# Taxonomy and biology of *Quambalaria* spp. infecting eucalypts in South Africa

by

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**DECLARATION**

I, Seamus William Morgan, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted for any degree at this or any other university.

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**Mister Seamus William Morgan**

(November 2018)

**DEDICATION**

I dedicate this dissertation to my grandmother Erna MacLean. You were always a stable voice and a solid rock in my life and I miss you every day. I will always strive to do better and keep you in my heart.

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**Preface**

The Microstromatales is an order within the class Exobasidiomycetes of the Basidiomycota, which contains three families, the Microstomataceae, Volvocisporiaceae and Quambalariaceae. The family of interest in this particular study is the Quambalariaceae, which includes a single genus, *Quambalaria*. The genus currently includes seven species, *Quambalaria pitereka, Q. eucalypti, Q. simpsonii, Q. coyrecup, Q. cyanescens, Q. purpurascens* and *Q. fabacearum*. Most of these species have been isolated from leaves, shoots or stems of eucalypts or other trees. Some causing serious leave and shoot dieback or stem cankers, especially in commercial monoculture plantations in Australia and abroad. *Quambalaria cyanescens* is the only one that has also been found in other niches such as bark beetle galleries on hardwoods or as opportunistic human pathogens.

To date, only *Q. eucalypti* has been found in South Africa, mainly causing damage to clonal hedges in commercial eucalypt nurseries. The premise of this study was the discovery of *Quambalaria*-like infections in seed capsules of a number of Eucalyptus species growing in a small plantation in Pretoria that was established to serve as source of food to koala bears in the Pretoria Zoo.

The **first chapter** of this dissertation comprises of a comprehensive review of the literature pertaining to the genus *Quambalaria* looking at its taxonomic history, pathogenicity and distribution. **Chapter two** focuses on the description of a new species isolated form *Eucalyptus* seed capsules and the first report of *Quambalaria cyanescens* from South Africa.

Questions have been raised as to whether members of this genus are capable of sexual reproduction. In recent years, genome sequencing has been used to characterize mating loci and determine the mating strategies of multiple fungal species that were once suggested to be asexual. In **chapter three**, we discuss the processes involved in sequencing and assembly of the first whole genome sequence for a species in the Microstromatales. The genome sequence was generated from an isolate representing *Quambalaria eucalypti*. In **chapter four**, this genome sequence was investigated to determine whether the genes necessary for mating were present. This was done by exploring the genome using sequences of mating related genes from other, related basidiomycete taxa.

# Chapter 1

## Taxonomy, pathology and economic importance of *Quambalaria* species: A review

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## 1.0 Abstract

The genus *Quambalaria* (Microstromatales, Exobasidiomycetes) includes six species, five of which are pathogens of *Eucalyptus* and *Corymbia* species. The only exception is *Q. cyanescens*, which has been shown to be non-pathogenic to plants, but can be an opportunistic human pathogen. The symptoms on trees include shoot and leaf blight, which is characteristic of *Q. eucalypti* and *Q. pitereka* and stem cankers caused by *Q. coyrecup.* The studies in this dissertation arose from the discovery of a *Quambalaria*-like species infecting *Eucalyptus* seed capsules. The complete body of research on *Quambalaria* is reviewed here, focusing on the taxonomy, pathogenicity, host range and geographical distribution. The review revealed a lack of knowledge on basic aspects of its biology, especially in terms of its mating behaviour and sexual reproduction. For this reason, these two aspects in related in species of the Ustilaginomycetes were also considered. This revealed that whole genome sequences and some knowledge regarding mating genes in14 species of Ustilaginomycetes have been published. This knowledge provided a framework in which a whole genome sequence of a *Quambalaria* species could be explored for the presence of genes related to sexual reproduction. It is suggested that such knowledge could shed light on many unknown aspects of the reproduction and life cycle of *Quambalaria* species.

## 2.0 Introduction

Eucalypts (species of *Eucalyptus* and *Corymbia*) as well as *Pinus* and *Acacia* are among the most widely used plantation trees, with eucalypts covering more than 20 million hectares in 2013 (Iglesias Trabado 2008), particularly in the tropics and Southern Hemisphere. These trees have consequently become one of the most important and fastest growing sources of fibre, particularly for the pulp and paper industry in the world (Brockerhoff *et al.* 2013). The health of these forests, both natural and managed, is more threatened today by insect pests and pathogens than they have ever been before (Hunter *et al.* 2009; Jacobs & Nesser 2005; Morin *et al.* 2012; Slippers *et al.* 2015; Wingfield *et al.* 2008b; Wingfield *et al.* 2015). The threats to forest health are rapidly changing, particularly those that arise from direct and indirect anthropogenic influences. These threats include fungal pathogens and insect pests (**Figure 1**).

Diseases and pests of forest crops such as eucalypts are either native and have acquired the ability to infect/infest these trees or they have been introduced, typically from the country of the host tree's origin, which is Australia in the case of *Eucalyptus* spp.(Branco *et al.* 2015). An example of the latter scenario would be the fungal pathogen *Quambalaria eucalypti* that was first reported in South Africa in 1993 (Wingfield 1993) and was most probably introduced into South Africa through the importation of seed from Australia (Roux *et al.* 2006, Wingfield *et al.* 2008a).

The first insect pest of eucalypts to be reported in South Africa was the Eucalyptus Weevil, *Gonipterus scutellatus* in 1916 (Tooke 1953). Eucalypt pest insects have continued to emerge in regions where these trees are non-native at a rate of approximately one insect species every 6.3 years outside of its native range from 1873 to 1986. However, after 1986 there was a sharp increase in the number of pests, with the number of insect pests being detected outside of their native range increasing to one species every 1.4 years (**Figure 2**) (Hurley *et al.* 2016). This exponential increase in the number of introduced invasive insect species after 1986 corresponds with the global increase of the planted area of eucalypt forest plantations (Hurley *et al.* 2016).

Phytosanitary protocols are the major line of defence available to limit the global spread of pests and pathogens. There is evidence suggesting that phytosanitary measures can reduce the rate of introductions of invasive species into new environments, if applied in a strict manner (Hlasny 2012; Roques 2010). Countries where this approach has shown to be successful include New Zealand and Australia, both of which are geographically isolated and

relatively wealthy (Eschen *et al.* 2015; Hlasny 2012; Roques 2010). But implementation of these measures on a global scale is limited. For example, poorer countries are unlikely to be able to afford the institution of biosecurity actions in order to accomplish effective barring to the same extent as the countries mentioned above (Hulbert 2016; Hurley *et al.* 2017). Even where the best possible phytosanitary measures have been put into practice, serious new pest problems continue to arise (Carnegie *et al.* 2010). The limitations of even the best quarantine procedures are effectively illustrated in the example of the accidental introduction of myrtle rust, *Puccinia psidii,* into Australia, despite considerable knowledge of this pathogen and considerable efforts to exclude it (Carnegie *et al.* 2010; Carnegie & Cooper 2011; Morin *et al.* 2012).

## 2.0 The Genus *Quambalaria*

The genus *Quambalaria*, which currently consists of six species, was described in 2000 (Simpson 2000) to accommodate the species *Sporothrix pitereka, S. pusilla* and *S. eucalypti* These were reclassified under *Quambalaria* based on various morphological traits (**Table 1**) and pathogenicity to eucalypts. Due to these traits and the dense growth of white conidiophores on agar media and the host, and the lack of distinct denticles on the conidiogenous cells, Simpson (2000) argued that these taxa could no longer be grouped with *Sporothrix* spp. as it was defined in the Ophiostomatales.

Based on the observed absence of dolipore septa in their hyphae, it was proposed that the newly defined *Quambalaria* spp. could possibly reside in either one of the basidiomycete orders Exobasidiales or Ustilaginales (Simpson 2000). In order to further classify *Quambalaria,* phylogenetic analyses of the large ribosomal subunit (LSU) region were performed by De Beer *et al* (2006). Their analysis indicated that the genus *Quambalaria* belongs in the basidiomycete order Microstromatales. Furthermore, they confirmed with Transmission Electron micrographs that the species in this genus had dolipore septa. This was in contrast to the view of Simpson (2000) who suggested that they were absent. This was also notably different from the ultrastructure of the septal pores of species belonging to the families Microstromataceae and Volvocisporiaceae. Therefore, to accommodate the species with dolipores, a new family was described, Quambalariaceae. Thus, the Microstromatales now includes taxa that have septa with simple pores, as well as taxa with dolipores or septa without pores (De Beer *et al.* 2006).

In addition to *Q. pitereka* and *Q. eucalypti*, De Beer *et al.* (2006) transferred *Sporothrix cyanescens* to *Quambalaria*, but treated *Q. pusilla* as of uncertain status. This was because

DNA sequences could not be obtained to determine the phylogenetic placement of this species. Subsequent to the study of De Beer *et al.* (2006), *Q. coyrecup* (Paap *et al.* 2008), *Q. simpsonii* (Cheewangkoon *et al.* 2009), *Q. fabacearum* (Bezerra *et al.* 2018), and *Q. purpurascens* (De Beer 2012), have been described as novel species in *Quambalaria*. De Beer (2012) also showed that *Q. simpsonii* is a synonym of *Q. pusilla*, bringing the total number of currently accepted species in the genus to six. All of these are discussed in greater detail below, where their taxonomy, pathogenicity, hosts and geographical distribution is considered for each of them.

### *3.1 Quambalaria pitereka* (J. Walker & Bertus) J.A. Simpson

*Quambalaria pitereka,* the type species of the genus *Quambalaria,* was first reported causing damage to *Corymbia maculata* (as *Eucalyptus maculata*) seedlings in nurseries in New South Wales, Australia, in the 1950's (Walker & Bertus 1971). Walker & Bertus (1971) described this fungus as *Ramularia pitereka*, in this study, he noted that that most *Ramularia* species occur on herbaceous hosts while *Q. pitereka* occurred on the Myrtaceae. Crous (1998) excluded *R. pitereka* from the family Mycosphaerellaceae in his revision of the *Mycophaerella* species associated with *Eucalyptus.* Braun (1998) later treated *R. pitereka* in *Sporothrix* based on the conidial scar morphology. Simpson (2000) proceeded to designate *S. pitereka* (Braun) as the type species of his new genus, *Quambalaria*.

#### 3.1.1 Host species and distribution

Extensive surveys conducted by Pegg *et al*. (2008) showed that that *Q. pitereka* is highly prevalent on spotted gums (*Corymbia* spp.). This fungus is commonly found plantations in subtropical and tropical regions of eastern Australia, Queensland and New South Wales (Pegg *et al.* 2008). The species has also been reported in *Corymbia* plantations in Western Australia (Paap *et al.* 2008), and has been isolated from the genera *Blakella* and *Angophora* in New South Wales (Simpson 2000).

Tolerance to Quambalaria shoot blight (QSB) varies among spotted gum species and provenances (Dickinson *et al.* 2004). *Corymbia variegata* provenances that originate in areas with higher rainfall and nearer to the coast, are less susceptible to *Q. pitereka* than those originating from inland areas where lower annual rainfall occurs. This has resulted in the continuation of the planting of spotted gum using less-susceptible provenances (Pegg *et al.* 2009).

*Quambalaria pitireka* was first thought only to occur in Australia where it is native but it has since between identified on *Corymbia citriodora* in the Guangdong Province of China (Chen *et al.* 2017; Zhou *et al.* 2007). During the initial outbreak in the Guangdong Province, more than seven hectares of plantations were severely damaged. This was confirmed using the sequence of the internally transcribed spacer region (ITS) region of the ribosomal rRNA (Zhou *et al.* 2007). The species is thus considered a potential threat to the eucalypt industry in China. The spread of *Q. pitereka* from the east coast to the west coast of Australia and to China has been due to the expansion of eucalypt plantations in Australia, and the import of seed to China from Australia (Wingfield *et al.* 1993; Roux *et al.* 2006; Zhou *et al.* 2007)

### 3.1.2 Pathology

Of all the species of *Quambalaria*, *Q. pitereka* is the best studied in terms of its pathology and underlying mechanisms of infection. For this reason, these aspects of this species is discussed in more detail here than for the other species.

*Quambalaria pitireka* was first reported to cause shoot distortion, leaf spots and stem lesions (**Figure 3**) on *Corymbia (Eucalyptus) maculata* (Walker & Bertus 1971). Such infections have a profoundly negative effect on the growth and form of young spotted gum trees, especially in the first two years of growth (Self 2002). However, as plantation forestry expanded, the disease occurred more widespread, causing considerable damage (Carnegie 2007).

The use of spotted gum as priority species declined in sub-tropical regions of Australia due to the severe damage caused by QSB and that resulted in poor tree form, and occasionally, the death of trees. In Queensland and northern New South Wales, *Corymbia* plantations are grown close to indigenous stands of spotted gum, with mature trees often found within a plantation. In these areas, *Q. pitereka* sporulates on the immature foliage of seedlings (**Figure 4**) and saplings and young adult foliage of mature trees which makes these native stands the most probable source of infection for plantation grown trees (Pegg *et al.* 2008).

The symptoms of QSB seen on the foliage of *Corymbia* hybrids in sub-tropical regions of Australia are similar to those observed on spotted gum (Pegg *et al.* 2008). However, the disease was observed to be limited to new shoots and expanding foliage, with infection seldom resulting in stem death or loss of apical dominance (Pegg *et al.* 2008). Yet, in species trials in north Queensland, *Q. pitereka* infection resulted in necrotic lesions on mature foliage of *C. torelliana* hybrids, with premature leaf senescence resulting from high levels of infection (Pegg *et al.* 2008). Sporulation on the hybrids, however, was restricted to

the abaxial leaf surfaces and usually occurred only in the lesions centres. The lesions were surrounded by a water-soaked margin, which was also not observed on the spotted gum trees. The reason for this symptom is unclear but it may be as a result of host reaction to infection or the climatic conditions during infection and disease development in tropical north Queensland (Pegg *et al.* 2008).

Infected shoots on seedlings and young trees affected by QSB typically have a shiny white appearance due to the massive development of the fungus under the cuticle (Pegg *et al.* 2008). These white pustules are composed of a dense layer of conidiophores, which are hyaline, up to 50µm long and 2-2.5µm wide and non-septate, are up to 100µm in diameter and closely packed within the diseased area (Walker & Bertus 1971).

The germination of *Q. pitereka* conidia is similar to that found for the myrtle rust pathogen, *Austropuccinia psidii*, and *Q. eucalypti* (Pegg *et al.* 2009b). The conidia of *A. psidii* require high levels of humidity or the presence of free water at temperatures above 15°C and below 30°C to germinate. The same conditions are required for germination of *Q. pitereka* spores on plant tissue. A notable distinction between conidia germination of *A. psidii* and *Q. pitereka* is that *Q. pitereka* is not prohibited by light, only slowed down. The germination and fungal growth and pre-penetration of the host usually occurs during the warmer spring and summer months in the tropical and sub-tropical regions of Australia as conditions are optimal during this period (Pegg *et al.* 2009b). Rainfall is common during these times of the year and the day and night temperatures are in the optimal range for spore germination. Young, susceptible leaves and shoots become abundant on species of *Corymbia* and *Eucalyptus* that experience growth flushes due to rising temperature and rainfall. Penetration of the host occurs when the spores adhere to the leaf surface and the hyphae move into the host through stomata and wounds and remain intercellular until the host tissue dies (Pegg *et al.* 2009b). *Quambalaria pitereka* has been shown to be able to penetrate the host tissue through stomata (**Figure 4**) or wounds without the use of specialized penetration structures. This was confirmed when *Corymbia* and *Eucalyptus* were artificially inoculated with *Q. pitereka* and *Q. eucalypti* and were examined using Scanning Electron Microscopy (Pegg *et al.* 2009b).

Pegg *et al.* (2008) showed that *Q pitereka* is able to grow on and penetrate tissues of a range of *Eucalyptus* spp. that are non-host plants for this pathogen. However, growth was arrested soon after penetration implying that host defences do not activate immediately (**Figure 7**). These results were confirmed when *Q. pitereka* was used to inoculate

*Eucalyptus,* and the resulting non-sporulating lesions suggested a non-compatible pathogen-host interaction (Pegg *et al.* 2011).

Degradation of the cuticle of *C. variegata* during infection by *Q. pitereka* suggests that *Quambalaria* spp. may produce an adhesive substance which contains degradative enzymes. The properties of adhesion of *Quambalaria* spores to the stem and leaf surfaces is currently unknown but it is possible that a mucilaginous coating is activated by moisture (Pegg *et al.* 2009b). This property has been described for the rust *Uromyces fabae* by Struck (2006), an adequate moisture level is required during the infection process as it is required to hydrate the fungal propagules. This leads to a rapid release of mucilage that aids in the adhesion of the spores to numerous substrates. The composition of the mucilaginous substance is unknown and while fungal adhesives are usually water-insoluble glycoproteins, their composition can vary between species, genera and family (Tucker & Talbot 2001).

In 2008, *Q. pitereka* was isolated from cankers on woody stems of *C. citriodora* as well as from stems wounds caused by hail damage on *C. variegata, C. citriodora, C. henryi* and *C. maculata* (Pegg *et al.* 2008). Simpson (2000), however, observed that *Q. pitereka* was also present in the apparently healthy woody tissue of *C. maculata, C. henryi* and *C. variegata* indicating that this fungus may be an endophyte. Therefore, it is worth considering that the presence of the pathogen in the woody stems may serve as a mechanism for its long-term survival.

Pegg *et al.* (2008) first reported the presence of *Q. pitereka* on *C. torelliana* in tropical regions of north Queensland where it was found in plantations and native forest ecosystems. This finding had a significant impact on breeding programmes. Hybrids of *C. torelliana* and spotted gum species, such as *C. maculata,* displayed resistance to infection by *Q. pitereka*. This was supported by the observation that until 2008 the pathogen has not been observed in trial plantings or on amenity trees of *C. torelliana* in the subtropical regions (Pegg *et al.* 2008). From a plantation point of view, *Corymbia* hybrids have been shown to perform significantly better than their parent material and did not appear to be affected by *Q. pitereka* at the small plantation level (Lee 2007).

