given names which reflect their phylogenetic similarities. A reference within the name could be included to indicate that no morphology had been observed for that species. Enabling such naming would prevent databases such as GenBank from becoming cluttered with near useless unknown sequences and allow the naming of the growing abundance of habitually sterile fungi.

SEVT are extreme forms of subtropical rainforests which experience seasonal rains and are often exposed to drought (McDonald 1996). The high number of unique fungal endophytes observed in this study may be indicative of benefits conferred by the endophytes such as improved drought tolerance. It has often been argued that the presence of endophytes affords some benefit to the host in the form of improved fitness and resilience (Rodriguez and Redman 2008). The presence of fungal endophytes

in plants exposed to drought conditions has been shown to decrease water loss by up to 30% and the presence of particular endophytes also conferred an increased tolerance to cold (Redman *et al.* 2011; Rodriguez and Redman 2008). The Darling Downs region is latitudinally lower than typical rainforest areas and plants in this area are often exposed to drought and intense periods of cold (temps <5^{o}C; BOM 2013; McDonald 2010). The ability of plants within SEVT to withstand these conditions may be being assisted by their association with these unique fungal endophytes. Further research could be conducted to examine the difference in endophyte IR in plants exhibiting signs of stress in SEVT compared to those showing signs of improved fitness.

2.6 Conclusion

This study has demonstrated that the dry rainforests of southeast Queensland house a broad diversity of previously undescribed fungal endophytes. This ecosystem, is under continued threat from clearing and weed invasion (McDonald 2010). Currently less than 17% of the original habitat remains and only 25% of this occurs within protected areas (McDonald 2010). SEVT host an array of novel fungi species and possibly genera whose precise ecological role has not even begun to be explored. Studies such as this highlight the need for increased protection and preservation of potentially valuable natural resources. Permanent loss of these sites will impact on what appears to be a significant storehouse of fungal endophyte diversity.

3 Specificity of fungal endophytes from *Geijera salicifolia* in SEVT

3.1 Introduction

Specificity in fungal associations may focus on two areas: Host specificity, where a particular fungal species or genus will only associate with a specific host; or fungal specificity, where the host will associate with a specific set of fungi (Dearnaley *et al.* 2012). Given the ubiquitous and typically generalist nature of fungi, host specificity is relatively rare (Arnold and Lutzoni 2007; Brodeur 2012; Bruns *et al.* 2002; Davey *et al.* 2013). Fungal specificity, on the other hand, is much more commonly observed and may be seen at many different levels. For example, particular fungal species, genera or even families may associate exclusively or commonly with particular plant species, genus or family (Carroll 1988; Den Bakker *et al.* 2004; Graham and Dearnaley 2012; Lindblad 2000). Specificity can also be further narrowed to include tissue specificity such as particular fungi or suites of fungi observed in stems, leaves or roots of particular plants (Blackwell 2011; Davey *et al.* 2013; Schafer and Yoder 1994; Yuan *et al.* 2009). Undoubtedly, as our capacity to distinguish fungal subspecies and strains improves, so too will the observance of even more situations where specificity occurs (Arnold 2007; Brodeur 2012).

The adaptive significance of specificity in plant-fungal associations is poorly understood. However, Den Bakker *et al.* (2004) found that fungal specialists were able to obtain more carbon from their host than generalists. This is particularly the case within those fungi which associate with members of the Orchidaceae (Dearnaley *et al.* 2012). The benefits for plants seem to be less clear since associating with a generalist would mean that seed germination and growth would not be restricted (Bruns *et al.* 2002; Den Bakker *et al.* 2004). However, in the case of mycorrhizal associations (Den Bakker *et al.*

2004; Molina *et al.* 1997); (Taylor *et al.* 2003) or the mutual carbon exchange observed in the Orchidaceae (Cameron *et al.* 2006), clear nutritional benefits to the host plant may be observed. Specificity in these cases may lead to a more efficient exchange of nutrients and increased seed viability, thereby giving rise to improved overall plant fitness (Dearnaley *et al.* 2012). Fungal specificity often occurs therefore, more frequently when it serves some physiological role such as in mutualistic exchanges, or biotrophic pathogenesis (Blackwell 2011; Brodeur 2012; Den Bakker *et al.* 2004; Lindblad 2000; Otero *et al.* 2002; Otero *et al.* 2007). Two interesting exceptions should be noted here, obligate mycoheterotrophic plants are known to form highly specific associations with mycorrhiza but 'cheat' their fungal partner out of any real benefits (Hynson and Bruns 2010). Arbuscular mycorrhizal (AM) plants, on the other hand, do not form specific fungal associations despite only a relatively small number of species of fungi being known to associate with a over 300,000 species of AM plants (Parniske 2008).

While most fungi are typically generalists in nature, fungal specificity may occur in particular specialised ecological niches (Arnold and Lutzoni 2007; Brodeur 2012; Bruns *et al.* 2002; Davey *et al.* 2013). Ectomycorrhizal fungi such as *Rhizopogon* or *Suillus* are well known to interact almost exclusively with the Pinaceae family (Den Bakker *et al.* 2004; Molina *et al.* 1997). Many further examples of specific mycorrhizal associations have also been observed, particularly within the Orchidaceae (Bougoure *et al.* 2009; Dearnaley *et al.* 2012; Graham and Dearnaley 2012; Irwin *et al.* 2007; Otero *et al.* 2002; Otero *et al.* 2007). Graham and Dearnaley (2012) observed a single *Ceratobasidium* species forming associations with the epiphytic orchid *Sarcochilus weinthalii*. Similarly, Irwin *et al.* (2007) also observed specific interactions occurring between two different

Ceratobasidium species and the terrestrial orchid *Pterostylis nutans* across a wide region of eastern Australia. In the case of the Orchidaceae, fungal specificity may further vary depending on whether the orchid is a seed or a mature adult plant (Dearnaley *et al.* 2012; Dearnaley 2007; Taylor *et al.* 2003). It should be noted that specific mycorrhizal associations are not restricted to the Orchidaceae but also occur within many plant families (Taylor *et al.* 2003). Pathogenic fungi also form typically specific host interactions which can be observed occurring across a broad range of fungal lineages (van der Does and Rep 2007). In some cases, such as the rusts and smuts, this may even involve multiple hosts in the completion of the life cycle of the fungi (Barrett *et al.* 2008; Paine *et al.* 1997).

Specificity within endophytic fungi has been observed in recent years (Arnold and Lutzoni 2007; Otero *et al.* 2002; Otero *et al.* 2007). While many endophytes are described as generalists, these are typically isolated using conventional plating methods which tend to favour generalists with broad host ranges and an ability to grow rapidly and easily on agar (Arnold and Lutzoni 2007). However examinations of those fungi which cannot be cultured paints a very different species composition and would imply that specificity may frequently be missed (Arnold 2007; Guo *et al.* 2001). Different assemblages of fungi are often observed depending on the tissue being examined and these may exhibit a level of tissue specificity (Helander *et al.* 1993; Suryanarayanan *et al.* 2011). Class 3 endophytes, those largely restricted to above ground tissues, are rarely thought to exhibit specificity (Rodriguez *et al.* 2009). More research is still needed to determine the extent to which specificity occurs within fungal endophyte associations.

3.2 Aims

This study aimed to determine if fungal specificity was occurring within a particular key plant species of SEVT.

The following questions were addressed in this chapter:

1. Does *Geijera salicifolia* exhibit fungal specificity?
2. Are there differences in overall diversity between endophytes from *Geijera salicifolia* plants at different locations of SEVT?
3. Does the species assemblage of *Geijera salicifolia* differ to those found in other plant species within SEVT?

A combination of molecular and traditional methods of assessment were used to determine overall fungal taxon composition within this host plant over five sites. This data was then further compared with the diversity observed in the study outlined in Chapter 2.

3.3 Methods

3.3.1 Sample collection

Leaf samples from 22 plants of *Geijera salicifolia* were collected from SEVT across five sites in the months of March in 2010 and 2011 (Figure 4). A total of six leaves were collected from each tree; two leaves each from high in the canopy, mid level and from the lowest branches. Six trees were sampled from Birdwood, five from Boodua and Highfields, four from Mt Kingsthorpe and two from Mt Tyson. These five sites were all listed as regional ecosystem type 11.8.3; SEVT on Cainozoic igneous rock. The purpose of this site selection was to minimise any variation that may be due to habitat type.

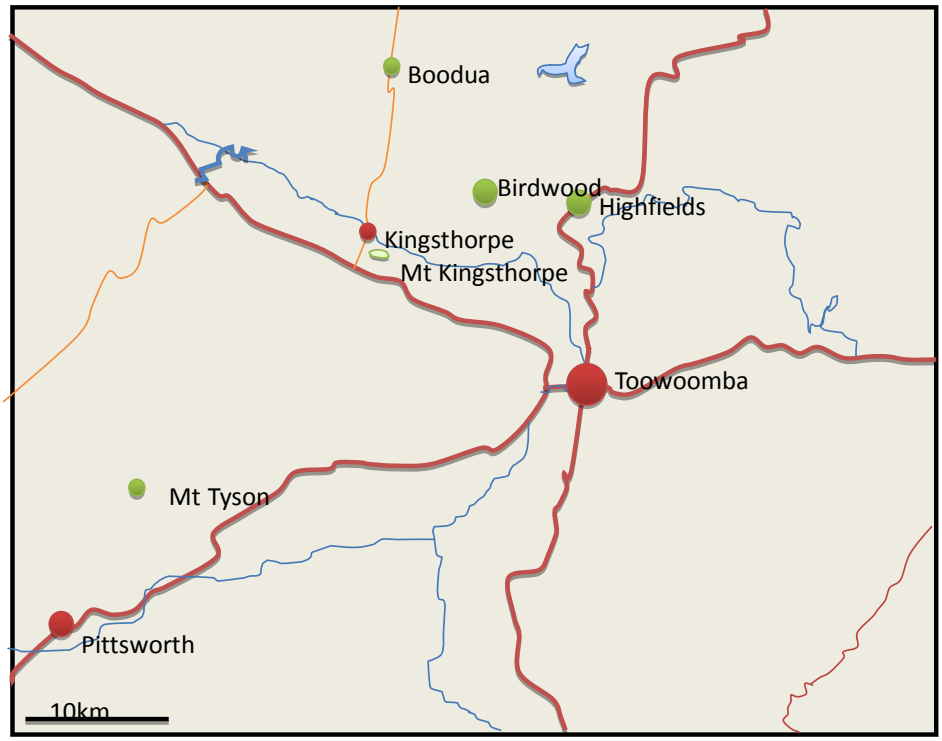

Figure 4 Location of SEVT sampled in this study. Sites indicated in green.

Endophytes were isolated by using a variation of the method developed by Boddington (2009) as outlined in chapter 2. Leaves were surface sterilised to remove any fungal epiphytes by first washing vigorously in tap water three times and then by soaking in sterile milli-q water for at least three minutes. After immersing in 95% ethanol for 90 seconds, leaves were rapidly passed through a flame and then pressed onto a control plate prior to sampling. This was to ensure that the surface sterilisation procedure had been successful. Eight tissue segments were removed using a sterile hole punch and arranged onto a potato dextrose agar (PDA) containing 1.5mg/ml sterile streptomycin and tetracycline. This was conducted in duplicate for each sample. Plates were then incubated in the dark at 23^{o}C and checked regularly for growth. When hyphae were observed to grow from the leaf material, they were transferred to a fresh PDA plate without antibiotics.

Colonisation Rates (CR) were determined based on %= N_i/N_s X 100 and as a measure of species richness, isolation rates (IR) were based on N_e/N_s. Where N_i=number of samples with at least one isolate, N_e=number of endophytes collected and N_s= total number of samples (Douanla-Meli and Langer 2012; Fróhlich *et al.* 2000).

3.3.2 Fungal identification

Fungal endophytes were identified based on a combination of morphological characteristics and molecular methods. Fungi which produced spores were identified using the following guides: (Bhimba *et al.* 2012; Carvalho *et al.* 2012; Ellis 1960). In some cases, fungal endophytes were only able to be identified to the genus or family level (Huang *et al.* 2009). Sterile fungal isolates were moved to 4^{o}C to encourage the production of spores. If colonies still did not sporulate, isolates were identified using molecular methods (see below).

To determine if unculturable endophytes were present, leaf samples from *G. salicifolia* from all sites were kept and DNA extracted and PCR amplified using fungal specific primers for further analysis (as per below)

3.3.3 DNA extraction

DNA was extracted from all endophytes and leaf discs within 24 hours of collection or in the case of fungal cultures, once suitable growth was observed. DNA was extracted using the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich) following the manufacturers' instructions. DNA from leaf samples was additionally purified prior to PCR amplification to remove tannins and other inhibitory compounds using a DNA purification kit (Macherey Nagel, Cheltenham, Australia) following the manufacturer's instructions for large fragment DNA.

DNA extracted from the plant tissue appeared brown in colour. When the initially extracted sample was run on an agarose gel, DNA appeared undegraded, however PCR amplification was unsuccessful. Serial dilutions of samples showed that lack of PCR amplification was not a DNA concentration issue. When a known, previously successfully amplified sample of fungal DNA was combined with the plant/fungal DNA (extracted from the plant tissue) it could no longer be amplified, indicating the presence of inhibitors in the plant/fungal DNA. Small molecule inhibitors such as salts were removed through the use of a Millipore Drop Dialysis 0.025μM V membrane (Millipore, MA, USA) however inhibition was still observed. Given the continued brown colour of the solution, it is therefore most likely that tannins were present and responsible for the PCR inhibition. Several attempts to clean the DNA were then undertaken involving several different purification kits including PowerCleanDNA clean-up Kit (Mo-bio, US); Diffinity tips (Fisher Biotec, Wembley WA); Nucleospin (Macherey and Nagel, Cheltenham, Australia) as per instructions and with suggested modifications for purification of large fragment DNA). When none of these were successful in removing the inhibitors from the DNA, additives to the PCR reaction were tried. This included BSA and DMSO at starting concentrations of 400ng/ml-160μg/ml but these were also unsuccessful in allowing PCR amplification.

Extracted fungal DNA was amplified using fungal specific primers. PCR amplification involved adding 1μL of the extracted DNA to 7μL sterile milli-Q water, 10μL of the PCR ready mix (Sigma-Aldrich) and 1μL of each of the fungal specific primer ITS1F (Gardes and Bruns 1993) and the ITS4 primer (White *et al*. 1990).

All initial PCR reactions were performed in duplicate and a control tube containing 1µl of sterile H_2O instead of DNA was also included. Samples were amplified with 35 cycles of 95°C for one minute, 50°C for one minute and 72°C for one minute, with a final elongation step of ten minutes at 72°C. 2µl of the PCR product was then run on a 2% electrophoresis gel and viewed under UV light using a Quantum ST4 gel documentation system (Vilber Lourmat, Fisher Biotec) and photographed using a Quantum capture ST4® image acquisition and analysis software package.

PCR products were purified in preparation for sequencing using a DNA purification kit (Macherey Nagel or Diffinity purification tips (Fisher Biotec) as per the manufacturer's instruction. 2µl of the resulting purified DNA was then run on a 2% w/v agarose gel to check the concentration of the purified sample.

3.3.4 Sequencing of isolated DNA

Samples containing between 17ng and 30ng of DNA were sent to the Brisbane laboratory of the Australian Genome Research Facility (AGRF) for sequencing. Upon receipt of the returned sequences, samples were viewed using the Chromas® 2.0 program to check for contamination. A BLAST search using the National Centre for Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov/> was used to determine closest species matches.

3.3.5 Statistical analysis

A t-test was used to first determine if there were any significant differences between the sampling years and a univariate analysis was used to determine differences in IR of endophytes between site and within sites as well as species abundance using SPSS (IBM_Corp. Released 2012). The homogeneity of variance was tested and Tukey post

hoc tests were used to confirm the suitability of the data. Data was checked for normalcy prior to analysis and those which did not display a normal distribution were then log transformed.

3.4 Results

187 fungal endophytes were isolated from 132 leaves. These were collected from 22 plants of *Geijera salicifolia* across the five sites. The overall mean CR was 80.3% and overall mean IR was 0.7. 40 isolates were identified via morphological characteristics. Of the remaining 148, DNA was successfully obtained from all samples, although the ITS region could not be amplified for 16 samples. These non-amplified isolates had no obvious identifying morphological features (Figure 5) and were classed as mycelia sterilia. The culture characteristics of these samples, such as hyphae colour, growth rate, growth form, and the ability to stain the agar, appeared very different from each other and these were therefore classed as separate taxa (Figure 5).

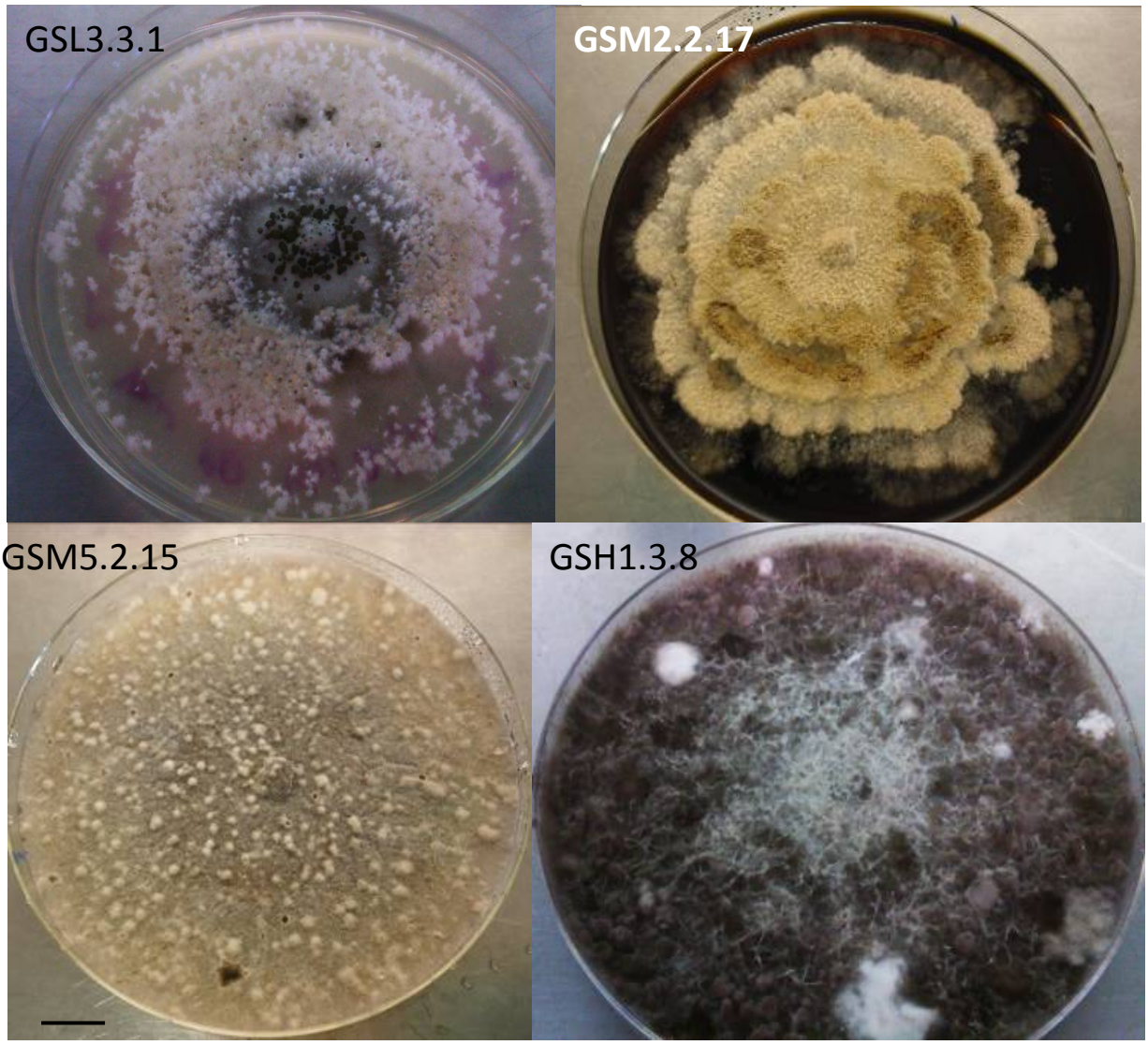

Figure 5 Example of the diverse culture characteristics of isolates classed as mycelia sterilia. Scale bar = approximately 1cm

Preliminary analysis found no significant differences in isolates collected in the two different sampling years (F=2.355, DF=64, P=0.408; see appendices Table B.18) so further analysis of the data was performed. A univariate analysis found no significant differences between numbers of fungi isolated at each of the sites or from within the canopy (high mid low; site: P= 0.351 DF=4, canopy location P= 0.305 DF=2, site and canopy location P= 0.305 DF=8; see appendices Table B.19). On average, 2.79

(SE=0.232) isolates were collected at each site and location within the canopy (Figure 6) with an average of 8.5 (SE=0.76) isolates per tree.

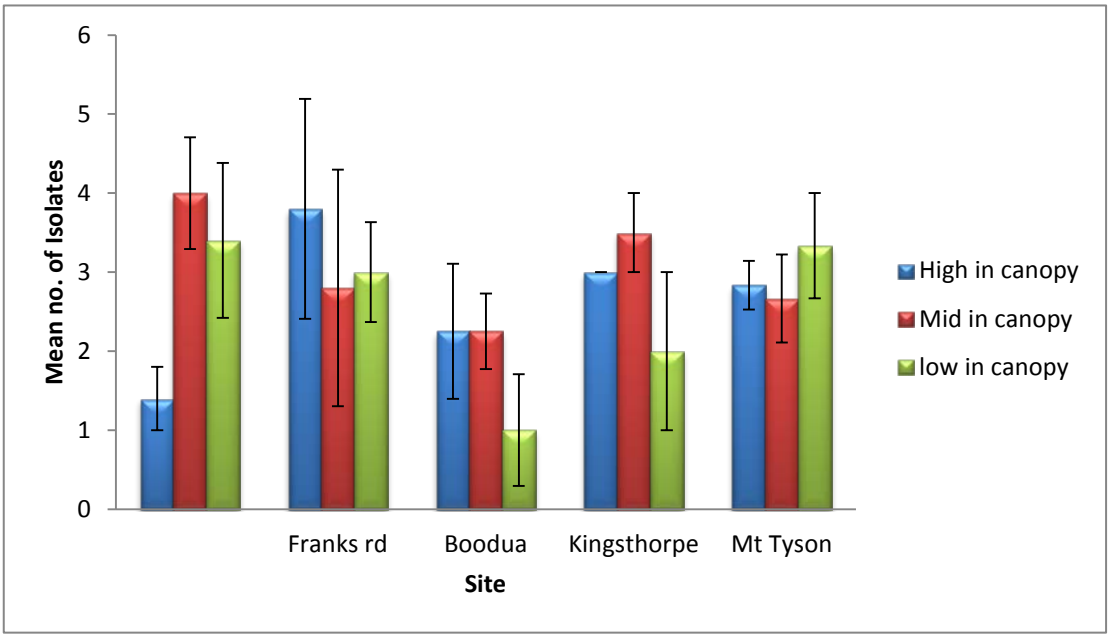

Figure 6 Average number (±SE) of isolates collected per site and location within each site.

The most commonly isolated genera were *Guignardia* (48), *Nigrospora* (23), *Xylaria* (12), and *Preussia*/*Sporomiella* (7). *Phoma*, *Phomopsis* and *Neofussicoccum* were also isolated five times each and species from the order Pezizales were isolated 24 times (Table 6). A univariate analysis of the top six most common species found statistically more *Guignardia* were present across the five sites (F=3.6, DF=5, P=0.14; see appendices Table B. 20).

Of the 48 isolates of *Guignardia,* 15 were identified via morphology and the remaining 33 were identified through molecular methods. Of these, 27 had sequence similarities of less than 95% to other known sequences of *Guignardia* in GenBank (Table 6). 22 isolates had a sequence similarity of 93% or less to the same isolate of *Guignardia bidwellii* (HM049171.1). Comparison of these sequences found that similarity between

isolates was between 98-100%. Six isolates had a closest match to various *G. mangiferae* sequences in GenBank (Table 6). Closest matches ranged from 93% for two isolates (GSM1.2.8 and GSH2.1.10) to 98-100% similarity for the remaining four. When these two were compared to each other via BLAST 2 sequence analysis they were found to be 98% similar to each other but only 90% similar to the remaining four taxa. When GSM1.2.8 and GSH2.1.10 were compared to other isolates with higher sequence similarities to *G. bidwellii* they were found to be 99% similar. Two isolates (GSH5.4.3 and GSH5.5.11) had a 95% sequence similarity to FJ418187.1 *G. vaccinii* (Table 6). When these sequences were compared to each other however, they were found to be 87% similar. The remaining two isolates were 93% similar to AB454283.1 *G. ardisiae* and were found to be 99% similar to each other (Table 6). Based on sequence similarity analysis, six species of *Guignardia* were therefore isolated in this study.

All *Nigrospora* isolates that were identified through sequence analysis were found to be between 98-99% similar to other *Nigrospora* sequences in GenBank. These were either *N. oryzae* or *N. sphaerica.* Given that both these species are now considered to be *Khuskia oryzae*, only one species of *Nigrospora*/ *Khuskia* was therefore observed.

Table 6 Endophytic fungi isolated from *G.salicifolia* with closest GenBank match where applicable.

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Birdwood	1	Low	GSL5.1.1	JN207335.1 *Nigrospora* sp.	525/538	98%	*Nigrospora* sp.	KF128862
Birdwood	1	Low	GSL5.1.2	AF485074.1 Pezizales sp.	558/582	96%	*Sarcosomataceae* sp.	KF128863
Birdwood	1	Low	GSL5.1.3				*Nigrospora* sp.	
Birdwood	1	Low	GSL5.1.4	EU272486.1 *Nigrospora oryzae*	532/542	98%	*Nigrospora* sp.	KF128874
Birdwood	1	Mid	GSM5.1.5	HM123589.1 Fungal sp.	565/594	95%	*Sarcosomataceae* sp.	KF128875
Birdwood	1	Mid	GSM5.1.6				*Xylaria* sp.	
Birdwood	1	Mid	GSM5.1.7	HQ608062.1 *Nigrospora oryzae*	495/507	98%	*Nigrospora* sp.	KF128876
Birdwood	1	Mid	GSM5.1.8	HQ630982.1 *Nigrospora* sp.	491/497	99%	*Nigrospora* sp.	KF128877
Birdwood	1	High	GSH5.1.9	JN185456.1 *Nigrospora oryzae*	423/430	98%	*Nigrospora* sp.	KF128878
Birdwood	1	High	GSH5.1.10	AF485074.1 Sarcosomataceous sp.	541/572	95%	*Sarcosomataceae* sp.	KF128879
Birdwood	1	High	GSH5.1.11	JQ717319.1 *Acremonium* sp.	541/558	97%	*Acremonium* sp.	KF128880
Birdwood	2	Low	GSL5.2.12				*Xylaria* sp.	
Birdwood	2	Low	GSL5.2.13	JQ760520.1 Pezizomycetes sp.	517/556	93%	*Sarcosomataceae* sp.	KF128881
Birdwood	2	Low	GSL5.2.14				Mycelia sterilia 10	
Birdwood	2	Mid	GSM5.2.15				Mycelia sterilia 13	
Birdwood	2	High	GSH5.2.16				Mycelia sterilia 5	
Birdwood	2	High	GSH5.2.17	JX298897.1 *Absconditella sphagnorum*	440/473	93%	*Absconditella* sp.	KF128882
Birdwood	3	Low	GSL5.3.1				*Guignardia* sp.	
Birdwood	3	Low	GSL5.3.2	JQ760277.1 Sarcosomataceae sp.	488/533	92%	*Sarcosomataceae* sp.	KF128832

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Birdwood	3	Low	GSL5.3.3	HM068372.1 *Pestalotiopsis* sp	611/618	99%	*Pestalotiopsis* sp.	KF128833
Birdwood	3	Mid	GSM5.3.4	EU167596.1 *Mycosphaerella coacervata*	534/540	99%	*Mycosphaerella coacervata*	KF128849
Birdwood	3	Mid	GSM5.3.5	AY555731.1 *Muscodor albus*	590/612	96%	*Muscodor* sp.	KF128812
Birdwood	3	Mid	GSM5.3.6				Mycelia sterilia 14	
Birdwood	3	High	GSH5.3.7				Mycelia sterilia 6	
Birdwood	3	High	GSH5.3.8				Mycelia sterilia 7	
Birdwood	3	High	GSH5.3.9				Mycelia sterilia 8	
Birdwood	4	Low	GSL5.4.1	HM222948.1 *Xylaria* sp.	490/543	90%	*Xylaria* sp.	KF128834
Birdwood	4	Mid	GSM5.4.2	HQ889723.1 *Nigrospora sphaerica*	540/544	99%	*Nigrospora* sp.	KF128850
Birdwood	4	High	GSH5.4.3	FJ418187.1 *Guignardia vaccinii*	513/535	95%	*Guignardia* sp.	
Birdwood	4	High	GSH5.4.4				*Guignardia* sp.	
Birdwood	4	High	GSH5.4.5	AB454283.1 *G. ardisiae*	599/644	93%	*Guignardia* sp.	KF128813
Birdwood	5	Low	GSL5.5.1	JQ743587.1 *G. mangiferae*	622/626	98%	*Guignardia* sp.	KF128835
Birdwood	5	Low	GSL5.5.2	HM049170.1 *G.bidwellii*	582/639		*Guignardia* sp.	
Birdwood	5	Low	GSL5.5.3	EU236704.1 *Phomopsis* sp.	536/555	97%	*Phomopsis* sp.	KF128836
Birdwood	5	Low	GSL5.5.4				*Guignardia* sp.	
Birdwood	5	Low	GSL5.5.5	HM049170.1 *Guignardia bidwellii*	582/639	91%	*Guignardia* sp.	KF128837
Birdwood	5	Low	GSL5.5.6				*Guignardia* sp.	
Birdwood	5	Mid	GSM5.5.7	AY555731.1 *Muscodor albus*	599/623	96%	*Muscodor* sp.	KF128852
Birdwood	5	Mid	GSM5.5.8	AB041241.1 *Guignardia mangiferae*	606/606	100%	*Guignardia* sp.	KF128853