### 3.2 *Quambalaria cyanescens* (De Hoog & De Vries) Z.W. de Beer

#### 32.1 Taxonomy

*Quambalaria cyanescens* was first isolated from human skin in the Netherlands in 1973 and described as *Sporothrix cyanescens* De Hoog and De Vries (De Hoog & De Vries 1973). Later, *S. cyanescans* was confirmed to have dolipores septa and it was suggested that the

species might be the anamorph of a basidiomycete (Smith 1985). Based on this fact and the presence of the basidiomycetous coenzyme Q-10 system (Suzuki & Nakase 1986, Laurie *et al*. 2012), Moore (1987) reclassified *S. cyanescens* in the newly erected genus *Cerinosterus.* Using partial LSU-rDNA sequences and the nutritional profile, *Cerinosterus cyanescens*, was shown to be a close relative of *Microstroma juglandis* (Microstromatales; Exobasidiomycetes) (Middelhoven *et al.* 2000), and therefore substantially different from the type species of the genus *Cerinosterus*. In order to resolve this problem, a new genus *Fugomyces* was established for *Fugomyces cyanescens* (Sigler & Verweij 2003). Subsequently, De Beer *et al.* (2006) treated the species in *Quambalaria*. Based on DNA sequences of the ITS region, *Q. cyanescens* was shown to form a monophyletic group with *Q. eucalypti* and *Q. pitereka,* confirming its placement in the genus. This observation was supported by the septal pore ultrastructure of *Q. cyanescans* (**Figure 5**) (De Beer *et al.* 2006). Since *F. cyanescens* was the type species for *Fugomyces*, this genus is now treated as synonym of *Quambalaria* (De Beer *et al.* 2006).

### 3.2.2 Host species and distribution

*Quambalaria cyanescens* was first isolated from air and human skin samples in the Netherlands in the 1970's (De Hoog & De Vries 1973). It occupies a broad range ecological niches such as air, water, soil and plants (De Beer *et al.* 2006). *Quambalaria cyanescens* has also been reported from many different parts of the world including the USA, India, Israel and Spain (Kolarík *et al.* 2006).

*Quambalaria cyanescens* is rarely encountered as a pathogen of humans. Data collected from clinical cases suggest that this fungus may possibly be an opportunistic pathogen isolated primarily from individuals who are immunocompromised (Sigler *et al.* 1990). The species has also been reported to be associated with pseudoepidemic nosocomial pneumonia as an environmental contaminant (Jackson *et al.* 1990), and was recently isolated from a female patient after augmentation mammoplasty (Fan *et al.* 2014). Another report of *Q. cyanescens* is from the Northern Territory in Australia, the fungus was isolated from one biopsy sample of a forearm lesion caused by *Sporothrix schenckii*, although it is thought to not be the primary cause of infection (Subedi *et al.* 2014).

In an interesting phenomenon, *Q. cyanescens* has been identified from and shown to have an association with bark beetles collected from a range of hardwood host trees, including *Tilia*, *Quercus* and *Ficus* spp. in Hungary, Bulgaria and the Mediterranean (Kolarík *et al.* 2006). It is also the first fungus in the Ustilaginomycetes to be associated with bark beetles. It has been suggested that the galleries of bark beetles are a niche for many

heterobasidiomycetes, but the way in which these fungi interact with the beetles and other insects or fungi in the bark beetle habitat remains unknown (Kolarík *et al.* 2006). It might be that *Q. cyanescens* is a mycoparasites of ambrosia and blue-stain fungi typically associated with the beetles (Six 2005). However, in preliminary trials, *Q. cyanescens* did not display mycoparasitic activity (Kolarík *et al.* 2006). The entomopathogenicity of this fungus has not been studied. It was isolated from living adults or larvae, rather than from cadavers, which suggests that it might be harmless to bark beetles (Kolarík *et al.* 2006).

### 3.2.3 Pathology

In terms of its presence on trees, the first record of *Q. cyanescens* on eucalypts in Australia came from New South Wales in 2006 from *Corymbia callophylla* (Paap 2006). It was subsequently identified from *C. calophylla* and *C. ficifolia* in Western Australia, where it sporulates on shoots and newly emerging leaves together with *Q. pitereka* (Pegg *et al.* 2008). Paap *et al.* (2008) isolated the species from stem cankers alongside *Q. coyrecup.* This again raises the possibility that it might be a mycoparasite or it could also merely be a commensal in these niches.

*Quambalaria cyanescens* has been reported from trees outside of the eucalypt family, in association with silver birch (*Betula pendula*) in Russia (Antropova *et al.* 2014). Most recently it was found in association with decline symptoms of grapevine in Iran, where studies suggested it might be a trunk pathogen of the grapevines (Narmani & Arzanhou 2019). However, during infection trails on *Corymbia*, *Q cyanescens* did not prove to be pathogenic (Paap 2006). Although the fungus is commonly isolated from plants, it is not considered to a tree pathogen of significance (De Beer *et al.* 2006; Paap *et al.* 2008).

### 3.3.4 Other research

*Q. cyanescens* produces the antibiotic sesquiterpene globulol, which is widespread in the plant kingdom. (+)-Globulol has been detected in many plant oil extracts that have mainly been prepared from fresh leaves and bark, buds (*Cinnamomum zeylanicum*, Lauraceae), roots, and rhizomes (*Ferula hermonis*, Apiaceae; *Angelica sylvestris*, Apiaceae) (Stodůlková *et al.* 2008). Members of the family Myrtaceae also possess the capacity to produce (+)-globulol, e.g. *Eucalyptus* (Salgado *et al.* 2003). The presence of (+)-globulol in essential oils prepared from the leaves of *Eucalyptus urophylla* (Salgado *et al.* 2003) has been shown to be toxic to the fungal pathogens *Fusarium oxysporum, Botrytis cinerea* and *Bipolaris sorokiniana* (Stodůlková *et al.* 2008). Potent antimicrobial activity was exhibited by the crude extract isolated from a colour-pigmented endophytic *Quambalaria* species (which is closely

related to *Q. cyanescens*) against a variety of human and plant pathogens, such as *Shigella dysenteriae, Escherichia coli* and *Candida albicans* (Stodůlková *et al.* 2008; Padhi 2013).

In addition to the above-mentioned antibiotic, a novel active metabolite naphthoquinone (quambalarine A), which displayed broad antifungal and antibacterial activity, was isolated from *Q. cyanescens* (Císa *et al.* 2015). Another secondary metabolite, Quambalarine B (QB), was also extracted from *Q. cyanescens* (Stodulkova *et al.* 2016). This compound has been shown to have potential anticancer activity. At micromolar concentrations, it has been reported to inhibit the proliferation of several model leukemic cell lines (Jurkat, NALM6, and REH), whereas higher concentrations induce cell death (Stodulkova *et al.* 2016).

### 3.3 *Quambalaria eucalypti* (M.J. Wingfield) J.A. Simpson

#### 3.3.1 Taxonomy

The fungus known today as *Quambalaria eucalypti* was first observed on diseased *Eucalyptus* leaves in South Africa in 1987 and after which is was described as *Sporothrix eucalypti* (Wingfield *et al.* 1993). Simpson (2000) transferred *S. eucalypti* to the new genus, *Quambalaria* at the time when he treated the taxonomy of *Q. pitereka*.

Morphologically *Q. eucalypti* is characterized by the aerial hyphae that are hyaline, smooth, loosely aggregated, irregular, and 1-2 µm wide. The conidiogenous cells are scattered, integrated or terminal in side branches, the cells vary in size and in shape and are usually uniform in width or widest at the swollen apex, seldom tapering to a narrow apex with the apical part forming the conidia through sympodial growth. The conidia are hyaline, non-septate, smooth and thin walled (Wingfield *et al.* 1993, Simpson 2000).

#### 3.3.2 Host and distribution

*Quambalaria eucalypti* was initially reported in KwaZulu-Natal, South Africa on a *Eucalyptus grandis* clone (Wingfield *et al.* 1993). It has since been reported on *E. nitens* from the Mpumalanga Province (Roux *et al.* 2006) where infection resulted in extensive leaf and shoot dieback, and stem cankers on 1-year-old trees (**Figure 6**). Some of the symptoms of the disease resembled Quambalaria Shoot Blight (QSB) caused by *Q. pitereka*. However, this was the first time it had been reported to occur on the stems of larger trees (Roux *et al.* 2006). *Q. eucalypti* is the only species of the genus reported from South Africa. It is considered to cause a disease of importance due to the damage that can result from infection, but is not considered a major threat (Roux *et al.* 2006; Wingfield *et al.* 2008a). *Quambalaria eucalypti* has been reported on *E. grandis, E. longirostrata, E. grandis* × *E.*

*camaldulensis, E. microcorys* and *E. dunnii* (Pegg *et al.* 2008). In these cases, the pathogen was isolated from leaf spots on trees in commercial plantations, tree trials and private gardens in subtropical and tropical regions of Australia (Pegg *et al.* 2008), where it is has been suggested to have originated (Wingfield *et al.* 1993; Roux *et al.* 2006; Zhou *et al.* 2007). The pathogen has been reported from Brazil, causing leaf and shoot lesions on *E. globulus* and substantial problems during the clonal propagation of *Eucalyptus* (Alfenas *et al.* 2001).

*Quambalaria eucalypti* has been found in It Uruguay, where it was first reported infecting i twigs of *E. globulus* (Bettucci *et al.* 1999). In 2007, the pathogen was found in Uruguay associated with leaf lesions on the native tree *Myrceugenia glaucescens* (Perez *et al.* 2008). This is an example of a host shift and is of great concern as *Q. eucalypti* had been hypothesised to be host specific on *Eucalyptus* (Wingfield *et al.* 2013). This host shift is entirely possible as *E. globulus* and *M. glaucescens* fall into closely related tribes, Eucalypteae and Myrteae respectively, within the Myrtaceae (Wilson *et al.* 2005).

The first report of *Q. eucalypti*, in Europe was from Portugal on *E. globulus* where it resulted in stem cankers and dieback of seedlings (Bragança *et al.* 2015). The most recent report of this pathogen has been on the young shoots and leaves of *E. urophylla x E. grandis* clones in China (Chen *et al.* 2017).

### 3.3.3 Pathology

*Quambalaria eucalypti* was first identified on an *E. grandis* clone, TAG12, in South Africa (Wingfield *et al.* 1993). The fungus was isolated from brown necrotic wounds on the actively growing shoots and leaves of trees. The most common, visible, signs of the fungus are white masses of powdery spores on leaf lesions and stems (Wingfield *et al.* 1993). Due to the apparent host shift that occurred in Uruguay, this fungus is now considered a potentially serious pathogen. However, in South Africa, it was not considered to be of significant importance until it was reported causing shoot dieback, leaf spots and dark, moist, sunken stem lesions, with visible white spore masses, on *E. nitens* (Roux *et al.* 2006).

The infection process of *Q. eucalypti* is very similar to that of *Q. pitereka*. The primary difference between these two species is that *Q. eucalypti* does not infect *Corymbia* spp., the same way that *Q. pitereka* does not infect *Eucalyptus* spp. Pegg *et al* (2008) observed that *Q. eucalypti* was able to grow and penetrate non-host plants, such as *C. variegata,* however growth was arrested soon after infection (**Figure 7**). The same phenomenon has been observed in *Q. pitereka* (Pegg *et al.* 2008).

### 3.4 *Quambalaria pusilla* (Braun) J.A. Simpson (= *Q. simpsonii* Cheewangkoon & Crous)

#### 3.4.1 Taxonomy

*Quambalaria pusilla* was first identified on *Eucalyptus camaldulensis* in Thailand and was described as *Sporothrix pusilla* based on the conidial scars and hila, that were neither darkened nor thickened, though slightly refractive (Braun 1998). There were no acropetal conidial chains, and the primary conidia sometimes produced secondary conidia in a direct manner or the secondary conidia are produced on a short, minutely denticulate conidiophore (Braun 1998). The taxonomic status of the fungus was questioned by De Beer *et al.* (2006) in the absence of a living culture and DNA sequences. A dried culture of the type specimen was discovered and the its status as a distinct species of *Quambalaria* was confirmed with DNA sequences (De Beer 2012)

Cheewangkoon *et al.* (2009) described the novel species, *Q, simpsonii* from *Eucalyptus tintinnans* from the Northern Territory in Australia (**Figure 8**). However, the authors did not compare the new species to *Q. pusilla*, because DNA sequences were not available for that species. The DNA sequences produced by De Beer (2012) for *Q. pusilla*, however, revealed that *Q. simpsonii* and *Q. pusilla* represent the same species. The older of the two names has nomenclatural preference and these authors reduced *Q. simpsonii* to synonymy with *Q. pusilla*. The most recent report of this species has been from stem cankers, caused by *Teratosphaeria zuluensis*, of *E. urophylla x E. grandis* from across the Guangdong and Hainan Provinces of China (Chen *et al.* 2017). This species was consistently isolated from these cankers, but it is still unknown whether this species is a pathogen or has some ecological interaction with *T. zuluensis* (Chen *et al.* 2017).

### 3.5 *Quambalaria coyrecup* T. Paap

#### 3.5.1 Taxonomy

*Quambalaria coyrecup* was originally known as *Sporothrichum destructor*, and was initially observed on *Corymbia ficifolia* in Western Australia in the 1920's (Beard 1963; Cass Smith 1970; Shearer 1994). However, since no formal description was provided for the species (Walker & Bertus 1971), the name remained invalid. Paap *et al*. (2008) found the species causing cankers associated with gummosis (**Figure 9**) on marri, *Corymbia ficifolia*, and formally described it as *Q. coyrecup*.

In culture, *Q. coyrecup* produces slender sterile hyphae with hyaline walls, branched and obscurely septate (Paap *et al.* 2008). The conidiogenous cells are very similar to the sterile hyphae; the cells are usually long, arising terminally and occasionally laterally, unbranched, cylindrical, and usually straight and bearing a single stellate whorl, or it can occasionally form a spiral of a few or up to 13 conidia, or conidia forming by sympodial growth from the apical part. The conidia are hyaline, non-septate, and smooth, may be vacuolated, cylindrical or oblong and rounded on both ends. The conidia are sessile with a basal scar on one or both ends and often give rise to one or more obovoid or narrowly ellipsoidal secondary conidia through budding or through short conidiogenous cells (**Figure 10**) (Paap *et al.* 2008).

### 3.5.2 Hosts and distribution

*Quambalaria coyrecup* was first observed in 1928 in Western Australia on *C. ficifolia* (Beard 1963). From there it rapidly spread through the Perth region during the early 1930s infecting *Corymbia ficifolia* resulting in a severe canker disease. *Quambalaria coyrecup* still occurs in Western Australia, especially in the Perth metropolitan (Yulia *et al.* 2014).

Host range studies on *Q. coyrecup* (as "*S. destructor*") were performed using artificial inoculation with several isolates obtained from *C. ficifolia* (Cass Smith 1970). The closely related *Corymbia calophylla* (marri), an endemic species to the southwest of Western Australia, was also susceptible to the pathogen (Cass Smith 1970). In 1939, the first stem cankers of *C. calophylla* were recorded in Pickering Booth, with *Q. coyrecup* (as "*S. destructor*") as the causal agent. Naturally infected native stands of *C. calophylla* were identified in surveys by Cass Smith (1970) in numerous parts of the state (**Figure 11**). The cankers discovered on *C. calophylla*, closely resembled those observed on *C. ficifolia,* especially the cankers on the smaller branches. Therefore, reciprocal inoculations were performed on *C. calophylla* and *C. ficifolia* seedlings with isolates from both hosts. The experiments yielded very similar results with both tree species displaying similar symptoms and both the fungal isolates were found to be microscopically identical (Cass Smith 1970). Between the 1960s and the 1980s, the canker disease on *C. calophylla* has become more severe and widespread throughout the southwest of Western Australia, which has led to decline and death of this tree species (**Figure 12**)(Shearer 1994). Cankers were only observed on *C. ficifolia* when planted outside of its native range near to the native range of *C. callophylla*. This lead to the hypothesis that this pathogen is endemic on these species and results only in infection when these species are moved out of their native ranges (Paap *et al.* 2008). In a recent survey, canker incidence has been seen to increase on *C. callophylla*. This is thought to be due to the increase in mean temperature and decrease in

the mean rainfall of Western Australia as well as the increasing incidence of *Phytophthora* infecting *C. callophylla* (Paap *et al.* 2017).

### 3.5.3 Pathology

*Quambalaria coyrecup* is the only species of *Quambalaria* to cause cankers on eucalypts, specifically *C. ficifolia*, resulting in stem swelling and severe bark splitting. The fact that *Q. coyrecup* causes cankers is interesting as the majority of tree- canker causing fungi are ascomycetes. This is in contrast to other species in the Microstromatales and their relatives that are foliar pathogens (De Beer *et al.* 2006).

Cankers are only formed after infection by *Q. coyrecup* through natural or artificial wounds, indicating that wounding is required for infection and disease development to occur (Yulia *et al.* 2014). This finding by Yulia *et al*. (2014), supports what is currently thought to be the mode of infection for stem canker causing fungi, as most fungi that cause cankers are unable to directly penetrate the bark and are wound parasites (Blanchette & Biggs 1992). It has been shown that the canker symptoms are a result of callus formation and that this condition occurs only when the vascular cambium had been infected.

On eucalypts infection of the cambium is typically confirmed with the detection of kino (Yulia *et al.* 2014). Kino is produced by these trees in response to cambium disturbance as it is rich in a range of polyphenolics including ellagitannins. These compounds have fungistatic properties and are produced following pathogenic invasion of the phloem and death of the cambium in the stems (Tippett *et al.* 1985; Old & Davidson 2000). The invasion of sapwood often leads to the discoloration of the wood directly underneath the canker. This reduces water movement through the colonized wood and leads to wilting of the leaves distal to the canker. Infection leads to hypertrophy (cell enlargement) and necrosis (cell death) which results in the formation of cankers. The production of hypertrophic cells is considered to be a response by the plant to limit fungal infection, as these cells will form a barrier to infection. In *C. ficifolia*, thickened cells constitute these barrier zones and can be detected through UV illumination as the thickened cells are autofluorescent. Antimicrobial and water impermeable substances such as lignin, suberin and other polyphenolics are accumulated in the first layer of the barrier zone cells as these cells are the closest to the pathogen (Smith *et al.* 2007; Yulia *et al.* 2014).

The canker disease caused by *Q. coyrecup* occurs on trees that inhabit a wide range of rainfall regions, soil and vegetation types, and girdling and killing of the host can occur

rapidly. It is therefore important to determine the cause of and to develop options for the control of the disease (Paap *et al.* 2008).

### 3.6 *Quambalaria fabacearum* J.D.P. Bezerra

*Quambalaria fabacearum* was isolated and identified as an endophyte of *Mimosa tenuiflora*, which belongs to the Fabaceae, in Brazil (Bezerra *et al.* 2018). Apart from this report, nothing is known about the distribution or other host trees of this species.

Unlike other *Quambalaria* spp., this species produces pale yellowish pigment when grown in culture on PDA. The mycelium is superficial to partly immersed with hyaline hyphae which are smooth, loosely septate and branched. The conidiogenous cells are cylindrical, straight to curved, integrated or terminal, forming conidia via sympodial growth. The conidiogenous cells are denticulate, pointed or flattened and inconspicuous. Conidiophores are similar to the hyphae and are scattered, terminal or as short side branches, cylindrical, hyaline and smooth. The conidia are smooth, non-septate, intermittently guttulate, and hyaline. The primary conidia are ellipsoidal to fusiod or obovoid with basal scars at one or both ends, which often give rise to several obovoid or slightly ellipsoidal secondary conidia (Bezerra *et al.* 2018).

### 3.7 *Quambalaria purpurascens* Z.W. de Beer, Marinc. & G.S. Pegg *nom. prov.*

*Quambalaria purpurascens* was first reported as *Quambalaria* sp. from asymptomatic *Leptospermum junipae* in Victoria, Australia (Amin *et al.* 2010). Similar isolates were obtained during a study by De Beer (2012) from leaf spots observed on *Angophora costata* in New South Wales. Together, these isolates formed a well-supported lineage, which was distinct from the other *Quambalaria* species. De Beer (2012) provided a provisional name for the taxon that will be submitted for publication in due course. This species is thus known only from Australia, and nothing more is known about its pathogenicity or host range.

*Quambalaria purpurascens* produces a deep purple pigment when grown on artificial media, similar to that of *Q. cyanescens.* The mycelium is superficial, with colonies appearing powdery and flat. The conidiogenous cells are smooth and hyaline, discrete or integrated, intercalary or terminal, reduced to short denticles directly on vegetative hyphae at times, cylindrical, straight, unbranched, with parts consisting of denticles, mostly at the apex and swollen or throughout the upper half of the cell without swelling. Conidiophores are semi-

macronematous or micronematous. The conidia are smooth, non-septate, slightly pointed at the base, hyaline, ellipsoidal and guttulated (De Beer 2012)

## 4.0 Fungal pathogens of *Eucalyptus* seed capsules

Several different species of fungi have been isolated and reported from the seed capsules of eucalypt trees. Unfortunately their effect on seed production is largely unknown (Drake 1974, 1981; Webb 1983; Sharma *et al.* 1985). The following section treats the information presently available on the association between *Quambalaria* and *Eucalyptus* seed capsules. During the 1970's, a survey was conducted of south-east Queensland to assess the productive success of three species of *Eucalyptus* (*E. crebra, E. melanophloia, E. populnea*), and of hybrids of *E. crebra* and the two other species. During this survey, a species of *Ramularia* was reported to be causing damage to the developing capsules. This resulted in the loss of seed in all five genotypes (Drake 1974, 1981). The infection of the capsules with *Ramularia* must have occurred at an early stage as young capsules were heavily infected shortly after flowering (Drake 1974). The infected capsules opened early and the contents were covered in a white mass of spores and mycelium. The seed loss for the different genotypes ranged from 2.3% for *E. crebra* to 83% for *E. melanophloia x E. crebra*. As *E. crebra, E. melanophloia* and *E. melanophloia x E. crebra* have been reported as host for *Quambalaria (Ramularia) pitereka*, which occurs in south-east Australia, it has been suggested that the infections recorded by Drake was also caused by *Q. pitereka* (Brown 2000). However, at the time of the study of Brown (2000), only *Q. pitereka* was known from Australia. As neither isolates nor further data are available of the fungus isolated from the seed capsules, it impossible to confirm the true identity of the species described by Drake.

A decline in seed production of 5-year old *E. camaldulensis* as a result of capsule disease was also reported in India in 1985 (Sharma *et al.* 1985). It was suggested that the infections were caused by *Colletotrichum gloeosporioides (= Glomerella cingulata)* and a *Torula* sp. This disease occurred only on *E. camaldulensis* and resulted in the operculum becoming leathery and dried up while remaining attached to the capsule, the stamens became brown and curled, and it subsequently spread to the capsules (Sharma *et al.* 1985).

Seed abortion, capsule lesions, necrosis of infected capsules, and twig die-back are some of the symptoms associated with *Dothiorella (Fusiccocum) eucalypti* infection on *E. camaldulensis* (Webb 1983). These disease symptoms have been reported in southern Florida were it resulted in seed crop failure. This was followed by the implementation of the use of vegetative cuttings for the commercial planting of *E. camaldulensis* in the area.

Infection of the capsules usually followed the colonization of the flower parts which then spread to the pedicels and peduncles and finally to the twigs, causing dieback (Webb 1983).

## 5.0 Whole genome sequencing and sexual reproduction in the Microstromatales

No whole genome sequence has been published for any species of *Quambalaria*. Such information would contribute substantially to a deeper understanding on the life cycles, and pathogenicity of these fungi. Furthermore, only asexual states have been described for these fungi and nothing is known about the mating systems or mating genes of any *Quambalaria* species, neither for any of their closest relatives. In this section of the review I briefly consider the higher taxonomic placement of the Microstromatales, and link this information to taxa for which whole genome sequences are available. Knowledge gained in terms of mating genes for these related species is also considered.

The Microstromatales, which includes the families Microstromataceae, Volvocisporiaceae and Quambalariaceae (De Beer *et al.* 2006), is one of the orders of the Exobasidiomycetes (Wang *et al.* 2015). This class includes a large number of species; of which many are plant pathogens. The best-known examples of these are species of *Tilletia* and *Exobasidium*. Species of *Tilletia* cause disease on rice (Biswas 2003), wheat (Carris *et al.* 2006; Mitra, 1931) and rye (Castlebury & Carris 1999) and species of *Exobasidium* cause disease on blueberry (Brewer *et al.* 2014), lingonberry (Burt 1915) and *Rhododendron* spp. (Nagao *et al.* 2003). No studies based on whole genome sequences have been published to date for any species in the Exobasidiomycetes.

The Exobasidiomycetes forms part of the subphylum Ustilaginomycotina (**Figure 13**) (McLaughlin *et al.* 2009). The closest relatives to this class for which studies based on whole genome sequences have been published are indicated with arrows in **Figure 14**. These include the human pathogens in the genus *Malassezia* in the Malasseziomycetes, and the biotrophic maize pathogens, *Ustilago maydis* and *Sporisorium reilianum,* and *Ustilago hordei,* a pathogen of barley (**Figure 14**) (Wang *et al.* 2015).

Whole genome sequencing of these pathogens has highlighted the power of comparative genomics of related taxa to gain insight into synteny, the factors related to reproduction, such as the mating type genes, and other lifestyle characteristics (Que *et al.* 2014). Below I briefly consider the mating behaviour of the species for which whole genome sequences are available. These include *Ustilago maydis* (Kämper *et al.* 2006), *Ustilago hordei* (Laurie *et al.* 2012), *Sporisorium reialianum* (Schirawski *et al.* 2010), *Sporisorium scitamineum* (Que *et al.*

2014), *Malassezia globosa* (Xu *et al.* 2007) and *Malassezia sympodialis* (Gioti *et al.* 2013). This provides, context and background information for future exploration and comparison of a *Quambalaria* genome.

All *Malassezia* spp. form part of the dominant members of the human skin microbiota. A number of these species has been found to be associated with pityriasis versicolor, eczema and dermatitis of canines, dandruff of humans, and occasional systemic infections (Saunders *et al.* 2012). Fourteen species of *Malassezia* have been described from various animals as well as humans (Hort & Mayser 2011). A common feature among the species of this genus is the requirement of exogenous lipids for growth, with *Malassezia pachydermis* as the only exception. Another distinctive feature of this genus are the thick cell walls, that comprises of ~70% sugars, ~10% protein, and 15-20% lipids (Nagata *et al.* 2012).The cell wall is enclosed by a lipid capsule-like layer, which is thought to play a role in host interactions (Mittag 1995). No sexual stage has been described for any species of *Malassezia.*

*Ustilago maydis* is an important pathogen of maize The species is a well-established model organism for the smut fungi. This fungus causes stunted plant growth and results in reduced yield, which leads to extensive economic losses (Kämper *et al.* 2006). *Ustilago maydis* is a dimorphic fungus and has yeast-like saprophytic growth in its haploid phase. The initiation of sexual development occurs when two of the haploid cells fuse which is controlled by a tetrapolar mating system which consists of the *a* and *b* mating type loci (Morrow & Fraser 2009).

*Ustilago hordei* causes covered smut of oats and barley and is considered an economically important disease with a worldwide distribution. Infective hyphae are formed when two basidiospores of different mating types fuses, and then infects the plant during the seedling stage. Symptoms only appear when the infected meristem differentiates into the floral tissue (Hu *et al.* 2002)

*Sporisorium reilianum* is the fungal agent that is responsible for the disease known as head smut in corn (Al-Sohaily *et al.* 1963; Martinez *et al.* 2002). This disease occurs around the world and results in significant economic losses. The fungus infects the corn plant through the roots during the seedling stage. The symptoms only become visible when the plant begins to flower, when the formation of phyllody in the inflorescences is observed. This fungus causes crop losses as it replaces the inflorescences, or cobs, with teliospore masses, which are then dispersed by the wind upon release (Ghareeb *et al.* 2011). The life

cycle of *S. reilianum* is similar to that of *U. maydis* in that it has a yeast-like stage and a parasitic stage. The parasitic stage arises from the fusion of two hyphae of opposite mating types that then infect the plant through the roots (Martinez *et al.* 2002).

*Sporisorium scitamineum* is the causal agent of sugarcane smut disease and has become a global problem for the sugarcane industry. This disease causes stunted growth, thin stalks and an outgrowth of fungus from the stalk known as culmicolous. The black/gray outgrowth from the terminal bud or lateral shoots, known as a 'smut whip' is this disease's most noticeable characteristic (Hoy *et al.* 1986). Millions of teliospores are contained within the whip and are responsible for the rapid spread of this disease. During sexual crossing, infective dikaryotic hyphae are formed through the fusion of sporidia that are compatible (Que *et al.* 2014).

### 5.1 Genomic characteristics and synteny

Synteny is the conservation of blocks of order within two sets of chromosomes that are being compared with each other. In comparison to other plant pathogens, the genome of *Ustilago maydis,* is small. It has been suggested that the reason for this might be the lack of introns, that are not present in up to 70% of its genes (Kämper *et al.* 2006). Another reason for its small size might be the apparent absence of expanded gene families (Kämper *et al.* 2006). It has been suggested that the large scale intron loss occurred through recombination of reverse-transcribed transcripts with the genomic copy (Roy & Gilbert 2006).

In contrast to *U. maydis,* the *Ustilago hordei* genome is the largest of those amongst the smut fungi, with extensive repetitive elements contributing to its size. Despite the synteny that *U. hordei* has with other smut fungus genomes, the repetitive elements have shaped the genome evolution and consequently has impacted the biology of the fungus (Lee *et al.* 1999, Bakkeren *et al.* 2006)

The genomes of *U. maydis* and *U. hordei* display a high level of synteny (**Figure 15**), a level that is only slightly lower than that observed between *U. maydis* and *Sporisorium reilianum* (Laurie *et al.* 2012; Schirawski *et al.* 2010). During the mapping of the chromosomes, three major re-arrangements were observed (Laurie *et al.* 2012). These involved chromosome (chr) 1, 2 and 9 of *U. hordei* and chr 1, 5 and 20 of *U. maydis.* The co-linearity of the chromosomes among the three species is a higher between *U. hordei* and *S. reilianum* than between *U. hordei* and *U. maydis.* This observation suggests that the arrangement of *S. reilianum* may be the ancestral arrangement of the chromosomes in the three species derived from their common ancestor (Laurie *et al.* 2012). If this arrangement is indeed ancestral, then following

relocation events occurred in *U. maydis* and *U. hordei* respectively: the *a* locus moved from chr 20 to chr 5, and the *b* locus moved from chr1 to chr 20. This re-arrangement had profound effects on the biology of *U. hordei*. The placement of the *a* and *b* mating type loci together on the one chromosome, enabled the development of a bipolar mating system (Laurie *et al*. 2012).

### 5.2 Reproduction and Mating

Mating in *U. hordei* is regulated by one mating type locus (MAT) which has two alleles, MAT-1 and MAT-2 (Bakkeren *et al.* 2008). These two loci are more than 450 kb in length, with MAT-1 being comprised of 47 genes that are spread across the locus with large stretches of long terminal repeats (LTRs) between them. Approximately 50 % of the region is made up of transposable elements (TEs). The two loci are delimited by the *a* locus, a pheromone and pheromone receptor pair, and the *b* locus, a pair of transcription factors that contain homeodomains (Bakkeren *et al.* 2008).

This bipolar mating system of *U. hordei* promotes inbreeding which leads to increased homozygosity within the population. This reduces the chances for ectopic recombination transposable elements. This system may be beneficial as it fixes transposable elements in a population (Blumenstiel 2011; Laurie *et al.* 2012). This is in contrast to tetrapolar mating systems (e.g. *S. reilianum*), where outcrossing increases heterozygosity leading to a higher risk of ectopic recombination of related transposable elements. *U. hordei* utilizes bipolar mating in conjunction with RNA inference and repressive chromatin features and DNA methylation, supported by repeat-induced point mutation (RIP), to provide a means in which to control the copious transposable elements in its genome (Blumenstiel 2011; Laurie *et al.* 2012). Transposable elements can still escape the control mechanisms and accumulate in loci where recombination is suppressed. Genes at these loci could then be affected as well as their evolution within the population. This effect is of particular interest in terms of effector genes that interact with plant hosts (Laurie *et al.* 2012).

Like *U. hordei*, *S. scitamineum* has a bipolar mating system, in which there is linkage of the *a* and *b* loci and the mating- type locus (MAT) segregates as one locus, which is unlike *U. maydis* (Laurie *et al.* 2012). The *a* locus encodes a lipopeptide with pheromone and pheromone membrane receptor, that are responsible for compatible hyphal fusion and cell recognition. The *b* locus encodes a number of transcription factors that regulate the expression of genes responsible for maintaining dikaryotic hyphal growth in plants. Through comparative genomics, the mating-type regions of bipolar and tetrapolar smut fungi have been shown to not be fundamentally different. The genes of these *a* and *b* mating-type

complexes are found in both bipolar and tetra-polar smuts as well as related species (Que *et al.* 2014).

The genus *Malassezia* has no known sexual stage. However, using comparative genomics with the smut fungus *U. maydis*, *M. globosa* was found to contain gene regions that have a resemblance to the *a* and *b* loci of *U. maydis* (Xu *et al.* 2007). These genes also share similar organisation to the *a* and *b* loci of the bipolar *U. hordei* (Bakkeren *et al.* 2006), as in the pheromone and pheromone receptor genes, and the homeodomain containing transcription factor genes that are similar to bW and bE, appear on the same supercontig (**Figure 16**) (Xu *et al.* 2007). it has consequently been hypothesized that this region may be MAT loci of approximately 170kb in length. These loci, however, do not contain the same repetitive elements or many of the same genes of the *U. hordei* MAT loci. When comparative genomics was performed on this gene region using the more distantly related *Cryptococcus neoformans*, orthologs of the pheromone receptor gene and CID1 were found. These genes suggest the presence of a sexual stage for *Malassezia*. Other orthologs were also identified during the comparison, such as genes involved in sporulation, meiosis, and pheromone signalling (Xu *et al*. 2007).

When a genomic comparison was performed between *M. globosa* and *M. sympodialis,* a high level of synteny was observed (Gioti *et al.* 2013). *A* and *B* mating type loci were also identified through sequence alignment and appeared to be 141kb apart. In contrast to that expected of a bipolar mating system, both the *A* and *B* MAT locus alleles of *M. sympodialis* displayed extensive flanking synteny with the equivalent *M. globosa* MAT locus regions (Gioti *et al.* 2013). This is not the case in *U. hordei*, where the *A* and *B* MAT regions are separated by a region of highly rearranged sequences of about 430 to 500 kb in size (Lee *et al.* 1999).

Further evidence to suggest that the mating system of *Malassezia* does not appear to fit either bipolar or tetrapolar systems, was shown in the sequencing of the *A* and *B* MAT alleles from a population sample of isolates (Gioti *et al.* 2013). Two distinct *a* alleles, *a1* and *a2,* were identified in terms of both sequence conservation and gene orientation. Three *b* alleles, *b1, b2* and *b3,* were identified based on N-terminal substitutions or in the protein-protein interaction domains of the two homeodomain factors bE and Bw. With the discovery of biallelism for the *A* locus and triallelism for the *B* MAT locus, it has been suggested that recombination is possible between these regions (Gioti *et al.* 2013).

## 6.0 Conclusions

Some species of *Quambalaria* have recently emerged as important tree pathogens. The discoveries of fungi resembling *Quambalaria* from *Eucalyptus* seed capsules in South Africa and on which this dissertation is based have raised several questions. The first of these concerns the identification of the newly discovered *Quambalaria* spp from seed capsules. The identity of the *Quambalaria* isolates discovered in the seed capsules of *Eucalyptus* form the first part of this dissertation This will be based on sequences of the LSU, and the ITS containing two spacers (ITS1 and ITS2) flanking the nuclear ribosomal 5.8S gene. Both these gene regions were applied in the first comprehensive phylogeny of the genus (De Beer *et al* 2006) and the subsequent phylogenetic studies (Bragança *et al.* 2015; Paap *et al.* 2008; Zhou *et al.* 2007). In addition, the applicability of the elongation factor (EF) gene region for the addition phylogenetic support for distinguishing species in this group (Schoch *et al.* 2014; Stielow *et al.* 2015) will be considered

A review of the current knowledge regarding *Quambalaria* clearly shows that, little is understood of the biology and ecology of these fungi. For example, no sexual state has been observed for any of the known *Quambalaria* species. Yet, DNA sequences showed that there is substantial genetic diversity within species like *Q. pitereka* (Pegg *et al*. 2011). This raises the second question addressed in this dissertation ie whether sexual reproduction and outcrossing occurs in any of the *Quambalaria* species Considering the knowledge of mating systems of relatives of *Quambalaria*, has shown that this question would best be answered by generating a whole genome sequence for a *Quambalaria* species. This sequence could then be explored for mating genes using the sequences of mating genes from related species of Ustilaginomycotina.