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Birdwood	5	Mid	GSM5.5.9	JQ760520.1 Pezizomycetes sp.	569/601	95%	*Sarcosomataceae* sp.	KF128854
Birdwood	5	Mid	GSM5.5.10	AY816311.1 *Guignardia mangiferae*	617/618	99%	*Guignardia* sp.	KF128851
Birdwood	5	High	GSH5.5.11	FJ418187.1 *G. vaccinii*	509/531	95%	*Guignardia* sp.	KF128814
Birdwood	5	High	GSH5.5.12	AF485074.1 Sarcosomataceous sp.	559/589	95%	*Sarcosomataceae* sp.	KF128815
Birdwood	5	High	GSH5.5.13	JQ760638.1 Pezizomycetes sp.	572/601	95%	*Sarcosomataceae* sp.	KF128816
Birdwood	5	High	GSH5.5.14	EU573703.1 *Apodospora peruviana*	515/526	98%	*Apodospora peruviana*	KF128817
Birdwood	6	Low	GSL5.6.1	JN207296.1 *Nigrospora* sp.	531/532	99%	*Nigrospora* sp.	KF128838
Birdwood	6	Low	GSL5.6.2	HQ829058.1 *Pyronema* sp.	570/571	99%	*Pyronema* sp.	KF128839
Birdwood	6	Low	GSL5.6.3	JF502436.1 Sarcosomataceae sp.	541/562	96%	*Sarcosomataceae* sp.	KF128840
Birdwood	6	Mid	GSM5.6.4	JQ760432.1 Sarcosomataceae sp.	565/598	94%	Sarcosomataceae sp.	KF128855
Birdwood	6	Mid	GSM5.6.5				Mycelia sterilia 15	
Birdwood	6	Mid	GSM5.6.6	AF485074.1 Sarcosomataceae sp.	559/584	96%	Sarcosomataceae sp.	KF128856
Birdwood	6	High	GSH5.6.7	AY681176.1 *Neurospora terricola*	581/589	99%	*Neurospora terricola*	KF128818
Birdwood	6	High	GSH5.6.8	HQ829058.1 *Pyronema* sp.	502/536	94%	*Pyronema* sp.	KF128819
Boodua	1	Low	GSL2.1.1	JN418768.1 *Preussia* sp.	492/496	99%	*Preussia* sp.	KF128767
Boodua	1	Low	GSL2.1.2				*Nigrospora* sp.	
Boodua	1	Mid	GSM2.1.3	JQ760747.1 Pezizomycetes sp.	494/521	95%	*Sarcosomataceae* sp.	KF128771
Boodua	1	Mid	GSM2.1.4	HQ392721.1 *Neofusicoccum luteum*	560/566	99%	*Neofusicoccum luteum*	KF128772
Boodua	1	Mid	GSM2.1.5	HQ130663.1 *Sporormiella* sp.	512/520	98%	*Preussia* sp.	KF128773
Boodua	1	Mid	GSM2.1.6				*Xylaria* sp.	
Boodua	1	Mid	GSM2.1.7	HQ130664.1 *Sporormiella* sp.	532/543	98%	*Preussia* sp.	KF128774

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Boodua	1	Mid	GSM2.1.8	AF485074.1 Sarcosomataceous sp.	559/586	95%	*Sarcosomataceae* sp.	KF128775
Boodua	1	Mid	GSM2.1.9	DQ923533.1 *Neofusicoccum corticosae*	552/559	99%	*Neofusicoccum corticosae*	KF128776
Boodua	1	High	GSH2.1.10	JF261459.1 *Guignardia mangiferae*	546/590	93%	*Guignardia* sp.	KF128858
Boodua	1	High	GSH2.1.11				*Nigrospora* sp.	
Boodua	1	High	GSH2.1.12	HM049170.1 *Guignardia bidwellii*	586/631	93%	*Guignardia* sp.	KF128768
Boodua	1	High	GSH2.1.13	HM049170.1 *Guignardia bidwellii*	586/632	93%	*Guignardia* sp.	KF128769
Boodua	1	High	GSH2.1.14	HM049170.1 *Guignardia bidwellii*	585/630	93%	*Guignardia* sp.	KF128770
Boodua	1	High	GSH2.1.15				*Guignardia* sp.	
Boodua	2	Low	GSL2.2.26	JF694936.1 *Nigrospora* sp.	520/530	98%	*Nigrospora* sp.	KF128783
Boodua	2	Low	GSL2.2.27				*Nigrospora* sp.	
Boodua	2	Mid	GSM2.2.16				*Xylaria* sp.	
Boodua	2	Mid	GSM2.2.17				Mycelia sterilia 11	
Boodua	2	Mid	GSM2.2.18	AB524025.1 *Xylaria grammica*	553/565	98%	*Xylaria grammica*	KF128777
Boodua	2	Mid	GSM2.2.28	HM049170.1 *Guignardia bidwellii*	586/633	93%	*Guignardia* sp.	KF128784
Boodua	2	Mid	GSM2.2.29	AB440105.1 Xylariaceae sp.	526/527	99%	Xylariaceae sp.	KF128785
Boodua	2	High	GSH2.2.19	HM049170.1 *Guignardia bidwellii*	587/632	93%	*Guignardia* sp.	KF128778
Boodua	2	High	GSH2.2.20	HM049170.1 *Guignardia bidwellii*	584/633	92%	*Guignardia* sp.	KF128779
Boodua	2	High	GSH2.2.21	HM049170.1 *Guignardia bidwellii*	587/635	92%	*Guignardia* sp.	KF128780
Boodua	2	High	GSH2.2.22				Mycelia sterilia 2	
Boodua	2	High	GSH2.2.23	HQ607802.1 *Preussia* sp.	528/543	97%	*Preussia* sp.	KF128781

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Boodua	2	High	GSH2.2.24				*Nigrospora* sp.	
Boodua	2	High	GSH2.2.25	JQ316440.1 Fungal endophyte	528/545	97%	Sordariomycete	KF128782
Boodua	3	Low	GSL2.3.1	HQ832828.1 *Nigrospora* sp	538/542	99%	*Nigrospora* sp.	KF128869
Boodua	3	Low	GSL2.3.2	AY219880.1 *Lecythophora* sp.	501/534	94%	*Lecythophora* sp.	KF128870
Boodua	3	Low	GSL2.3.3				*Nigrospora* sp.	
Boodua	3	Low	GSL2.3.4				*Xylaria* sp.	
Boodua	3	High	GSH2.3.5				*Xylaria* sp.	
Boodua	3	High	GSH2.3.6				Mycelia sterilia 3	
Boodua	3	High	GSH2.3.7				*Coniochaeta* sp.	
Boodua	3	High	GSH2.3.8	HQ599602.1 *Devriesia fraseriae*	528/549	96%	*Devriesia* sp.	KF128864
Boodua	3	High	GSH2.3.9	EU826926.1 Uncultured soil fungus clone	519/542	96%	Hyphomycete	KF128865
Boodua	4	Low	GSL2.4.1	DQ885897.1 *Microdiplodia hawaiiensis*	546/558	98%	*Microdiplodia* sp.	KF128824
Boodua	4	Low	GSL2.4.2	DQ885897.1 *M.hawaiiensis*	545/561	97%	*Microdiplodia* sp.	KF128825
Boodua	4	High	GSH2.4.3	JF499844.1 *Teratosphaeria cf. bellula*	503/535	94%	*Teratosphaeria* sp.	KF128826
Boodua	5	Low	GSL2.5.1	HM049170.1 *Guignardia bidwellii*	587/631	93%	*Guignardia* sp.	KF128827
Boodua	5	Low	GSL2.5.2	HM049170.1 *Guignardia bidwellii*	587/632	93%	*Guignardia* sp.	KF128828
Boodua	5	Low	GSL2.5.3	HM049170.1 *Guignardia bidwellii*	586/631	93%	*Guignardia* sp.	KF128829
Boodua	5	Low	GSL2.5.4	HM049170.1 *Guignardia bidwellii*	588/633	93%	*Guignardia* sp.	KF128830
Boodua	5	Low	GSL2.5.5	AJ507323.4 *Phaeococcomyces*	572/641	89%	*Phaeococcomyces* sp.	KF128831

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
				chersonesos				
Boodua	5	Mid	GSM2.5.6	HQ599596.1 *Strelitziana eucalypti*	550/622	88%	*Chaetothyriales* sp.	
Boodua	5	Mid	GSM2.5.7	JN225892.1 *Phaeomoniella* sp	577/596	97%	*Phaeomoniella* sp.	KF128848
Highfields	1	Low	GSL1.1.1				*Nigrospora* sp.	
Highfields	1	Low	GSL1.1.2	JN712460.1 *Diaporthe leucospermi*	510/512	99%	*Phomopsis* sp.	KF128866
Highfields	1	Low	GSL1.1.3				*Acremonium* sp.	
Highfields	1	Low	GSL1.1.4	FJ538333.1 *Guignardia mangiferae*	583/586	99%	*Guignardia* sp.	KF128867
Highfields	1	Low	GSL1.1.5	HM595506.1 *Phomopsis* sp.	517/536	96%	*Phomopsis* sp.	KF128868
Highfields	1	Mid	GSM1.1.6				*Nigrospora* sp.	
Highfields	1	Mid	GSM1.1.7				*Epiccocum* sp.	
Highfields	1	Mid	GSM1.1.8				Mycelia sterilia	
Highfields	2	Low	GSL1.2.1	HM049170.1 *Guignardia bidwellii*	581/633	92%	*Guignardia* sp.	KF128759
Highfields	2	Low	GSL1.2.2	GQ169494.1 *Cladosporium* sp.	529/530	99%	*Cladosporium* sp.	KF128761
Highfields	2	Low	GSL1.2.3	HM049170.1 *Guignardia bidwellii*	588/633	93%	*Guignardia* sp.	KF128762
Highfields	2	Low	GSL1.2.4				*Guignardia* sp.	
Highfields	2	Low	GSL1.2.5	AY620999.1 *Phomopsis* sp.	569/578	98%	*Phomopsis* sp.	KF128763
Highfields	2	Mid	GSM1.2.6	HM049170.1 *Guignardia bidwellii*	591/637	93%	*Guignardia* sp.	KF128764
Highfields	2	Mid	GSM1.2.7	HM049170.1 *Guignardia bidwellii*	581/631	92%	*Guignardia* sp.	KF128765
Highfields	2	Mid	GSM1.2.8	JF261459.1 *Guignardia mangiferae*	546/584	93%	*Guignardia* sp.	KF128857
Highfields	2	Mid	GSM1.2.9	EU715609.1 *Xylaria* sp.	565/572	99%	*Xylaria* sp.	KF128872
Highfields	2	Mid	GSM1.2.10	HM049170.1 *Guignardia bidwellii*	588/635	93%	*Guignardia* sp.	KF128760

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Highfields	2	Mid	GSM1.2.11				*Guignardia* sp.	
Highfields	2	High	GSH1.2.12				*Sarcosomataceae* sp.	
Highfields	2	High	GSH1.2.13				*Xylaria* sp.	
Highfields	3	Low	GSL1.3.1	HM049170.1 *Guignardia bidwellii*	596/640	93%	*Guignardia* sp.	KF128820
Highfields	3	Low	GSL1.3.2	HQ607898.1 *Preussia* sp.	531/541	98%	*Preussia* sp.	KF128821
Highfields	3	Low	GSL1.3.3				*Guignardia* sp.	
Highfields	3	Low	GSL1.3.4				*Guignardia* sp.	
Highfields	3	Low	GSL1.3.5				*Guignardia* sp.	
Highfields	3	Mid	GSM1.3.6	HM049170.1 *Guignardia bidwellii*	591/638	93%	*Guignardia* sp.	KF128841
Highfields	3	Mid	GSM1.3.7	AY327476.1 *Xylaria hypoxylon*	767/810	95%	*Xylaria* sp.	
Highfields	3	High	GSH1.3.8				Mycelia sterilia 1	
Highfields	3	High	GSH1.3.9	EU482261.1 *Xylaria* sp.	549/549	100%	*Xylaria* sp.	KF128808
Highfields	4	Low	GSL1.4.1	HQ832835.1 *Nigrospora* sp.	550/553	99%	*Nigrospora* sp.	KF128822
Highfields	4	Mid	GSM1.4.2	HM049170.1 *Guignardia bidwellii*	594/639	93%	*Guignardia* sp.	KF128842
Highfields	4	Mid	GSM1.4.3	HQ631023.1 *Epicoccum* sp.	547/549	99%	*Epiccocum* sp.	KF128843
Highfields	4	Mid	GSM1.4.4				*Guignardia* sp.	
Highfields	4	Mid	GSM1.4.5	AB470886.1 *Phoma herbarum*	520/522	99%	*Phoma herbarum*	KF128844
Highfields	4	Mid	GSM1.4.6	AB470886.1 *Phoma herbarum*	505/519	97%	*Phoma sp.*	KF128845
Highfields	4	High	GSH1.4.7	DQ682580.1 *Fusarium* sp.	546/553	99%	*Fusarium* sp.	KF128809
Highfields	4	High	GSH1.4.8	JN225913.*1 Coniochaeta* sp.	548/555	99%	*Coniochaeta* sp.	KF128810
Highfields	5	Low	GSL1.5.1	HM123375.1 Sarcosomataceae sp	560/582	96%	*Sarcosomataceae* sp.	KF128823

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Highfields	5	Mid	GSM1.5.2	HM123375.1 Sarcosomataceae sp	509/527	97%	*Sarcosomataceae* sp.	KF128846
Highfields	5	Mid	GSM1.5.3	JQ809680.1 *Guignardia* sp.	631/636	99%	*Guignardia* sp.	KF128847
Highfields	5	Mid	GSM1.5.4				*Guignardia* sp.	
Highfields	5	Mid	GSM1.5.5				*Guignardia* sp.	
Highfields	5	High	GSH1.5.6	FJ037749.1 *Preussia* sp.	526/538	98%	*Preussia* sp.	KF128811
Mt Kingsthorpe	1	Low	GSL3.1.1	AB625411.1 *Xylaria grammica*	547/559	98%	*Xylaria grammica*	
Mt Kingsthorpe	1	Mid	GSM3.1.2				*Nigrospora* sp.	
Mt Kingsthorpe	1	High	GSH3.1.3	AB741616.1 Xylariaceae sp.	485/534	91%	Xylariaceae sp.	KF128786
Mt Kingsthorpe	1	High	GSH3.1.4				*Nigrospora* sp.	
Mt Kingsthorpe	2	Mid	GSM3.2.1	DQ865095.1 *Preussia africana*	506/509	99%	*Preussia africana*	JN418775
Mt Kingsthorpe	2	Mid	GSM3.2.2	EF445349.1 *Neofusicoccum vitifusiforme*	386/387	99%	*Neofusicoccum vitifusiforme*	KF128873
Mt Kingsthorpe	3	Low	GSL3.3.1				Mycelia sterilia 9	
Mt Kingsthorpe	3	Low	GSL3.3.2				*Guignardia* sp.	
Mt Kingsthorpe	3	Low	GSL3.3.3				*Guignardia* sp.	
Mt Kingsthorpe	3	Mid	GSM3.3.4	AF324336.1 *Muscodor albus*	586/616	95%	*Muscodor* sp.	KF128788
Mt Kingsthorpe	3	Mid	GSM3.3.5	JF449549.1 uncultured pleosporales	444/498	89%	Pleosporales	KF128789
Mt Kingsthorpe	3	Mid	GSM3.3.6	EF394822.1 *Mycosphaerella acaciigena*	522/526	99%	*Mycosphaerella acaciigena*	KF128790
Mt Kingsthorpe	3	High	GSH3.3.7	JQ732911.1 *Mycosphaerella crystallina*	533/539	99%	*Mycosphaerella crystallina*	KF128791
Mt Kingsthorpe	3	High	GSH3.3.8	AF485074.1 Sarcosomataceous sp.	561/591	95%	*Sarcosomataceae* sp.	KF128792

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Mt Kingsthorpe	3	High	GSH3.3.9	EF394823.1 *Mycosphaerella acaciigena*	519/523	99%	*Mycosphaerella acaciigena*	KF128793
Mt Kingsthorpe	3	High	GSH3.3.10	AB454283.1 *Guignardia ardisiae*	579/621	93%	*Guignardia* sp.	KF128787
Mt Kingsthorpe	4	Low	GSL3.4.1	AJ300332.1 *Cladosporium oxysporium*	381/382	99%	*Cladosporium oxysporium*	KF128794
Mt Kingsthorpe	4	Low	GSL3.4.2	DQ912693.1 *Phoma glomerata*	426/428	99%	*Phoma* sp.	KF128795
Mt Kingsthorpe	4	Low	GSL3.4.3	AF324336.1 *Muscodor albus*	590/593	99%	*Muscodor* sp.	
Mt Kingsthorpe	4	Mid	GSM3.4.4	DQ219433.1 *Nigrospora oryzae*	313/316	99%	*Nigrospora* sp.	
Mt Kingsthorpe	4	Mid	GSM3.4.5	KC311486.1 *Phoma aliena*	517/520	99%	*Phoma aliena*	
Mt Kingsthorpe	4	Mid	GSM3.4.6	AY620999.1 *Phomopsis* sp.	543/546	99%	*Phomopsis* sp.	
Mt Kingsthorpe	4	High	GSH3.4.7	FJ478134.1 *Nigrospora sphaerica*	531/538	98%	*Nigrospora* sp.	KF128792
Mt Kingsthorpe	4	High	GSH3.4.8				Mycelia sterilia 4	
Mt Kingsthorpe	4	High	GSH3.4.9	HM049170.1 *Guignardia bidwellii*	580/625	93%	*Guignardia* sp.	KF128793
Mt Tyson	1	Low	GSL4.1.1	JQ760747.1 Pezizomycetes sp.	495/520	95%	*Sarcosomataceae* sp.	KF128796
Mt Tyson	1	Low	GSL4.1.2	JN943389.1 *Cochliobolus cynodontis*	571/573	99%	*Cochliobolus cynodontis*	KF128797
Mt Tyson	1	Low	GSL4.1.3	JN207335.1 *Nigrospora* sp.	536/546	98%	*Nigrospora* sp.	KF128798
Mt Tyson	1	Mid	GSM4.1.4	HQ392721.1 *Neofusicoccum luteum*	555/558	99%	*Neofusicoccum luteum*	KF128799
Mt Tyson	1	Mid	GSM4.1.5				Mycelia sterilia 12	
Mt Tyson	1	Mid	GSM4.1.6	HQ392721.1 *Neofusicoccum luteum*	558/562	99%	*Neofusicoccum luteum*	KF128800
Mt Tyson	2	Low	GSL4.2.1	HQ130716.1 *Phoma* sp.	518/518	100%	*Phoma* sp.	KF128801
Mt Tyson	2	Low	GSL4.2.2	HQ130716.1 *Phoma* sp.	517/518	99%	*Phoma* sp.	KF128802
Mt Tyson	2	Low	GSL4.2.3	AF485074.1 Sarcosomataceous sp.	554/562	99%	*Sarcosomataceae* sp.	KF128871

Site	Tree no.	Location	Code	Closest Match	Identity	%	Proposed Identity	Genbank No.
Mt Tyson	2	Low	GSL4.2.4	JQ760277.1 Pezizomycetes sp.	474/522	91%	*Sarcosomataceae* sp.	KF128803
Mt Tyson	2	Mid	GSM4.2.5	EU552114.1 *Conoplea fusca*	580/619	94%	*Sarcosomataceae* sp.	KF128804
Mt Tyson	2	High	GSH4.2.6	JQ694104.1 *Diplodia* sp.	541/546	99%	*Diplodia* sp.	KF128805
Mt Tyson	2	High	GSH4.2.7	AF485074.1 Sarcosomataceous sp.	571/583	98%	*Sarcosomataceae* sp.	KF128806
Mt Tyson	2	High	GSH4.2.8	AF485074.1 Sarcosomataceous sp.	545/568	96%	*Sarcosomataceae* sp.	
Mt Tyson	2	High	GSH4.2.9	HQ829064.1 *Cladosporium* sp.	529/531	99%	*Cladosporium* sp.	KF128859
Mt Tyson	2	High	GSH4.2.10	HQ630993.1 *Paraphaeosphaeria* sp.	540/557	97%	*Paraphaeosphaeria* sp.	KF128860
Mt Tyson	2	High	GSH4.2.11	JQ781836.1 *Cladosporium tenuissimum*	514/514	100%	*Cladosporium tenuissimum*	KF128861

3.5 Discussion

This study represents the first detailed examination of fungal endophytes within the plant genus *Geijera*. 187 fungal endophytes from 22 plants of *Geijera salicifolia* from five separate SEVT were identified. No differences were observed in endophyte isolation rates between the various sites or at various heights within the canopy. The species observed in this study were similar to those identified in the initial diversity study outlined in chapter 2. Both studies found endophyte community compositions differed to those commonly observed in other studies. As discussed in chapter 2, novel species assemblages could in part be explained by the uniqueness of the habitat being examined (Espinosa Garcia and Langenheim 1990; Sakayaroj *et al.* 2010). It is interesting that closer examination of one plant species within SEVT supports the patterns of species observed in the broader study (with the possible exception of the higher number of *Guignardia* species). Furthermore, since this included a much broader range of sampling locations, this would further suggest that the endophytes sampled in this study are representative of overall patterns in SEVT.

The CR observed in this study was similar to other studies of plants within the Rutaceae, of which *Geijera* is a member. In a recent paper, Douanla-Meli *et al*. (2013) recorded a CR of 82.3% in *Diospyros crassiflora*. Similarly Glienke-Blanco *et al*. (Glienke-Blanco *et al.* 2002) reported a CR of 81% in *Citrus* spp. while Duran *et al*. (Denison 1969) observed a slightly lower value of 72% in *Citrus limon*. Studies examining fungal endophytes in other plant families found similar results to those in the Rutaceae. For example, Yuan *et al*. (Yuan *et al.* 2009) found leaf CR to be 69% in the Orchidaceae, while Arnold and Herre (Arnold and Herre 2003) found a leaf CR of 85% within the Malvaceae. The endophytic CR observed in this study are similar to those commonly observed within

rainforest trees (Douanla-Meli and Langer 2012; Fróhlich *et al.* 2000; Gamboa and Bayman 2001; Toofanee and Dulymamode 2002) and reflect those outlined in chapter 2. Arnold and Lutzoni (2007) showed that endophyte CR were strongly correlated to latitude. Wetter, more tropical regions contain the highest diversity which decreased as sites moved away from the equator (Arnold and Lutzoni 2007). The results of this study would suggest that CR of *G. salicifolia* do not differ greatly from CR observed in other rainforest species and are most likely a reflection of the overall CR within SEVT.

A major objective of this study was to determine any patterns of fungal specificity which may be occurring within *G. salicifolia* and potentially SEVT as a whole. Four groups of fungi appeared frequently in both studies, these were *Nigrospora*, *Guignardia*, *Preussia* and members of the Sarcosomataceae. *Guignardia* represented the most abundant fungal taxon in this study (26%) and was found to be occurring statistically more often than all other species. *Guignardia* species have frequently been recorded as endophytes in the Rutaceae. For example, in their study of three different citrus plants, Glienke-Blanco *et al.* (2002) recorded 27% of isolates belonged to the genus *Guignardia*. Given the large isolate numbers observed in this study, together with the known associations that *Guignardia* have been observed to form with other members of the Rutaceae, it would appear that *G. salicifolia* is exhibiting a level of specificity with several *Guignardia* species.

Guignardia was first described in 1892 by Viala and Ravaz and is the anamorphic name of the genus *Phyllosticta* (Wikee *et al.* 2011). However, as yet, very few *Guignardia* have been appropriately matched to their *Phyllosticta* counterparts (Su and Cai 2012). *Guignardia* are typified by the presence of dark brown globose or subglobose ostiolate

ascomata, a pseudoparenchymatous centrum, and distinct mucilaginous appendages which may occur at one or both ends of the spore (Hyde 1995). They commonly occur as endophytes with a broad host range and geographical observance (Peres *et al.* 2007; Rodrigues *et al.* 2004) and may also be latent pathogens activating in adverse environmental conditions (Barnett and Hunter 1998{Wikee, 2013 #467). *G. criticarpa* has been associated as the causative agent of black spot, a pathogen of citrus plants (Glienke *et al.* 2011; Okane *et al.* 2003; Peres *et al.* 2007), although Durán *et al.* (1969) observed it also occuring endophytically. Several *Guignardia* spp. have also been found to be pathogens of other plants such as mistletoe and *Aesculus* sp. (Pastirakova *et al.* 2009; Sultan *et al.* 2011). *Guignardia* has also been commonly observed as a generalist endophyte most typically in the form of *G. mangiferae* (Glienke-Blanco *et al.* 2002; Wulandari *et al.* 2009). Most research to date has focused on those species pathogenic to citrus plants (Glienke *et al.* 2011; Okane *et al.* 2003; Peres *et al.* 2007; Wulandari *et al.* 2009) or *G. mangiferae* (Glienke *et al.* 2011; Peres *et al.* 2007; Rodrigues *et al.* 2004) with comparatively little research examining other species which occur purely as endophytes in citrus (Glienke-Blanco *et al.* 2002; Ho *et al.* 2012). This study indicates that at least six species of *Guignardia* were inhabiting *G*. *salicifolia* plants, suggesting that the endophytic life mode is quite common for the *Guignardia* genus (Peres *et al.* 2007; Rodrigues *et al.* 2004).

A number of species of *Guignardia* have previously been observed in Australia. *G. criticarpa,* the causal agent of black spot in citrus plants, was first recorded in Sydney, Australia in 1895 but was not described until 1948 (Kiely 1948). *G. bidwelli* has been observed occurring pathogenically on grapevines (Sosnowski *et al.* 2012). When the anamorphic phase, *Phyllosticta*, is considered, many more species have been observed.

For example *P. annulicaudata, P. ashtonii, P. blackwoodiae, P. hemibrunnea*, *P. dianellicola* and *P. telopeae* were discovered on six separate Australian plants (Yip 1988, 1989). *P. beaumarisii* and *P. tortilicaudata* sp. nov. were observed occurring as pathogens of *Muehlenbeckia* sp and *Atherosperma moschatum* respectively (Paul and Blackburn 1986; Yip 1987). These species do not have corresponding sequences in GenBank with the exception of *G. criticarpa* and *G. bidwelli* and only short sequences of *P. beaumarisii* and *P. telopeae.* Given the relatively low sequence information occurring in GenBank for *Guignardia* or *Phyllosticta* it is difficult to determine, without morphological data, whether the species observed in this study represent new occurrences for Australia or whether they are indicative of previously described species. More research would need to be conducted to elucidate this.

Nigrospora taxa were also frequently isolated in this study. In the initial diversity study (chapter 2) this genus represented 28% of all endophytic isolates observed. However, in this study, the abundance of *Nigrospora* was considerably less and only accounted for 12% of the total species observed. In the initial diversity study, the high numbers of *Nigrospora* were attributed both to the ubiquitous nature of the microbe and also the unseasonably high rainfall observed during the sampling period (Das and Gupta-Bhattacharya 2008; Meterology 2010). The *G. salicifolia* plants in this study were sampled in the following two years when the region was only experiencing moderately high rainfall patterns (BOM 2011, 2012). This decrease in rainfall might explain the differences observed between the two studies and may reflect a more typical occurrence pattern for this ubiquitous genus within SEVT. Furthermore, given that *Nigrospora* are ubiquitous fungi, it is unlikely that the abundance observed in this study is due to specificity.

Of particular interest was the frequent observance of isolates with low sequence matches to members of the Pezizales, in particular Sarcosomataceae. A similar result was observed in the initial diversity study and may be indicative of a pattern existing more broadly within SEVT and may be an example of habitat type specificity. Members of the Sarcosomataceae, known as cup-fungi, are easily identified due to the prominent, often brightly coloured, cup like fruiting bodies that are produced on the ground or on decaying leaf and bark material (Jumpponen and Jones 2009; Unterseher *et al.* 2011). Sarcosomataceae belong to the class Pezizomycetes of which only one order is known, Pezizales, from which many edible truffles arise (Tedersoo *et al.* 2013). Characteristically, Sarcosomataceae occur as saprotrophs but they have also been shown to form ectomycorrhizal associations (Tedersoo *et al.* 2006), and on rare occasions have been observed occurring endophytically, although usually not in the prevalence observed in this study. Recently, Soca-Chafre *et al.* (2011) isolated one unidentified Sarcomosataceae species from *Taxus globosa,* while Weber isolated one species from the twig of *Cistus salvifolius* (Suryanarayanan *et al.* 2002). Despite Pezizales being considered to be common class 3 endophytes by Rodriguez *et al*. (2009), most studies such as Köpcke *et al*. (2002), Higgins *et al*. (2007) and Wang *et al.* (2005) have only found one or two species of Sarcomosataceae occurring endophytically. However Sarcomosataceae species are well recognised as dominating species composition within the lichens and in particular endolichens (Arnold *et al.* 2009). Therefore the high number of isolates growing endophytically observed in this study indicates a largely undescribed growth form for the Sarcosomataceae.

Endophytic Pezizales have been largely undescribed until relatively recently (Tedersoo *et al.* 2013). A recent paper by U'Ren *et al*. (2010) examined the species compositions of

endolichen and endophytes in various host species of rainforest plants. Interestingly, while Pezizales were discovered to be the dominate order (43.2%) within the endolichenic fungi, this was largely not the case for endophytes. One notable exception occurred in the mosses where significantly more Pezizales (>70%) were observed than in endolichens (U'Ren *et al.* 2010). Given the saprotrophic nature of many of the Sarcosomataceae, perhaps this indicates a mode of transmission to and from the leaf litter due to easy spore dispersal. The results of this study would suggest that their presence within the leaf litter may be due to their endophytic nature during different life phases.

Pezizales are prevalent within temperate regions of the northern hemisphere in the form of Pyrenomataceae however this family is rare in the southern hemisphere (Tedersoo *et al.* 2010). Tedersoo *et al*. (2013) however, notes that it is generally the Sarcosomataceae that are represented in the southern hemisphere. The high prevalence of such an under explored taxon as was observed in this study, further highlights the need to protect unexplored Australian ecosystems. As a result of the fertile soil in which they preside, SEVT are rapidly being cleared (Ecosystem_Conservation_Branch 2007; McDonald 2010). This study was able to sample only a small subset of the geographical locations of SEVT. In order to fully understand the distribution of Pezizales in such ecosystems, more research is urgently needed. No doubt countless novel fungi are lost when unique ecosystems such as these are cleared. Furthermore, given the reputed slow growing nature of species of Pezizales on agar (Tedersoo *et al.* 2013) their presence in many culture-based endophytic studies could easily be missed. Interestingly, in this study the isolates grew quickly on agar, covering a plate within 3 months, which may in part explain their occurrence in this study. As

molecular studies are being increasingly used to examine unculturable endophytes, the observation of this particular fungal order may increase and a more complete ecological picture may begin to develop.

This study has focused on endophytes isolated using the plating method. *In planta* molecular methods are known to enhance our understanding of endophyte diversity since they are able to identify isolates currently unable to grow in culture (Anderson and Cairney 2004; Arnold *et al.* 2007; Avis *et al.* 2010; Hughes *et al.* 2001). This was attempted in this study but was not successful due to the presence of PCR inhibitors in the extracted DNA. *G. salicifolia* is a highly aromatic plant that may be identified in part by the strong lavender smell that its leaves produce when crushed (Harden GJ *et al.* 2009). *G. salicifolia* was selected for this study as it is listed as a key species for SEVT, is easy to correctly identify and was known to occur commonly in all five sites sampled (McDonald 1996; McDonald 2010). Unfortunately, this choice of plant species resulted in an inability to utilise the extracted DNA due to the presence of Rutaceae specific inhibitors. In part, this may have been affected by the type of DNA extraction kit used. The Extract-N-Amp Plant PCR Kit (Sigma-Aldrich) is an excellent rapid extraction kit which was found to produce high quantities of good quality DNA when used for isolated fungal DNA. However, since it does not have a purification step during the extraction process and leaf material is left in the final solution, inhibitors such as tannins were found to commonly remain. Specialized post extraction purification kits were found to be ineffective in removing the inhibitors. Using numerous other methods such membranes to filter the DNA and adding various neutralising agents were also found to be ineffectiv. Problems such as this could potentially be averted by using kits which

filter out plant material in the first few steps of DNA extraction. Unfortunately, time did not permit the re-sampling and re-extraction of plant DNA for this study.