## 7.0 References

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## 8.0 Figures and Tables

**Table 1.** Characteristics based on which Simpson (2000) described and distinguished the new genus *Quambalaria* from *Ramularia* and *Sporothrix*, in which the three *Quambalaria* species was treated previously.

| **Character** | ***Quambalaria*** | ***Ramularia s. str.*** | ***Sporothrix s. str.*** |
|---|---|---|---|
| Stromatic tissue | Present, hyaline | Sometimes present, hyaline or faintly pigmented | Absent |
| Condiophores | Solitary to caespitose | Solitary to fasciculate | Solitary |
| Conidiogenous cells hyaline | Yes | Usually, or with basal pigment | Yes |
| Growth sympodial | Yes | Yes | Yes |
| Conidia | Solitary | Solitary or catenate | Solitary |
| Thickened spore scars | No | Slightly to strongly thickened | Inconspicuous |
| Conidial scars darkened | No | Always, usually strongly | No |
| Refractive spore scars | Slightly to strongly refractive | Strongly refractive | No |
| Spore scars protruding on small geniculations on condiogenous cells | Yes | Yes | No |
| Denticles | Minute | No | Usually prominent |
| Secondary conidia | Present but not on all primary conidia | None | Formed in some species |
| Teleomorph | Unknown | Mycosphaerellaceae | Ophiostomataceae |

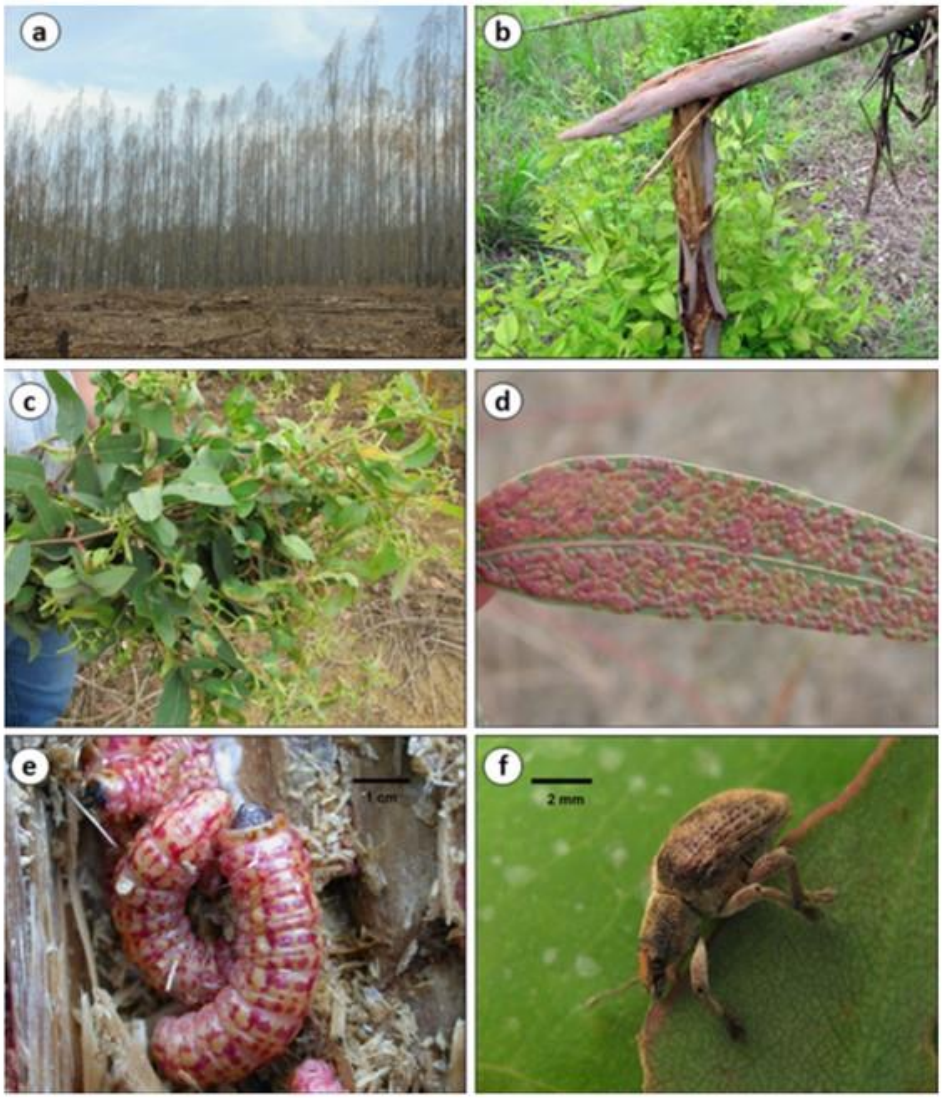


**Figure 1**. Examples of insect pests of commonly found in Eucalypt plantations. (a) Early leaf senescence caused by infestation of *Thaumastocoris peregrinus*; (b) wind break due to tunneling of *Phorocantha* larvae; (c) galling caused by *Leptocybe invasa*; (d) leaf galled by *Ophelimus maskelli*; (e) larvae of *Coryphodema tristis* boring in wood; (f) adult *Gonipterus scutellatus* feeding on leaf (Wingfield *et al.*, 2013).

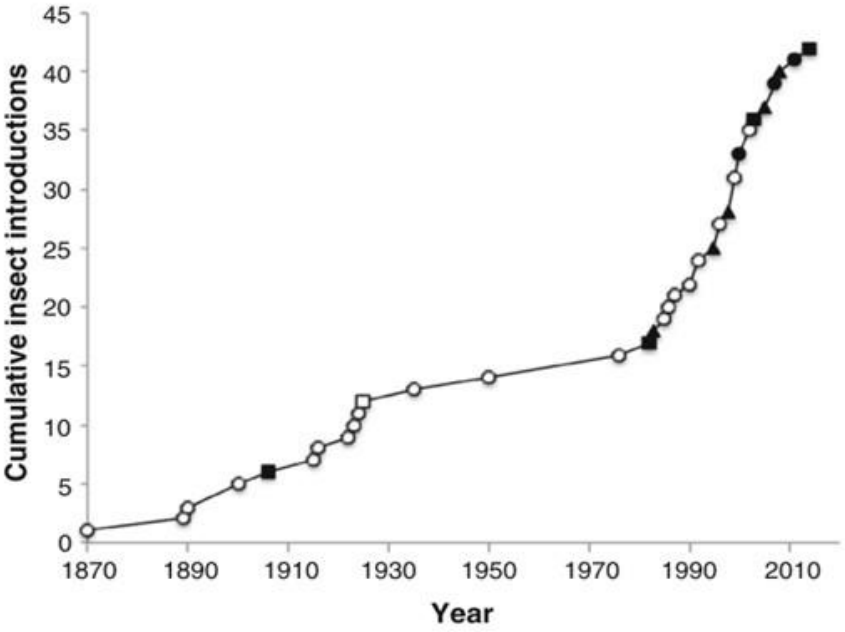


**Figure 2.** Cumulative introduction of insect pests feeding on eucalypts outside their native range in Australasia. Symbols indicate the country of first detection: New Zealand (open circle), South Africa (filled square), Argentina (open square), USA (filled triangle) and Europe (UK, Italy and France; filled circle). The rate of introduction of new pests has increased rapidly since 1986 (Hurley *et al.*, 2016).


**Figure 3.** *Quambalaria pitereka* affects new foliage causing spotting, necrosis and distortion of (a) expanding leaves and (b) green stems. Diseased shoots are covered in white masses of conidia and conidiophores which rupture through the waxy leaf cuticle (Pegg *et al.*, 2009a).

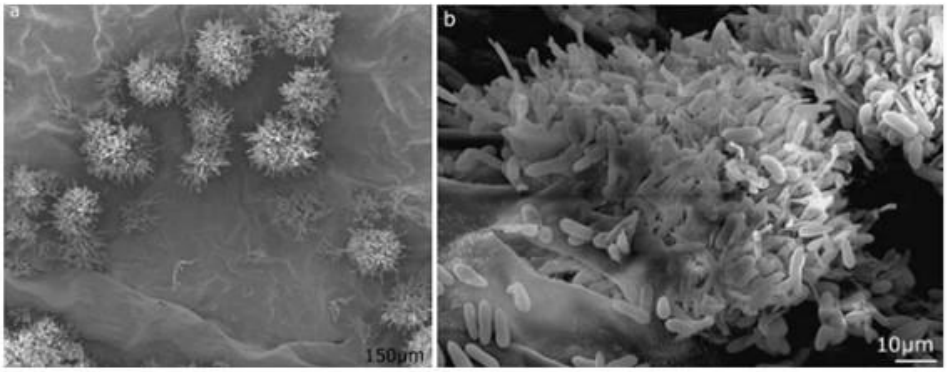


**Figure 4.** Scanning electron micrograph showing conidiophores and conidia of *Quambalaria pitereka* (a) arising from leaf stomata and (b) rupturing through the epidermal layer on juvenile stem of *Corymbia variegata* (Pegg *et al.*, 2008).

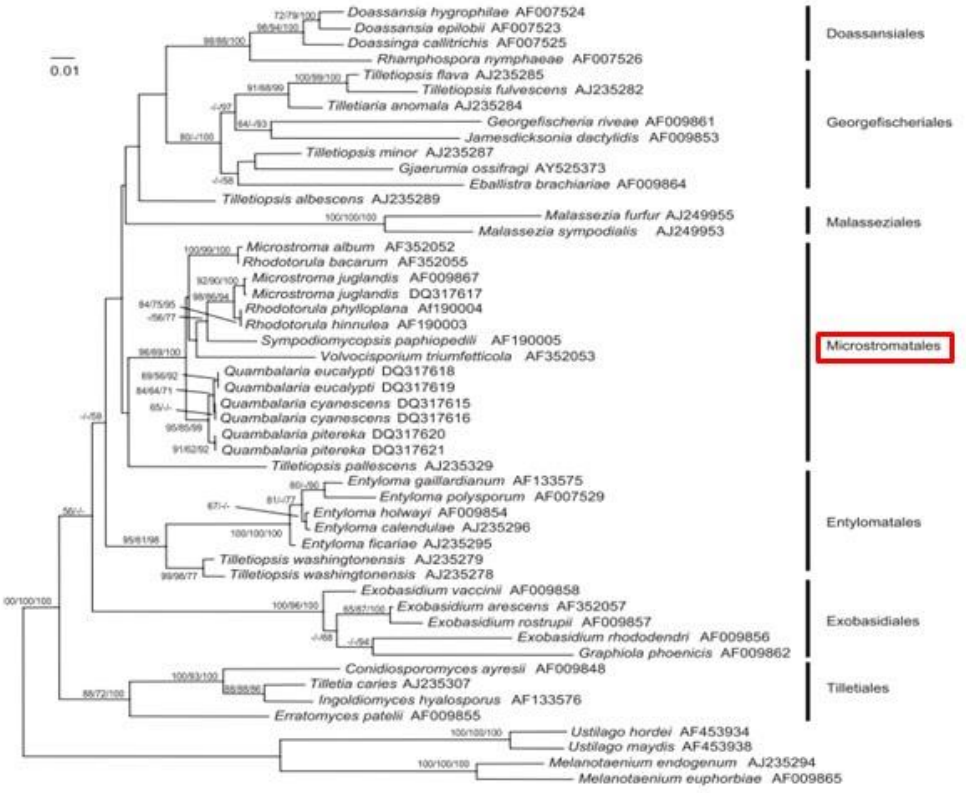


**Figure 5**. Phylogram obtained by neighbour-joining analysis of the nuclear LSU region sequences of species in the Microstromatales. The topology was rooted with four members of the Ustilaginomycetidae (de Beer *et al.*, 2006).


**Figure 6;** Symptoms of *Quambalaria eucalypti* infection on *Eucalyptus* spp. (a) developing leaf spots on a *E. grandis* × *E. camaldulensis* clone, (b) leaf spots with white fungal spore masses, (c) leaf spot, blight and shoot die-back, (d) stem lesions and white spore masses on a *E. nitens* stem (Roux *et al.,* 2006).

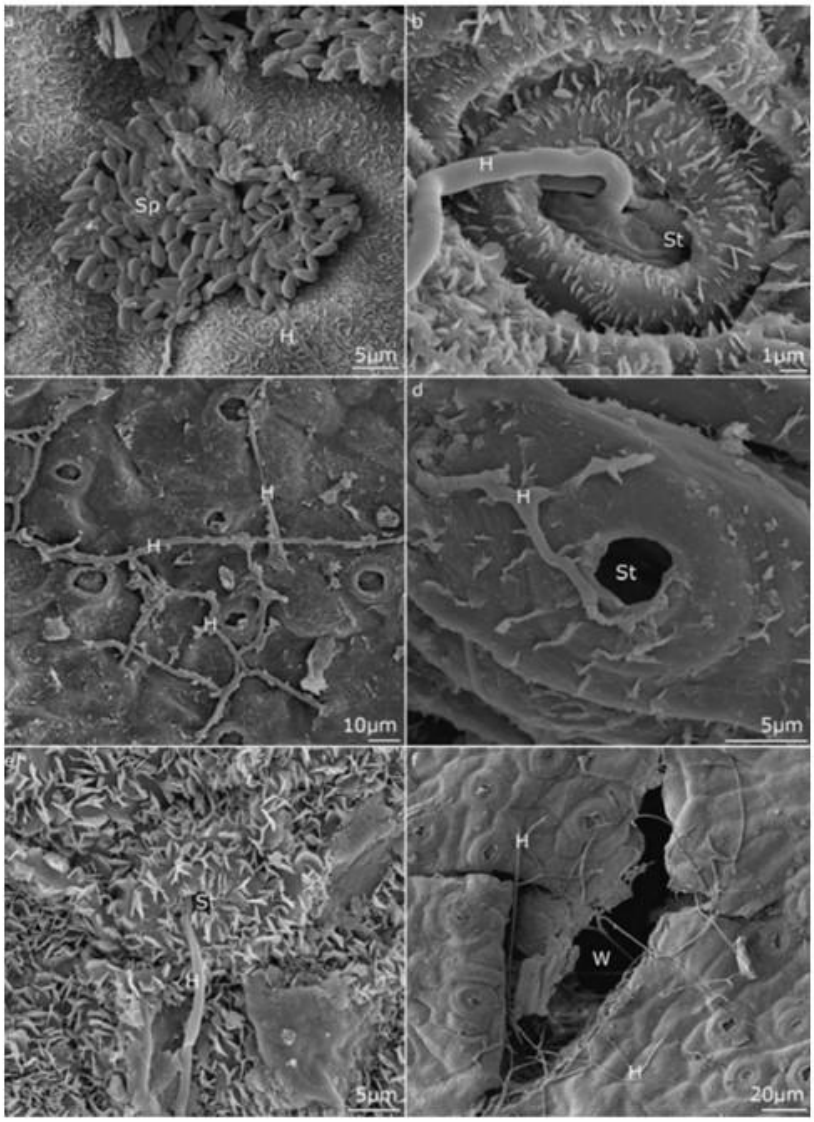


**Figure 7.** Germination of *Q. eucalypti* (a) and *Q. pitereka* (b, c, d) conidia (Sp) and growth of hyphae (H) occurred on host species outside their known range. (a) *Q. eucalypti* produced an accumulation of secondary conidia directly from primary conidia or from short germ tubes on *C. variegata* leaves. *Q. pitereka* hypha (H) penetrating a stomata (St) on juvenile *C. torelliana* leaf (b), *Q. pitereka* conidia germinated producing hyphae that grew on the surface of a *E. grandis* leaf (c), juvenile stem of *C. variegata* (d) and penetration of the stomata (St) of *E. dunnii* (e). *Q. eucalypti* hypha (H) penetrating an artificially created wound (W) on the surface of a *E. grandis* leaf (f) (Pegg *et al.*, 2008).

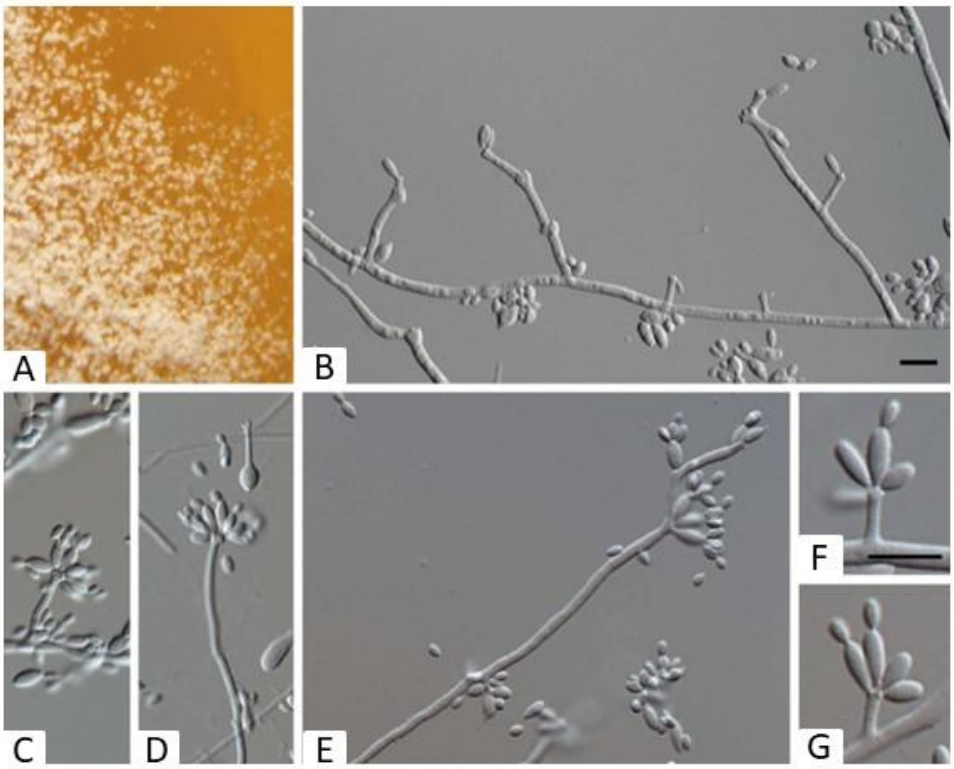


**Figure 8;** *Quambalaria simpsonii*. A. Colony on MEA; B-G. hyphae, conidiogenous cells and conidia. — Scale bars = 10 µm (Cheewangkoon *et al*., 2009).

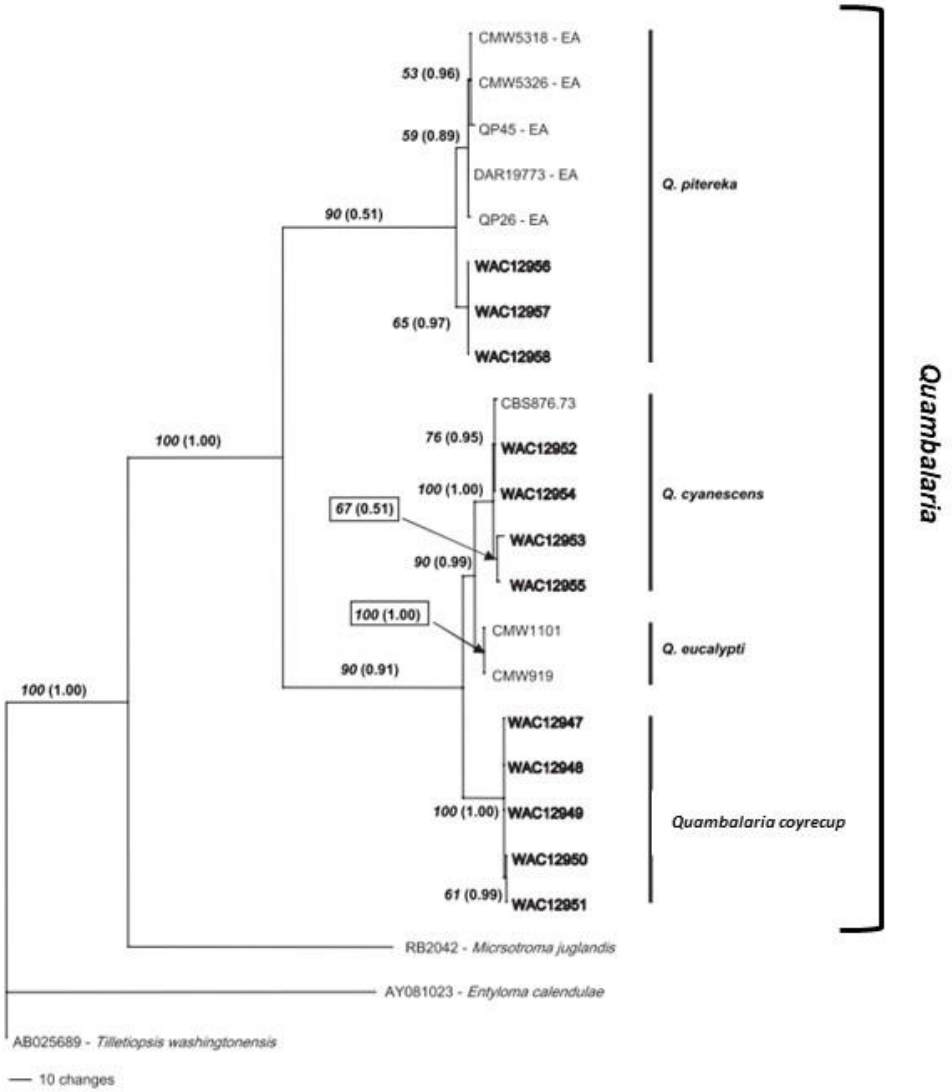


**Figure 9.** Modified phylogenetic tree of combined LSU and ITS sequence data from Paap *et al.* (2008) that shows the distinction between *Q. coyrecup* and the other three species of *Quambalaria* that were known at the time.

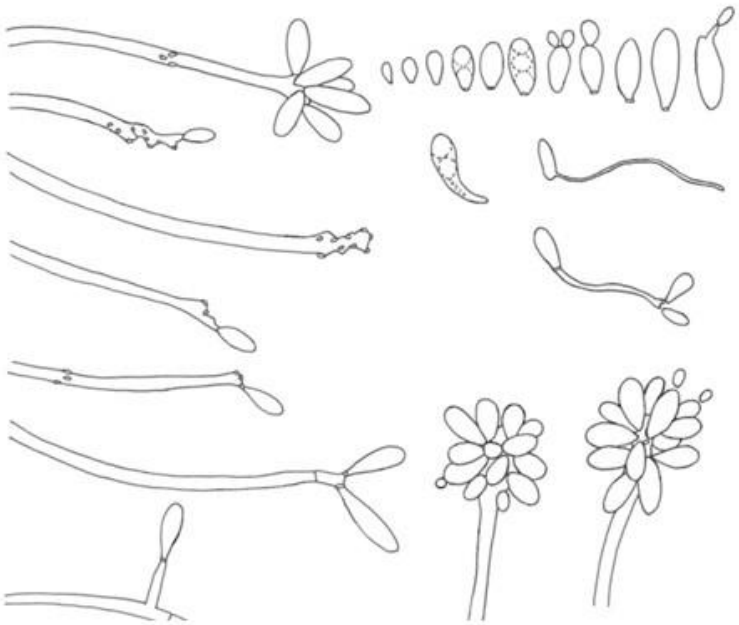

**Figure 10;** *Quambalaria coyrecup* with denticulate conidiogenous cells, primary and secondary conidia, and germinated conidia. Bars [10 mm] (Paap *et al.* 2008)

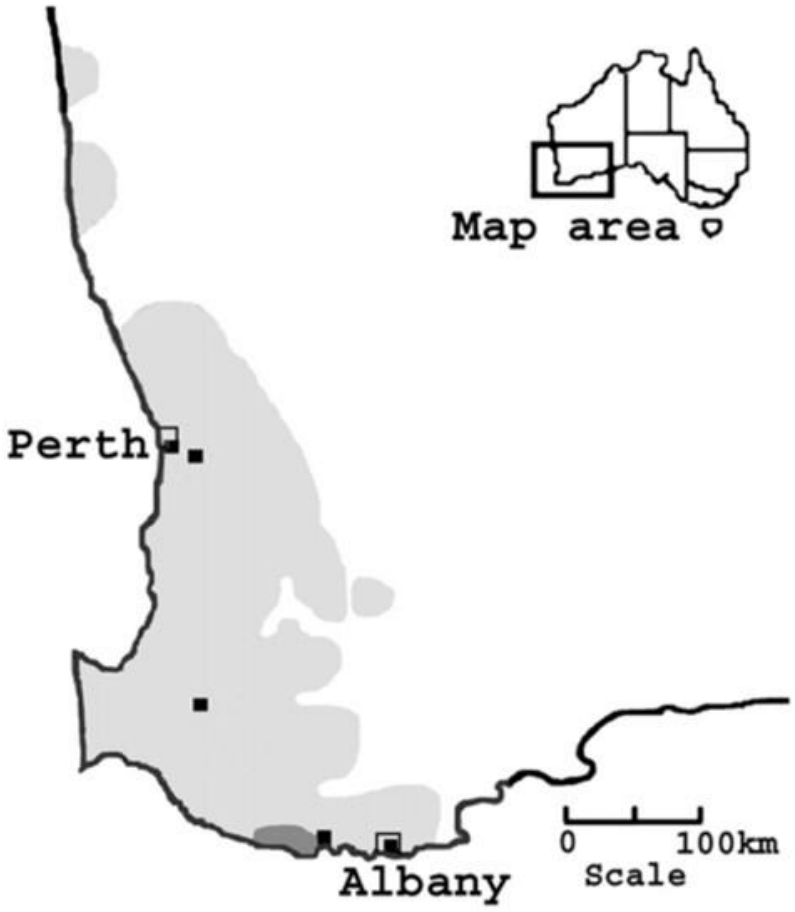


**Figure 11.** Distribution of *Corymbia calophylla* (light grey) and *C. ficifolia* (dark grey) in the southwest of Western Australia. Locations where cankers of *C. calophylla* were first recorded by Cass Smith (1970) (black dots) (Paap *et al.*, 2008).

**Figure 12.** Severe perennial target cankers, resulting from infection with *Q. coyrecup* of *Corymbia calophylla* causing tree decline **(**Paap *et al.* 2008).

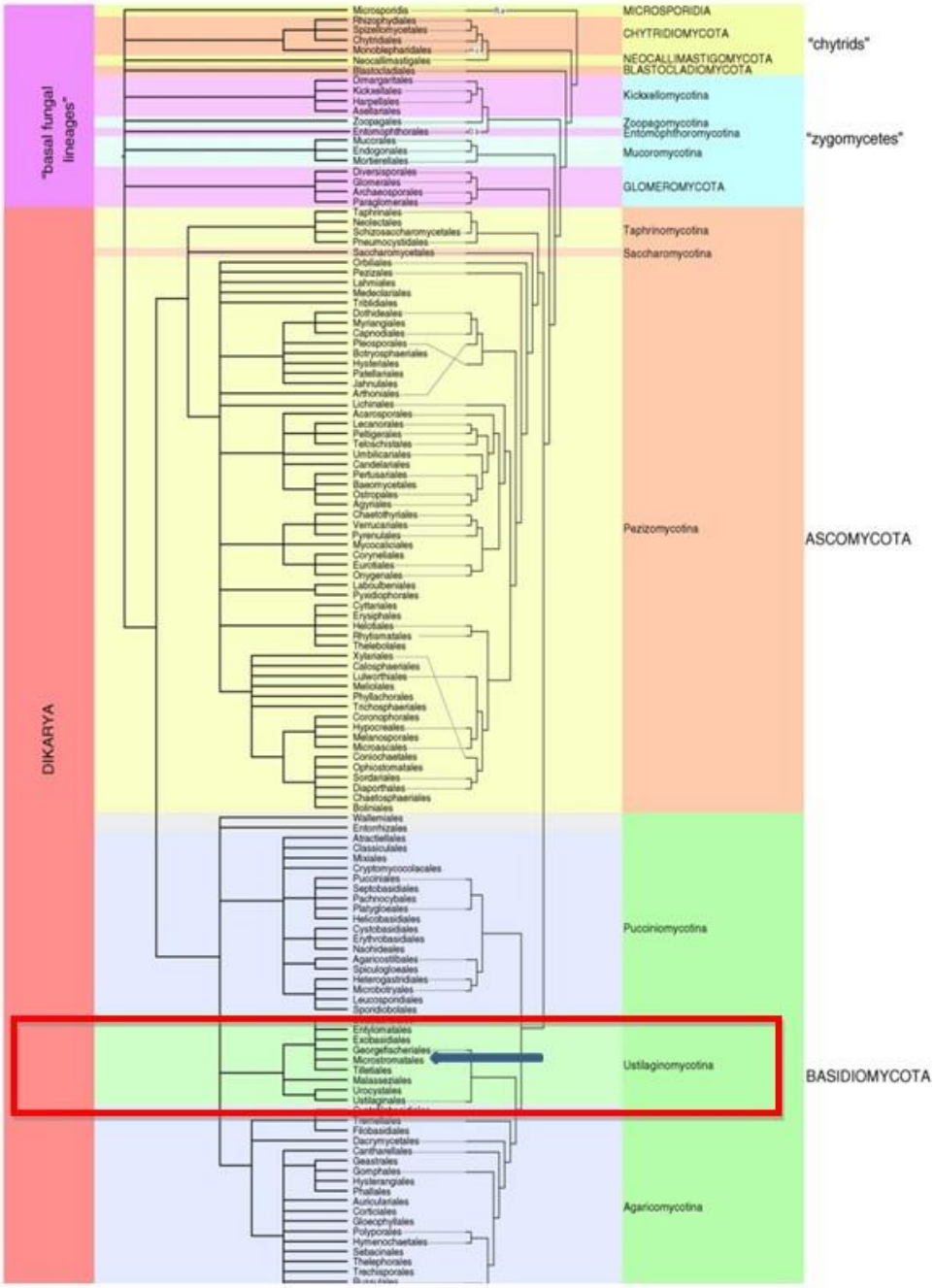


**Figure 13.** Phylogeny and classification of Fungi. The Ustilaginomycotina outlined in red. Microstromatales indicated with the blue arrow. Modified from McLaughlin *et al.* (2009).

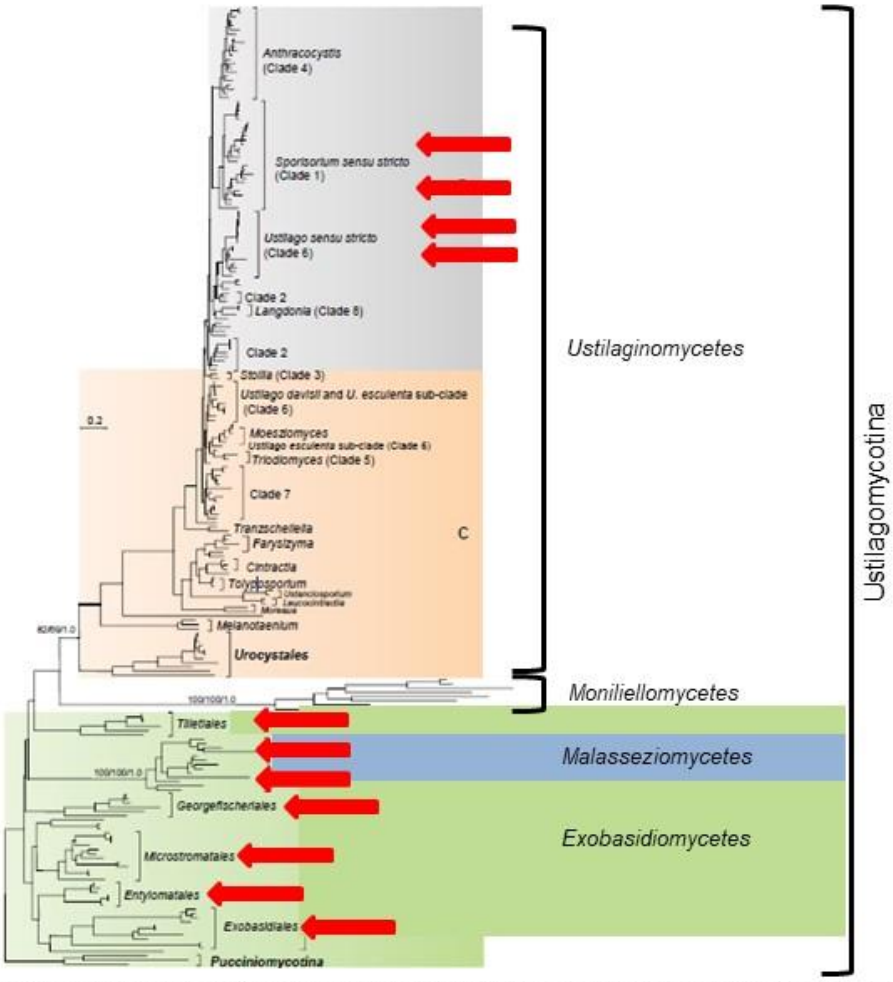


**Figure 14.** Phylogenetic tree constructed using maximum likelihood analysis from the combined sequences of the LSU rRNA D1/D2 domains and ITS1+2 regions (including 5.8S rRNA gene) depicting the phylogenetic relationships of yeast taxa with teleomorphic taxa within *Ustilaginomycotina* modified from Wang *et al.* (2015). This phylogenetic tree shows the position of the species within the *Ustilaginomycotina* which have published whole genome sequences, indicated by the red arrows.

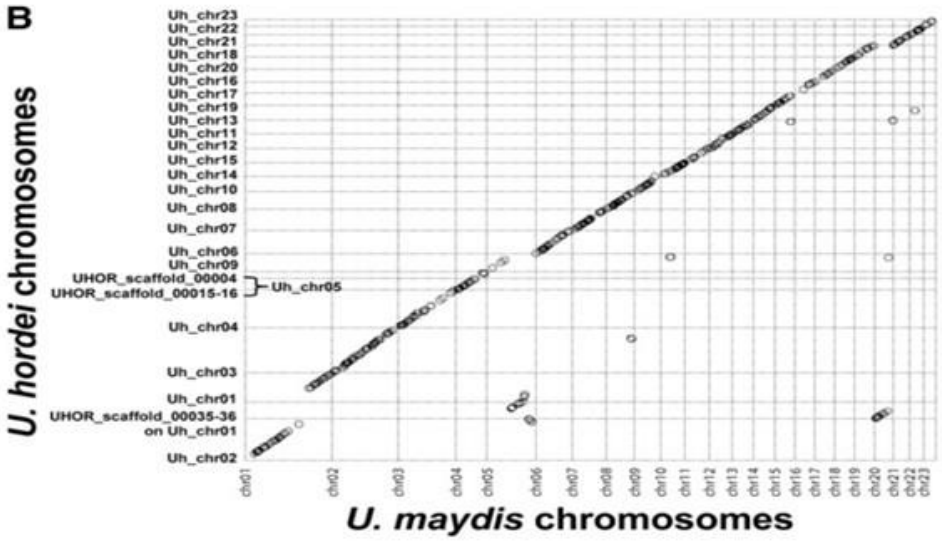


**Figure 15.** Dot plot of the synteny occurring between the chromosomes of *Ustilago maydis* and the assembled supercontigs of *Ustilago hordei*. This result shows that there is a high degree of synteny between these two smuts (Laurie *et al* 2012).

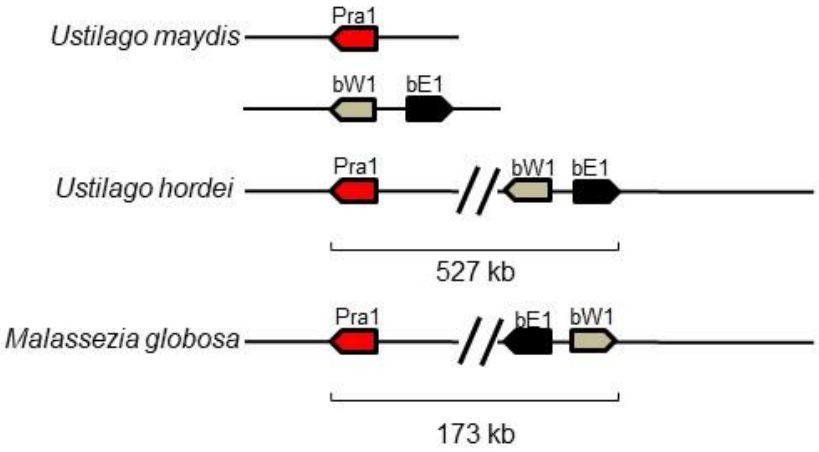


**Figure 16.** Schematic representation of the mating loci of *Ustilago hordei, Ustilago maydis,* and *Malassezia globosa* redrawn from Xu *et al.* (2008). pra1: pheromone receptor 1; bW1: homeodomain transcription factor b west 1; bE1: homeodomain transcription factor b east 1.

# Chapter 2

## *Quambalaria ernaiae* nom. prov. from *Eucalyptus* seed capsules and a first report of *Q. cyanescens* from South Africa

**Abstract**

The genus *Quambalaria* (Microstromatales, Exobasidiomycetes) includes six species. Five of these cause disease of *Eucalyptus* and *Corymbia* spp. and are considered to be native to Australia. *Quambalaria cyanescens*, is most commonly found as an epiphyte on Myrtaceae trees, but is also found in bark beetle galleries of hardwoods, and is known as an opportunistic human pathogen. *Quambalaria pitereka* and *Q. eucalypti* have been accidently introduced to various countries where it causes diseases of *Eucalyptus* spp. The aim of this study was to identify *Quambalaria* isolates obtained from *Eucalyptus* seed capsules and diseased shoots in South Africa, using DNA sequences for the Internal Transcribed Spacer (ITS) regions, as well as the Elongation factor one alpha (EF-1α) gene regions. Phylogenetic analyses of the sequence data revealed that the isolates represent a lineage distinct from all known species of *Quambalaria*, described here as *Q.ernaiae* nom. prov. The isolates from diseased shoots were those of *Q. cyanescens* representing the first report of this species from South Africa.