It is widely recognised that a combination of molecular and culture-based methods are needed in order to gain a full understanding of endophytic diversity (Anderson and Cairney 2004; Chobba *et al.* 2013; Duong *et al.* 2006; Guo *et al.* 2001; Manter and Vivanco 2007; Nikolcheva and Bärlocher 2004b). Furthermore, when molecular-based methods are used, higher proportions of basidiomycetes are often isolated (Crozier *et al.* 2008; Guo *et al.* 2001) and different species assemblages are often observed (Chobba *et al.* 2013). Unfortunately, the issues surrounding PCR inhibitors in this study meant that this aspect of the project could not be undertaken. Future research could examine whether there were any significantly different patterns of endophytic species within *G. salicifolia.* This would also indicate whether the high diversity seen in this study is an accurate representation of the abundance of endophytes within *G. salicifolia* and even SEVT as a whole.

3.6 Conclusions

The results of this study have found that specificity is most likely occurring between *Guignardia* species and *G. salicifolia.* It is further hypothesised that the high frequency of endophytic and unique Sarcosomataceae may also be an example of specificity occurring within SEVT. This is an intriguing observation and further highlights the importance of protecting unique habitat types such as SEVT. More research is needed in order to evaluate the presence of non-culturable endophytes in *G. salicifolia*, to further elucidate whether any tissue endophyte specificity may also be occurring and to determine what precise ecological role the Pezizales taxa are serving within SEVT.

4 Further taxonomic characterisation of endophytic taxa from SEVT

4.1 Introduction

Fungal taxonomy has a history dating back to the early 16th century (Shenoy *et al.* 2007; Sutton 1980). Originally considered members of the plant kingdom, early botanists found classification of fungi difficult due to their ability to reproduce both asexually and sexually under different conditions (Shenoy *et al.* 2007); a problem with classification which persists today (Seifert *et al.* 2000; Subramanian 1962; Subramanian 1983; Sutton 1980). Fungi only known by their asexual phase were found to be particularly difficult to name and were first given generic names by the Italian botanist Micheli (1679-1737), (Rodolico 1974). This ability to switch between modes of reproduction led to the development of the dual classification system (Shenoy *et al.* 2007). This method of naming remained in place for nearly 200 years but, with the introduction of molecular taxonomic approaches, has recently been changed in favour of a one fungus, one name system (Taylor 2011; Wingfield *et al.* 2012).

Molecular biology techniques have revolutionised fungal taxonomy (Taylor 2011). Some of the major drawbacks to traditional morphological-based methods are that phenotypic features may be difficult to observe and are often unreliable in defining species boundaries (Mitchell 2010). Such hurdles can be largely overcome when using molecular-based methods such as PCR and DNA sequencing (Holder and Lewis 2003; Mitchell 2010). The ability to infer phylogenetic relatedness through the use of DNA sequences has made it unnecessary to obtain sexual life stages of fungi when only asexual morphology has been observed (Reynolds and Taylor 1992). Furthermore, when multiple anamorphic phases have been identified for one species, phylogenetic placement is still possible (Mitchell 2010). Given this, the last 20 years has seen a

dramatic change in our understanding of the genetic relationships of fungi, with molecular-based taxonomic methods being routinely used for fungal identification (Begerow *et al.* 2010; Bellemain *et al.* 2010; Hibbett *et al.* 2011).

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is the most commonly used region for molecular taxonomic identification of fungi (Álvarez and Wendel 2003; Begerow *et al.* 2010; Eberhardt 2010). In fungi, the ITS region typically refers to the spacer regions and 5.8s gene which exists between the genes coding for the small ribosomal subunit (18s) and the large ribosomal subunit (26S) (Baldwin *et al.* 1995; Schoch *et al.* 2012). Genetic changes in the two spacer regions, designated ITS 1 and ITS 2, is thought to occur relatively slowly within individuals, but varies from species to species, making it an ideal source for phylogenetic analysis (Schoch *et al.* 2012). Over the last 20 years the ITS sequence has become the DNA region of choice for almost all fungi (Schoch *et al.* 2012; Seifert 2009). This prevalence of use, and apparent relative reliability (Schoch *et al.* 2012) has resulted in its proposal as the DNA barcode for the fungal kingdom (Begerow *et al.* 2010; Bellemain *et al.* 2010; Hibbett *et al.* 2011; Schoch *et al.* 2012; Seifert 2009).

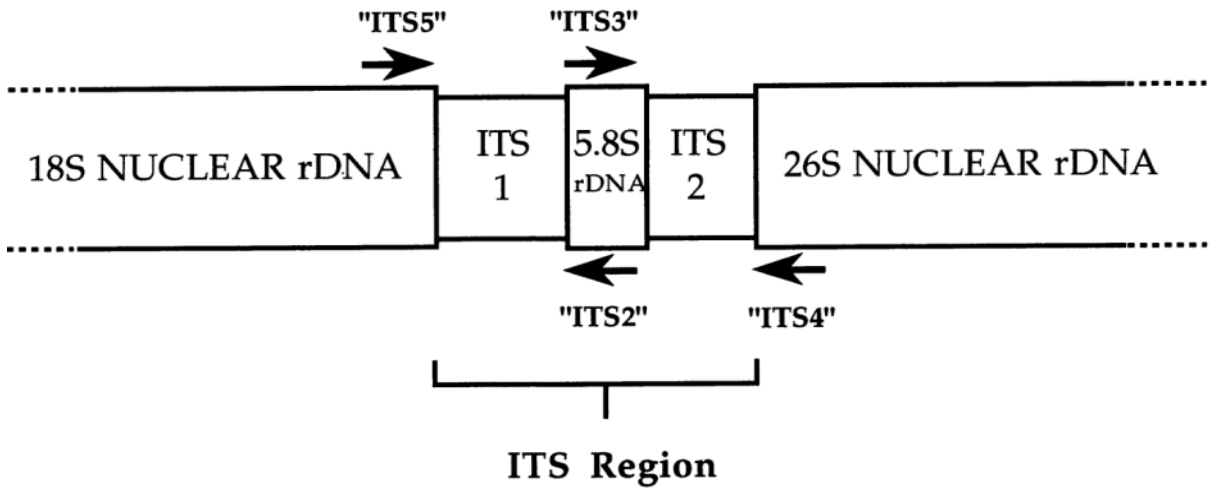

Figure 7 The ITS regions of the nuclear rDNA used for fungal taxonomy purposes, including all three gene regions (Baldwin et al. 1995).

Despite the many recent advances in identification of fungi via molecular-based methods, two major aspects remain problematic. The first is that a wealth of fungi only observed as 'mycelia sterilia' require further taxonomic characterisation via difficult to obtain morphological features. The second is that a large number of environmental samples or uncultured fungi are only known by their DNA (O'Brien *et al.* 2005). Increase in the use of molecular-based methods and the prevalence of the use of the ITS DNA region has seen an abundance of unidentified environmental sequences appearing in databases such as GenBank (Brock *et al.* 2009). Under the current nomenclature code (International Code of Botanical Nomenclature; ICBN), no formal way of identifying these cultures exists (Begerow *et al.* 2010; Hibbett *et al.* 2011). At best, samples can be phylogenetically analysed and identified to the genus level but an almost equal number can barely be correctly placed in families or even orders (Begerow *et al.* 2010; Johnston *et al.* 2006). The use of multigene phylogenetic analysis of the more complex lineages has the potential to provide some solutions to this issue, although until modifications to the code occur which can encompass such fungi, no real solutions can be attained.

Phylogenetic analysis relies on various statistical approaches to examine differences in base pairs of sequenced DNA. Current methods can be divided into two: traditional methods and Bayesian-based methods (Holder and Lewis 2003). Of the traditional methods, the two most commonly used are the Neighbour Joining (NJ) and the Maximum Likelihood (ML) algorithms (Baldauf 2003; Mitchell 2010). In the case of more closely related datasets, NJ trees provide a rapid and relatively accurate assessment of relatedness (Mitchell 2010). ML trees have been found to provide a more accurate tree than the NJ, particularly for more distantly related datasets, but are at times computationally prohibitive (Holder and Lewis 2003). Both methods rely on 'bootstrapping', repeatedly applying the algorithm (typically 1000 times) to the same set of data, in order to give a statistical indication of tree reliability (Baldauf 2003; Hall 2004). Bootstrapping values above 70% are generally considered good (Holder and Lewis 2003) and only those above 50% should be recorded (Baldauf 2003). Bayesian models use a similar analytical approach to ML methods and are even more computationally demanding (Mitchell 2010; Simmons *et al.* 2004). Bayesian approaches attempt to correct some of the statistical weaknesses of ML methods by using parametric tests instead of the nonparametric bootstrapping method to assess probability and reliability of branches (Ronquist and Deans 2010). The biggest issue with this method is that the computational requirements may be prohibitive (Mitchell 2010).

The addition of multiple gene regions within the analysis may be used to further resolve problematic fungal phylogenies (Gadagkar *et al.* 2005). Typical extra gene regions used are the small subunit (SSU) and the large subunit (LSU) of the nuclear ribosomal DNA region and the unrelated protein coding β tubulin gene (Gadagkar *et al.* 2005; Gueidan *et al.* 2007; Miller and Huhndorf 2005; Rintoul *et al.* 2012). LSU is commonly selected

due to the ease with which it may be amplified as well as the availability of universal primers. The β tubulin gene is often used in comparative studies where examination of unlinked genes is required for more difficult phylogenies. In this case the intron poor 3' end is considered suitable in aiding the resolution of ascomycete lineages (Miller and Huhndorf 2005).

4.2 Aims

The aim of this study was to more completely characterise the phylogeny of four prominent endophytic taxa from SEVT. It also examined more fully the question, are there any novel fungal taxa in SEVT? OK The groups focussed on included *Nigrospora*, *Preussia*, *Guignardia* and the Pezizales. In order to resolve the taxonomic placement of some species, three gene regions were included in the analysis. In some situations this was not possible or clear phylogenetic characterisation already existed, in which case one or two gene regions were used.

4.3 Methods

Sequences of four commonly occurring SEVT endophytic taxa were selected for more detailed phylogenetic analysis. Data from both the *G. salicifolia* endophytic specificity study (chapter 3) and the initial diversity study (chapter 2) were included. The ITS region within the order Pezizales has been well resolved and so taxa from this order were only analysed based on this DNA region. Due to a lack of useful sequence information in GenBank, *Nigrospora* was analysed based on the ITS rDNA region. *Guignardia* and *Preussia* were analysed based on the ITS and LSU region and in the case of *Preussia*, the β tubulin gene was also included. Each gene region was analysed separately before being combined to produce one tree.

4.3.1 Amplification and sequencing of isolated DNA

After DNA had been extracted from pure fungal cultures, PCR was conducted with the Extract-N-Amp™ Plant PCR kit (Sigma-Aldrich, Castle Hill, NSW, Australia). The fungal specific ITS primers included the ITS1F forward primer (Gardes and Bruns 1993) and ITS4 reverse primer (White *et al.* 1990). LSU primers used included the primer pair LROR forward primer 5'ACC CGC TGA ACT TAA GC'3 and LR7 reverse primer 5'TAC TAC CAC CAA GAT CT'3 with primer LR5 5'CCT GAG GGA AAC TTC G'3 used for sequencing (Vilgalys and Hester 1990). The β-tubulin primers included BT1819R 5'TTC CGT CCC GAC AAC TTC GT'3 and BT2196 5'CTC AGC CTC AGT GAA CTC CAT'3 (Miller and Huhndorf 2005).

All initial PCR reactions were performed in duplicate and a control tube containing 0.5µl of sterile H_2O instead of DNA was also included. All primers were amplified under the same conditions of 35 cycles of 95°C for one minute, 50°C for one minute and 72°C for one minute, with a final elongation step of ten minutes at 72°C. 2µl of the PCR product was then run on a 2% electrophoresis gel and viewed under UV light using the Quantum ST4 gel documentation system (Vilber Lourmat, Fisher Biotec). This was then photographed using the Quantum capture ST4® image acquisition and analysis software package.

In preparation for sequencing, PCR products were purified using a DNA purification kit as per the manufacturers' instructions (Macherey Nagel, Cheltenham, Australia). In order to check the concentration of the purified sample, 2µl of the resulting purified DNA was then run on a 2% w/v agarose gel. After purification, samples containing between 17ng and 30ng of DNA were then sent to the Brisbane laboratory of the

Australian Genome Research Facility (AGRF) for sequencing. Returned sequences were viewed using the Chromas® 2.0 program to check for contamination. A BLAST search using the National Centre for Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov/> was used to determine closest species matches. As per Hibbett *et al.* 2011, a cut off of 97% sequence similarity in conjunction with phylogenetic analysis was used to identify separate species. Delineation ofseparate genera was based on this information plus whether taxa formed a separate, well supported clade and had a sequence similarity of less than 93% (Hibbett *et al.* 2011).

4.3.2 Phylogenetic analysis

Phylogenetic analysis was performed with MEGA version 5 (Tamura *et al.* 2011). Sequences were aligned using Muscle (Edgar 2004) and a neighbour-joining tree (Saitou and Nei 1987) was constructed from the alignment file using the Maximum Composite Likelihood method (Tamura *et al.* 2011) and bootstrapping of 1000 replicates (Felsenstein 1985). Where possible, only vouchered CBS or AFTOL sequences in GenBank were included for comparison since these sequences have had their identity confirmed through independent means.

Nigrospora samples were initially grouped based on morphological characteristics. These were: colour of hyphae, ability to grow into the agar, agar colouration and presence or absence of aerial hyphae. All morphological comparisons were based on cultures after 4 weeks of growth on PD agar. The cultures were assembled into 14 morpho-groups (Table 7). DNA from representatives of each group were sequenced and phylogenetically analysed using Mega5 (Tamura *et al.* 2011).

4.4 Results

4.4.1 Phylogenetic characterisation of *Nigrospora* spp.

By far the most abundant genus, *Nigrospora* spp. were isolated 89 times in the study (23 isolates from the specificity study and 66 from the diversity study). Sporulating cultures were loosely placed into 9 morphtypes based on the colour of the hyphae, presence or absence of aerial hyphae and the apparent ability to grow into or colour PDA (Table 7). DNA was successfully amplified from 41 isolates. Isolates ADH2.15, CAL2.3 and CMH2.14 had sequence similarities between 95-97% to other known sequences in GenBank (Table 7). The remaining 38 isolates all returned closest matches of greater than 98% to various *Nigrospora* sequences in GenBank (Table 7). Isolates belonging to seven morphotypes were observed to sporulate (Figure 8). Spores belonging to morphotype 2 were found to be considerably smaller (<2μm) than the other morphotypes observed (>3μm; Figure 8).

Molecular phylogenetic analysis grouped the isolates of *Nigrospora* into two major clades which contained either known samples of *N. sphaerica* (Clade 1) or *N. oryzae* (Clade 2; Figure 9). Morphotypes 1, 4, 6, 7 and 8 only clustered within clade 1 and with other known samples of *N. sphaerica*. Morphotype 2 clustered with other known samples of *N. oryzae* forming clade 2. Morphotypes 3, 5 and 9 clustered within both clades 1 and 2 (Figure 9).

Table 7 Morphotypes of *Nigrospora* isolates and closest Genbank matches where applicable

Code	Hyphae colour	Growth into agar	Colour agar	Aerial hypha	Morpho-group	Closest match	Identity	% similarity	Proposed identity	GenBank No.
CMH2.14*	Black	Black	Y	N	1	HM565952.1	464/484	95%	*Khuskia oryzae*	KF227832
ADH2.15	Black	Black	N	N	2				*Khuskia oryzae*	
CMH2.15*	Black	Black	Y	Y	2	GU934549.1	422/423	99%	*Khuskia oryzae*	KF227833
ADM2.10	Black	Tan	N	Y	3				*Khuskia oryzae*	
BSH2.13	Black	Tan	N	Y	3	JN903534.1	464/466	99%	*Khuskia oryzae*	KF227808
BSL2.4	Black	Tan	N	N	3				*Khuskia oryzae*	
CIM1.7	Black	Tan	N	N	3				*Khuskia oryzae*	
CLL1.6	Black	Tan	N	Y	3	HQ248210.1	472/481	98%	*Khuskia oryzae*	KF227829
CLM1.8	Black	Tan	N	N	3	HM565952.1	503/511	98%	*Khuskia oryzae*	KF227830
CMH2.13	Black	Tan	N	N	3	HQ608063.1	487/488	99%	*Khuskia oryzae*	KF227831
EAM2.10	Black	Tan	N	Y	3	JF819161.1	515/525	98%	*Khuskia oryzae*	KF227838
CAL2.3	Black	N	N	N	4	JF817271.1	461/472	97%	*Khuskia oryzae*	KF227815
PAM2.6	Black	N	N	N	4				*Khuskia oryzae*	
ADH2.14	Tan	Tan	Y	Y	5	EU579801.1	540/542	99%	*Khuskia oryzae*	
ADL2.7	Tan	Tan	N	fluffy	5	FJ478134.1	538/541	99%	*Khuskia oryzae*	
ADM2.8	Tan	Tan	N	fluffy	5				*Khuskia oryzae*	
BPM2.4	Tan	Tan	N	fluffy	5	HQ608063.1	507/507	100%	*Khuskia oryzae*	KF227806
BSH2.11	Tan	Tan	N	N	5				*Khuskia oryzae*	
BSM2.7	Tan	Tan	N	Y	5				*Khuskia oryzae*	
CAH2.10	Tan	Tan	N	N	5				*Khuskia oryzae*	
CMM2.12	Tan	Tan	N	N	5	HQ608030.1	536/537	99%	*Khuskia oryzae*	

Code	Hyphae colour	Growth into agar	Colour agar	Aerial hypha	Morpho-group	Closest match	Identity	% similarity	Proposed identity	GenBank No.
E.AH2.11	Tan	Tan	N	N	5				*Khuskia oryzae*	
EAH2.12	Tan	Tan	N	Y	5				*Khuskia oryzae*	
EAL2.1	Tan	Tan	N	Y	5				*Khuskia oryzae*	
EAL2.3	Tan	Tan	N	N	5				*Khuskia oryzae*	
EAL2.6	Tan	Tan	N	N	5				*Khuskia oryzae*	
ADH2.12	White	black	N	Y	6	JQ936183.1	536/537	99%	*Khuskia oryzae*	
ADH2.13	White	black	N	Y	6	JQ936183.1	532/535	99%	*Khuskia oryzae*	
ADL2.1	White	black	N	N	6				*Khuskia oryzae*	
ADL2.3	White	black	N	Y	6				*Khuskia oryzae*	
ADL2.5	White	black	N	Y	6	JQ246358.1	493/494	99%	*Khuskia oryzae*	KF227804
CMM2.10	White	black	N	fluffy	6	JQ936183.1	539/539	100%	*Khuskia oryzae*	
NVH2.12	White	black	N	Y	6	HQ608030.1	534/535	99%	*Khuskia oryzae*	
NVH2.13	White	black	N	Y	6				*Khuskia oryzae*	
NVL2.2	White	black	N	N	6				*Khuskia oryzae*	
ADL2.2	White	Tan	N	N	7	HQ889723.1	447/447	100%	*Khuskia oryzae*	KF227798
BPL2.1	White	Tan	N	Y	7	HQ608030.1	512/513	99%	*Khuskia oryzae*	KF227804
BSH2.10	White	Tan	N	N	7				*Khuskia oryzae*	
BSM2.8	White	Tan	N	N	7				*Khuskia oryzae*	
CLM1.7	White	Tan	N	fluffy	7				*Khuskia oryzae*	
CML2.2	White	Tan	N	Y	7				*Khuskia oryzae*	
CMM2.11	White	Tan	N	N	7	HQ608030.1	483/485	99%	*Khuskia oryzae*	KF227836
EAL2.2	White	Tan	N	N	7				*Khuskia oryzae*	

Code	Hyphae colour	Growth into agar	Colour agar	Aerial hypha	Morpho-group	Closest match	Identity	% similarity	Proposed identity	GenBank No.
EAM2.8	White	Tan	N	fluffy	7				*Khuskia oryzae*	
EAM2.9	White	Tan	N	Y	7				*Khuskia oryzae*	
GSL2.3	White	Tan	N	fluffy	7				*Khuskia oryzae*	
GSL1.1	White	Tan	N	Y	7				*Khuskia oryzae*	
SLH2.14	White	Tan	N	N	7				*Khuskia oryzae*	
SLH.2.15	White	Tan	N	Y	7				*Khuskia oryzae*	
SLL2.1	White	Tan	N	N	7				*Khuskia oryzae*	
SLL2.2	White	Tan	N	N	7				*Khuskia oryzae*	
SLM2.6	White	Tan	N	N	7				*Khuskia oryzae*	
SLM2.9	White	Tan	N	N	7				*Khuskia oryzae*	
BSM2.6	White/tan	Tan	-	-	8	HQ607936.1	515/515	100%	*Khuskia oryzae*	KF227810
ELV3.14	White/tan	Tan	N	N	8	HQ607936.1	509/509	100%	*Khuskia oryzae*	KF227842
ELV3.8	White/tan	Tan	N	N	8				*Khuskia oryzae*	
NVM2.3	White/tan	Tan	N	fluffy	8				*Khuskia oryzae*	
SLM2.5	White/tan	Tan	N	N	8				*Khuskia oryzae*	
SLM2.7	White/tan	Tan	N	N	8				*Khuskia oryzae*	
ADM2.9	White	N	N	N	9				*Khuskia oryzae*	
BSL2.5	White	N	N	N	9	GU073125.1	471/476	98%	*Khuskia oryzae*	KF227809
CML2.6	White	N	N	N	9	JQ936183.1	543/545	99%	*Khuskia oryzae*	
GSM1.6	White	N	N	Y	9				*Khuskia oryzae*	

*both samples were clearly different when viewed on a plate

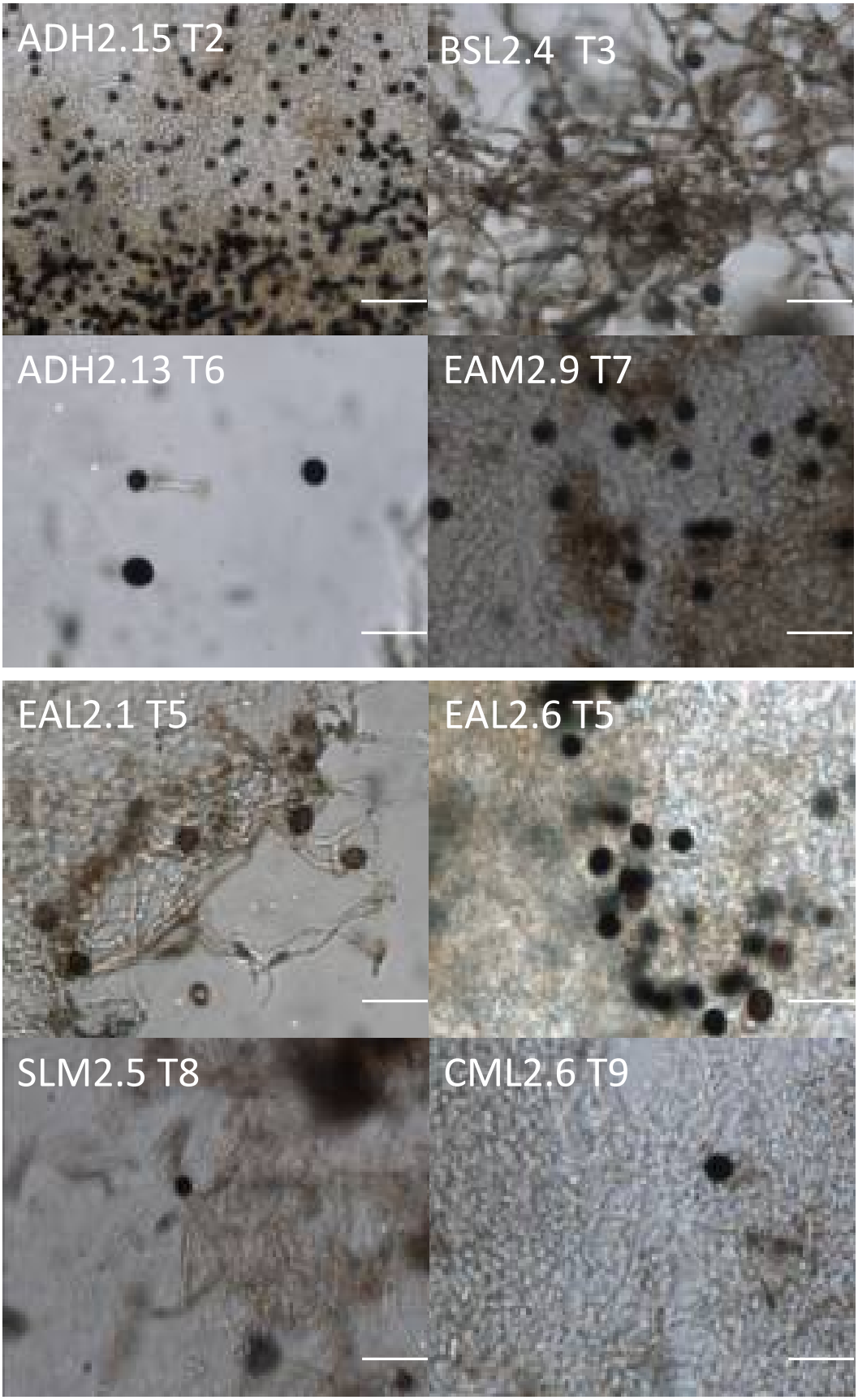

Figure 8 Spore size of isolates of various morphotypes. Scale bar= 50μm. T=morphotype number

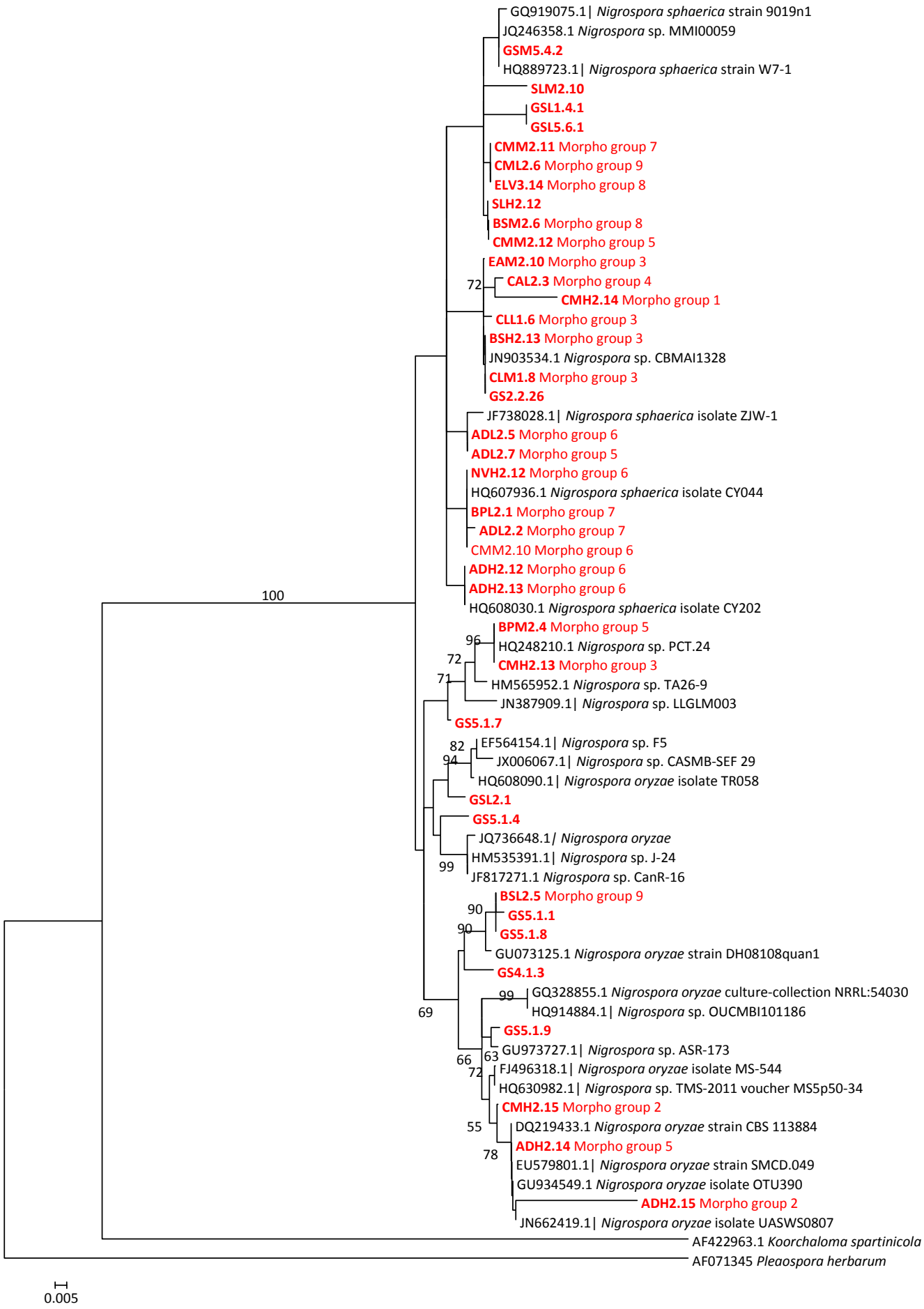

Figure 9 Neighbour-joining phylogenetic tree of *Nigrospora* spp. ITS region with 1000 bootstrap support.

4.4.2 Phylogenetic characterisation of *Preussia* spp.

A total of 25 isolates of *Preussia* spp. were obtained from 13 host plants belonging to nine different species (Table 8). Ten of these were found to have less than 97% identity to any known sequence in the ITS region when compared against GenBank using the BLAST search option (Table 8). When these isolates were compared to each other, ADM2.11 and NVM2.5 were found to be 98% similar over 360 bp and ELV3.11 and ELV3.2 were found to be 99% similar over 400 bp. Of the remaining fifteen isolates, four had identity to *Preussia africana* (99% over 507-509 bp; Table 8). Eight isolates had identity to *Preussia isomera* and three isolates had identity to three separate unnamed *Preussia* spp. (98-99% over 514-426 bp; Table 8).

Table 8 Host and closest GenBank matches of *Preussia* taxa isolated in this study (table looks a bit squashed)

Code	Host	Closest match	Identity	%	Proposed identity	GenBank No.
ADL2.4	*Alectryon diversifolius*	EU551184.1 *Sporormiella isomera*	493/498	98%	*Preussia isomera*	JN418766
ADL2.6	*Alectryon diversifolius*	FJ210518.1 *Preussia* sp.	422/426	99%	*Preussia* sp.	JN418765
ADM2.11	*Alectryon diversifolius*	GQ203763.1 *Preussia isomera*	335/352	95%	*Preussia* sp.1a	JN418767
BSH2.9	*Bursaria spinosa*	EU551195.1 *Preussia Africana*	504/507	99%	*Preussia* aff. *africana*	JN418768
CAH2.11	*Cassine australis*	AY943053.1 *Sporormiella isomera*	491/496	98%	*Preussia isomera*	JN566153
CAH2.9	*Cassine australis*	EU551184.1 *Sporormiella isomera*	497/503	98%	*Preussia isomera*	JN418769
CAL2.1	*Cassine australis*	EU551184.2 *Sporormiella isomera*	448/450	99%	*Preussia isomera*	JN418770
EAL2.4	*Erythroxylum australe*	HQ637314.1 *Preussia pilosella*	483/499	96%	*Preussia* sp.2	JN566152
EAL2.5	*Erythroxylum australe*	GU212409.1 Pezizomycotina sp.	419/451	92%	*Preussia* sp.3	JN418771
EAL2.7	*Erythroxylum australe*	DQ468033.1 *Preussia pilosella*	448/468	95%	*Preussia* sp.4	JN418772
ELV3.11	*Eustrephus latifolius*	HQ602666.2 *Preussia* sp.	382/426	89%	*Preussia* sp.5	JN418774
ELV3.2	*Eustrephus latifolius*	HQ602666.1 *Preussia* sp.	422/471	89%	*Preussia* sp.5	JN418773
ELV3.4	*Eustrephus latifolius*	JQ760353. 1 Dothidiomycete	434/468	93%	*Preussia* sp 7	

Code	Host	Closest match	Identity	%	Proposed identity	GenBank No.
GSM3.1	*Geijera salicifolia*	DQ865095.1 *Preussia Africana*	506/509	99%	*Preussia* sp.	JN418775
GSL1.3.2	*Geijera salicifolia*	HQ607898.1 *Preussia* sp.	531/541	98%	*Preussia* sp.	KF128821
GSL2.1.1	*Geijera salicifolia*	JN418768.1 *Preussia* sp.	492/496	99%	*Preussia* sp.	KF128767
GSH1.5.6	*Geijera salicifolia*	FJ037749.1 *Preussia* sp.	526/538	98%	*Preussia* sp.	KF128811
GSH2.2.23	*Geijera salicifolia*	HQ607802.1 *Preussia* sp.	528/543	97%	*Preussia* sp. 8	KF128781
GSM2.1.5	*Geijera salicifolia*	HQ130663.1 *Sporormiella* sp.	512/520	98%	*Preussia isomera*	KF128773
GSM2.1.7	*Geijera salicifolia*	HQ130664.1 *Sporormiella* sp.	532/543	98%	*Preussia isomera*	KF128774
NVM2.5	*Notelaea venosa*	EU551195.1 *Preussia Africana*	480/503	95%	*Preussia* sp.1b	JN418776
NVM2.6	*Notelaea venosa*	EU551184.1 *Sporormiella isomera*	497/499	99%	*Preussia isomera*	JN418777
PAL2.1	*Pittosporum angustifolium*	EU551184.2 *Sporormiella isomera*	507/511	98%	*Preussia isomera*	JN418778
PAL2.3	*Pittosporum angustifolium*	HQ130664.1 *Preussia* sp.	416/439	95%	*Preussia* sp.6	JN418779
PPV3.6	*Pandorea pandorana*	FJ210521.1 *Preussia* sp.	508/514	98%	*Preussia* sp.	JN418780

When sequences were phylogenetically analysed using the three gene analysis (for GenBank accession codes see appendices Table C.21), they formed seven separate clades (Figure 10). Clade 1 comprised isolates ADM2.4, CAL2.1, CAH2.9, CAH2.11, GSM2.1.5, GSM2.1.7, NVM2.6, and PAL2.1. The closest GenBank match for five of these sequences was to *P. isomera* (EU551184.1). CAH2.11 had a closest match to a different isolate of *P. isomera* (AY943053.1) and GSM2.1.5 and GSM2.1.7 had a closest match to a *Sporormiella* sp. (HQ130663.1). Clade 1 also included the isolate PAL2.3 but this is most likely a separate *Preussia* species as it was only 95% similar to the other members of the clade and had a velvety yellow appearance compared to the orange glossy colonies of the other isolates (Figure 11). The sequences making up clade six were compared using the Blastn-2 alignment tool and despite being morphologically similar (Figure 11Figure 12), all four isolates were found to be less than 97% similar suggesting they represent different species. While two isolates (BSH2.9, GSM3.1) showed a sequence match of 99% to *P. africana*, these were different submissions to GenBank and did not group together when phylogenetically analysed. Interestingly, isolates ADH2.11 and NVM2.5 did not cluster together when all three gene regions were analysed but clustered together with good bootstrap support when the LSU and β tubulin gene regions were considered independently (Figure 13; Figure 14). However, given that these sequences were 98% similar when aligned using the Align Multiple Sequences tool in GenBank, isolates were labelled as *Preussia* sp1a and *Preussia* sp1b respectively. Good congruence was observed across the three gene regions for *Preussia* isolates which formed clades 1, 2, 5 and 6 (Figure 10, Figure 13, Figure 14; Appendices Table C.21). In particular, isolates ELV3.2 and ELV3.11 which formed clade 6, consistently separated out early from all other *Preussia* sequences.

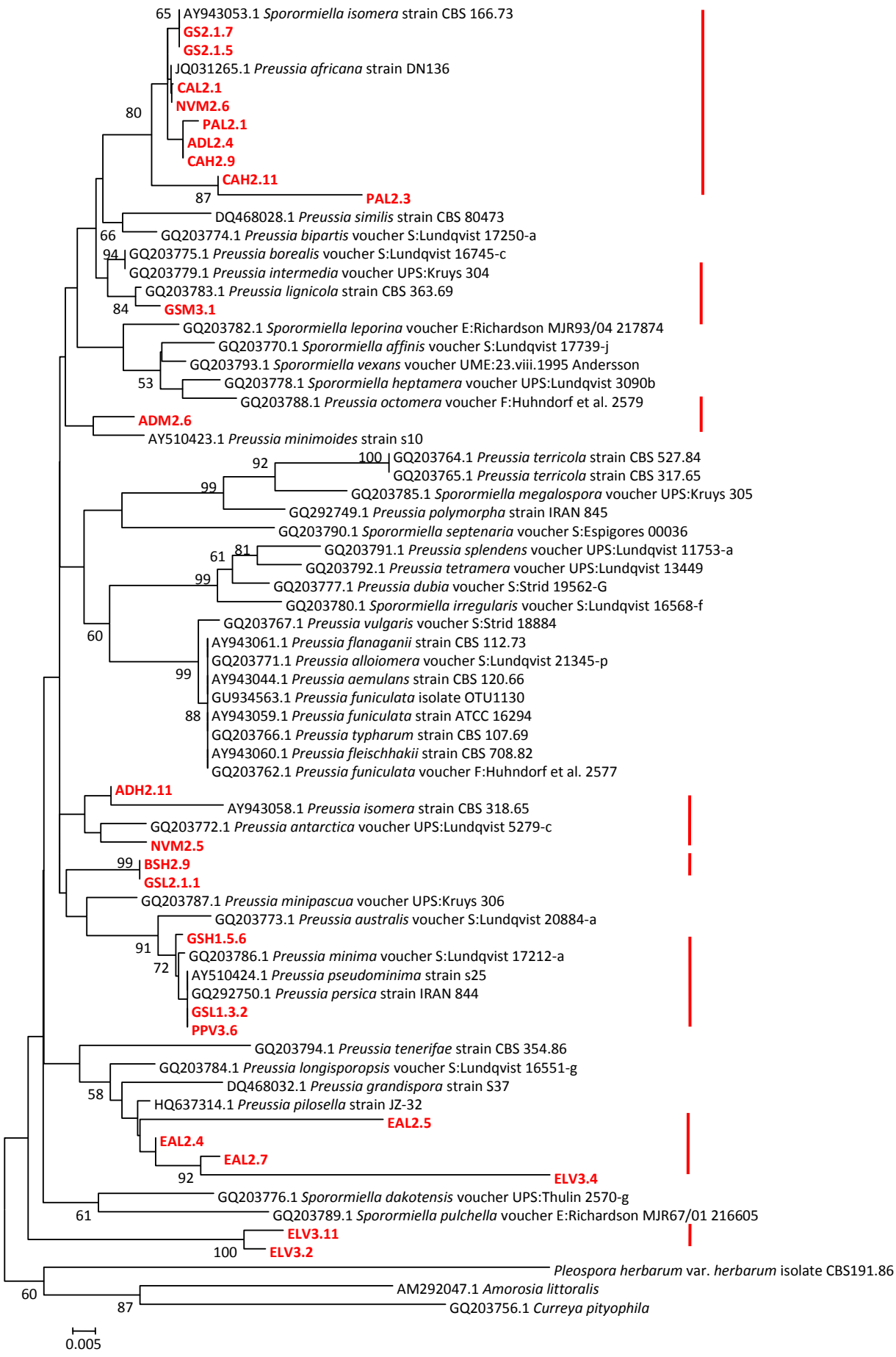

Figure 10 Multi-gene (ITS, LSU and β-tubulin) Neighbour-joining tree of *Preussia* spp. with 1000 bootstrap support. Red text indicates samples collected in this study while the red lines indicate clades

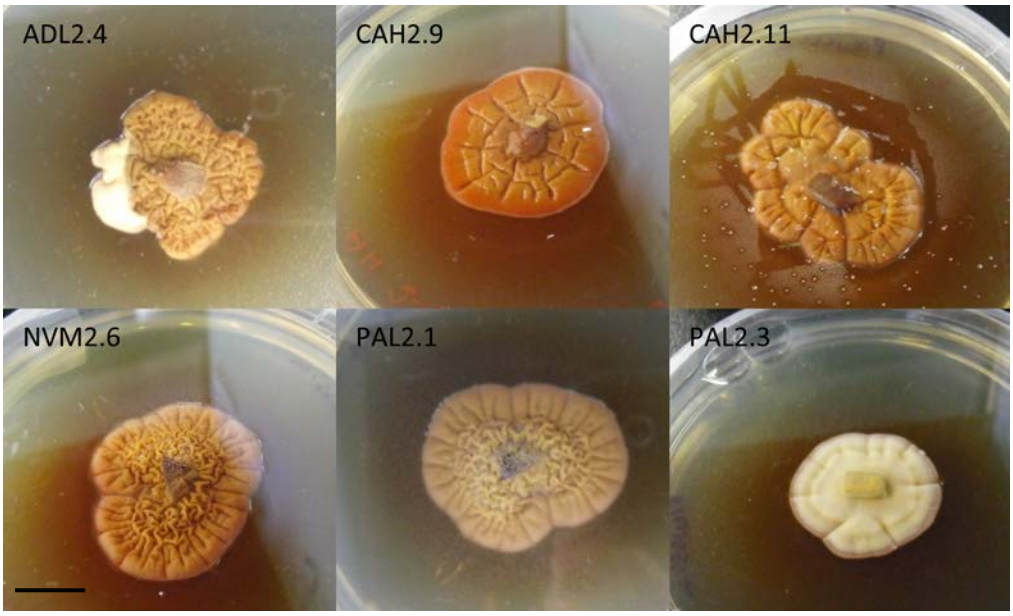

Figure 11 Morphological appearance of the same age cultures of *Preussia* spp. in clade 1. Scale bar = approximately 2cm

Figure 12 Morphological appearance of the same age cultures of *Preussia* spp. in clade 6. Scale bar = approximately 2.5cm

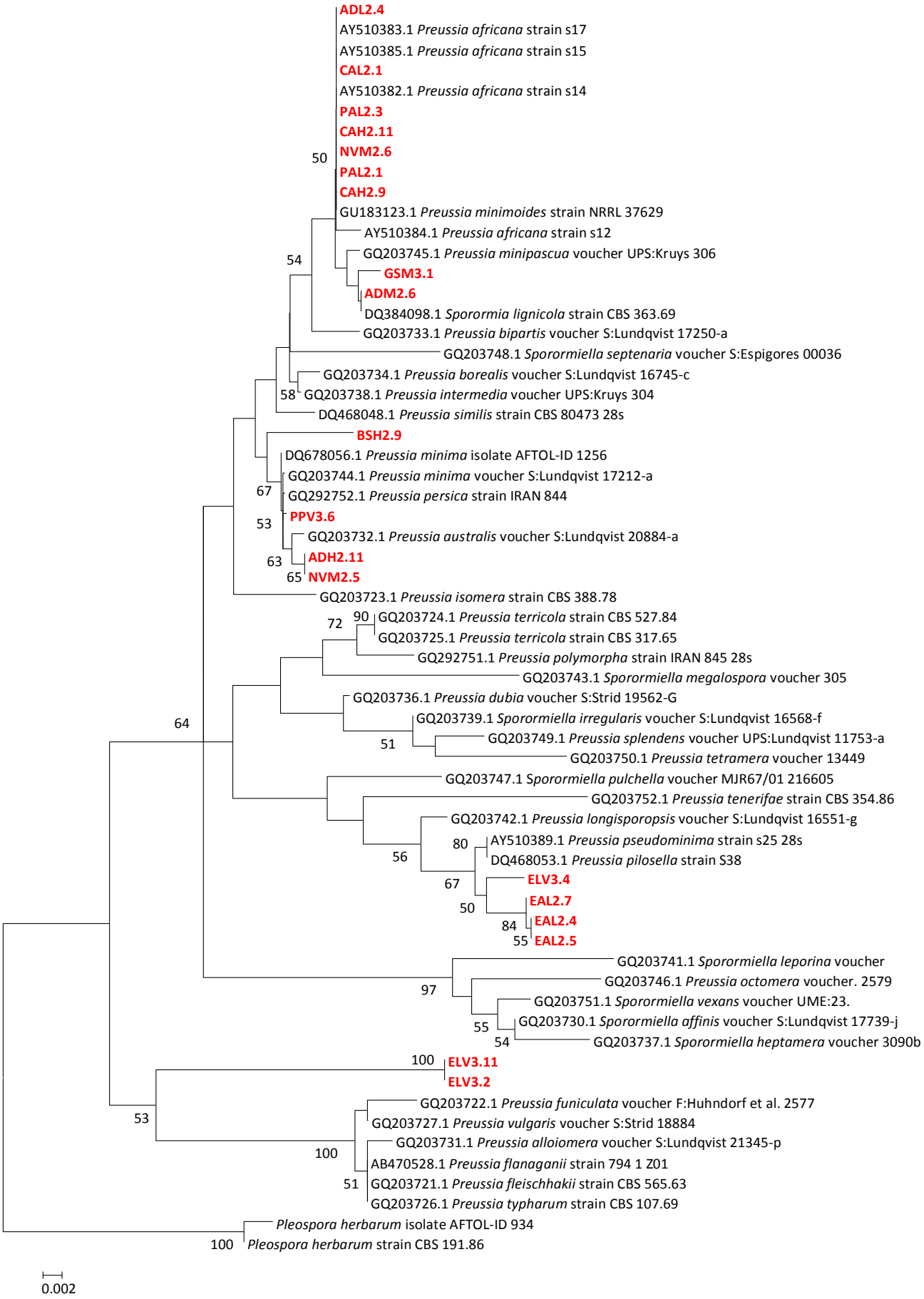

Figure 13 Neighbour-joining tree of *Preussia* spp based on LSU sequences. Isolates from this study are in red

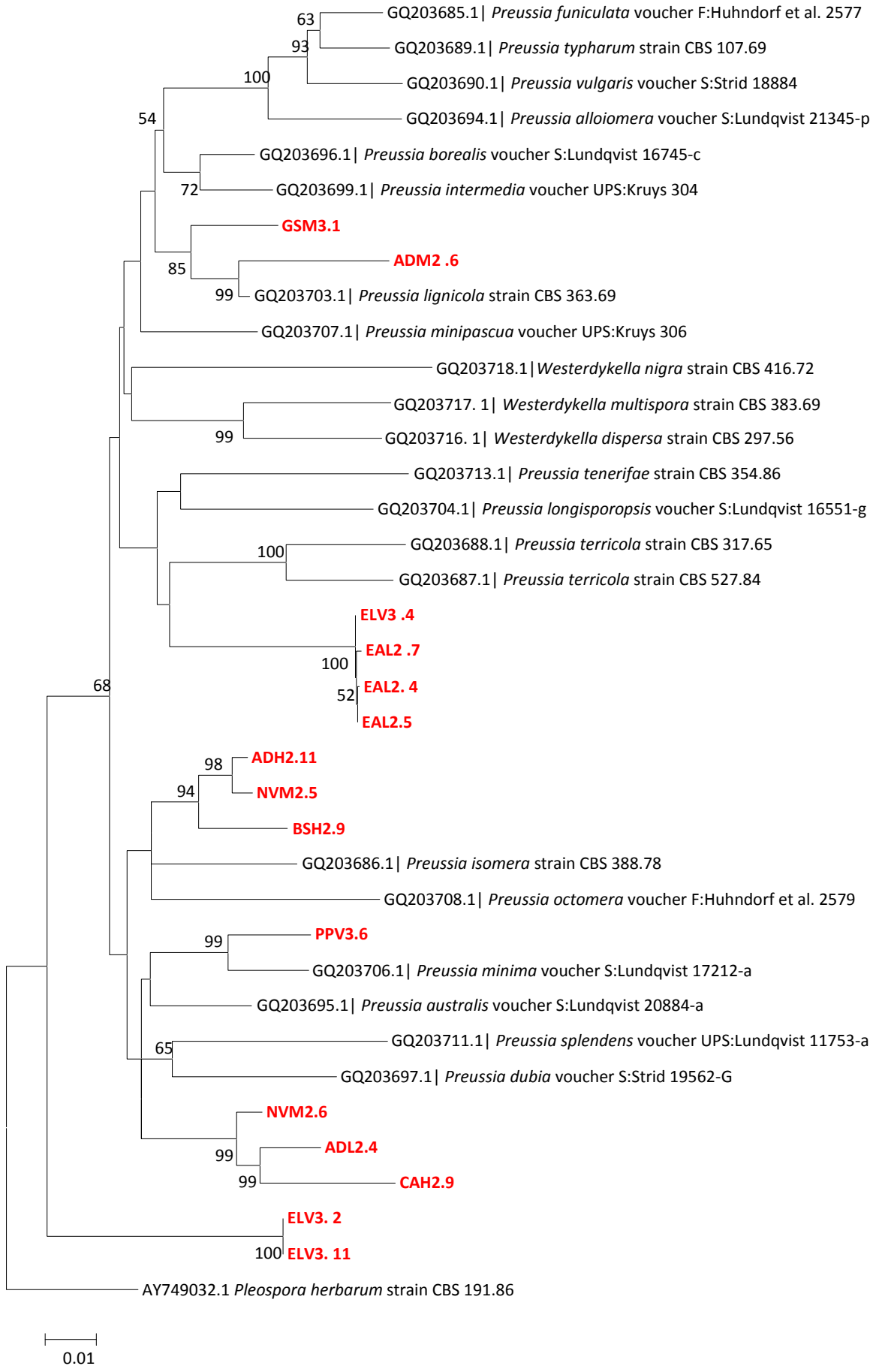

Figure 14 Neighbour-joining tree of *Preussia* spp based on beta tubulin sequences. Isolates from this study are in red

4.4.3 Phylogenetic characterisation of *Guignardia* spp.

Guignardia species weres isolated nine times from five different host species (six plants in total) in the initial diversity study (Chapter 2) and were found to occur at all three sites. A further 48 isolates were obtained from four of the sites in the *G. salicifolia* endophytic specificity study (Chapter 3) bringing the total number of isolates to 57. DNA was successfully obtained from 41 isolates. Of these, 33 had a less than 97% similarity to any other sequence in GenBank (Table 9). Most sequences returned a closest match of either *G. mangiferae* or *G. bidwellii* except for four isolates which had closest sequence matches to *G. ardisiae* (2) and *G. vaccinii* (2). Isolates CIL1.2, EAH2.13, ELV3.18, EXH1.23 and GSL1.4 were closest to the FJ538333.1 sequence of *G. mangiferae,* however samples CIL1.2 and ELV3.18 only had a sequence similarity of 95%. Samples ELV3.6, ELV3.18 and ELV3.19 had sequence similarities of greater than 99% when aligned using the align multiple sequences tool in GenBank. When these sequences were phylogenetically analysed, they clustered closely together (Figure 15). Therefore, these isolates were considered to be the same species and were given the name *Guignardia* sp 4. 26 isolates clustered closely together when phylogenetically analysed (Clade 1; Figure 15) and were given the name *Guignardia* sp 1. Isolate GSH5.5.11 formed a separate branch when phylogenetically analysed and had a sequence similarity of 95%, as did isolate CIL1.2 (Figure 15; Table 9). As such, they were given the name *Guignardia* sp 2 and *Guignardia* sp 5 respectively. Isolate CAH2.14 clustered with ELV3.6, ELV3.18 and ELV3.19 but when aligned using the align multiple sequences tool it was only found to be 95% similar and so was recorded as *Guignardia* sp 3. Isolates EAH2.13 EXH1.23, GSM5.5.10, GSM5.5.8, GSL1.4, GSL1.5.3 and GSL5.5.1 all returned closest matches to other *G. mangiferae* sequences of between 98 and 99%,

however, when these sequences were phylogenetically analysed they clustered with other sequences from *G. psidii* as well. For this reason, they were named *Guignardia* sp 6. There was good congruence between ITS and LSU gene regions for clades labelled sp. 1, 2 and 4 (Figure 15, Figure 16). No representatives from sp. 3 were analysed using the LSU sequence region. However, given the very small number of LSU sequences in Genbank, it is difficult to gain reliable data and so the ITS and LSU were not combined (Figure 16; see appendices Table C.21 for GenBank ascension codes). Unicellular hyaline ovoid conidia with an apical hyaline tail, as long or slightly longer than the conidia, were observed from representatives of species 1 (Figure 17). Conidia appeared slightly indented with a mucilaginous sheath and were 20µm x 10µm in diameter. When grown on PDA, black pycnidia formed amongst grey to white hyphae. Isolates from the other species appeared morphologically dissimilar when grown on PDA but produced similar black pycnidia (Figure 17).

Table 9 Host and closest GenBank matches of *Guignardia* taxa isolated in this study

Code	Host	Closest match	Identity	% similarity	Proposed identity	Genbank No.
CAH2.14	*Cassine australis*	HQ874762.1 *Guignardia mangiferae*	383/396	97%	*Guignardia* sp3	
CIL1.2	*Croton insularis*	FJ538333.1 *Guignardia mangiferae*	592/625	95%	*Guignardia* sp5	
EAH2.13	*Erythroxylum australe*	FJ538333.1 *Guignardia mangiferae*	595/597	99%	*Guignardia* sp6	
ELV3.18	*Eustrephus latifolius*	EU677818.1 *Guignardia mangiferae*	563/592	95%	*Guignardia* sp4	
ELV3.19	*Eustrephus latifolius*	FJ538333.1 *Guignardia mangiferae*	592/620	95%	*Guignardia* sp4	
ELV3.6	*Eustrephus latifolius*	EU677811.1 *Guignardia mangiferae*	569/596	95%	*Guignardia* sp4	
EXH1.23	*Elattostachys xylocarpa*	FJ538333.1 *Guignardia mangiferae*	591/592	99%	*Guignardia* sp6	
GSH2.1.10	*Geijera salicifolia*	JF261459.1 *Guignardia mangiferae*	546/590	93%	*Guignardia* sp1	KF128858
GSH2.1.12	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	586/631	93%	*Guignardia* sp1	KF128768
GSH2.1.13	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	586/632	93%	*Guignardia* sp1	KF128769
GSH2.1.14	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	585/630	93%	*Guignardia* sp1	KF128770

Code	Host	Closest match	Identity	% similarity	Proposed identity	Genbank No.
GSH2.2.19	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	587/632	93%	*Guignardia* sp1	KF128778
GSH2.2.20	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	584/633	92%	*Guignardia* sp1	KF128779
GSH2.2.21	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	587/635	92%	*Guignardia* sp1	KF128780
GSH3.3.10	*Geijera salicifolia*	AB454283.1 *Guignardia ardisiae*	579/621	93%	*Guignardia* sp1	KF128787
GSH3.4.9	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	580/625	93%	*Guignardia* sp1	
GSH5.4.3	*Geijera salicifolia*	FJ418187.1 *Guignardia vaccinii*	513/535	95%	*Guignardia* sp1	
GSH5.4.5	*Geijera salicifolia*	AB454283.1 *Guignardia ardisiae*	599/644	93%	*Guignardia* sp1	KF128813
GSH5.5.11	*Geijera salicifolia*	FJ418187.1 *Guignardia vaccinii*	509/531	95%	*Guignardia* sp2	KF128814
GSL1.2.1	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	581/633	92%	*Guignardia* sp1	KF128759
GSL1.2.3	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	588/633	93%	*Guignardia* sp1	KF128762
GSL1.3.1	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	596/640	93%	*Guignardia* sp1	KF128820
GSL1.4	*Geijera*	FJ538333.1 *Guignardia*	583/586	99%	*Guignardia*	KF128867

Code	Host	Closest match	Identity	% similarity	Proposed identity	Genbank No.
	salicifolia	*mangiferae*			sp6	
GSL2.5.1	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	587/631	93%	*Guignardia* sp1	KF128827
GSL2.5.2	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	587/632	93%	*Guignardia* sp1	KF128828
GSL2.5.3	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	586/631	93%	*Guignardia* sp1	KF128829
GSL2.5.4	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	588/633	93%	*Guignardia* sp1	KF128830
GSL5.5.1	*Geijera salicifolia*	JQ743587.1 *Guignardia mangiferae*	622/626	98%	*Guignardia* sp6	KF128835
GSM1.2.10	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	588/635	93%	*Guignardia* sp1	
GSM1.2.6	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	591/637	93%	*Guignardia* sp1	KF128764
GSM1.2.7	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	581/631	92%	*Guignardia* sp1	KF128765
GSM1.2.8	*Geijera salicifolia*	JF261459.1 *Guignardia mangiferae*	546/584	93%	*Guignardia* sp1	KF128857
GSM1.3.6	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	591/638	93%	*Guignardia* sp1	KF128841
GSM1.4.2	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	594/639	93%	*Guignardia* sp1	KF128842

Code	Host	Closest match	Identity	% similarity	Proposed identity	Genbank No.
GSM1.5.3	*Geijera salicifolia*	JQ809680.1 *Guignardia mangiferae*	631/636	99%	*Guignardia* sp6	KF128847
GSM2.2.28	*Geijera salicifolia*	HM049171.1 *Guignardia bidwellii*	586/633	93%	*Guignardia* sp1	KF128784
GSM5.5.10	*Geijera salicifolia*	AY816311.1 *Guignardia mangiferae*	617/618	99%	*Guignardia* sp6	KF128851
GSM5.5.8	*Geijera salicifolia*	AB041241.1 *Guignardia mangiferae*	606/606	100%	*Guignardia* sp6	KF128853
MDC2.3	*Myoporum debile*	JF261459.1 *Guignardia mangiferae*	534/573	93%	*Guignardia* sp1	

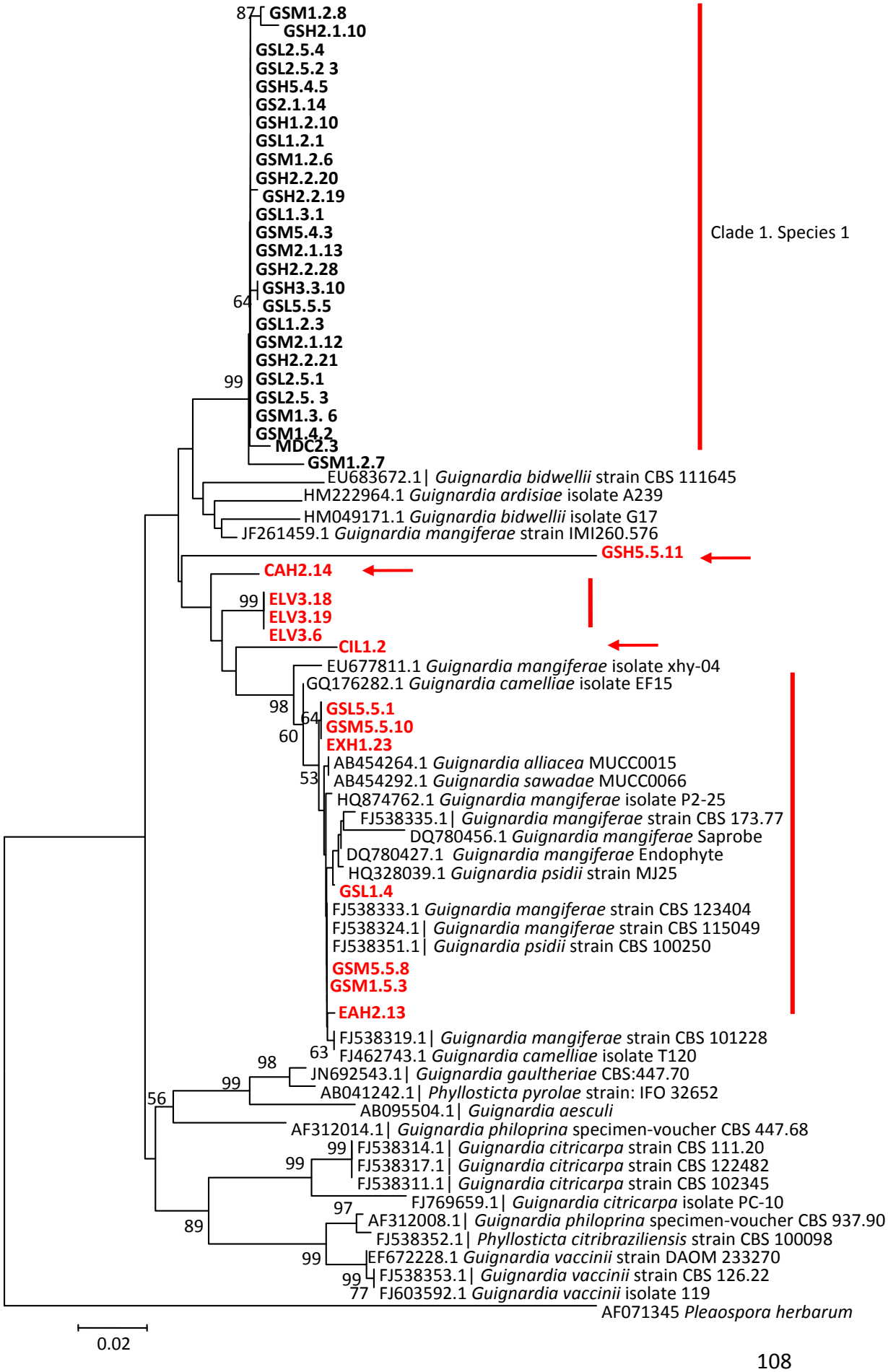

Figure 15 Neighbour-joining tree of the ITS region for *Guignardia* sp. Red lines and arrows indicate species groupings isolated in this study.