## Introduction

*The genus Quambalaria* was described to accommodate three fungal species, *Sporothrix pitereka, S. pusilla* and *S. eucalypti,* that cause leaf and shoot blight of commercially propagated *Eucalyptus* and *Corymbia* spp. (Simpson 2000). Based on LSU sequences and the ultrastructure of the septal pores, De Beer *et al.* (2006) showed that *Quambalaria* resides in a distinct family Quambalariaceae, in the basidiomycete order Microstromatales. In addition to the three species previously treated as *Sporothrix*, De Beer *et al.* (2006) showed *S. cyanescens* resides in *Quambalaria*. Subsequent to the study of De Beer *et al*. (2006), three additional species were described in the genus from eucalypts in Australia. These include *Q. coyrecup* (Paap *et al.* 2008), *Q. simpsonii* (Cheewangkoon *et al*. 2009), and *Q. purpurascens* (De Beer 2012). De Beer (2012) showed that DNA sequences of the holotype specimen of *Q. pusilla* was identical to those of the ex-holotype culture of *Q. simpsonii*, and reduced the latter species to synonymy with *Q. pusilla*.

*Quambalaria pitereka* (treated as *Ramularia pitereka*) was the first species in this genus to be described, causing shoot distortion, leaf spots and stem lesions on *Corymbia* trees in Australia (Walker & Bertus 1971). This pathogen was initially thought to be restricted to Australia (Pegg *et al.* 2005, 2008), but has later also been reported on *Corymbia* trees in the Guangdong Province of China where it an accidentally introduced pathogen (Zhou *et al.* 2007).

*Quambalaria eucalypti* was first reported in 1987 as *Sporothrix eucalypti* causing leaf lesions on *Eucalyptus grandis* clones in South Africa (Wingfield *et al.* 1993) and has also been reported to cause shoot dieback on *E. nitens* (Roux *et al.* 2006). Although it was first identified in South Africa, *Q. eucalypti* is thought to have originated in Australia (Wingfield *et al.* 1993, De Beer *at al.* 2006). It has since been reported in Brazil (Alfenas *et al*. 2001), Australia (Pegg *et al.* 2008), Thailand and Laos (De Beer 2012), and Portugal, where it causes stem cankers and dieback on Eucalyptus globulus seedlings (Bragança *et al*. 2015). In Uruguay, it has undergone a host shift to infect Myrtaceae native to that country *(Perez et al*. 2008).

*Quambalaria pusilla* was first identified on *Eucalyptus* camaldulensis leaves in Thailand in 1998 (Braun 1998). Its synonym, *Q. simpsonii* was isolated from *Eucalyptus tintinnans* in the Northern Territory Australia (Cheewangkoon *et al.* 2009). *Q. pusilla* has most recently also been reported from Laos (De Beer 2012).

*Quambalaria coyrecup* is known only from the Northern Territory and Western Australia where it causes a serious disease of *Corymbia calophylla* (marri). It is the only *Quambalaria*

species known to cause stem cankers, with swelling or severe bark splitting that results from infections (Paap *et al.* 2008).

*Quambalaria cyanescens* was first isolated from human skin in the Netherlands and is known to be an opportunistic pathogen of humans (De Hoog & De Vries 1973; Sigler *et al*. 1990). It has also been isolated from bark beetle galleries in hardwood trees, such as oak (Kolarík *et al.* 2006). *Quambalaria cyanescens* is found in a wide range of ecological niches such as air, water, soil and plants (De Beer *et al.* 2006) with reports from the USA, India, Israel and other countries in the Mediterranean (Kolarík *et al.* 2006, De Beer 2012).

*Quambalaria fabacearum* was first reported as an endophyte of *Mimosa tenuiflora* in Brazil (Bezerra *et al.* 2018). Very little is known about this species outside of this report.

*Quambalaria purpurascens* nom. prov. was first reported as an unnamed species and an endophyte of asymptomatic *Leptospermum junipae* in Victoria, Australia (Amin *et al.* 2010). The DNA sequences of this fungus matched those isolated form leaf spots observed on *Angophora costa*. It was subsequently given a provisional name of *Q. purpurascens* nom. prov. (De Beer 2012), and is in the process of being described.

All currently known *Quambalaria* species have been reported from eucalypts in Australia, where they are apparently native (Wingfield *et al.* 2015). *Quambalaria pitereka, Q. eucalypti,* and *Q. pusilla* are the only species to have been found on eucalypts outside of Australia (De Beer 2012). *Q. cyanescens* is the only exception with a wide, almost global distribution both as an opportunistic human pathogen and in bark beetle galleries of non-Myrtaceous hardwoods (De Beer 2012).

In view of South Africa's long history of *Eucalyptus* forestry, it is surprising that only one species of *Quambalaria*, *Q. eucalypti*, has been recorded in this country (**Figure 1**). South Africa has commonly imported seed from Australia for plantation development. This is likely to have been the pathway of introduction of *Q. eucalypti* into the country and it is consistent with the fact that the first record of the pathogen was from a nursery (Wingfield *et al.* 1993; Roux *et al.* 2006).

Recently, seed capsules of *Eucalyptus sideroxylon* growing in a plantation in Pretoria exhibited white fungal growth. The morphology of the fungus superficially resembled that of a *Quambalaria* spp. In addition, *Eucalyptus* shoots from the KwaZulu-Natal region, with symptoms resembling those caused by *Q. eucalypti* were submitted to the FABI Diagnostic Clinic for analyses. The aims of this present study were to identify these fungi based on DNA sequences and morphology.

## Materials and Methods

### Isolates

The apparent *Quambalaria* sp. on the seed capsules of from the *Eucalyptus sideroxylon* was isolated by Dr W. Botha of the Plant Protection Research Institute (PPRI), Roodeplaat, Pretoria (**Figure 2**). These seed capsules were from *Eucalyptus sideroxylon* trees in a stand of trees belonging to the Pretoria Zoo and had been collected by Dr Stephan Neser during seed collection exercises (**Table 1**). The diseased shoots displaying symptoms of Quambalaria Shoot Blight (QSB), originated from *Eucalyptus grandis* nursery hedges in the KwaZulu Natal Province.

Infested plant material was agitated above the surface of 2% Malt Yeast extract Agar (MYA) Petri dishes to release the fungal spores. The Petri dishes were then incubated at 26˚C for 14 days (Roux *et al.* 2006). In addition, white spore masses were lifted from infected leaf tissue using sterile needles from the lesions on the leaves and transferred to 2% Malt Yeast extract Agar (MYA). Once the cultures commenced growth, they were purified by transferring hyphal tips from individual colonies to clean 2% MYA plates using a sterilized needle under a dissection microscope in order to obtain a pure culture. Pure cultures were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Institute (FABI) at the University of Pretoria.

### DNA extraction, PCR and Sequencing

Two isolates from the diseased shoots displaying QSB symptoms and seven isolates from the seed capsules were selected for DNA extraction. Approximately 10 mg of mycelium was scraped from the surface of each MYA plate and transferred to a 1.5 ml Eppendorf tube. 60 µl of PrepMan Ultra™ (Applied Biosystems) was added to each tube. The tubes were then incubated for 2 min at 60 ˚C on a heating block. After incubation the mycelium was macerated thoroughly and further incubated for 7 minutes at 60˚C. The samples were then centrifuged at 13 400 rpm for 6 minutes and the supernatant was transferred to a sterile Eppendorf tube and diluted with 200µl Tris-HCL (10 mM) buffer. Of the diluted DNA solution, 1-6 µl was used as template for PCR reactions and the remainder was stored at -20˚C. This variation in volume was due to the different DNA concentrations obtained during the extraction process. The DNA concentration was determined using Nanodrop Spectrophotometry.

PCR was performed using primers ITS1-F and ITS4 (White *et al.* 1990), QuamEF-R and QuamEF-F (De Beer 2012) in order to amplify the primary fungal barcoding region, Internal Transcribed Spacer (ITS) regions of the ribosomal DNA operon (Schoch *et al.* 2012), as well as the secondary barcode for fungi, the Translation Elongation Factor 1-α gene (EF) (Stielow

*et al.* 2015). The reaction mixtures, with a volume of 25 µl each, consisted of 5 µl of 5X MyTaq™ PCR reaction buffer (comprising of 5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline), 1 unit MyTaq™ DNA polymerase, 0.5 µl of each primer (10 mM), 13.85µl-17.85 µl SABAX Pour Water (Adcock Ingram) and 1-6 µl of extracted DNA. Amplifications were performed in an Viriti thermocycler (Applied Biosystems) using the following conditions: an initial denaturation step at 95 ˚C for 3 min, followed by 35-37 cycles of 94˚C for 30 s, 52-55 ˚C annealing for 30 s, 72 ˚C extension for 60 s and a final extension step at 72 ˚C for 8 min. Quality of PCR products was then assessed on 1 % TAE gel using electrophoresis.

PCR products were cleaned using the Exosap protocol (Thermo Fisher Scientific). 8µl of Exosap was added to the PCR product and the reaction was performed in an Viriti thermocycler using the following conditions: a 15 minute step at 37 ˚C followed by a 15 minute step at 80 ˚C. The amplified products were sequenced with the Big Dye® Terminator 3.1 cycle sequencing premix kit (Applied Biosystems) employing the forward and reverse primers used for PCR and were analysed on an ABI PRISIM® 3100 Genetic Analyzer (Applied Biosystems). Consensus sequences were constructed with ContigExpress, a component of Vector NTI Advance 11 (Invitrogen). These sequences were subjected to BLAST searches in order to confirm identity of the isolates at a genus level.

**Phylogenetic Analyses**

Phylogenetic analyses were performed on the ITS and EF sequences data separately. Data sets for phylogenetic analyses were compiled in MEGA 7 (Tamura *et al.* 2013). Sequence data obtained were used along with reference sequences obtained from NCBI GenBank, with sequences for *Microstroma album* and *Rhodotorula bacarum* used as outgroups for the ITS analyses, and *Microstroma* as the outgroup during EF analyses.

ITS and EF sequences were aligned using the online version of MAFFT v. 7 (Katoh 2013). Maximum likelihood (ML) analyses were done using raxmlGUI (Silvestro & Michalak 2012) with the GTR+G+I substitution model selected. Ten parallel runs with four threads and 1000 bootstrap replications were conducted. Bayesian inference (BI) analyses were performed using MrBayes v. 3.2.2 (Ronquist *et al.* 2012) employing the GTR+G+I substitution model. Ten parallel runs, each with four chains, were conducted. Trees were sampled at every 100th generation for 5 M generations. After sampling, 25 % of trees were discarded as a burn-in phase and posterior probabilities were calculated from all the remaining trees.

**Growth Study**

Optimum growth temperature was determined by culturing isolates of taxon 1 on MEA for 10 days in the dark at 5-40˚C in 5˚C intervals. An agar disc (7mm diameter) obtained from the leading edge of a growing colony was placed in the centre of each plate. Two isolates were used, CMW 49449 and CMW 50252, with three replicates per temperature and three replicaes per isolate. Colony diameter was measured after 10 days by taking two perpendicular measurements of each colony and calculating the mean growth rate in mm (Wingfield *et al.* 1993)

## Results

### Isolates

In total, four isolates were obtained study from *E. sideroxylon* seed capsules and two isolates were obtained from diseased shoots, all of which resembled *Quambalaria* spp.

### Phylogenetic Analyses

Analyses of the Maximum Likelihood and Bayesian Inference trees produced from the sequence data showed seven distinct lineages (**Figures 3 and 4**). Six of these lineages represented the known species of *Quambalaria*. The isolates obtained from the seed capsules formed a well-supported lineage, labelled Taxon 1 (**Figures 3 and 4**) in both ITS and EF1-α trees. This lineage is distinct from all known species in *Quambalaria*.

Isolates obtained from the diseased shoots from KwaZulu-Natal resided in the first and third lineages in both the ITS and EF1-α trees (**Figures 3 and 4**). The first lineage included the ex-type of *Q. cyanescens* while the third lineage included the ex-type of *Q. eucalypti* (**Figures 3 and 4**).

The ITS sequence representing the ex-type of *Q. fabacearum* from the study of Bezerra *et al.* (2018), grouped within the clade representing *Q. cyanescens*. No EF sequence is available for *Q. fabacearum*.

### Taxonomy

DNA sequence analyses revealed that the isolates of *Quambalaria* from *Eucalyptus* seed capsules reside in a distinct lineage representing an undescribed species. This taxon is therefore named and described as a novel species below.

### Taxon 1

***Quambalaria ernaiae*** S.W. Morgan, Z.W. de Beer, S. Marincowitz **nom. prov.**

**MB pending (Figure 3, 4 and 5).**

*Etymology*: named for Erna MacLean, the grandmother of the first author, who passed away during the course of this study.

*Conidiophores* micronematous, semi-macronematous.

*Conidiogenous cells* hyaline, single or in cluster, terminal or lateral, occasionally born directly on vegetative hyphae, cylindrical, 9.5–27 µm long, 1.5–3 µm wide with little change from base to apex, smooth, fertile upper part often showing zig-zag growth, with denticles (1–3 µm wide) (**Figure 5**).

*Conidia* hyaline, ellipsoidal, 3.5–7 × 3.5–7.5 µm (average 5.5 × 5.5 µm), aseptate, smooth.

*Colonies* on MYA showing circular growth, smooth edge, sponge-like texture, showing darkening of media around the colony. Optimum temperature for growth temperature 25 °C, growing to a diameter of 15.75 mm after 10 days, with minimum growth at 15 °C and maximum growth at 30 °C, in the dark.

*Type material*. SOUTH AFRICA, Gauteng, Pretoria, Soutpansberg road, from seed capsules of *Eucalyptus sideroxylon* collected by S. Neser and isolated by W. Botha in 2016 (Holotype PREM 61939), culture ex-holotype CBS 142946 = CMW 49449.

*Other specimens examined*. SOUTH AFRICA, Gauteng, Pretoria, Soutpansberg road, from seed capsules of *Eucalyptus sideroxylon* collected by S. Neser and isolated by W. Botha in 2016 (Holotype PREM 61939), CMW 50252.

## Discussion

Phylogenetic analyses of sequences of two gene regions generated in this study revealed the presence of a novel *Quambalaria* species on *Eucalyptus* seed capsules in South Africa, provisionally described here as *Q. ernaiae.* In addition, the one isolate obtained from the diseased shoots was shown to be *Q. cyanescens* and this represents a first report of this species from South Africa. Consequently, together with *Q. eucalypti*, three of the seven species in this genus are now known to occur on *Eucalyptus* in South Africa.

The new species identified through phylogenetic analyses in this study was isolated from seed capsules of various *Eucalyptus* species. Most *Quambalaria* species are associated with leaf or shoot infections of *Eucalyptus* or *Corymbia* trees (Paap *et al.* 2008; Pegg *et al.* 2009), and this is also true for *Q. eucalypti*, the only species previously known to occur in South Africa (Wingfield *et al.* 1993; Wingfield *et al.* 2008).

*Quambalaria pitereka* is the only species that has been reported from *Eucalyptus* flowers and seed capsules (Marbus *et al.* 2011). A '*Ramularia* sp.' was reported to infect seed capsules and cause seed loss in three species, *Eucalyptus crebra, E. drepanophylla* (*E. crebra*) and *E. melanophloia*, along with a possible hybrid of *E crebra x E. melanophloia* in the 1970s in south-east Queensland, Australia (Drake 1981, 1974). The infected capsules opened early and the contents were covered in a white mass of spores and mycelium. These *Eucalyptus* species have been reported to be hosts for *Quambalaria (Ramularia) pitereka*. Therefore, Brown (2000), suggested that the '*Ramularia* sp.*'* might have been *Q. pitereka.*

*Quambalaria cyanescens* is the most widely distributed of the *Quambalaria* fungi, and is the only species not known as a pathogen of eucalypts. The first report of *Q. cyanescens* on eucalypts was from New South Wales (Australia) in 2006 where it was isolated from *Corymbia callophylla* (Paap 2006). More recently, this fungus was found on *C. calophylla* and *C. ficifolia* in Western Australia, where it sporulates on shoots and newly emerging leaves together with *Q. pitereka* (Pegg *et al.* 2008). Paap *et al.* (2008) showed that *Q. cyanescens* also occurs in stem cankers alongside *Q. coyrecup*. The present *Q. cyanescens* was found within *Q. eucalypti* lesions on *Eucalyptus grandis*. This raises the question as to whether there is an association between these species of fungi within these lesions and what role *Q. cyanescens* might play in the pathogenicity of *Q. eucalypti*.

It is interesting to note that the ITS sequence of the recently described *Q. fabacearum* (Bezerra *et al.* 2018), grouped among *Q. cyanescens* sequences in our analyses. in the analyses of Bezerra *et al.* (2018) only two *Q. cyanescens* sequences were included. It has been shown that the latter species is genetically highly variable (De Beer 2012). To clarify the taxonomic position of *Q. fabacearum*, we suggest an EF sequences should be generated.

It is a well-known that fungi infecting trees in commercially managed plantations in non-native environments can behave differently and be more pathogenic than in their native environments (Wingfield *et al.* 2015). Nothing is known regarding the potential importance of the newly discovered *Quambalaria* spp. in South Africa. Pathogenicity trials should be conducted to consider this question.

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## Figures

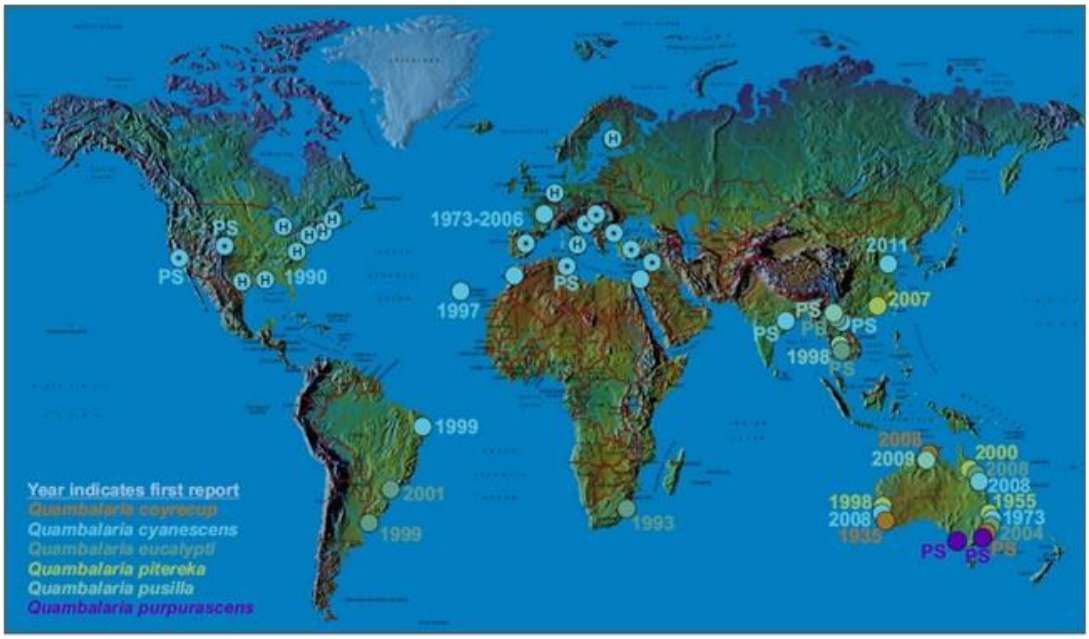



**Figure 1**. Global distribution of *Quambalaria*. The year of the first report of each species from a country. H = isolated from human tissue; * associated with insect
(Unpublished data)

**Figure 2.** Seed capsules of *Eucalyptus sideroxylon* showing white mycelium and spore masses resulting from *Quambalaria* infections (Photos: S. Neser).