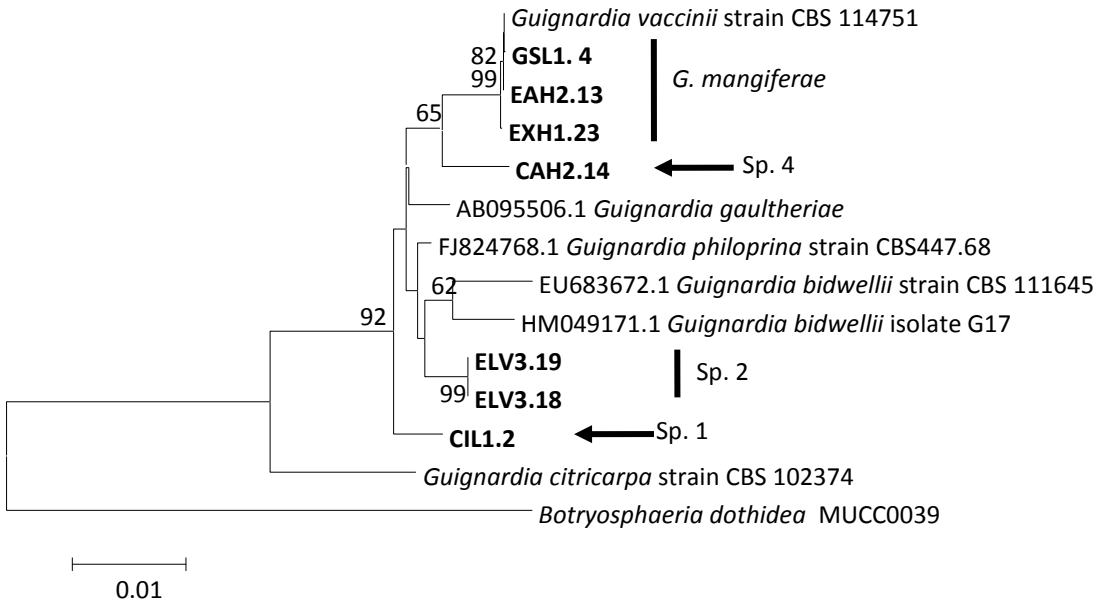

Figure 16 Neighbour-joining tree of the LSU region for *Guignardia* sp.

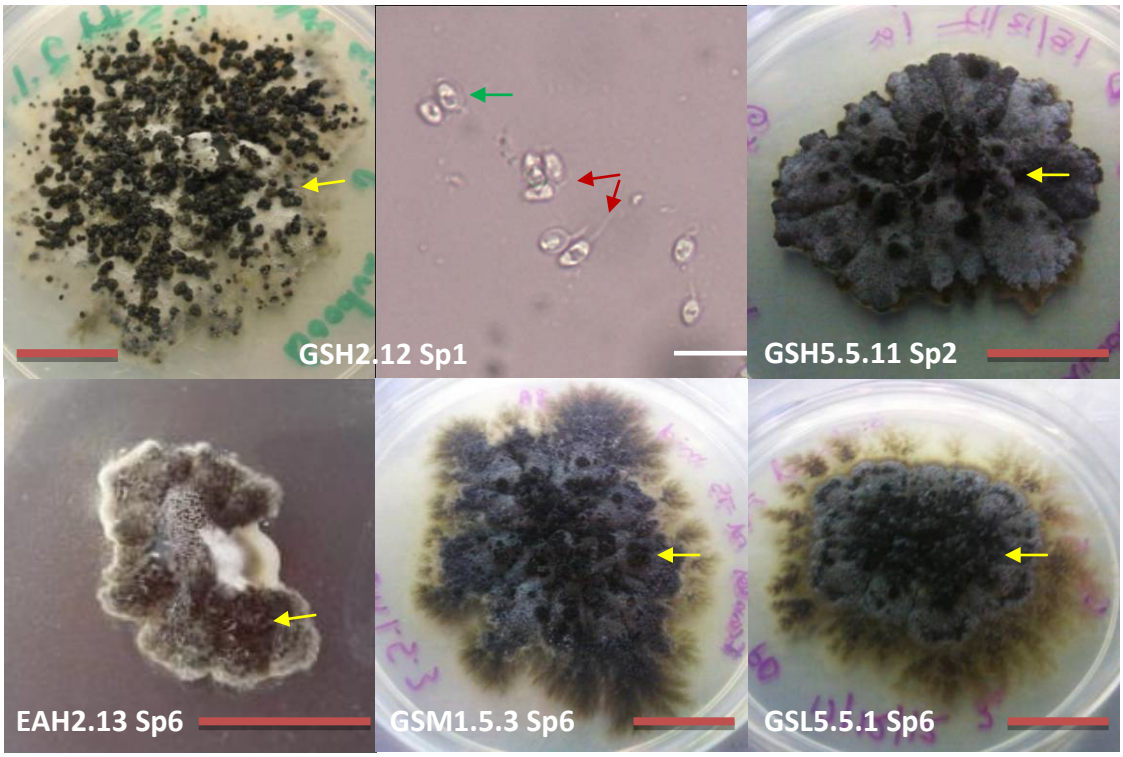

Figure 17 Morphological characteristics of three species of *Guignardia*. White scale bar=50µm. Red scale bar= 2cm. Yellow arrows indicate the black pycnidia common to all Guignardia isolates. Red arrows indicate the characteristic flagellum? attached to spores while the green arrow indicates the mucilaginous sheath.

4.4.4 Phylogenetic characterisation of Pezizales taxa

In total, 38 isolates in this project (including 24 from the *G. salicifolia* specificity study) were found to belong to the order Pezizales (Table 10). Three of these isolates had sequence similarities of less than 90% with identified species in Genbank and a further ten were between 91% and 93% (Table 10). 20 isolates had sequence similarities between 94% and 97% (Table 10). 34 sequences were found to belong to three families within the order Pezizales. Three isolates clustered within the Pyronemataceae and had a 99% match to other *Pyronema domesticum* sequences in Genbank. Isolate GSH2.9 had a 96% sequence similarity to *Paurocotylis pila* (Pyronemataceae) and isolate GSM5.1.5 had a 95% sequence similarity to an unknown Pyronemataceae; however, both these sequences formed a clade which separated very early in the Pyronemataceae clade (Appendices Figure C.27). Isolate AE3.2 had 96% sequence similarity to *Oedocephalum adhaerens* (*incertae sedis*). Phylogenetic analysis of the remaining 32 sequences found they formed well supported monophyletic clades, to the genus level belonging to two families (Figure 18). 29 isolates clustered with the Sarcosomataceae forming three separate clades (Figure 18). Clade 1 had 17 isolates, six of which had a closest match of between 89% and 94% to *Conoplea fusca* (EU552114.1). Of the remaining 11 in clade 1, nine had sequence similarities of 97% or less and twowere 98% similar to the same unidentified Sarcosomataceae endophyte (AF485074.1, Figure 18). When all 17 sequences were analysed using the align multiple sequences tool in GenBank, they were less than 97% similar to each other. This sequences similarity matched the phylogenetic analysis which formed a further 9 clades (Species 1-9, Genus 1; Figure 18). Isolate GSL5.6.3 formed a clade of its own (Figure 18). A further 11 isolates all

clustered together, had 93% sequence similarity to previously unclassified Pezizales and formed a well-supported clade which separated early within the Sarcosomataceae (Genus 2, Figure 18). When these 11 sequences were analysed using the align multiple sequences tool in GenBank, they were also found to be less than 97% similar to each other. The sequence similarity matched the phylogenetic analysis which formed a further 5 clades labelled species 1-5 (Genus 2; Figure 18). The remaining three isolates clustered with the Sarcoscyphaceae also forming a separate clade with good bootstrap support (Genus 3, Figure 18). Isolates from Genus 1 and 2 appeared similar when grown on PDA while the isolates from Genus 3 appeared similar to each other but were morphologically dissimilar to the isolates which clustered within Sarcosomataceae (Figure 19).

Table 10 Host and closest GenBank matches of Pezizales isolated in this study

Code	Host	Closest Match		%	Proposed identity	Genbank No.
AE2.1.1	*Alphitonia excelsa*	HM016895.1 *Pyronema domesticum*	431/440	98%	*Pyronema* sp.	KF227800
AE2.1.2	*Alphitonia excelsa*	JQ760765.1 Pezizomycetes sp.	407/426	96%	Sarcosomataceae sp.4 Genus 2	KF227801
AEH3.2	*Alphitonia excelsa*	FJ695215.1 *Oedocephalum adhaerens*	398/415	96%	Pezizales sp. 1	KF227802
AEL3.1	*Alphitonai excelsa*	HQ829058.1 *Pyronema domesticum*	473/474	99%	*Pyronema* sp.	KF227803
BPM2.5	*Brachychiton populneus*	EU552114.1 *Conoplea fusca*	470/510	92%	Sarcosomataceae sp.1 Genus 1	KF227807
CAH2.13	*Cassine australis*	JQ760750.1 Pezizomycetes sp.	511/550	93%	Sarcosomataceae sp. Genus 2	KF227812
CIM1.4	*Croton insularis*	U66009.1|PCU66009 *Pithya cupressina*	524/572	92%	Sarcoscyphaceae sp.1 Genus 1	KF227824
CIM1.1.6	*Croton insularis*	U66009.1|PCU66009 *Pithya cupressina*	530/580	91%	Sarcoscyphaceae sp.1 Genus 1	KF227825
ELV3.1.16	*Eustrephus latifolius*	EU552114.1 *Conoplea fusca*	531/590	90%	Sarcosomataceae sp.5 Genus 1	KF227844
EMH2.1.3	*Ehretia membranifolia*	JQ760747.1 Pezizomycetes sp.	468/497	93%	Sarcosomataceae sp.3 Genus 2	KF227853
EXH1.1.14	*Elattostachys xylocarpa*	EU552114.1 *Conoplea fusca*	506/564	90%	Sarcosomataceae sp.7 Genus 1	KF227855

Code	Host	Closest Match		%	Proposed identity	Genbank No.
EXH.1.1.21	*Elattostachys xylocarpa*	JQ761977.1 Pezizomycetes sp.	506/509	92%	Sarcoscyphaceae sp.1 Genus 1	KF227856
EXL1.1.16	*Elattostachys xylocarpa*	HQ829058.1 *Pyronema* sp.	564/565	99%	*Pyronema* sp.	KF227859
EXM1.11	*Elattostachys xylocarpa*	EU552114.1 *Conoplea fusca*	551/616	89%	Sarcosomataceae sp.7 Genus 1	KF227865
GSH2.9	*Geijera salicifolia*	EU826926.1 Uncultured soil fungus clone	519/542	96%	Pyronemataceae sp. 1	KF128865
GSH3.3.8	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	561/591	95%	Sarcosomataceae sp.8 Genus 1	KF128792
GSH4.2.7	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	571/583	98%	Sarcosomataceae sp.7 Genus 1	KF128806
GSH4.2.8	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	545/568	96%	Sarcosomataceae sp.1 Genus 1	
GSH5.1.10	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	541/572	95%	Sarcosomataceae sp.4 Genus 1	KF128879
GSH5.5.12	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	559/589	95%	Sarcosomataceae sp.2 Genus 1	KF128815
GSH5.5.13	*Geijera salicifolia*	JQ760638.1 Pezizomycetes sp.	572/601	95%	Sarcosomataceae sp.5 Genus 2	KF128816

Code	Host	Closest Match		%	Proposed identity	Genbank No.
GSL1.5.1	*Geijera salicifolia*	HM123375.1 Sarcosomataceae sp	560/582	96%	Sarcosomataceae sp.1 Genus 1	KF128823
GSL4.1.1	*Geijera salicifolia*	JQ760747.1 Pezizomycetes sp.	495/520	95%	Sarcosomataceae sp.3 Genus 2	KF128796
GSL4.2.3	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	554/562	99%	Sarcosomataceae sp.7 Genus 1	KF128871
GSL4.2.4	*Geijera salicifolia*	JQ760277.1 Pezizomycetes sp.	474/522	91%	Sarcosomataceae sp.1 Genus 2	KF128803
GSL5.1.2	*Geijera salicifolia*	AF485074.1 Sarcosomataceous endophyte	558/582	96%	Sarcosomataceae sp.5 Genus 1	KF128863
GSL5.2.13	*Geijera salicifolia*	JQ760520.1 Pezizomycetes sp.	517/556	93%	Sarcosomataceae sp.2 Genus 2	KF128881
GSL5.3.2	*Geijera salicifolia*	JQ760277.1 Sarcosomataceae sp.	488/533	92%	Sarcosomataceae sp.1 Genus 2	KF128832
GSL5.6.3	*Geijera salicifolia*	JF502436.1 Sarcosomataceae sp.	541/562	96%	Sarcosomataceae sp.1	KF128840
GSM1.5.2	*Geijera salicifolia*	HM123375.1 Sarcosomataceae sp	509/527	97%	Sarcosomataceae sp.1 Genus 1	KF128846
GSM2.1.3	*Geijera salicifolia*	JQ760747.1Pezizomycetes sp.	494/521	95%	Sarcosomataceae sp.3 Genus 2	KF128771
GSM2.1.8	*Geijera salicifolia*	AF485074.1 Sarcosomataceous	559/586	95%	Sarcosomataceae sp.3 Genus 1	KF128775

Code	Host	Closest Match		%	Proposed identity	Genbank No.
		endophyte				
GSM4.2.5	*Geijera salicifolia*	EU552114.1 *Conoplea fusca*	580/619	94%	Sarcosomataceae sp.9 Genus 1	KF128804
GSM5.1.5	*Geijera salicifolia*	HM123375.1 Fungal sp.	565/594	95%	Pyronemataceae sp. 2	KF128875
GSM5.5.9	*Geijera salicifolia*	JQ760520.1Pezizomycetes sp.	569/601	95%	Sarcosomataceae sp.5 Genus 2	KF128854
GSM5.6.4	*Geijera salicifolia*	JQ760432.1Sarcosomataceae sp.	565/598	94%	Sarcosomataceae sp.2 Genus 2	KF128855
GSM5.6.6	*Geijera salicifolia*	AF485074.1 Sarcosomataceae sp.	559/584	96%	Sarcosomataceae sp.6 Genus 1	KF128856
SLL2.3	*Santalum lanceolatum*	EU552114.1 *Conoplea fusca*	501/549	91%	Sarcosomataceae sp.3 Genus 1	KF227884

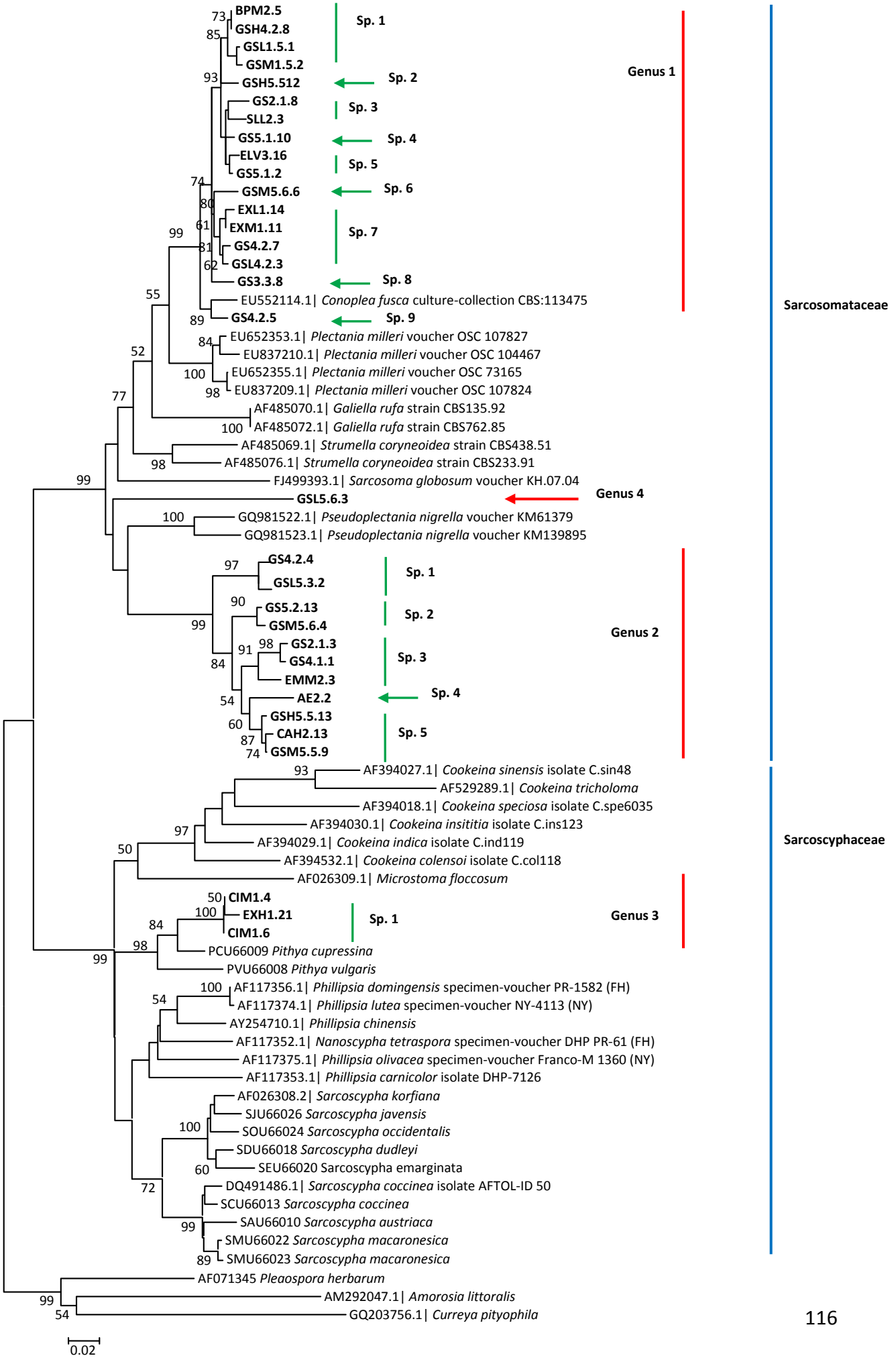

Figure 18 **Phylogenetic tree of Sarcosomataceae and Sarcoscyphaceae. Red lines indicate possible new genera, blue lines indicate family groupings. Red arrow indicates the single individual which may represent a new genus**

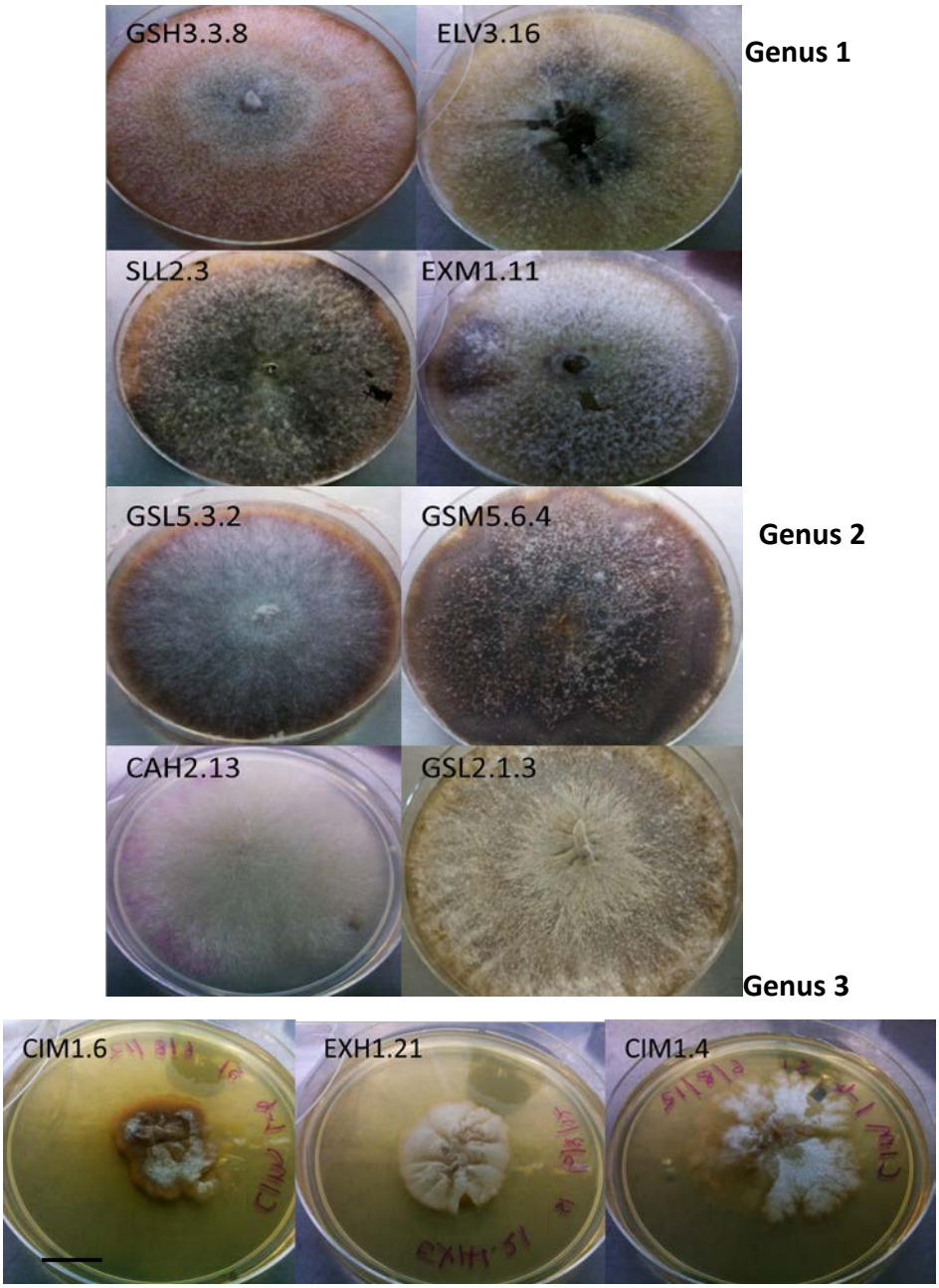

Figure 19 Morphological characteristics of the three potentially novel genera of Sarcosomataceae (Genus 1 and 2) and Sarcoscyphaceae (Genus 3)Scale bar = approximately 1cm

4.5 Discussion

The last 20 years has seen molecular-based methods revolutionise fungal taxonomy. The ease with which mycelial samples can now be sequenced and the increasing availability of comparative sequences in databases such as UNITE and GenBank has greatly aided fungal phylogenetic studies (Douanla-Meli and Langer 2012; Shenoy *et al.* 2007). Furthermore, molecular taxonomic approaches has led to the possibility of the

'one fungus one name' change to the ICBN (Douanla-Meli and Langer 2012; Hawksworth 2011; Taylor 2011). The ITS gene region is largely accepted as the genetic barcode for fungi, although there is acknowledgement that it is not useful for certain genera (Begerow *et al.* 2010; Bellemain *et al.* 2010; Douanla-Meli and Langer 2012; Eberhardt 2010; Meusnier *et al.* 2008; Nilsson *et al.* 2011; Seifert 2009). Several other gene regions may also provide useful taxonomic information, particularly when used in conjunction with the ITS and are useful for problematic genera (Gadagkar *et al.* 2005; Miller and Huhndorf 2005). The LSU occurs at the 5' end of the ITS region and is most commonly used in multigene studies but β tubulin and RBP2 protein coding regions are increasingly being used (Miller and Huhndorf 2005).

This study used a combination of ITS, LSU and β tubulin genes for the phylogenetic assessment of four endophytic taxa. The ITS region was found to provide excellent taxonomic clarity for the order Pezizales and sufficient resolution for *Nigrospora* at the species level. Good congruence was observed between the ITS and LSU for *Guignardia* however, this was limited due to a lack of comparative sequences in GenBank particularly for the LSU gene. The three gene tree provided the best resolution for *Preussia* although it was still insufficient to delineate to the level of species. It also highlighted the need to further clarify the taxonomic status of several genera within the Sporormiaceae. Recent work by Kruys and Wedin (2009) has shed some light on this, although it would appear that more research is needed. Overall, the most taxonomic clarity was provided by the ITS region and would support the use of this as a universal genetic barcode with the acknowledgement that certain genera are only resolved through multi gene analysis.

4.5.1 Phylogenetic characterisation of the *Nigrospora* isolates

According to the Index Fungorum, both *N. sphaerica* and *N. oryzae* are now named after their shared anamorph, *Khuskia oryzae*. A comparison of the isolates found within this study and those recorded as *N. sphaerica* or *N. oryzae* in GenBank found that they formed two distinct monophyletic clades. Examination of isolates which clustered within these clades found that there was a 95% sequence similarity between them. This would suggest that *K. oryzae* is actually a grouping of related species. Historically *N. sphaerica* or *N. oryzae* have been separated by the smaller spore size in the latter (Ellis 1971; Meredith 1961; Webster 1952). On the occasion when spore size was observed from isolates in this study, they were found to follow the patterns observed in the phylogenetic analysis. Given this morphological difference and the phylogenetic evidence from this study, it would appear that perhaps *N. sphaerica* or *N. oryzae* should be maintained as separate species.

4.5.2 Phylogenetic characterisation of the *Preussia* isolates

A diversity of *Preussia* spp. were isolated from SEVT. Because of the lack of sporulation of the isolates, molecular taxonomic methods involving sequencing of the ITS-rDNA, LSU-rDNA, and β tubulin regions and GenBank comparisons were used to identify fungal taxa. Of the 25 *Preussia* taxa obtained, 12 were able to be identified to species level while five had close similarity with two unidentified *Preussia* spp. Results from this study suggest that isolate BSH2.9 is not *Preussia africana* based on the combined analysis of all three gene regions but is not likely to be a new species. The remaining nine isolates were new *Preussia* records for GenBank.

Samples ADL2.4, CAL2.1, CAH2.9, NVM2.6 and PAL2.1 all returned the same closest match in GenBank (EU551184.1/EU551184.2 *Sporormiella isomera* syn. *Preussia isomera*), had similar morphological characteristics and grouped together when phylogenetically analysed. From this, it was concluded that they were different isolates of *P. isomera*. Isolates GSM2.1.5 and GSM2.1.7 also had similar morphological characteristics and grouped together with the other isolates of *P. isomera* and so were also given this name. Given that sample PAL2.3 had a divergent ITS, LSU and β tubulin sequence and had differing colony morphology, it is possible that this isolate is a related but different *Preussia* species. Samples GSM3.1 and BSH2.9 both returned closest matches to *P. africana* but when these samples were phylogenetically analysed, they did not group together. Furthermore, when the samples were compared to each other, they had a 92% sequence similarity. These results would suggest that they are in fact different species. Moreover, GSM3.1 clustered more closely with *P. lignicola*. From this, it is possible that GSM3.1 is an isolate of *P. lignicola* while BSH2.9 is most likely a previously isolated but undescribed *Preussia* sp.

Isolates ELV3.11 and ELV3.2 may potentially belong to a new genus. In all three gene regions examined, both isolates consistently formed a distinct clade which separated out early from other *Preussia* branches. Both isolates also had low sequence similarities (89%) to other known sequences in GenBank. This low similarity, together with the consistency observed between the three gene regions studied may indicate that they belong to a separate genus that is not currently represented in GenBank.

The results of this study also indicate that the ITS, LSU and β region are not sufficient in differentiating some species in this genus. Poor bootstrap support was consistently

observed in some clades and a polyphyletic structuring was observed at the genus level when *Westerdykella* and *Sporormiella* isolates were included in the analyses. There has been considerable difficulty in separating the genera *Preussia*, *Sporormiella* and *Spororominula* based on morphological characteristics in the past (Arenal *et al.* 2005; Ito and Nakagiri 1995; Kruys *et al.* 2006; Kruys and Wedin 2009). Substrate choice was considered an important factor in separating *Preussia* and *Sporormiella*, however studies have since shown this to be an inaccurate approach (Arenal *et al.* 2007; Kruys and Wedin 2009). It is now widely accepted that *Preussia*, *Sporormiella* and *Spororominula* are synonyms of each other and new species are given the name *Preussia* (Arenal *et al.* 2005, 2007; Guarro *et al.* 1997; Kruys and Wedin 2009). The results of this study concur with combining the three genera but also indicate that more work needs to be done in order to accurately resolve this genus.

Only a small number of *Preussia* spp. have previously been reported in Australia. The Australian plant pest database records three taxa; *P. minima* which was identified from seeds of chick pea and two isolates identified to the genus level from leaves of a bromeliad and an Asteraceae species. A recent paper by Peterson *et al.* (2009) recorded finding *P. africana, P. australis, P. minima* and *P. isomera* in the dung of Koalas. Some of the *P. isomera* samples isolated in this study clustered closely and had high sequence identity with the *P. isomera* (EU551184) from the study by Peterson *et al.* (2009). Bell (2005) has also recorded *P. cylindrica* and *P. funiculata* but although sequences for these taxa exist in GenBank, no matches were detected in this study. Bell also identified nine Australian *Sporormiella* spp. which all have corresponding sequences in GenBank but did not correlate with the samples obtained here. Six fungal isolates in this study had less than 97% identity to any *Preussia* species in GenBank thus suggesting that

there is greater diversity in this genus than currently reported in Australia. Further characterisation of *Preussia* sp. (FJ210518) and *Preussia* sp. (FJ210521) will conceivably add to the Australian species list for this fungal genus.

Members of the *Preussia* genus are most commonly coprophilous (Arenal *et al.* 2005; Kruys *et al.* 2006) but have also been isolated from wood, soil and dead plant matter (Arenal *et al.* 2005; Kruys and Wedin 2009; Poch and Gloer 1991). Less commonly, *Preussia* spp., have been collected from endophytic sources (Arenal *et al.* 2007; Peláez *et al.* 1998). As discussed in chapter 2, the results of this study would suggest that the *Preussia* endophytic life mode may be more frequent than previously thought.

Originally the difference between the genera *Preussia* and *Sporormiella* was thought to be the perithecial structure in the latter (Guarro *et al*. 1997). Recently, Kruys and Wedin (2009) argued for the merging of these genera based on detailed molecular and morphological examinations. *P. isomera* had been previously classed as *Sporormiella* due to its coprophilous growth habit (Kruys and Wedin 2009). However, in this study, isolates were observed growing endophytically, adding further support to the fact that growth form is not a suitable identifying feature, and that the *Preussia* and *Sporormiella* genera should be merged.

4.5.3 Phylogenetic characterisation of *Guignardia* isolates

56 isolates of *Guignardia* were observed overall within this study, predominately from the host plant *G. salicifolia.* The high occurrence frequency and ecological patterns of *Guignardia* were discussed more fully in chapter 3. Phylogenetic analysis suggested that six different species were observed between the two studies. Clade 1 was by far the most predominant species, with 26 isolates observed. This clade clustered separately to

other known species in GenBank. However, given that there are 256 recorded species for *Guignardia* in the Index Fungorum and only 10 are in GenBank, it is unlikely that any isolates observed in this study are new species for the genus.

There has been considerable debate regarding the precise number of species within the genus *Guignardia.* Currently the Index Fungorum lists 256 species, however in 2011 there were 335 species recorded (Wikee *et al.* 2011). In 2002, the anamorphic genus *Phyllosticta* was revised to include 141 members (Van der Aa *et al.* 2002) while Kirk and Ainsworth (Kirk and Ainsworth 2008) estimated only 91 species would be correct. As yet, very few *Guignardia* species have been linked to their *Phyllosticta* counterpart (Su and Cai 2012). This is in part due to many *Phyllosticta* species remaining asexual (Wikee *et al.* 2011). Given the changes under the code, only one name is now acceptable (Taylor 2011; Wingfield *et al.* 2012) and according to the Index Fungorum, *Guignardia* has been given precedence. However several authors have argued that the anamorphic stage, *Phyllosticta*, be given precedence due to its commonality and historical use (Glienke *et al.* 2011; Su and Cai 2012). Regardless of which name is accepted, currently described *Guignardia* need to be linked to their corresponding anamorph, most likely through the use of molecular methods. More research is needed in this area in order to sufficiently delineate the taxonomy of this genus.

4.5.4 Phylogenetic characterisation of the Pezizales isolates

This study observed 38 different isolates of Pezizales belonging to seven genera and comprising possibly 18 different species. Of these, 33 are likely to be new isolates and form four potentially new genera belonging to the Sarcosomataceae and Sarcoscyphaceae. 17 isolates were observed forming the well supported clade, genus 1,

with potentially nine different species observed. One individual was observed in genus 4 and 12 isolates occurred within genus 2, representing five different species. Similarly genus 3, which contained 3 isolates, formed a well-supported clade within the Sarcoscyphaceae that grouped separately but from a similar base branch to *Pithya*. Given the low sequence similarity (91-92%) of genus 3 to other *Pithya* isolates in GenBank, together with the phylogenetic evidence provided here, it would seem likely that this could also be a new genus within the Sarcoscyphaceae. All potentially new genera occurred on well supported branches and given that representatives of nearly all genera of the Sarcosomataceae and Sarcoscyphaceae appear in GenBank, it would seem likely that these are previously undescribed genera. One genus within the Sarcosomataceae and five genera of the Sarcoscyphaceae were unable to be included in the tree construction. However in all cases, these genera contain only one species, with the exception of *Kompsoscypha,* and are only found in European or North American areas (Kirk and Cooper 2007). Therefore, it is unlikely that the isolates in this study belong to these omitted genera.

Of the nine known genera belonging to Sarcosomataceae, four have been previously recorded in Australia (http://biocache.ala.org.au). These are *Plectania (*four species*)*, *Pseudoplectania nigrella, Strumella histerioidea* and *Urnula* sp. According to the Atlas of Living Australia (http://biocache.ala.org.au/), the vast majority of records occurred within Tasmania, although these taxa have also been observed in NSW along the coastline near Sydney, the eastern coastline of Victoria, several sightings near Perth WA and nine sightings in Queensland. Of these, only *Plectania campylospora* has been recorded in vine thickets or rainforests or in Queensland. Given that all of these genera are included in GenBank, and that none of them showed a close match to the samples

examined in this study, it would therefore seem highly likely that the results of this study are at least new records for Australia.

Similarly, four genera of the Sarcoscyphaceae have been recorded occurring in Australia. These are: *Sarcoscypha* (two species), *Cookeina* (three species), *Phillipsia* sp. and *Aurophora dochmia* (http://biocache.ala.org.au/). With the exception of *A. dochmia*, all of these species occur in GenBank. As *A. dochmia* is known only from the tropics(Denison 1969), the three taxa collected in this study may represent new records for Australia. Given that *A. dochmia* was collected from north Queensland, further research would need to be conducted in order to determine whether the taxa in this study represent a new species or a range expansion for *A. dochmia.*

Less than 97% DNA sequence similarity is often considered sufficient to delineate new species (Begerow *et al.* 2010; Hibbett *et al.* 2011; Hughes *et al.* 2009), although this is not universal for all fungal genera (Nilsson *et al.* 2008; Nilsson *et al.* 2009). Nilsson et al. (2008) argued that species variation within the ascomycota was 1.96% and therefore sequence similarities of less than 98% could be considered new species. Others have argued that a more conservative 95% cutoff should be used (Peay *et al.* 2007). Given the low sequence similarities and strong bootstrap support, it would seem likely that the 33 species which formed four distinct clades within the Sarcosomataceae and Sarcoscyphaceae are part of undescribed genera. This may be further supported by the fact that nearly all genera belonging to these two families were included in the phylogenetic assessment.

While a 3% sequence difference is routinely recognised as sufficient to delineate a new species for most fungi (Begerow *et al.* 2010; Nilsson *et al.* 2008), the variation indicative

of separating genera has been much less discussed. With the increase in uncultured environmental sequences in GenBank, some discussion has arisen regarding the need for at least temporary names for these sequences, representing novel species and genera (Horton *et al.* 2009; Nilsson *et al.* 2009; Ryberg *et al.* 2008). Currently, under the ICN code, no leeway exists for instances such as this. If morphological characteristics are not observed, as was the case in this study, assigning any kind of name to these potential new genera is impossible. Identification within GenBank must therefore be restricted to the family level. The ecological value of such phylogenetic studies therefore becomes limited since underlying patterns within ecosystems such as SEVT become more difficult to assess.

Genus 1 and 2 contained multiple species with more than 12 individuals each and were observed in both of the studies across all five sites. These were collected from several different trees belonging to eight different plant species suggesting that this is an ecosystem pattern rather than site or host specificity phenomenon. This observation is even more interesting considering that Pezizales are rarely observed as endophytes. As discussed more fully in chapter 3, more research is needed to examine what role these taxa may be playing within SEVT. However, the observation of these fungi occurring in a different ecological role to previously described Pezizales would further support their belonging to new genera.

4.6 Conclusion

This study has demonstrated that southeast Queensland SEVT house a diversity of novel endophytic *Preussia* spp, *Guignardia* spp. and Pezizales. This is a significant observation from a number of perspectives. Habitats such as SEVT are under continued threat from

clearing and weed invasion (McDonald 2010) with only 17% of the original habitat remaining. Permanent loss of these sites will impact on what appears to be a significant storehouse of previously undescribed fungal endophyte diversity. Both *Preussia* and Pezizales are also not typically observed as endophytes and results such as these highlight the need for continued investigations of other previously unexplored habitats to identify novel fungal life forms. More research is also needed to examine the precise ecological roles that the novel *Preussia* and Pezizales species and genera are serving within SEVT. Description of morphological features is also needed in order to further characterise and name these novel species.

5 Antimicrobial activity of endophytes from SEVT

5.1 Introduction

In recent years the problem of antibiotic resistance in nosocomial microbes, that is those originating in hospitals, has reached concerning levels (Guo *et al.* 2008a). With the rapid increase in such drug-resistant pathogens, the need for new antimicrobials has become critically important (Aly *et al.* 2011; Spellberg *et al.* 2008). Despite clinicians' best efforts to manage rates of resistance, new sources of potential drugs need to be regularly discovered in order to remain one step ahead of rapidly evolving microbial pathogens (Spellberg *et al.* 2008). Currently between 60-70% of all commercially used chemicals, many of which have important medicinal applications, are fungal in origin (Suryanarayanan *et al.* 2012). Given that fungal endophytes are potentially the most diverse but least explored group of fungi (Hawksworth 1991; Hawksworth 2001), these organisms offer the potential for many new drug discoveries. Most notable in recent years has been the discovery of taxol production in the endophytic fungus, *Taxomyces andreanae* (Stierle *et al.* 1993). Taxol is a highly effective anti cancer treatment (Wani *et al.* 1971) butprior to its discovery in fungi, treatments per patients were cost prohibitive and had considerable negative environmental impacts (Aly *et al.* 2011; Stierle *et al.* 1993). As a result of such discoveries, interest in fungal endophytes as sources of potential medicinal compounds has grown over the years. Much of this interest centres on the ability of endophytes to produce secondary metabolites.

Secondary metabolites are low molecular weight compounds produced by endophytic fungi in response to environmental stresses (Shwab and Keller 2008; Sumarah *et al.* 2010). These compounds, which tend to be encoded by gene clusters, have inhibitory or

toxic effects on other microorganisms (Shwab and Keller 2008). Up to 80% of endophytic secondary metabolites show bioactivity that may be of use medicinally, agriculturally or industrially (Schulz *et al.* 2002). Considering that over half of all compounds isolated from endophytes are novel (Tan and Zou 2001), it is unsurprising that interest in fungal endophytes as potential drug sources has increased dramatically (Berdy 2005; Lv *et al.* 2010; Strobel *et al.* 2004; Suryanarayanan *et al.* 2009; Tan and Zou 2001; Yu *et al.* 2010).

The methods used for obtaining and screening fungal endophytes can significantly alter the number of new antimicrobials obtained. The first factor to consider is the environment from which plants should be sampled. An environment continually exposed to stress, such as low rainfall areas, (Yu *et al.* 2010) will harbour endophytes with more novel compounds. Additionally, choosing an unexplored ecosystem, such as mangroves or marine environments, may also increase the likelihood of obtaining novel antimicrobials (Bhimba *et al.* 2012; Buatong *et al.* 2011; Schulz *et al.* 2002; Strobel and Daisy 2003). Thirdly, the plants from which endophytes are collected may also affect the number of antimicrobials discovered. A number of researchers have chosen plants with known medicinal properties with good results (Aly *et al.* 2011; Gao *et al.* 2005; Huang *et al.* 2008; Raviraja *et al.* 2006; Schulz *et al.* 2002; Tejesvi *et al.* 2007; Wiyakrutta *et al.* 2004). The final consideration is the method of extraction and testing of fungal endophytes. Most high throughput methods involve testing crude extracts of cultures growing in broth (Yu *et al.* 2010). The problem associated with this is that in most cases, fungi have not been stressed or exposed to competitive microbes during growth. Therefore, detection of novel chemicals is unsuccessful because the fungi have not been stimulated to produce them. Despite the ability to perform thousands of tests in a day,

rates of discovery using high throughput methods have decreased (Mishra *et al.* 2008), and are known for yielding false hits (Zhang *et al.*). Using the more laborious plate screening method (Amin *et al.* 2010; Boddington 2009), may enable a pre-selective step prior to bulk testing of crude extracts and help to eliminate some of the false leads. This pre-selection step may be further enhanced by the addition of dead microbial cells to the broth in order to induce a response from the fungus (Boddington 2009).

5.2 Aims

This study examined the antimicrobial activity of fungal endophytes isolated from SEVT and aimed to address the following questions:

1. Which SEVT fungal endophytes produce antimicrobials?
2. Is there a higher proportion of fungal endophytes producing antimicrobials in SEVT compared to other studies?
3. Are the active compounds fungicidal, bacteristatic or bactericidal?
4. What are the compounds involved?
5. What is the minimum inhibitory concentration of these compounds?

Isolates were initially screened via the method outlined in Boddington (2009) and Amin et al. (2010) and then further stressed in bulking broth by the addition of autoclaved bacterial and yeast? cells. Extracts of fungi with antimicrobial activity were fractionated via HPLC and pure compounds further characterised by NMR.

5.3 Methods

5.3.1 Initial screening of endophytic isolates

Fungal isolates grown as per the methods detailed in Chapter 2 were tested for bioactivity using a variation of the method outlined in Boddington (2009) and Amin et al. (2010). The centre of a Sensitest (STA, Sigma, Castle Hill, NSW, Australia) plate was inoculated with 0.5cm^3 of pure fungal culture on agar and grown in the dark at 22^{o}C. Once cultures were >2cm in diameter, bacteria were streaked onto the plate beginning at the edge of the fungal colony and continuing outwards (Figure 21). A total of six medically important bacteria and one fungus were tested, these were three gram positive bacteria;*Bacillus cereus* (ATCC 14579), *methicillin resistant Staphylococcus aureus* (ATCC 43300) and *Enteroccocus faecalis* (ATCC 19433); three gram negative bacteria : *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 25668), *Serratia marcescens* (ATCC 14756), and *Candida albicans* (ATCC 10231) as the fungal representative. All six bacteria and *C. albicans* were tested on each plate. Plates were then left to incubate overnight at 37^{o}C. If inhibition was observed, this was measured as the distance between the edge of the hypha and the beginning of the first bacterial/*Candida* colony. All plates were prepared in duplicate.

Figure 20 Method of testing fungal endophytes, F= fungus Numbers represent different pathogens

When large numbers of the same endophytic species were isolated, a subset of those isolates was screened. Isolates which were unable to grow on STA were also excluded from further analysis. If isolates were found to belong to a species whose medicinal characteristics have previously been well described e.g. *Penicillium*, they were also not taken further.

Isolates which showed some level of bioactivity were retested to ensure reproducibility. Endophytes which continued to show bioactivity were then selected for further analysis and grown in 10ml tubes containing Malt extract (ME) broth for one week with ambient light and gently shaken at 130rpm. If little growth was observed at this point (e.g. colony had not grown beyond 2.5cm in diameter), the isolate was discarded from further analysis.

Once fungal endophytes had been growing in ME for a week, samples were transferred to 500ml flasks for bulk growth. Initially, isolates were grown in 3x 500ml conical flasks with 250ml of ME broth which was then shaken at 150rpm for one month at 23^{o}C. However, this was found to increase the risk of contamination and that the temperature fluctuated up to 6^{o}C. As a result, later samples were grown under static conditions at

23^{o}C. In the final week, the temperature was reduced to 20^{o}C in order to slow growth and increase secondary metabolite production. After four days of growth, 1ml of autoclaved *Staphylococcus aureus* and *Enteroccocus faecalis* or *Candida albicans* cells were aseptically transferred to the growing cultures.

Once the samples had grown for four weeks, bioactive compounds were extracted using ethyl acetate (EtOAc). This was achieved by first filtering the broth through sterile cheesecloth to remove the mycelium. The remaining broth solution was then divided in half and added to two 500ml separatory funnels. 200ml of EtOAc was then added to each funnel. These were shaken vigorously and allowed to settle before collecting the EtOAc layer. This procedure was repeated and a 5ml sample of the broth solution was kept for testing. The remaining mycelium was scraped into a separate beaker and 200ml of EtOAc added. Mycelia were then crushed with a glass rod and left to soak in the beaker. After about 20 minutes, the EtOAc was filtered and added to the EtOAc sample obtained above. The total volume was then 1L per 500ml of starting broth. The EtOAc was then evaporated off the final sample using a rotary evaporator (Heidolph Laborota 4001, Brinkmann, Germany).

Bioactivity was re-confirmed by testing a small amount of the dried down crude sample reconstituted in 25%-50% Ethanol (EtOH), depending on solubility. Some crude extracts were reconstituted in 50% Di-methyl sulphoxide (DMSO) to a concentration of 500μg/ml however it was found that DMSO inhibited bacterial growth and so future samples were tested using either 50% or 25% EtOH, depending on extract solubility. Testing was achieved by well assay and was based on the standards produced by the Clinical Laboratory Standards Institute (CLSI (Clinical *et al.* 2006), and performed in

duplicate against MRSA, *E. faecalis, B.cereus, C. albicans, E.coli* and *P. aeruginosa*. Each well of the microtitre tray contained 50µl of Mueller Hinton (MH) broth, 50µl of bacteria suspended in 0.7% saline and 50µl of the crude extract or broth. Test pathogens were made up to a 0.5 McFarland standard. Controls included in each assay were as follows: Positive control = 50µl MH broth, 50µl appropriate antibiotic (amoxicillin, tetracycline, ciprofloxacin, vancomycin or amphotericin B at a final concentration of 4µg/ml), 50µl bacteria; Negative control= 50µl MH broth, 50µl sterile distilled water, 50µl bacteria; Contamination control = 50µl MH broth, 100µl of sterile distilled water and a solvent (EtOH) control = 50µl MH broth, 50µl solvent, 50µl bacteria. 50µl of the left over broth from each sample was also tested as an additional control in the well assay, to ensure bioactivity was not contained in this layer. Samples of the crude extract showing bioactivity against the pathogens were taken to Eskitis Institute for Cell and Molecular Therapies, Griffith University, Brisbane for fractionation.

5.3.2 Fractionation of crude extract

Samples were fractionated at Eskitis (Eskitis Instititute, Griffith University, Mount Gravatt, Queensland) with the assistance of Dr Rohan Davis. HPLC analysis was conducted by first dissolving 2mg of each fungal EtOAc extract into 200µl of DMSO of which 100µl was injected onto an analytical C18 Onyx HPLC column (4.6 mm × 150 mm). The HPLC system used consisted of a Waters 600 controller and pump (225µl pump-heads) and 996 PDA detector. A Preparative HPLC FLOM model 722 2-channel Gastorr degasser was connected between the HPLC solvent reservoirs and the HPLC gradient mixer and pump. A Gilson 215 fraction collector (5ml syringe) equipped with an 819 injector valve actuator with 200µl Rheodyne sample loop was used for analytical HPLC injections and fraction collection. Waters Millenium32 (versions 4.0) and Gilson 735

software (version 6.00) software were used on the HPLC and liquid handling systems, respectively. All solvents used for chromatography were Lab-Scan HPLC grade and the H_2O was Millipore Milli-Q PF filtered. Trifluoroacetic acid (TFA) was Fluka at a 99% purity (Sigma, Castle Hill, NSW, Australia). Evaporation of HPLC fractions was performed using a Christ Alpha-condenser connected to a Christ Beta-RVC centrifugal evaporator. Methyl 4-hydroxy benzoate 99% (Aldrich Cat #M50109), ethyl 4-hydroxy benzoate 99% (Aldrich Cat #111988), benzophenone 99% (Aldrich Cat #B930-0) and uracil 99% (Sigma Cat #U-0750) were used as positive controls for the HPLC fractionation protocol. Extracts were then fractionated using a methanol/water/0.1% trifluoroacetic acid gradient, conditions of which have been reported elsewhere (Camp *et al.* 2011). The total run time for each injection was 11 minutes with five fractions (5 x 1 minute) collected between 2 and 7 minutes. Following fractionation of all eight bioactive extracts, a standard mixture consisting of methyl 4-hydroxy benzoate, ethyl 4-hydroxy benzoate, benzophenone and uracil (all in 0.125 mg/ml in DMSO) was injected as a positive control for the HPLC process. Solvents were evaporated to dryness, with each fraction being resuspended in 50% or 25% EtOH, depending on solubility. Fractions were then retested for antimicrobial activity by the CLSI method as described above. Isolates with bioactive fractions were then regrown in larger volumes (8x 500ml flasks=2L) via the procedures outlined above.

Fractions were further separated using C18 silica semi-preparative HPLC analysis on a C18 flash column to obtain a pure compound and then Proton NMR (using either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer) was utilised for structural elucidation. Fractions or pure compounds were obtained using isocratic conditions of 100% aqueous TFA (1%) for 1 minute followed by a linear gradient to 99% MeOH/1%

TFA in 59 minutes at a flow rate of 9ml/minute. Sixty fractions (1 minute each) were collected. If further purification was needed, fractions were preabsorbed to C18 silica semi-preparative and then loaded onto a C18 flash column, using isocratic conditions of 80% H_2O/20% MeOH for 1 minute followed by a first step of a linear gradient to 60% H_2O/40% MeOH in 50 minutes, a second step of a linear gradient to 99% MeOH/1% TFA in 5mn and hold 5mn while in isocratic mode at a flow rate of 9ml/minute. NMR characterisation of compounds was undertaken by Dr Rohan Davis at the Eskitis.

5.3.3 Testing of pure compounds

The antimicrobial activity of pure compounds was tested against two microorganisms commonly associated with nosocomial infections; methicillin resistant *Staphylococcus aureus* (ATCC 43300) and *Candida albicans* (ATCC 10231). Antimicrobial activities were evaluated using a broth microdilution method based on the protocol outlined in the CLSI procedures (Wikler *et al.* 2006). In brief, cultures of MRSA and *C. albicans* were freshly cultured onto either Sensitest (Oxoid, Australia) or horse blood agar (Sigma, Castle Hill, NSW, Australia) and incubated overnight at 35^{o}C in preparation for testing. Test cultures were suspended in MH broth to a concentration of a 0.5 McFarland standard and used within 15 minutes of preparation. Test compounds were dissolved in a 25% ethanol/0.7% saline solution in a double dilution series beginning at 1mg/ml. The minimum inhibitory concentrations (MIC) for each organism was read as the concentrations in the first wells that showed no visible growth after incubation at 35°C at 18, 20 and 24 hours for MRSA and 18, 20, 24 and 48 hours for *C. albicans*. Vancomycin and methicillin (4μg/ml) were used as controls for MRSA while amphotericin B (4μg/ml) was used for *C. albicans*. Other controls included a broth contamination control and a solvent control. The resistance to methicillin of freshly

grown MRSA was confirmed prior to testing using oxacillin discs according to the Calibrated Dichotomous Sensitivity (CDS) test method (Bell *et al.* 1999).

5.4 Results

5.4.1 Initial screening against endophytic isolates

A total of 206/228 fungal isolates collected from SEVT were tested for antimicrobial activity. Some isolates were encountered frequently and so not all were screened. For example of the 66 *Nigrospora* spp. isolated, only 53 isolates were screened. Additionally, three of the seven *Xylaria* spp. isolated were not screened since this genus is relatively well characterised in terms of bioactive compounds (Stadler 2011). An additional six fungal isolates could not be screened due to their inability to grow on STA and so were excluded from further analysis.

Of the 206 isolates tested, 16% (r 33 isolates) showed some level of bioactivity in initial screens (Table 11). No statistical difference was observed for the number of bioactive fungi between the three sites as determined by one-way ANOVA ($F(2,20) = 1.042$, $p = 0.371$; appendices Table D. 22). Most commonly, activity was observed towards *C. albicans* and/or gram positive bacteria. Three isolates (EOM1.6, GSM2.2 and MDC2.3) showed activity towards gram negative bacteria (Table 11). Morphological and DNA analysis determined these samples to be a *Penicillium* sp., a *Lecythophora* sp. and a *Guignardia* sp. These samples were not processed further however, due to only minor inhibition observed in the case of the *Lecythophora* sp. and *Guignardia* sp. and the likelihood of a penicillin-based product in the *Penicillium* sp. Isolates ELV3.12 and ELV3.15 were found to be genetically identical when sequenced and so were considered to be the same species. Fifteen samples showed activity against *C. albicans*, 22 were found to inhibit MRSA and 17 showed activity against *E. faecalis* (Table 11).

Table 11. Results of initial screening test against nosocomial pathogens

Isolate code	Identity	*B. cereus* (ATCC 14579)	*E. coli* (ATCC 25922)	*E. faecalis* (ATCC 19433)	*P. aeruginosa* (ATCC 25668)	*S. marcescens* (ATCC 14756)	MRSA (ATCC 43300)	*C. albicans* (ATCC 10231)
ADL2.4	*Preussia isomera*	+	-	++	-	-	++	-
ADM2.11*	*Preussia* sp.1	++	-	++	-	-	+	++
BSH2.9*	*Preussia africana*	+	-	+	-	-	++	-
BSH2.12	Pithomyces	-	-	-	-	-	+	-
CAL2.1	*Preussia* sp.	+	-	+	-	-	++	-
CAL2.2	*Pestalotiopsis* sp.	-	-	-	-	-	++	-
CAH2.9*	*Preussia isomera*	-	-	+	-	-	++	-
CAH2.11*	*Preussia isomera*	++	-	+	-	-	++	++
EAL2.4*	*Preussia* sp.2	-	-	+	-	-	++	-
EAL2.5	*Preussia* sp.3	-	-	+	-	-	+	-
EAL2.7*	*Preussia* sp.4	+	-	+	-	-	+	-
EAH2.13*	*Guignardia* sp.	-	-	-	-	-	++	-
ELV3.4	*Preussia* sp.	-	-	++	-	-	++	+
ELV3.7	*Preussia* sp.	-	-	-	-	-	-	++
ELV3.11*	*Preussia* sp.5	-	-	-	-	-	+	-
ELV3.12/15	*Coniochaeta* sp.	-	-	-	-	-	-	++
EMH2.3*	Pezizales	-	-	-	-	-	-	+
EOM1.6	*Penicillium* sp.	+	++	-	-	++	-	++
EXH1.22	*Muscodor* sp.	-	-	++	-	-	-	-
GSM2.2	*Lecythophora* sp.	-	+	-	-	+	-	+

Isolate code	Identity	*B. cereus* (ATCC 14579)	*E. coli* (ATCC 25922)	*E. faecalis* (ATCC 19433)	*P. aeruginosa* (ATCC 25668)	*S. marcescens* (ATCC 14756)	MRSA (ATCC 43300)	*C. albicans* (ATCC 10231)
GSH2.7*	*Coniochaeta* sp.	-	-	-	-	-	++	+
MDC2.3	*Guignardia* sp.	-	+	-	-	+	-	+
MDC2.4	*Toxicocladosporium* sp.	-	-	+	-	-	+	+
NVM2.4	*Fimetariella rebenhorstii*	-	-	+	-	-	++	+
NVM2.6	*Preussia isomera*	-	-	+	-	-	++	-
NVH2.11	*Lecythophora* sp.	-	-	+	-	-	++	++
PAL2.1	*Preussia isomera*	-	-	+	-	-	-	-
PAL2.3*	*Preussia* sp.6	-	-	++	-	-	++	+
PAM2.6	*Nigrospora* sp.	-	-	-	-	+	-	-
PPV3.1	*Epiccocum* sp.	-	-	-	-	-	++	-
SFL3.1	*Periconia* sp.	-	-	-	-	-	+	-
SLH2.17*	*Pseudocercospera* sp.	-	-	-	-	-	-	+
Total		7	3	17	0	4	22	15

"+" indicates inhibition zones <5mm observed, and "++" indicates inhibition zones ≥5mm. *indicates samples selected for HPLC analysis.

5.4.2 Fractionation of crude extract

All 33 isolates which showed initial bioactivity were selected for further analysis and grown in 10ml tubes for one week. Of these, 11 did not grow well by the end of the week and so were excluded from future analysis. The remaining 21 were transferred to 3x 500ml conical flasks for bulk growth. Two isolates became contaminated during this step and a further five isolates failed to show any bioactivity when crude extracts were tested after bulk growth and so were not tested further. The remaining 15 samples were HPLC analysed (Table 11; Table 12). When the HPLC fractions were then retested, EAH2.13 failed to show any bioactivity in the tubes collected between 2 and 7 minutes (Table 12). Isolate PAL2.3 was grown twice under the same conditions but produced a different chromatogram pattern (Figure 21) and so was rejected for further analysis. Of the remaining fractions, 11 showed antifungal activity and 10 showed activity towards MRSA (Table 12).

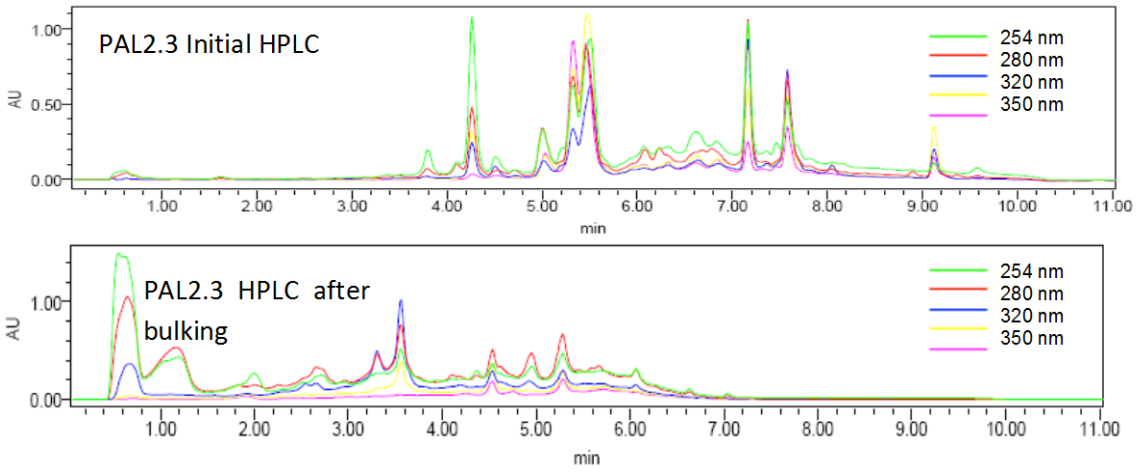

Figure 21 Isolate PAL2.3 showing different HPLC traces when grown under the same conditions.

Fifteen of the 33 isolates which showed initial bioactivity, belonged to the genus *Preussia*. During initial screening, all 15 isolates showed inhibitory activity against

MRSA*, E. faecalis, B. cereus* or *C. albicans* (Table 11). Crude extracts of eight of the isolates inhibited the growth of MRSA and *C. albicans*. The resulting HPLC fractions were re-tested for antimicrobial activity and bioactivity was found in samples corresponding to the region of separation from three to seven minutes (Table 12). Fungicidal activity was observed in fractions for all eight samples except BSH2.9 while bacteriostatic activity was observed against MRSA in fractions for all samples except ADM2.11 and PAL 2.3.

Table 12 HPLC fractions showing bioactivity against MRSA and *C. albicans*

Isolate code	Proposed identity	Fractions collected with activity against MRSA	Fractions collected with activity against *C. albicans*
ADM2.11	*Preussia* sp. (presumptive new taxa 1)	No fractions with activity against MRSA	6 min
BSH2.9	*Preussia aff. africana*	3,4,5 min	No fractions with activity against *C. albicans*
CAH2.9	*Preussia isomera*	3 & 5 min	2,4,5 min
CAH2.11	*Preussia isomera*	5 min	2,3,5,6 min
EAL2.4	*Preussia* sp. (presumptive new taxa 2)	5 min	No fractions with activity against *C. albicans*
EAL2.7	*Preussia* sp. (presumptive new taxa 4)	3 & 4 min	2,5,6 min
EAH2.13	*Guignardia* sp.	No fractions with activity against MRSA	No fractions with activity against *C. albicans*
ELV3.11	*Preussia* sp. (presumptive new taxa 5)	3 & 4 min	5 & 6 min
EMM2.3	Pezizales	No fractions with activity against MRSA	4 min
EXH1.22	*Muscodor* sp.	9 min	9min
GSL2.7	*Coniochaeta* sp.	3 & 4 min	4 min
MDC2.3	*Guignardia* sp.	7 &8 min	No fractions with activity against *C. albicans*
NVH2.11	*Lecythophora* sp.	5 min	4 min
PAL2.3	*Preussia* sp. (presumptive new taxa 6)	No fractions with activity against MRSA	3,4,5 min
SLH2.17	*Pseudocercospora* sp.	No fractions with activity against MRSA	4 min

5.4.3 Semi preparative carbon HPLC analysis of isolates

Due to a sufficient amount (>50mg) of crude extract being obtained, semi preparative carbon HPLC analysis was able to be conducted on four of the isolates (Figure 22). This was conducted on BSH2.9, EXH2.11 and CAH2.9 from crude extract collected from shaken cultures and CAH2.11 and BSH2.9 using static conditions for growth. Six pure compounds were successfully obtained from BSH2.9. Only those fractions of BSH2.9 from crude extract grown under static conditions continued to show bioactivity.