**Figure 3.** Phylogram resulting from ML analyses and BI analyses, using RaxML and MrBayes, of ITS sequences, including sequences generated in this study (bold type) together with selected reference sequences of all species of *Quambalaria*. T indicates ex-type isolates. Support values at branches resulted from 1000 bootstraps and only values above 65% and 0.8 are indicated.

ITS

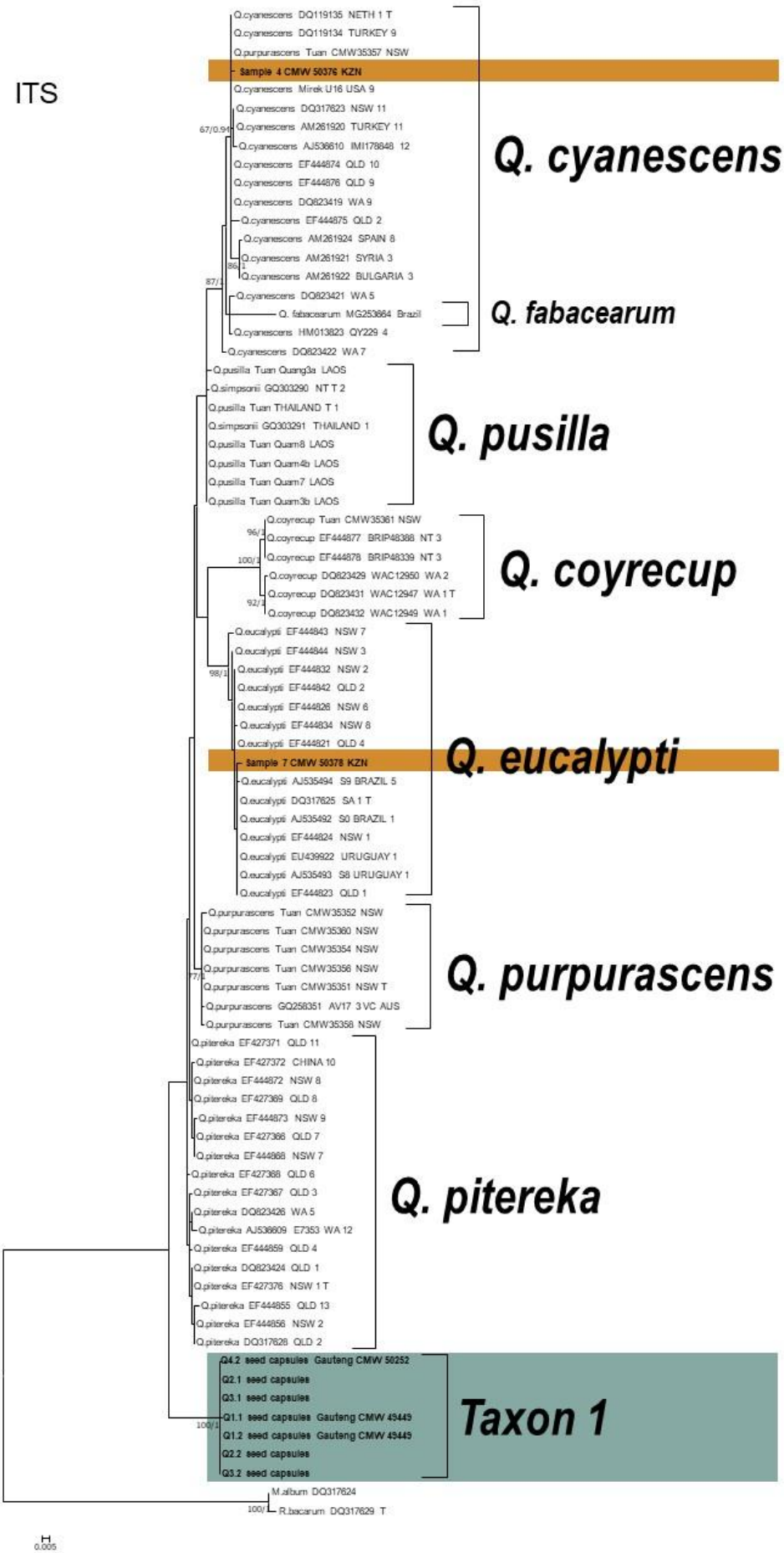
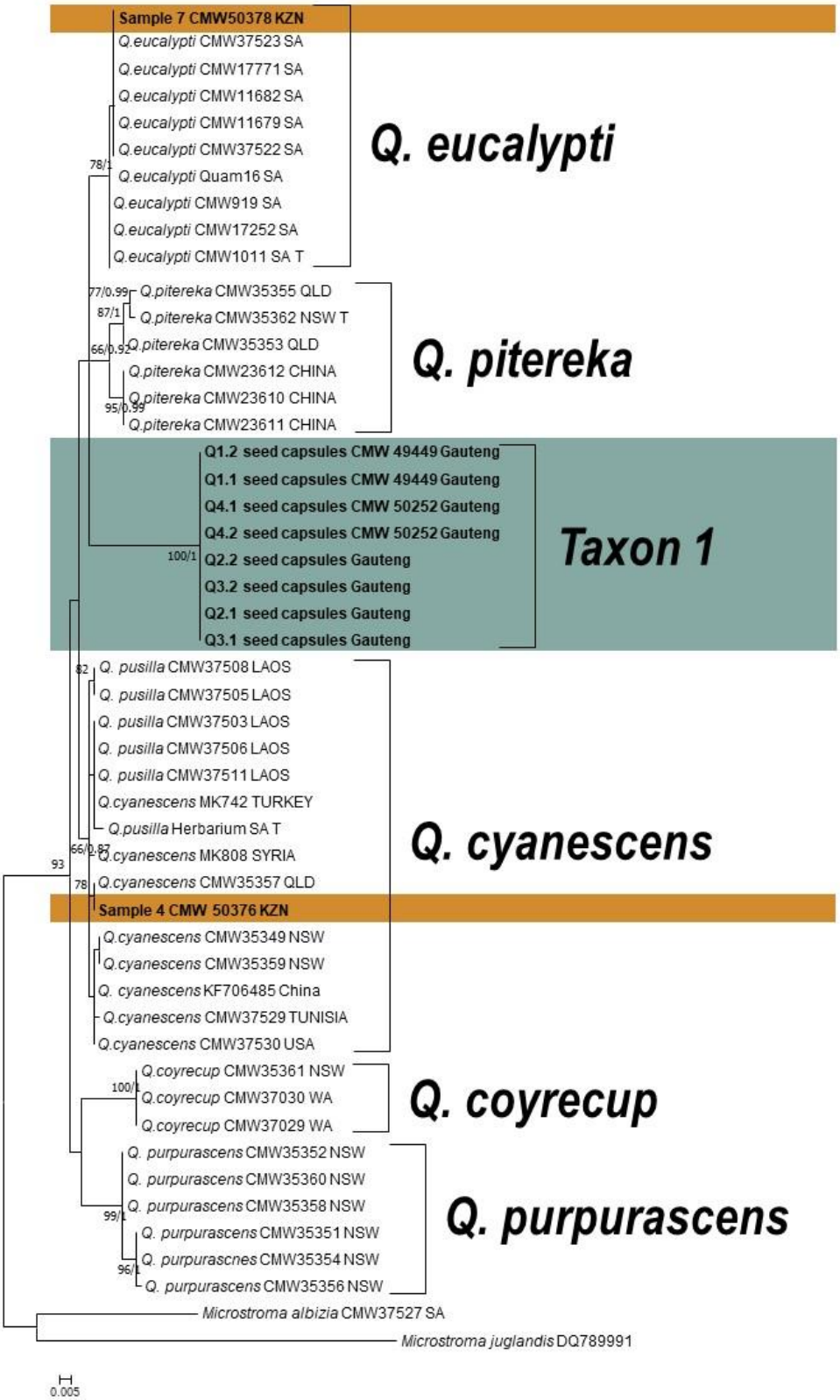


**Figure 4.** Phylogram resulting from ML analyses and BI analyses, using RaxML and MrBayes, of EF sequences, including sequences generated in this study (bold type) together with selected reference sequences of all species of *Quambalaria*. T indicates ex-type isolates. Support values at branches resulted from 1000 bootstraps and only values above 65% and 0.8 are indicated.

EF



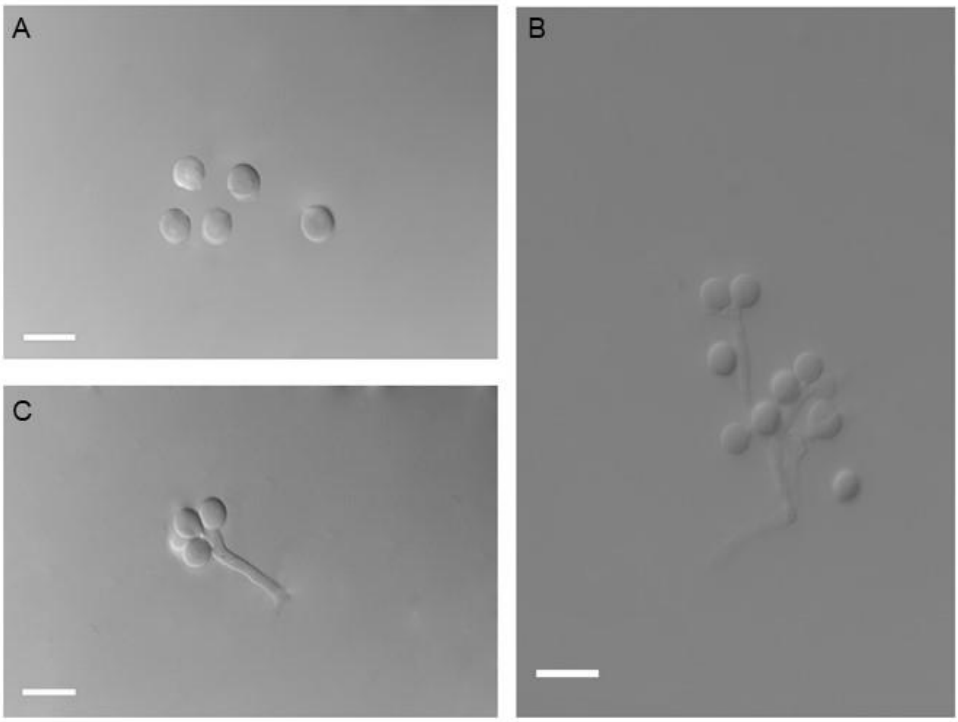



**Figure 6.** Morphological characteristics of *Quambalaria ernaiae* nom. prov. **A.** Conidia. **B, C.** Denticulate conidiogenous cell with conidia. All scale bars 10µm.

# Chapter 3

## Draft genome sequences of *Quambalaria eucalypti*

**Draft genome sequence of *Quambalaria eucalypti***

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## Introduction

The genus *Quambalaria* (Quambalariaceae, Microstromatales, Exobasidiomycetes, Ustilaginomycotina) includes mainly leaf and shoot pathogens of trees belonging to the Myrtaceae (De Beer *et al.* 2006; Pegg *et al.* 2009). The exceptions to this are *Q. coyrecup* which is a canker pathogen of marri (*Corymbia calophylla*) in Western Australia (Paap *et al.* 2008), and *Q. cyanescens* which is an opportunistic pathogen of primarily immunocompromised or debilitated humans (Kuan *et al.* 2015). The latter species is also frequently isolated from galleries of bark beetles infesting hardwoods, although its ecological role in these ecosystems remains enigmatic (Kolarík 2006). Of the leaf and shoot pathogens in the genus, *Q. pitereka* and *Q. eucalypti* are most important. *Quambalaria pitereka* affects *Corymbia* species in Australia (Pegg *et al.* 2009) and China (Zhou *et al.* 2008), while *Q. eucalypti* cause disease on *Eucalyptus* spp. in South Africa (Wingfield 1993), Brazil (Alfenas *et al.* 2001), Uruguay (Perez *et al.* 2008), Australia (Pegg *et al*. 2008) and Portugal (Bragança *et al.* 2015).

Research on species of the Quambalariaceae has mostly focussed on their classification and taxonomy (De Beer *et al.* 2006; Paap *et al.* 2008; Kijpornyongpan 2017), as well as their pathogenicity and impact on tree health (Pegg *et al.* 2009, 2011). However, little is understood about the life cycle and general biology of these fungi that are related to the smut fungi and human pathogenic members of the Malasseziales (De Beer *et al.* 2006; Wang *et al.* 2015). For example, it is not yet known whether the Quambalariaceae has the ability to reproduce sexually, and if so, what the mating system of these fungi encompasses.

Recently the first genome-based studies started exploring pathogenicity factors in the Ustilaginomycotina, and although two species in the Microstromatales were included in the comparative analyses, no representative of the Quambalariaceae was incorporated (Kijpornyongpan *et al.* 2018). Whole genome sequences have also been shown to be extremely valuable to study mating systems in smut fungi (Que *et al.* 2014), the Malasseziales (Xu *et al.* 2007), and the more distantly related Polyporales (James *et al.* 2013).

The aim of this study was thus to produce a draft genome sequence of *Q. eucalypti*. This genome sequence will allow for the exploration and comparative analyses of genes involved in pathogenicity and mating for this pathogen. Here we report the draft genome sequence of isolate CMW1101, an isolate representing the holotype (PREM 51089) of *Q. eucalypti*.

**Sequenced Strain**

South Africa: *Kwa-Zulu Natal*, Kwambonambi, *Eucalyptus grandis* clone TAG12, May 1987, M.J. Wingfield (CMW1101=CBS118844 ex-holotype isolate; PREM 51089 = holotype).

**Nucleotide accession number**

The draft genome sequence of *Quambalaria eucalypti* has been deposited at GenBank under the accession number RRYC00000000. The version presented here is RRYC01000000.

**Materials and Methods**

Genomic DNA was extracted from cultures grown on Malt Yeast Agar (2 % Malt extract, 0.5 % yeast extract; 2% agar, Biolab, Midrand, South Africa) using the method described by Duong *et al.* (2013). The genomic DNA was sent to Macrogen Inc. (South Korea), where one pair-end library with 500 bp insert size was prepared and sequenced on Illumia Hiseq 2500 to get 250 bp pair-end reads, aiming for 100 X coverage.

The raw sequencing reads were imported into CLC Genomics Workbench v. 7.5.1 (CLCBio. Aarhus), and default settings were used to both trim the reads for quality and to produce a *de novo* genome assembly using the trimmed reads. The completeness of the assembly was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO v. 1.1b1) tool using the Basidiomycota dataset (Simao *et al.* 2015). The number of protein coding genes was determined using Augustus v. 3.3.2 (Stanke *et al.* 2008) using pre-optimised species models for *Ustilago maydis*.

## Results and Discussion

The paired end sequencing yielded just over of 31 million reads. Assembly of the trimmed reads resulted in 966 contigs, with the largest contig being 225 583 bp, the smallest contig being 449 bp, with an average contig size of 24 384 bp and the N50 value was 62 600 bp. The genome size is estimated at around 23.5 Mb, estimated through the sum of the contig sizes, with a GC content of 60 %. This estimated size is in the larger size range of the Exobasidiomycetes, which typically range from 17 Mb to 19 Mb with the exception of *Tilletia caries* with a genome size of 29.5 Mb (Konishi 2013; Saika *et al.* 2014; Toome *et al.* 2014; Wang *et al.* 2015a; Kijpornyongpan *et al.* 2018). BUSCO analysis indicated an assembly completeness of 84.5 %. The assembly contained 1128 complete BUSCOs (1093 complete single- copy BUSCOs, 35 complete and duplicated BUSCOs), 129 fragmented BUSCOs and 78 missing BUSCOs out of a total 1335 BUSCO groups searched. AUGUSTUS predicted 7241 putative protein coding regions. The availability of the *Q. eucalypti* genome will enable the inclusion of this species as representative for the family Quambalariaceae in comparative studies with other members of the class Exobasidiomycetes. Such studies could focus on topics like the factors involved in pathogenicity, mating, evolution and more.

## Acknowledgements

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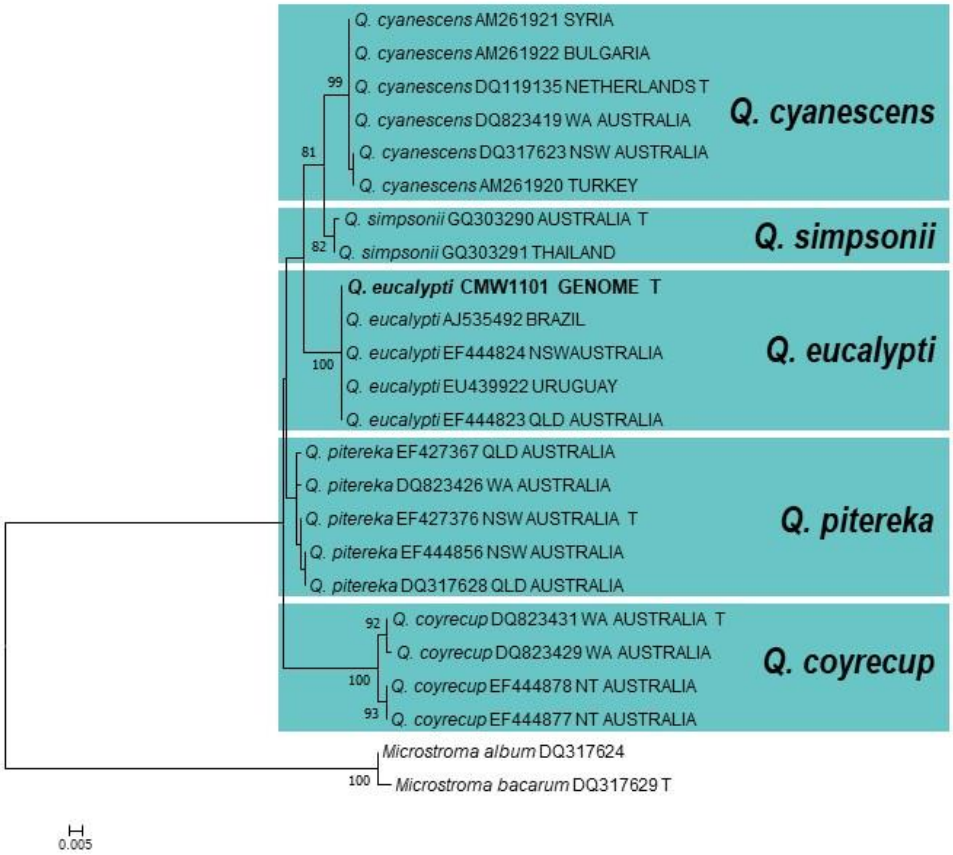


**Figure 1**. Phylogram resulting from a ML analyses using RaxML, based on ITS sequences of selected reference sequences representing all species of *Quambalaria*. The isolate from which the genomic DNA was extracted is indicated in bold type. T indicates ex-type isolates. NT = Northern Territory, NSW = New South Wales, WA = Western Australia, QLD = Queensland. Support values at branches resulted from 1000 bootstraps and only values above 75% are indicated.