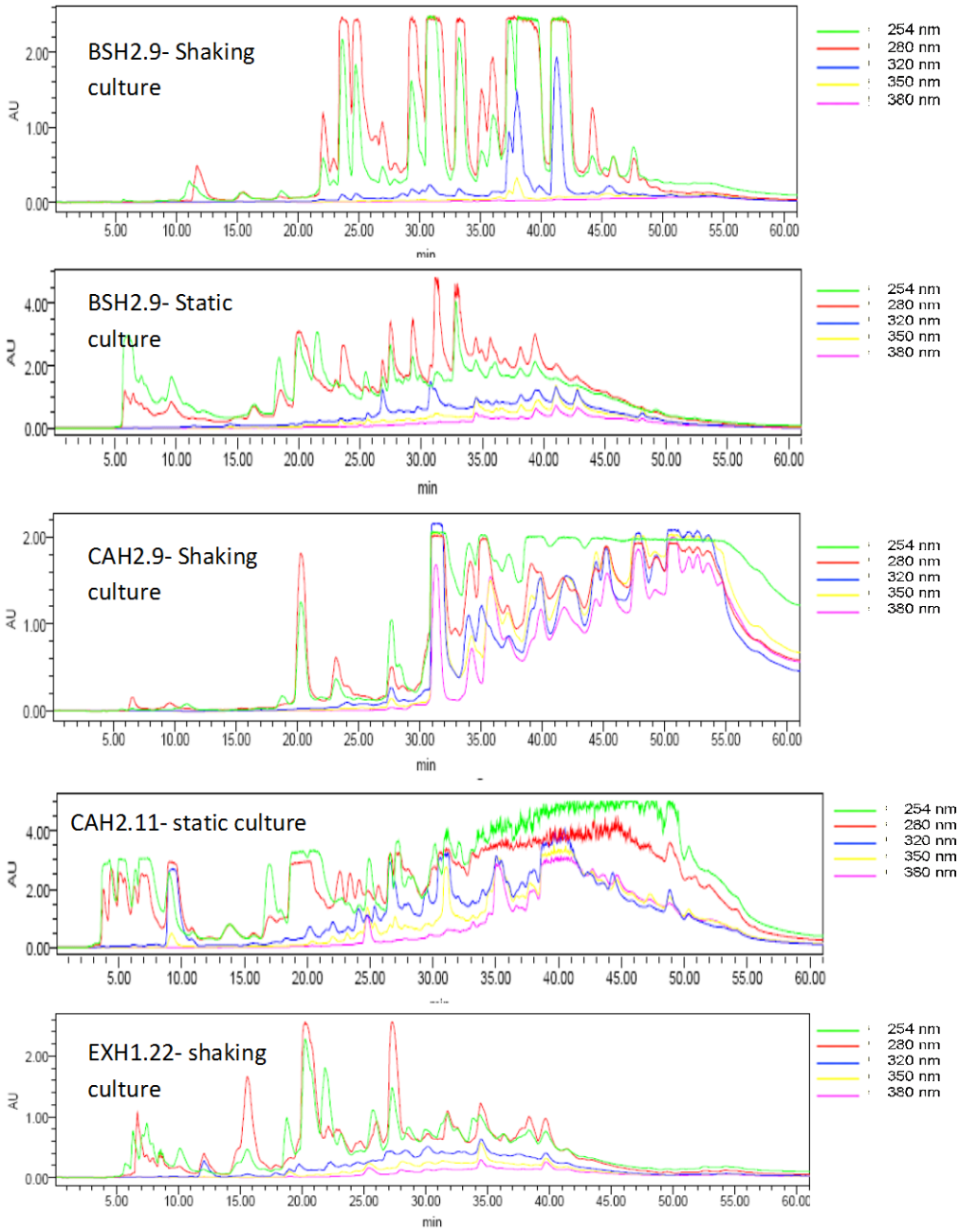

Figure 22 Semi preparative Carbon HPLC trace of four isolates. Mode of culture incubation is indicated.

Isolate BSH2.9 was grown in bulk using both static and shaking conditions. No bioactivity was observed in any of the HPLC fractions from crude extract which were shaken during culturing. When isolate BSH2.9 was grown again under static conditions the resulting chromatogram contained a similar pattern of peaks with the exception of the loss of a peak occurring at six minutes (Figure 23). When the resulting HPLC

fractions were retested, activity towards MRSA was observed in tubes corresponding to three, four and five minutes (Figure 23, Table 12).

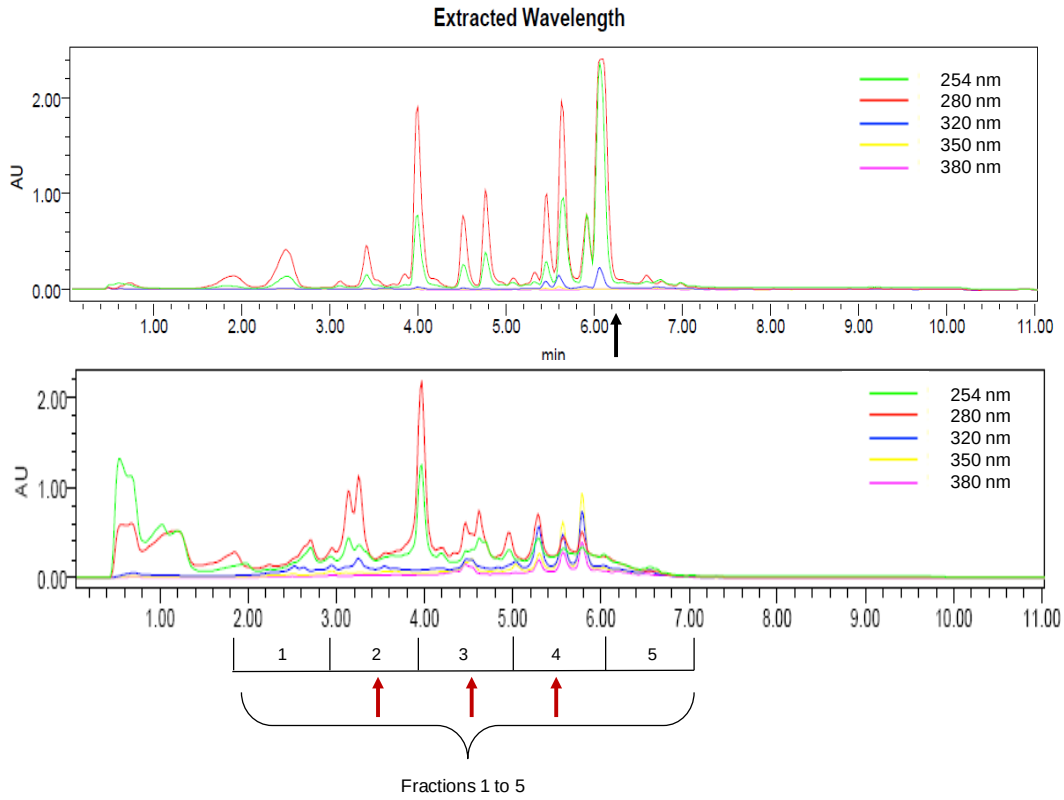

Figure 23 HPLC traces of BSH2.9. Black arrow indicates lost peak and location of one pure compound, red arrows indicate regions where activity towards MRSA was detected

A pure compound was obtained from the initial culture which corresponded to peaks observed at the six minute (Figure 23) and 42 minute mark respectively (Figure 24). 1mg of pure compound was obtained for testing against MRSA and *E. faecalis* however a serial dilution beginning at 200µg found no bioactivity. Structural elucidation identified the pure compound as a new structure related to bromomethylchlamydosporols A and B, which have both been known to have mild anti-microbial activity (Nenkep *et al.* 2010; Figure 24, Figure 25; see appendices Table D.23 for NMR data).

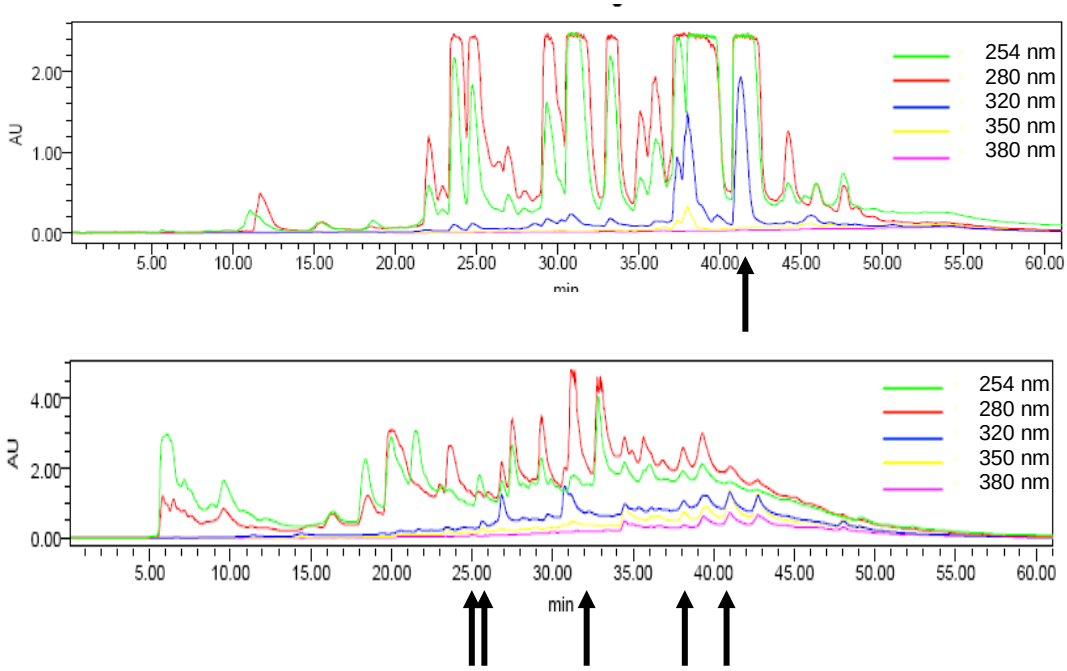

Figure 24 Semi preparative Carbon HPLC trace of BSH2.9 from the shaking and static culture methods respectively. Arrows indicate fractions from which pure compounds were obtained.

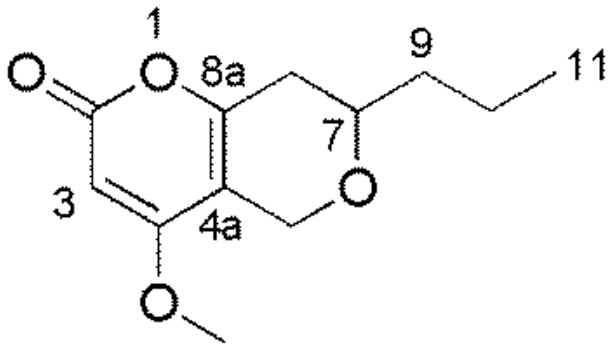

Figure 25 Chemical structure of pure compound obtained from BSH2.9 using the shaking method of incubation.

A further five pure compounds were obtained from BSH2.9 crude extract cultured via the static method (Figure 26). Pure compounds corresponded to semi prep fractions collected at 25, 31, 38 and 41 minutes (Figure 24). 1mg of pure compound was obtained for testing for three of the compounds (Figure 24). Of the two pure compounds which eluted at 25 minutes, only small amounts were obtained and therefore these could not be further tested. All three compounds tested showed bioactivity to *C. albicans* and two showed activity towards MRSA (Table 13). BSH2.9/31 and 38 had minimum inhibitory concentrations (MIC) against *C. albicans* of 0.25mg/ml at an incubation time of 24 hours

and no fungicidal activity when tested at 48 hours (Table 13). Pure compound BSH2.9/41 had a MIC of 0.06mg/ml at 24 hours and showed fungicidal activity at a concentration of 1mg/ml (Table 13). Compounds BSH2.9/ 31 and 41 had MIC of 0.007mg/ml and 0.03mg/ml respectively at 24hrs (see appendices Table D.24 and Table D.25 for antimicrobial data). Both did not exhibit bactericidal activity (Table 13).

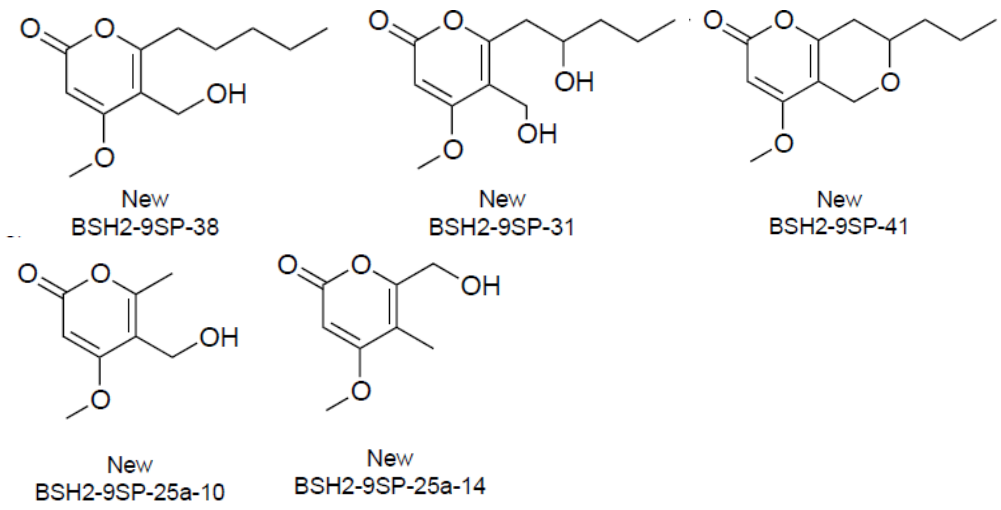

Figure 26 Pure compounds obtained from isolate BSH2.9 using the static culture method

Table 13 Minimum inhibitory concentrations (MIC) for the three pure compounds tested against MRSA and *C. albicans* at various time intervals

Compound tested	*C. albicans* MIC 18hrs	*C. albicans* MIC 24hrs	*C. albicans* MIC 48hrs	*C. albicans* MFC 48hrs	MRSA MIC 18hrs	MRSA MIC 24hrs	MRSA MBC
BSH2.9/ 31	0.12mg/ml	0.25mg/ml	0.5mg/ml	NA	0.004mg/ml	0.007mg/ml	NA
BSH2.9/ 38	0.25mg/ml	0.25mg/ml	0.5mg/ml	NA	NA	NA	NA
BSH2.9 /41	0.03mg/ml	0.06mg/ml	0.12mg/ml	1mg/ml	0.007mg/ml	0.03mg/ml	NA

NA= Not Applicable MBC =Minimum bactericidal concentration, MFC = Minimum fungicidal concentration

Structural elucidation of the five compounds found them to be novel polyketides which were related to each other. The five compounds were given the name Africanerones A-D respectively (Figure 26; Davis manuscript in preparation).

5.5 Discussion

The search for new antimicrobial compounds is a critical strategy in addressing the emergence of antimicrobial resistance (Berdy 2005; Coates and Hu 2006; Spellberg *et al.* 2008). Of particular concern in recent years has been the increase in observance of vancomycin-resistant staphylococci (Périchon and Courvalin 2009). Vancomycin is currently considered the drug of last resort for treating persistent staphylococci infections (Levine 2006). This is partially due to its effectiveness against such infections but also due to its toxicity to mammalian cells (Vaz *et al.* 2009).

Many of the drugs currently available for treating medically important bacteria have significant side effects. For example, amphotericin B, which is often the only effective antimycotic for treatment of systemic fungal infections (including *C. albicans*), is limited due to severe infusion-related nephrotoxicity and numerous other side-effects (Laniado-LaborÃ-n and Cabrales-Vargas 2009). As mentioned, vancomycin use to combat MRSA is also compromised by toxicity issues.

Interest in the use of fungal endophytes as potential new drug sources has gained momentum in the last several decades (Berdy 2005; Lv *et al.* 2010; Strobel *et al.* 2004; Suryanarayanan *et al.* 2009; Tan and Zou 2001; Yu *et al.* 2010). This interest has been heightened by reports which have shown that when endophyte-colonised plants are challenged by microbial pathogens they activate host defences more efficiently than non-symbiotic plants (Lv *et al.* 2010; Redman *et al.* 1999; Rodriguez *et al.* 2009). These host defences are induced by the fungal endophytes' production of secondary metabolites which then have the potential to be stimulated in culture and utilised commercially (Schulz *et al.* 2002; Sumarah *et al.* 2010).

The antimicrobial activities of 206 fungal endophytes from a previously unexplored ecosystem were examined in this study. Of these, 16% were found to exhibit some level of activity during preliminary screening. This is comparable to other studies which have found between 10% and 20% of tested isolates showed bioactivity during initial screening (Gong and Guo 2010). In a recent study of *Garcinia* plants in southern Thailand, it was found that 18.6% of endophytic isolates showed some level of bioactivity to at least either *S. aureus* or a *Candida* spp. (Phongpaichit and Rukachaisirikul 2006). Some studies have observed as little as 5% initial bioactivity (Carvalho *et al.* 2012) and others as much as 61% (Buatong *et al.* 2011).

13/33 isolates which showed initial antimicrobial activity belonged to the genus *Preussia,* a little studied and an under exploited taxon occurring worldwide. These fungi contained lipid-soluble compounds that inhibited the growth of MRSA and/or *C. albicans*. Chen *et al*. (2009) has previously shown that *Preussia* spp. contain bioactive compounds. These researchers isolated three new spirobisnaphthalene analogues from an undescribed *Preussia* sp. and showed that they inhibited *S. aureus*. While these analogues may have been present, they were not detected in this study.

Interestingly, isolates of *Preussia* that had similar ITS regions showed differing bioactivity. In particular, isolate ADM2.11 was found to have a 99% sequence similarity over 350bp with NVM2.5. However when these isolates were tested for bioactivity, sample ADM2.11 showed both antifungal and antibacterial activity while NVM2.5 did not. Variable isolate biochemistry has been shown by Peterson *et al*. (2009) who found that the two *P. africana* isolates they collected produced such differing enzymatic activity that it suggested they were potentially different species. Differing antimicrobial

activity among isolates of the same species has not been previously reported within the *Preussia* genus. However, such occurrences have been commonly reported for many other fungal species such as *Penicillium*, *Aspergillus* and *Furcatum* (Larsen *et al.* 2005). For example Phongpaichit and Rukachaisirikul (2006) found strains of the same species of *Fusarium* showed differing antimicrobial activity. Results such as this highlight the importance of testing all isolates of a particular species to gain an overview of bioactive production for a given fungal taxon.

Of particular interest was the bioactivity present in HPLC fractions from isolate BSH2.9 (*Preussia* sp.). Six novel pure compounds were identified, four of which were able to be tested. The first pure compound isolated was found to be closely related to bromomethylchlamydosporols A and B. Bromomethylchlamydosporols are halogenated analogues of the mycotoxin chlamydosporol (Nenkep *et al.* 2010). These are secondary metabolites more commonly associated with *Fusarium* spp. (Summerell and Leslie 2011). Nenkep *et al.* (2010) recently isolated bromomethylchlamydosporols from *Fusarium tricinctum* and found it to have mild antimicrobial properties against MRSA (Nenkep *et al.* 2010). No clinical use appears to have been identified for bromomethylchlamydosporols A and B, and this study represents the second observation of these compounds. Interestingly, *F. tricinctum* was isolated as an endophyte from a marine environment (Nenkep *et al.* 2010). However, the compound obtained from isolate BSH2.9 did not exhibit any antimicrobial activity when tested against *E. faecalis* or MRSA. Only a small amount of the pure compound was obtained and this prevented all pathogen species from being tested. Given that none of the fractions showed bioactivity when they were tested, it can be concluded that it is unlikely that the pure compound obtained is antimicrobial.

Of the remaining five novel compounds obtained, three were able to be tested and these displayed strong antimicrobial activity. Africanarone spelling? B and C had minimum inhibitory concentrations of 7μg/ml and 4μg/ml respectively. According to the CLSI standards, vancomycin has a MIC of 2μg/ml, while oxacillin and methicillin are 0.5μg/ml and 8μg/ml respectively (Clinical and Institute 2010). Structural elucidation of these compounds found them to be polyketides of the same structural class to which many important antibiotics such as tetracyclines, erythromycin and vancomycin also belong (Keating and Walsh 1999; Schulz *et al.* 2002); Davis paper in preparation}. Tetracyclines are a family of antibiotics which have had significant clinical use since the 1940's (Chopra and Roberts 2001). Vancomycin, as mentioned, is a drug of last resort which is of particular use against methicillin resistant bacteria (Levine 2006). As resistance is appearing in many important pathogen groups alternatives are urgently needed for treatment. The compounds isolated in this study have MIC's comparable to other known and medically significant drugs as well as sharing a similar chemical structure. Further research is needed to determine cell toxicity and mode of action for these compounds.

Of interest was the anti-fungal activity displayed by all three compounds towards *C. albicans.* Amphotericin b, the most commonly available anti-*Candida* treatment has a MIC of 1g/ml (Institute 2008). Fluconazole, another option for treatment, is even higher at 64g/ml (Institute 2008). All three of the compounds tested in this study, had MIC's which ranged from 60μg/ml to 250μg/ml after 24hrs, well below the effective concentrations of drugs currently in use. These early results appear promising and warrant further research. Of particular importance would be determining at what concentration these compounds are toxic to mammalian cells. Many human and mouse

cell lines are available for testing, such as L929 mouse fibroblast and NHK (normal human keratinocytes). These may then be tested using standardised 96 well assay procedures such as Crystal Violet Dye Exclusion (CVDE) and the MTT cell viability assay (Boddington 2009). This next step would need to be undertaken in order to determine if the promising results seen thus far may have any significant clinical use.

Interestingly isolate BSH2.9 displayed no activity towards *C. albicans* during any of the preliminary screening. This is not entirely surprising given that compounds Affricanarone B and C showed inhibition towards MRSA at as low as 7μg/ml and 4μg/ml respectively whereas activity towards C*. albicans* was observed at a much higher concentration, beginning at 30μg/ml when checked at 18hrs. This would suggest that the initial method of screening used here failed to pick up the anti- fungal activity due to low concentrations of the compound.

The differences in bioactive compound production between cultures that were shaken and those kept under static conditions indicate that different culture methods can influence fungal isolate biochemistry. This is not entirely surprising since it is well known that factors such as temperature, pH, light and the type of broth used also affect fungal secondary metabolite production (Amna *et al.* 2006; Miller 2001; Schneider *et al.* 2008). It does however, highlight the importance of testing various culturing factors in order to optimise the conditions suitable for the production of compounds of interest. Further research could be conducted to determine what these conditions may be and whether it can be streamlined for certain groups of fungi such as endophytes or mycorrhizal fungi.

5.6 Conclusion

This study examined the antimicrobial activity of 206 fungal endophytes from SEVT. Six novel pure compounds were isolated and four were able to be tested for activity against nosocomial pathogens. The bioactivities of three of these compounds appear within similar ranges to other known antibiotics used to treat MRSA and significantly lower concentrations than those currently used to treat *C. albicans*. These results appear promising and further research is needed to determine their cell toxicity and mode of actions.

Bioactivity of fungal endophytes within SEVT was found to be high in this study. Furthermore, many of these bioactive isolates appear to be new species. Given that SEVT have been extensively cleared in the past and are under continued threat from grazing, urban encroachment and future clearing, this would provide even more impetus to protecting these habitats. This study examined only a small subset of the fungi within SEVT. No information is yet known regarding the bioactivity of other indigenous saprobic, epiphytic or mycorrhizal fungi present in these habitats. Further research in SEVT could potentially reveal an additional wealth of medicinally useful fungi.

6 Conclusions and Future Directions

This study examined 416 fungal endophytes from five sites of SEVT. It represents the first investigation of fungi within SEVT and demonstrates that the dry rainforests of southeast Queensland are a large store house of novel fungal endophytes. Furthermore, it has shown that many of these fungal endophytes are likely new species and genera with several belonging to families not commonly observed as endophytes. While the numbers of endophytes isolated may not have been as high as other studies, diverse range of fungal endophyte species was observed. It has been well argued that endophytes may assist their host in withstanding many environmental pressures such as drought and herbivory and perhaps the high numbers observed in this study are a reflection of this. Future research could examine the difference in endophyte colonisation rate in plants displaying signs of stress in SEVT compared to those with visible signs of improved fitness. This research largely focused on endophytes from leaves of trees, further research could also examine whether species patterns are similar in different tissue and host types.

Isolation rates between plants varied greatly in this study. *Eustrephus latifolius* was found to harbour the largest diversity of fungal endophyte taxa. Given that these results were based on culturable endophytes only, future research could examine the diversity within these host species using molecular analysis of in planta DNA. It has been widely recognised that a dual approach of culture and molecular methods are needed in order to gain a thorough understanding of fungal endophyte diversity. Future research could examine those fungal endophytes unable to grow in culture. It could then compare the overall species compositions to discover any differences in diversity. Examination of the

same plants across several sites could also be undertaken in order to ascertain whether these results are reflections of SEVT as a whole.

An array of novel taxa was observed in this study with over half of the fungal endophytes isolated potentially representing new species. Perhaps most intriguingly was the high frequency of unique, endophytic Sarcosomataceae. The Sarcosomataceae observed in this study may not just represent new species but possibly new genera due to the novelty of their DNA sequences. Despite several attempts to induce sporulation for these species, no taxonomically useful reproductive features were successfully obtained. Further research would need to be undertaken in order to ascertain whether the Sarcosomataceae observed in this study are in fact new species and to further ascertain what their precise ecological role may be. Future research could also examine the diversity of endophytes in neighbouring ecosystem types in order to determine whether the array of novel taxa observed in this study are unique to SEVT or are more representative of the Darling Downs.

This study also observed fungal specificity occurring between *Guignardia* species and *G. salicifolia*. This observation may suggest that *Guignardia* species are more commonly associated with Rutaceae plants than previously thought. The overall diversity of fungal endophytes isolated from *G. salicifolia* reflected those found in other plants within SEVT. No differences in CR and IR were observed between the five sites examined. It is therefore possible that the diversity of endophytes observed within *G. salicifolia* is a reflection of overall diversity within SEVT. More research examining other plant hosts within SEVT could be conducted in order to confirm this.

The antimicrobial activity of 206 fungal endophytes from SEVT were examined in this study with 33 endophytes found to be displaying antimicrobial activity. The overall bioactivity of fungal endophytes in SEVT was found to be high and many of these bioactive endophytes were new species. Of particular interest were the six novel pure compounds isolated from *Preussia* spp. Four were able to be tested for activity against nosocomial pathogens, three of which showed promising bacteristatic and fungicidal activity towards MRSA and *C. albicans* respectively. The compounds involved were found to be novel polyketides which were named Affricanarones A-E. Two of these were found to have low MIC's which suggests further research of these compounds is warranted. These results are of particular importance since current treatment options for the nosocomial pathogens are limited.

SEVT store a wealth of ecologically and medically important fungi. Unfortunately, more than 85% of these habitats have already been cleared and the remaining patches are under continued threat from grazing, urban encroachment, weed invasion and future clearing. Studies such as this highlight the importance of actively preserving and protecting such potentially valuable natural resources. Furthermore, this study was only able to examine a small proportion of the fungal diversity within SEVT. Currently, very little information exists regarding saprobic, pathogenic, mycorrhizal and epiphytic fungi in these areas. More research is urgently needed in order to gain a better understanding of the fungi within SEVT. Permanent loss of sites such as this will greatly impact on what appears to be a significant storehouse of mycological diversity.

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8 Appendices

8.1 Appendices A (Chapter 2)

Table A.14 List of all endophytic taxa isolated in the diversity study.

Code	Species	GenBank No.
ADH2.12	*Nigrospora* sp.	
ADH2.13	*Nigrospora* sp.	
ADH2.14	*Nigrospora* sp.	
ADH2.15	*Nigrospora* sp.	
ADH2.16	*Phoma* sp.	
ADH2.17	*mycelia sterilia*	
ADL2.1	*Nigrospora* sp.	
ADL2.2	*Nigrospora* sp.	KF227798
ADL2.3	*Nigrospora* sp.	
ADL2.4	*Preussia* sp.	JN418766
ADL2.5	*Nigrospora* sp.	KF227799
ADL2.6	*Preussia* sp.	JN418765
ADL2.7	*Nigrospora* sp.	
ADM2.10	*Nigrospora* sp.	
ADM2.11	*Preussia* sp.	JN418767
ADM2.8	*Nigrospora* sp.	
ADM2.9	*Nigrospora* sp.	
AE2.1	*Pyronema domesticum*	KF227800
AE2.2	*Pezizales* sp.	KF227801
AEH3.2	*Oedocephalum* sp.	KF227802
AEH3.3	*mycelia sterilia*	
AEL.3.1	*Pyronema* sp.	KF227803
BPL2.1	*Nigrospora* sp.	KF227804
BPL2.2	*Phoma* sp.	KF227805
BPL2.3	*mycelia sterilia*	
BPM2.4	*Nigrospora* sp.	KF227806
BPM2.5	*Conoplea* sp.	KF227807
BSH2.10	*Nigrospora* sp.	

Code	Species	GenBank No.
BSH2.11	*Nigrospora* sp.	
BSH2.12	*Pithomyces* sp.	
BSH2.13	*Nigrospora* sp.	KF227808
BSH2.9	*Preussia* sp.	JN418768
BSL2.1	*mycelia sterilia*	
BSL2.2	*Monilia* sp.	
BSL2.3	*Cladosporium* sp.	
BSL2.4	*Nigrospora* sp.	
BSL2.5	*Nigrospora* sp.	KF227809
BSM2.6	*Nigrospora* sp.	KF227810
BSM2.7	*Nigrospora* sp.	
BSM2.8	*Nigrospora* sp.	
CAH2.10	*Nigrospora* sp.	
CAH2.11	*Preussia* sp.	JN566153
CAH2.12	unknown fungi	KF227811
CAH2.13	*Pezizales* sp.	KF227812
CAH2.14	*Guignardia* sp.	KF227813
CAH2.9	*Preussia* sp.	JN418769
CAL2.1	*Preussia* sp.	JN418770
CAL2.2	*Pestalotiopsis* sp.	KF227814
CAL2.3	*Nigrospora* sp.	KF227815
CAL2.4	*Penicillium* sp.	
CAL2.5	*Hypoxylon* sp.	KF227816
CAM2.6	*Xylaria* sp.	
CAM2.7	*Trichoderma* sp.	
CAM2.8	*Trichoderma* sp.	
CIL1.1	*Colletotrichum* sp.	KF227817
CIL1.2	*Guignardia* sp.	KF227818
CIM1.13	*Xylaria* sp.	
CIM1.14	*Fimetariella* sp.	KF227819
CIM1.15	*Hypoxylon* sp.	KF227820
CIM1.16	*Hypoxylon* sp.	KF227821

Code	Species	GenBank No.
CIM1.17	*Cercophora* sp.	KF227822
CIM1.3	*Biscogniauxia* sp.	KF227823
CIM1.4	*Pithya* sp.	KF227824
CIM1.5	*mycelia sterilia*	
CIM1.6	*Pithya* sp.	KF227825
CIM1.7	*Nigrospora* sp.	
CIM1.8	*Monilia* sp.	
CLH1.10	*Phomopsis* sp.	KF227826
CLH1.11	*Phomopsis* sp.	KF227827
CLH1.12	*Biscogniauxia* sp.	KF227828
CLH1.9	*Ceratophorum* sp.	
CLL1.1	*Phomopsis* sp.	
CLL1.2	*Pestalotiopsis* sp.	
CLL1.3	*Epiccocum* sp.	
CLL1.4	*Cladosporium* sp.	
CLL1.5	*mycelia sterilia*	
CLL1.6	*Nigrospora* sp.	KF227829
CLM1.7	*Nigrospora* sp.	
CLM1.8	*Nigrospora* sp.	KF227830
CMH2.13	*Nigrospora* sp.	KF227831
CMH2.14	*Nigrospora* sp.	KF227832
CMH2.15	*Nigrospora sp.*	KF227833
CML2.1	*mycelia sterilia*	
CML2.2	*Nigrospora* sp.	
CML2.3	*Sordaria* sp.	
CML2.4	*Acremonium* sp.	KF227834
CML2.5	*Bipolaris* sp.	
CML2.6	*Nigrospora* sp.	
CML2.7	*Neurospora* sp.	KF227835
CMM2.10	*Nigrospora* sp.	
CMM2.11	*Nigrospora* sp.	KF227836
CMM2.12	*Nigrospora* sp.	

Code	Species	GenBank No.
CMM2.8	*Sordaria* sp.	
CMM2.9	*Cladosporium* sp.	
EAH2.11	*Nigrospora* sp.	
EAH2.12	*Nigrospora* sp.	
EAH2.13	*Guignardia* sp.	KF227837
EAL2.1	*Nigrospora* sp.	
EAL2.2	*Nigrospora* sp.	
EAL2.3	*Nigrospora* sp.	
EAL2.4	*Preussia* sp.	JN566152
EAL2.5	*Preussia* sp.	JN418771
EAL2.6	*Nigrospora* sp.	
EAL2.7	*Preussia sp.*	JN418772
EAM2.10	*Nigrospora* sp.	KF227838
EAM2.8	*Nigrospora* sp.	
EAM2.9	*Nigrospora* sp.	
ELV3.1	*Xylaria* sp.	KF227839
ELV3.10	*Xylaria* sp.	
ELV3.11	*Preussia* sp.	JN418774
ELV3.12	*Coniochaeta* sp.	KF227840
ELV3.13	*Chaetomium globosum*	KF227841
ELV3.14	*Nigrospora* sp.	KF227842
ELV3.15	*Coniochaeta* sp.	KF227843
ELV3.16	*Conoplea* sp.	KF227844
ELV3.17	*Phoma* sp.	
ELV3.18	*Guignardia* sp.	KF227845
ELV3.19	*Guignardia* sp.	KF227846
ELV3.2	*Preussia* sp.	JN418773
ELV3.3	*Xylaria* sp.	KF227849
ELV3.4	*Preussia* sp.	KF227850
ELV3.5	*Cladosporium* sp.	
ELV3.6	*Guignardia* sp.	KF227851
ELV3.7	*Xylaria* sp.	KF227852

Code	Species	GenBank No.
ELV3.8	*Nigrospora* sp.	
ELV3.9	*Cladosporium* sp.	
EMH2.3	*Pezizales* sp.	KF227853
EMM2.1	*Epiccocum* sp.	
EMM2.2	*mycelia sterilia*	
EOL1.1	*Pestalotiopsis* sp.	
EOL1.2	*Microdiplodia* sp.	KF227854
EOM1.4	Pycnidial sp.	
EOM1.3		
EOM1.5	*mycelia sterilia*	
EOM1.6	*Penicillium* sp.	
EOM1.7	*Acremonium* sp.	
EXH1.12	*Cladosporium* sp.	
EXH1.13	*Sordaria* sp.	
EXH1.14	*Conoplea sp.*	KF227855
EXH1.20	*Botryodipodia* sp.	
EXH1.21	*Pithya* sp.	KF227856
EXH1.22	*Muscodor* sp.	KF227857
EXH1.23	*Guignardia* sp.	KF227858
EXL1.1	*Sordaria* sp.	
EXL1.10	*Sordaria* sp.	
EXL1.16	*Pyronema sp.*	KF227859
EXL1.15		
EXL1.17	*Phomopsis* sp.	KF227860
EXL1.18	*Acremonium* sp.	
EXL1.2	*Phomopsis* sp.	
EXL1.3	*Halorosellinea* sp.	KF227861
EXL1.4	*Biscogniauxia* sp.	KF227862
EXL1.5	*Cladosporium* sp.	
EXL1.6	*Phomopsis* sp.	KF227863
EXL1.7	*Cladosporium* sp.	
EXL1.8	*Cladosporium* sp.	

Code	Species	GenBank No.
EXL1.9	*Phomopsis* sp.	KF227864
EXM1.11	*Conoplea sp.*	KF227865
EXM1.19	*Microdiplodia* sp.	KF227866
GSH2.5	*Xylaria* sp.	
GSH2.6	*mycelia sterilia*	
GSH2.7	*Coniochaeta* sp.	
GSH2.8	*Devriesia* sp.	KF128864
GSH2.9	Uncultured soil fungus clone	KF128865
GSL1.1	*Nigrospora* sp.	
GSL1.2	*Phomopsis sp.*	
GSL1.3	*Acremonium* sp.	
GSL1.4	*Guignardia* sp.	
GSL1.5	*Phomopsis* sp.	KF128868
GSL2.1	*Nigrospora sp.*	
GSL2.2	*Lecythophora* sp.	KF128870
GSL2.3	*Nigrospora* sp.	
GSL2.4	*Xylaria* sp.	
GSM1.6	*Nigrospora* sp.	
GSM1.7	*Epiccocum* sp.	
GSM1.8	*mycelia sterilia*	
GSM3.1	*Preussia africana*	JN418775
GSM3.2	*Fusicoccum vitifusiforme*	
MDC2.1	*mycelia sterilia*	
MDC2.2	*mycelia sterilia*	
MDC2.3	*Guignardia* sp.	KF227867
MDC2.4	*Toxicocladosporium protearum* 97%	KF227868
MDC2.5	*Paraphaeosphaeria* sp.	KF227869
NVH2.10	*mycelia sterilia*	
NVH2.11	*Lecythophora* sp.	KF227870
NVH2.12	*Nigrospora* sp.	
NVH2.13	*Nigrospora* sp.	

Code	Species	GenBank No.
NVH2.8	*mycelia sterilia*	
NVH2.9	*Xylaria* sp.	
NVL2.1	*Gelasinospora* sp.	KF227871
NVL2.2	*Nigrospora* sp.	
NVM2.3	*Nigrospora* sp.	
NVM2.4	*Fimetariella* sp.	KF227872
NVM2.5	*Preussia* sp.	JN418776
NVM2.6	*Preussia* sp.	JN418777
NVM2.7	*Cladosporium* sp.	KF227873
PA.L2.1	*Preussia* sp.	JN418778
PA.L2.2	*mycelia sterilia*	
PA.L2.3	*Preussia* sp.	JN418779
PA.L2.4	*Xylaria* sp.	KF227875
PA.L2.5	*mycelia sterilia*	
PAH2.7	Pezizomycotina	KF227874
PAM2.6	*Nigrospora* sp.	
PPV3.1	*Pithomyces* sp.	
PPV3.2	*Aureobasidium* sp.	KF227876
PPV3.3	*Pestalotiopsis* sp.	
PPV3.4	Pleosporales	KF227877
PPV3.5	*Aureobasidium* sp.	KF227878
PPV3.6	*Preussia* sp.	JN418780
PPV3.7	*mycelia sterilia*	
SFL3.1	*Periconia* sp.	KF227879
SFL3.2	*mycelia sterilia*	
SLH2.11	*mycelia sterilia*	
SLH2.12	*Nigrospora* sp.	KF227880
SLH2.13	*Colletotrichum* sp.	KF227881
SLH2.14	*Nigrospora* sp.	
SLH2.15	*Nigrospora* sp.	
SLH2.16	*Phoma* sp.	KF227882
SLH2.17	*Pseudocercospora* sp.	KF227883

Code	Species	GenBank No.
SLL2.1	*Nigrospora* sp.	
SLL2.2	*Nigrospora* sp.	
SLL2.3	*Conoplea* sp.	KF227884
SLL2.4	*mycelia sterilia*	
SLM2.10	Nigrospora sp.	KF227885
SLM2.5	*Nigrospora* sp.	
SLM2.6	*Nigrospora* sp.	
SLM2.7	*Nigrospora* sp.	
SLM2.8	*Fimetariella* sp.	KF227886
SLM2.9	*Nigrospora* sp.	

Table A. 15 Raw data for testing differences between leaves with and without growth across the three sites (including samples believed to be over sterilised)

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Leaves with growth	.097	2	21	.908
Leaves without	.248	2	21	.783

ANOVA

		df	F	Sig.
Leaves with growth	Between Groups	2	2.233	.132
	Within Groups	21		
	Total	23		
Leaves without	Between Groups	2	4.472	.024
	Within Groups	21		
	Total	23		

Dependent Variable	(I) Site	(J) Site	Std. Error	Sig.
Leaves with growth	Highfields	Mt Kingsthorpe	.93687	.267
		Boodua	.76361	.966
	Mt Kingsthorpe	Highfields	.93687	.267
		Boodua	.81418	.119
	Boodua	Highfields	.76361	.966
		Mt Kingsthorpe	.81418	.119
Leaves without	Highfields	Mt Kingsthorpe	.79282	.432
		Boodua	.64621	.290
	Mt Kingsthorpe	Highfields	.79282	.432
		Boodua	.68900	.022
	Boodua	Highfields	.64621	.290
		Mt Kingsthorpe	.68900	.022

Table A.16 Raw data for testing differences between leaves with and without growth across the three sites (not including samples believed to be over sterilised)

	N	Mean	Std. Deviation	Std. Error
Highfields	5	13.0000	6.78233	3.03315
Mt Kingsthorpe	5	7.2000	8.52643	3.81314
Boodua	13	10.3846	5.29998	1.46995
Total	23	10.2609	6.38334	1.33102

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
.259	2	20	.774

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	84.558	2	42.279	1.042	.371
Within Groups	811.877	20	40.594		
Total	896.435	22			

(I) Location	(J) Location	Std. Error	Sig.
Highfields	Mt Kingsthorpe	4.02958	.341
	Boodua	3.35282	.719
Mt Kingsthorpe	Highfields	4.02958	.341
	Boodua	3.35282	.616
Boodua	Highfields	3.35282	.719

(I) Location	(J) Location	Std. Error	Sig.
Highfields	Mt Kingsthorpe	4.02958	.341
	Boodua	3.35282	.719
Mt Kingsthorpe	Highfields	4.02958	.341
	Boodua	3.35282	.616
Boodua	Highfields	3.35282	.719
	Mt Kingsthorpe	3.35282	.616

Table A.17 ANOVA of differences in fungal infection rates of fungi located high, mid and low in the canopy.

	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
no of isolates	.105	50	.200*	.889	50	.000
no. of species	.166	50	.001	.914	50	.001
no. of genera	.188	50	.000	.939	50	.012

All sig values are below 0.05 so a log of the values was taken before further analysis

	df	Mean Square	F	Sig.
Between groups	2	.128	1.807	.175
Within groups	47	.071		
Total	49			

(I) location	(J) location	Std. Error	Sig.
high	mid	.09266	.932
	low	.09266	.341
mid	high	.09266	.932
	low	.09124	.180
low	high	.09266	.341
	mid	.09124	.180

8.2 Appendices B (Chapter 3)

Table B.18 T-Test of the sampling years

Group Statistics

	Year	N	Mean	Std. Deviation	Std. Error Mean
No. of Isolates	2010/11	30	3.00	2.181	.398
	2012	36	2.61	1.609	.268

Table B.19 Univariate analysis of differences between fungi located high, mid and low in the canopy of *G. salicifolia* and compared across 5 sites

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Log of isolates

F	df1	df2	Sig.
1.733	14	43	.084

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + location high mid low + Site + locationhigh1mid2low3 * Site

Tests of Between-Subjects Effects

Dependent Variable: Log of isolates

Source	df	F	Sig.
Location high mid low	2	1.222	.305
Site	4	1.139	.351
Location high mid low * Site	8	1.231	.305
Error	43		

a. R Squared = .246 (Adjusted R Squared = .001)

Table B. 20 Univariate analysis the 6 most commonly observed fungal genera of *G. salicifolia* across 5 sites. Statistically significant values are highlighted.

Tests of Between-Subjects Effects, Dependent Variable: no of isolates

Source	df	F	Sig.
Corrected Model	5	3.636	.014
Intercept	1	29.777	.000
fungi	5	3.636	.014
Error	24		

a. R Squared = .431 (Adjusted R Squared = .312)

Estimated marginal means of the common fungi at the five sites

Dependent Variable: no of isolates

fungi	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Guignardia	9.600	1.631	6.234	12.966
Nigrospora	4.400	1.631	1.034	7.766
Sarcosomataceae 1	2.400	1.631	-.966	5.766
Preussia	1.400	1.631	-1.966	4.766
Xylaria	2.400	1.631	-.966	5.766
Sarcosomataceae 2	1.600	1.631	-1.766	4.966

Post Hoc Tests

Common fungi at the five sites

Multiple Comparisons, Dependent Variable: No of Isolates

Tukey HSD

(I) fungi2	(J) fungi2	Mean Difference (I-J)	Std. Error	Sig.
Guignardia	*Nigrospora*	5.2000	2.30651	.251
	Sarcosomataceae1	7.2000*	2.30651	.047
	Preussia	8.2000*	2.30651	.018
	Xylaria	7.2000*	2.30651	.047
	Sarcosomataceae2	8.0000*	2.30651	.022
Nigrospora	*Guignardia*	-5.2000	2.30651	.251
	Sarcosomataceae1	2.0000	2.30651	.951
	Preussia	3.0000	2.30651	.782
	Xylaria	2.0000	2.30651	.951
	Sarcosomataceae2	2.8000	2.30651	.826
Sarcosomataceae1	*Guignardia*	-7.2000*	2.30651	.047
	Nigrospora	-2.0000	2.30651	.951
	Preussia	1.0000	2.30651	.998

	Xylaria	.0000	2.30651	1.000
	Sarcosomataceae2	.8000	2.30651	.999
Preussia	*Guignardia*	-8.2000*	2.30651	.018
	Nigrospora	-3.0000	2.30651	.782
	Sarcosomataceae1	-1.0000	2.30651	.998
	Xylaria	-1.0000	2.30651	.998
	Sarcosomataceae2	-.2000	2.30651	1.000
Xylaria	*Guignardia*	-7.2000*	2.30651	.047
	Nigrospora	-2.0000	2.30651	.951
	Sarcosomataceae1	.0000	2.30651	1.000
	Preussia	1.0000	2.30651	.998
	Sarcosomataceae2	.8000	2.30651	.999
Sarcosomataceae2	*Guignardia*	-8.0000*	2.30651	.022
	Nigrospora	-2.8000	2.30651	.826
	Sarcosomataceae1	-.8000	2.30651	.999
	Preussia	.2000	2.30651	1.000
	Xylaria	-.8000	2.30651	.999

Based on observed means.

The error term is Mean Square(Error) = 13.300.

*. The mean difference is significant at the .05 level.

8.3 Appendices C (Chapter 4)

Table C.21 GenBank Ascension codes for *Preussia* and *Guignardia* isolates

Code	Proposed identity	GenBank No. ITS	GenBank No. LSU	GenBank No. β-tubulin
ADL2.4	*Preussia isomera*	JN418766	KF269193	KF269219
ADL2.6	*Preussia* sp.	JN418765	KF269194	KF269220
ADM2.11	*Preussia* sp.1a	JN418767	KF269192	KF269218
BSH2.9	*Preussia* affn. *africana*	JN418768	KF269195	KF269221
CAH2.11	*Preussia isomera*	JN566153	KF269196	
CAH2.9	*Preussia isomera*	JN418769	KF269197	KF269222
CAL2.1	*Preussia isomera*	JN418770	KF269199	
EAL2.4	*Preussia* sp.2	JN566152	KF269202	KF269223
EAL2.5	*Preussia* sp.3	JN418771	KF269203	KF269224
EAL2.7	*Preussia* sp.4	JN418772	KF269204	KF269225
ELV3.11	*Preussia* sp.5	JN418774	KF269205	KF269226
ELV3.2	*Preussia* sp.5	JN418773	KF269206	KF269227
ELV3.4	*Preussia* sp 7	KF227850	KF269207	KF269228
GSM3.1	*Preussia* sp.	JN418775	KF269212	KF269229
GSL1.3.2	*Preussia* sp.	KF128821		
GSL2.1.1	*Preussia* sp.	KF128767		
GSH1.5.6	*Preussia* sp.	KF128811		
GSH2.2.23	*Preussia* sp. 8	KF128781		
GSM2.1.5	*Preussia isomera*	KF128773		
GSM2.1.7	*Preussia isomera*	KF128774		
NVM2.5	*Preussia* sp.1b	JN418776	KF269213	KF269230
NVM2.6	*Preussia isomera*	JN418777	KF269214	KF269231
PAL2.1	*Preussia isomera*	JN418778	KF269215	
PAL2.3	*Preussia* sp.6	JN418779	KF269216	
PPV3.6	*Preussia* sp.	JN418780	KF269217	KF269232
CAH2.14	*Guignardia* sp3	KF227813	KF269198	
CIL1.2	*Guignardia* sp5	KF227818	KF269200	
EAH2.13	*Guignardia* sp6	KF227837	KF269201	

Code	Proposed identity	GenBank No. ITS	GenBank No. LSU	GenBank No. β-tubulin
ELV3.18	*Guignardia* sp4	KF227845	KF269208	
ELV3.19	*Guignardia* sp4	KF227846	KF269209	
ELV3.6	*Guignardia* sp4	KF227851		
EXH1.23	*Guignardia* sp6	KF227858	KF269210	
GSH2.1.10	*Guignardia* sp1	KF128858		
GSH2.1.12	*Guignardia* sp1	KF128768		
GSH2.1.13	*Guignardia* sp1	KF128769		
GSH2.1.14	*Guignardia* sp1	KF128770		
GSH2.2.19	*Guignardia* sp1	KF128778		
GSH2.2.20	*Guignardia* sp1	KF128779		
GSH2.2.21	*Guignardia* sp1	KF128780		
GSH3.3.10	*Guignardia* sp1	KF128787	KF269182	
GSH3.4.9	*Guignardia* sp1			
GSH5.4.3	*Guignardia* sp1			
GSH5.4.5	*Guignardia* sp1	KF128813		
GSH5.5.11	*Guignardia* sp2	KF128814		
GSL1.2.1	*Guignardia* sp1	KF128759		
GSL1.2.3	*Guignardia* sp1	KF128762		
GSL1.3.1	*Guignardia* sp1	KF128820		
GSL1.4	*Guignardia* sp6	KF128867	KF269211	
GSL2.5.1	*Guignardia* sp1	KF128827		
GSL2.5.2	*Guignardia* sp1	KF128828		
GSL2.5.3	*Guignardia* sp1	KF128829		
GSL2.5.4	*Guignardia* sp1	KF128830		
GSL5.5.1	*Guignardia* sp6	KF128835		
GSM1.2.10	*Guignardia* sp1			
GSM1.2.6	*Guignardia* sp1	KF128764		
GSM1.2.7	*Guignardia* sp1	KF128765		
GSM1.2.8	*Guignardia* sp1	KF128857		
GSM1.3.6	*Guignardia* sp1	KF128841		

Code	Proposed identity	GenBank No. ITS	GenBank No. LSU	GenBank No. β-tubulin
GSM1.4.2	*Guignardia* sp1	KF128842		
GSM1.5.3	*Guignardia* sp6	KF128847		
GSM2.2.28	*Guignardia* sp1	KF128784		
GSM5.5.10	*Guignardia* sp6	KF128851		
GSM5.5.8	*Guignardia* sp6	KF128853		
MDC2.3	*Guignardia* sp1	KF227867		

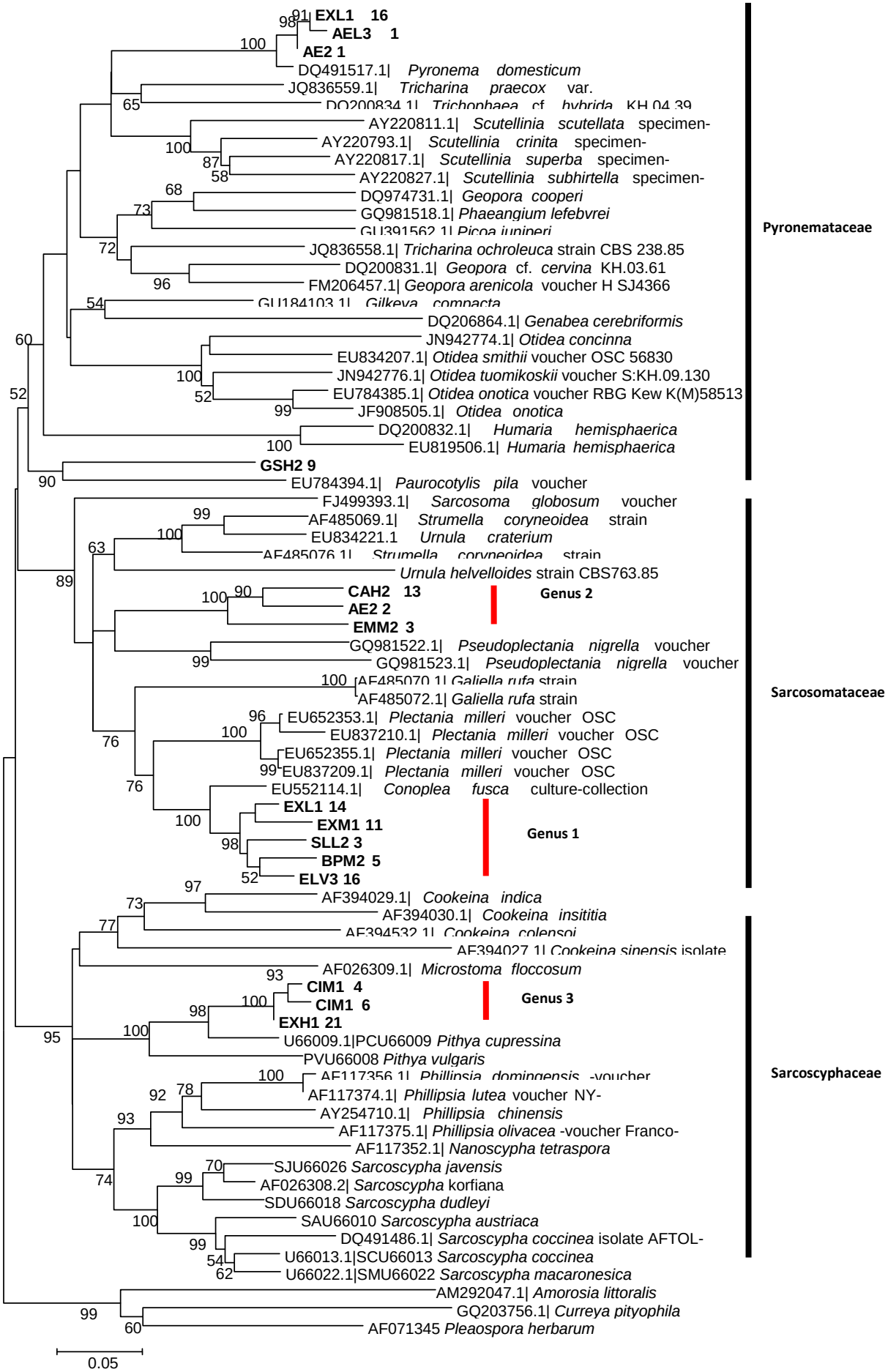

Figure C.27 Neighbour-joining tree of the ITS region of all three families with 1000 bootstrap support.

***G. salicifolia* samples from study 3 not included**

8.4 Appendices D (Chapter 5)

Table D. 22 ANOVA of fungi showing bioactivity across the three sites.

ANOVA

no._bioactive

	df	Mean Square	F	Sig.
Between groups	2	4.208	1.857	.182
Within groups	20	2.266		
Total	22			

(I) Location	(J) Location	Std. Error	Sig.
Highfields	Mt Kingsthorpe	.95208	.555
	Boodua	.79218	.158
Mt Kingsthorpe	Highfields	.95208	.555
	Boodua	.79218	.789
Boodua	Highfields	.79218	.158
	Mt Kingsthorpe	.79218	.789

Table D.23 NMR data for non bioactive compound isolate from BSH2.9

Position	^{13}C, mult.	^{1}H, mult. (*J* in Hz)	COSY	HMBC	ROESY
2	164.2, C				
3	87.9, CH	5.43, s (2.4)		2, 4, 4a, 5	4-OCH_3
4	168.5, CH				
4-OCH_3	56.0, CH_3	3.79, s		3, 4	3
4a	107.6, C				
5	61.5, CH_2	4.55, d (15.0)	5, 8	4, 4a, 7, 8a	5
		4.30, dt (15.0, 2.5)	5, 8	4a, 8a	5, 7
7	73.8, CH	3.60, m	8, 9	5, 9, 10	5, 8
8	32.5, CH_2	2.42, brs	5, 7, 8	4a, 7, 8a, 9	7, 9, 10
		2.41, brs	5, 7, 8	4a, 7, 8a, 9	7, 9, 10
8a	156.5, C				
9	37.4, CH_2	1.64, m	7, 9, 10	7, 8, 10, 11	7, 8, 10, 11
		1.52, m	7, 9, 10	7, 8, 10, 11	7, 8, 10, 11
10	18.4, CH_2	1.46, m	9, 10, 11	7, 9, 11	7, 8, 9, 11
		1.38, m	9, 10, 11	7, 9, 11	7, 8, 9, 11
11	13.9, CH_3	0.93, t (7.0)	10	9, 10	9, 10

[a] Spectra were recorded in $CDCl_3$ (30 °C) at 500 MHz (^{1}H) and 125 MHz (^{13}C).

Table D.24 Well assays of three novel compounds at four different time intervals against *Candida albicans*

18hrs

	1 1mg/ml	2 0.5mg	3 0.25	4 0.12	5 0.06	6 0.03	7 0.015	8 0.007	9 0.004	10 0.002	Bacteria control	Antibiotic control
A BSH2.9.sp 38							+	+	+	+	+	
B							+	+	+	+	+	
C BSH2.9.sp 31					+	+	+	+	+	+	+	
D					+	+	+	+	+	+	+	
E BSH2.9.sp 41				+	+	+	+	+	+	+	+	
F				+	+	+	+	+	+	+	+	
Contamination control											+	
Solvent control	+	+	+	+	+	+	+	+	+	+	+	

20hrs

	1	2	3	4	5	6	7	8	9	10	Bacteria control	Antibiotic control
A BSH2.9.sp 38						+	+	+	+	+	+	
B						+	+	+	+	+	+	
C BSH2.9.sp 31				+	+	+	+	+	+	+	+	
D				+	+	+	+	+	+	+	+	
E BSH2.9.sp 41				+	+	+	+	+	+	+	+	
F				+	+	+	+	+	+	+	+	

Contamination control											+	
Solvent control	+	+	+	+	+	+	+	+	+	+	+	

24hrs

	1	2	3	4	5	6	7	8	9	10	Bacteria control	Antibiotic control
A BSH2.9.sp 38						+	++	++	++	++	++	
B						+	++	++	++	++	++	
C BSH2.9.sp 31				+	+	++	++	++	++	++	++	
D				+	+	++	++	++	++	++	++	
E BSH2.9.sp 41			+	++	++	++	++	++	++	++	++	
F			+	++	++	++	++	++	++	++	++	
Contamination control											++	
Solvent control	++	++	++	++	++	++	++	++	++	++	++	

48hrs

	1	2	3	4	5	6	7	8	9	10	Bacteria control	Antibiotic control
A BSH2.9.sp 38				+	++	++	++	++	++	++	++	
B				+	++	++	++	++	++	++	++	
C BSH2.9.sp 31	+	++	++	++	++	++	++	++	++	++	++	
D	+	++	++	++	++	++	++	++	++	++	++	

E BSH2.9.sp 41		+	+	++	++	++	++	++	++	++	++	
F		+	+	++	++	++	++	++	++	++	++	
Contamination control											++	
Solvent control	++	++	++	++	++	++	++	++	++	++	++	

Antibiotic control = 50µl MH broth, 50µl amphotericin b 4mg/ml, 50µl bacteria.

Bacteria control (50µl MH broth with bacteria, 50µl sterile distilled water

Contamination control = 50µl MH broth, 50µl of saline.

Solvent control = 50µl MH broth with bacteria, 50µl solvent in saline,

Table D.25 Well assays of three novel compounds at two different time intervals against MRSA

20hrs

	1 1mg/ml	2 0.5mg	3 0.25	4 0.12	5 0.06	6 0.03	7 0.015	8 0.007	9 0.004	10 0.002	Bacteria control	Antibiotic control
A BSH2.9.sp 38	+	+	+	+	+	+	+	+	+	+	+	
B	+	+	+	+	+	+	+	+	+	+	+	
C BSH2.9.sp 31									+	+	+	
D									+	+	+	
E BSH2.9.sp 41										+	+	
F										+	+	
Contamination control											+	
Solvent control	+	+	+	+	+	+	+	+	+	+	+	

24 hrs

	1	2	3	4	5	6	7	8	9	10	Bacteria	Antibiotic

											control	control
A BSH2.9.sp 38	++	++	++	++	++	++	++	++	++	++	++	
B	++	++	++	++	++	++	++	++	++	++	++	
C BSH2.9.sp 31							++	++	++	++	++	
D							+	+	++	++	++	
E BSH2.9.sp 41									++	++	++	
F								+	++	++	++	
Contamination control											++	
Solvent control	++	++	++	++	++	++	++	++	++	++	++	

Antibiotic control = 50μl MH broth, 50μl vancomycin at final concentration of 4μg/ml, 50ul bacteria.

Bacteria control (50μl MH broth with bacteria, 50μl sterile distilled water

Contamination control = 50μl MH broth, 50μl of saline.

Solvent control = 50μl MH broth with bacteria, 50μl solvent in saline.