# Chapter 4

## Insights into the mating genes of *Quambalaria eucalypti* and other Exobasidiomycetes

**Abstract**

*Quambalaria eucalypti* (Exobasidiomycetes, Microstromatales, Quambalariaceae) is a basidiomycete fungal pathogen of *Eucalyptus* in South Africa, Australia, South America, Europe and China. No sexual state is known for this species neither for any of the species in the Exobasidiomycetes. In this study, the genomes of *Q. eucalypti* and other species of Exobasidiomycetes were explored for the presence of mating type loci. Pheromone receptor genes and the homeodomain binding factor genes were identified in *Q. eucalypti* as well as the majority of the species studied. The pheromone gene was identified in several species including *Exobasidium vaccinii.* The first evidence for possible sexual reproduction in *Quambalaria eucalypti* and several other species of Exobasidiomycetes, by confirming the presence of mating type genes and seemingly intact MAT loci in these fungi.

## Introduction

*Quambalaria eucalypti* is a basidiomycete fungus (Exobasidiomycetes, Microstromatales, Quambalariaceae), that causes distortion and die-back of young leaves on offshoots of young *Eucalyptus* plants (Wingfield *et al.* 1993; De Beer *et al*. 2006). Damage caused by this pathogen is especially prevalent in forest nurseries and young plantations where it has been reported from South Africa, Portugal, Brazil, Uruguay, Laos and Thailand (Wingfield *et al.* 1993; Roux *et al.* 2006; Pegg *et al.* 2008; De Beer 2012; Bragança *et al.* 2015). *Q. eucalypti* also infects numerous *Eucalyptus* spp. in many parts of Australia (Pegg *et al*., 2008). Although the pathogen is generally not considered a major threat to forests and plantations in Australia, it can be important in commercially managed plantations (Pegg *et al*. 2008; Wingfield *et al*. 2008; Mafia *et al.* 2009).

It has been suggested that Australia is the centre of origin of *Q. eucalypti*, as is the case for all other known *Quambalaria* species (De Beer 2012). Although no comprehensive population genetics studies have been have been undertaken for this fungus, DNA sequences for two gene regions revealed that Australian isolates are genetically more variable than those from other countries (Pegg *et al*. 2008; Wingfield *et al*. 2008, De Beer 2012; Bragança *et al*. 2015 ). It is known that multiple introductions of foreign pathogens to new countries can increase the possibility of genetic recombination that might result in increased virulence or adaptations that might facilitate host shifts (Gladieux *et al*. 2014; Wingfield *et al*. 2017). The chances for genetic recombination increases substantially when sexual reproduction takes place, which typically requires the presence of different mating types in a population (Gladieux *et al*. 2014). At present no predictions can be made regarding the reproductive potential for *Q. eucalypti*, as nothing is known about the mating system or mating genes of this or any other *Quambalaria* species, neither for the other species in the Microstromatales.

The Microstromatales is one of seven orders in the Exobasidiomycetes (Wang *et al.* 2015; **Fig. 1**). This class includes a variety of species, several of which are plant pathogens. Of these, the best known examples are members of the genera *Exobasidium* (Exobasidiales) and *Tilletia* (Tilletiales). *Exobasidium* spp. infect of the Ericaceae family, which includes species of blueberry, cranberry and common heather (Burt 1915; Nannfeldt 1981; Nagao *et al*. 2003; Brewer *et al*. 2014). Species of *Tilletia*, commonly known as bunt fungi, are considered economically important pathogens as they infect cereal crops such as wheat, rice and rye (Mitra 1931; Castlebury & Carris 1999; Biswas 2003; Carris *et al.* 2006). Although the life cycle of *Tilletia* has been studied (Durán & Cromarty 1977; Goates &

Hoffman 1987), little is known about the molecular mechanisms governing sexual reproduction in these species and the rest of the Exobasidiomycetes.

More distantly related to the Exobasidiomycetes, but still part of the Ustilaginomycotina, are the Ustilaginomycetes and the Malasseziomycetes (Wang *et al*. 2014; **Figure 1**). The Ustilaginomycetes accommodates several well-known plant pathogenic smut fungi (Bauer *et al*. 2001), including the model fungus, *Ustilago maydis*. The life cycle, mating system and mating genes of this species have been studied in depth, especially because pathogenicity is so intricately coupled to sexual development in this and other smuts (Martínez-Espinoza 2002; Brefort *et al.* 2009; Liang *et al.* 2019; Olicón-Hernández *et al.* 2019). The Malasseziomycetes includes species that are most commonly associated with human and animal skin (Boekhout *et al*. 2010; Wang *et al*. 2014). This group of yeasts has thus also attained substantial research attention, including studies that resolved their mating genes (Dawson 2007; Xu *et al.* 2007; Coelho *et al.* 2013; Gioti *et al.* 2013; Wu *et al.* 2015).

Sexual reproduction in heterothallic basidiomycete fungi is regulated by two mating- type loci (MAT), the pheromone/pheromone receptor (P/R) locus and the Homeodomain (HD) locus (James *et al*. 2013). In the literature, a number of different names have been applied to these two loci. The P/R locus is referred to as the *A* locus in the Tremellomycetes (Kwon-Chung 1976), the *a* locus in the Ustilaginomycetes (Puhalla 1970) and the *B* locus in the Agaricomycetes (Whitehouse 1949). The HD locus is referred to as the *B* locus in the Tremellomycetes (Kwon-Chung 1976), the *b* locus in the Ustilaginomycetes (Puhalla 1970) and the A locus in the Agaricomycetes (Whitehouse 1949). The naming system for the Ustilaginomycetes has also been used to describe the mating loci of the Malasseziomycetes ( Xu *et al*. 2007; Gioti *et al*. 2013). For the purpose of this chapter, we shall apply the names used for mating loci in these two classes also to the Exobasidiomycetes. Thus, the *a* locus refers to the pheromone/pheromone receptor (P/R) locus, while the *b* locus refers to the Homeodomain (HD) locus.

The mating systems of both the Ustilaginomycetes and the Malasseziomycetes fit broadly with the general models for mating in basidiomycete fungi that include both bipolar and tetrapolar mating systems (Raudaskoski & Kothe 2010). Bipolar mating refers to systems where the *a* and *b* mating loci are linked on the same chromosome as a single heritable locus (Bakkeren *et al.* 2008). Species that possess this system include *Ustilago hordei* (Lee *et al.* 1999; Bakkeren *et al*. 2006) and *Sporisorium scitamineum* (Yoder *et al*. 1986; Yan *et al*. 2016). *Malassezia sympodialis* and *M. globosa* also possess a bipolar mating system with the *a* and *b* mating regions being physically linked in both species, at 140kb and 170kb apart

respectively (Xu *et al.* 2007; Gioti *et al*. 2013). In a typical bipolar mating system, both the *a* and *b* regions are biallelic. However, *M. sympodialis* displayed biallelism at the *a* region and triallelism at the *b* region. This type of mating system is referred to as pseudobipolar (Xu *et al.* 2007; Gioti *et al*. 2013). *Tilletia caries* and *T. controversa* have been shown to possibly possess bipolar mating systems (Holton & Kendrick 1957; Hoffmann & Kendrick 1969; Trail & Mills1990). However, the mating genes of neither of these species have been characterised.

In tetrapolar mating systems the *a* and *b* mating regions are not physically linked, occur on different chromosomes and therefore segregate independently from each other during meiosis (Bakkeren *et al.* 1992, 2006; Bakkeren & Kronstad 1993, 1994). Both *Ustilago maydis* and *Sporisorium reilianum* possess tetrapolar mating systems, with *U. maydis* containing two alleles for the *a* region and more than 25 for the *b* region (Puhalla 1970; Silva 1972). *S. reilianum* contains three alleles for the *a* region and about five alleles for the *b* region (Schirawski *et al*. 2005).

The genes encoded for by the two mating regions have been elucidated for the Ustilaginomycetes and the Malasseziomycetes (Xu *et al.* 2007; Schirawski *et al*. 2005, 2010; Laurie *et al*. 2012; Gioti *et al*. 2013; Que *et al*. 2014;). The *a* locus codes for a pheromone and pheromone receptor pair that are responsible for compatible hyphal fusion and cell recognition. The fungal spore recognises a pheromone of the opposite mating type and this leads to the activation of a MAPK (mitogen-activated protein kinase). This MAPK regulates the activity of Prf1, an important transcription factor involved in the activation of the pheromone-responsive (*b* locus) genes (Urban *et al.* 1996; Schirawski *et al.* 2005; Bakkeren *et al.* 2008; Brefort *et al.* 2009; Kües 2011).

The *b* locus encodes a pair of homeodomain transcription factors. The two transcription factors form a heterodimeric complex, which only occurs when the proteins are derived from different alleles, which occurs with two partners of opposite mating types (Kämper *et al*. 1995). This heterodimer regulates the expression of genes involved in maintaining dikaryotic hyphal growth in the host and other pathogenicity factors (Urban *et al.* 1996; Schirawski *et al.* 2005; Bakkeren *et al.* 2008; Brefort *et al.* 2009; Kües 2011).

We recently generated the genome sequence for an isolate of *Quambalaria eucalypti* (Wingfield *et al*. 2018b), specifically to explore the mating type genes to gain a better understanding of whether sexual reproduction indeed takes place in this species. The availability of unpublished mating loci sequences extracted from whole genome sequences

of 12 other Exobasidiomycete species (see **Figure 1**), provided an opportunity to expand to compare the mating genes of *Q. eucalypti* with those of these species. The mating genes and loci that have been characterized for several species Ustilaginomycetes and Malasseziomycetes, provided useful reference material for the present study.

## Materials and Methods

*Phylogenetic analyses*

LSU sequences of representative species of the Ustilaginomycotina were downloaded from NCBI Genbank. These sequences were aligned using the online version of MAFFT v. 7 (Katoh 2013). Maximum likelihood (ML) analyses were done using raxmlGUI (Silvestro & Michalak 2012) with the GTR+G+I substitution model selected. Ten parallel runs with four threads and 1000 bootstrap replications were conducted.

*Isolates and genome sequences*

Genome sequences for 13 species in the Microstromatales were obtained from the NCBI Genbank genome database. These included the following: *Quambalaria eucalypti* CMW1101 (Wingfield *et al*. 2018a), *Golubevia pallescens* (BCHO00000000), *Tilletia caries* (LWDD00000000) , *Tilletia controversa* (LWDE00000000), *Tilletia horrida* strain QB-1 (Wang *et al*. 2015), *Jaminaea rosea* CBS14051, *Ceraceosorus guamensis* CBS139631, *Meira miltonrushii* CBS12591, *Exobasidium vaccinii, Tilletiopsis washingtonensis* NRRL Y-63783, *Pseudomicrostroma glucosiphilum* CBS14053, *Acaromyces ingoldii* CBS140884 (Kijpornyongpan *et al*. 2018) and *Tilletiaria anomala* CBS436.72 (Toome *et al*. 2014).

*Identification of mating type loci*

The genomes of the 13 species were investigated to identify the contigs containing potential MAT genes. The tBLASTn tool of Bioedit 7.2.6.1 (Hall 1999; Gioti *et al.* 2013) was used to screen the assembled genomes for mating type genes using sequences of the MAT genes from *Malassezia globosa* CBS7966 (GenBank accession number: AAYY00000000; Xu *et al.* 2007) and Mitochondrial Intermediate Peptidase *(MIP)* gene, EDR15793 of *Laccaria bicolor* S238N-H82 (GenBank accession number: EDR15793). The scaffolds that had the greatest similarity to these reference sequences were extracted for each species and annotated using Augustus 3.3.2 (Stanke *et al*. 2008) using the pre-optimised species model of *Ustilago maydis*. The predicted protein sequences were then identified using BLASTp analysis using the BLASTp function in the NCBI database. Protein sequences that were identified to represent pheromone receptors and the HD factors, were further analysed using

InterProScan 69.0 (https://www.ebi.ac.uk/interpro/about.html), and the pheromone gene was further analysed using SignalP 4.1 (Petersen *et al*. 2011) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/ TMHMM-2.0/).

## Results

*Characterisation of the mating loci*

*a locus*

tBLASTx searches identified sequences on contig 168 of the assembled genome of *Q. eucalypti* that were highly similar to the pheromone receptor gene (*Pra*) sequences of *M. globosa.* BLASTp analyses predicted the protein structure of the *Pra* pheromone receptor gene. No other genes related to the *a* locus were identified on this contig. Similar results were observed for *G. pallescens* (contig 14)*, T. caries* (contig 1181)*, T. controversa* (contig 223)*, T. horrida* (contig 366)*, J. rosea, C. guamensis, M. miltonrushii, E. vaccinii, T. washingtonensis, P. glucosiphilum*, *A. ingoldii* and *T. anomala* (**Figure 2**)*.* These results were further corroborated by InterProScan, SignalP and TMHMM analyses (**Figure 4a**).

tBLASTx searches identified sequences that were very similar to the pheromone gene (*mfa*) sequences of *M. globosa* in the following species *J. rosea, C. guamensis, M. miltonrushii, E. vaccinii*, and *T. anomala*. The genome of *Exobasidium vaccinii* contained two copies of the *Pra* and the *mfa* genes (**Figure 2**). BLASTp of the predicted protein sequences identified the structure of the pheromone gene in each of the predicted sequences. These results were further corroborated by InterProScan analysis.

*b locus*

tBLASTx searches identified sequences on contig 142 of the assembled genome of *Q. eucalypti* with high similarity to the Homeodomain binding factors, bE1 and bW1, of *M. globosa*. Similar results were observed for *T. caries* (contig 842)*, T. controversa* (contig 2121, 2565)*, T. horrida* (contig 84)*, J. rosea, C. guamensis, M. miltonrushii, E. vaccinii, T. washingtonensis, P. glucosiphilum*, *A. ingoldii* and *T. anomala.* In the species *G. pallescens* only one of the homeodomain binding factors, bW, was identified on scaffold 13, while *M. miltonrishii* contained three copies of the HD1 gene (**Figure 3**). These results were corroborated by InterProScan analyses and contained the homeodomain binding regions and DNA binding regions typically associated with HD factors (**Figure 4b**).

Mitochondrial intermediate peptidase (*MIP*), a gene considered to apart of the *b* locus, was identified on contig 230 of the assembled genome of *Q. eucalypti*. *MIP* was also identified in,

*T. caries* (contig 278), *T. controversa* (contig 180), *T. horrida* (contig 8) and *G. pallescens* (contig 5). The *MIP* gene in *G. pallescens* was found to be very close to *Pra* on the *a* locus (**Figure 2**). A *Rak1* like ortholog, shown to be involved in the regulation of virulence and mating in *U. maydis*, was also identified on contig 142 of the *Q. eucalypti* genome. Analyses using InterProScan revealed the presence of the typical seven WD-40 repeat motifs known for this gene (Wang *et al*. 2011).

On the same contig (142) of the genome of *Q. eucalypti*. Three other genes commonly associated with the *b* mating locus were identified, *C1D1, Sec61* and *MFS* (Bakkeren 2008; James *et al.* 2013). Of the other species included in this study, only *T. anomala* and *T. washingtonensis* contained the *C1D1* ortholog.

**Discussion**

Investigation of whole genome sequences allowed for the identification of the putative *a* and *b* mating loci in*: Q. eucalypti, G. pallescens, T. caries, T. controversa, T. horrida, J. rosea, C. guamensis, M. miltonrushii, E. vaccinii, T. washingtonensis, P. glucosiphilum*, *A. ingoldii* and *T. anomala.* The implication of this is that a sexual state may occur for these fungi.

Characterisation of the genes found at the *a* mating locus elucidated this region in the genomes of the species considered in this study. The genome of *Q. eucalypti* as well as all other species used in this study were found to contain a homolog of the pheromone receptor gene (*Pra*). An STE3 region was identified was identified in this gene. This region encodes an amino acid sequences with high proportion of hydrophobic residues that group into seven transmembrane domains (Nakayama *et al.* 1985). These regions were observed when the predicted proteins, obtained from the BLASTp search, were analysed using TMHMM (**Figure 4a**). This STE3 region has been found to be generally conserved in other Basidiomycete fungi and in which pheromone receptors are known to occur (Gioti *et al*. 2013).

No pheromone gene was found in the genome of *Q. eucalypti*. However, this gene was identified in *J. rosea, C. guamensis, M. miltonrushii, E. vaccinii* and *T. anomala* (**Figure 2**). The reason for this gene not being identified in the other species considered in this study, including *Q. eucalypti*, is that the pheromone gene is small and has a high degree of specificity with one pheromone only fitting one pheromone receptor (Schirawski *et al*. 2005). Interestingly, *E. vaccinii* possesses two sets of both the *Pra* and pheromone genes.

Genes that typically make up the *b* mating locus were characterised in order to elucidate this region in the genomes of fungi considered in this study. Putative Homeodomain binding factors, bE1 and bW1 were identified in the genome sequences of *Q. eucalypti, T. caries, T. controversa, T. horrida, J. rosea, C. guamensis, M. miltonrushii, E. vaccinii, T. washingtonensis, P. glucosiphilum*, *A. ingoldii* and *T. anomala*. An exception to this was in the case of *G. pallescens* where only the HD2 (bW) was identified (**Figure 3**). However, the genome assembly for this species appeared to be poor, possibly accounting for this missing gene.

Analyses of the putative bE and bW proteins in *Q. eucalypti,* revealed a single homeodomain binding region in each of the predicted proteins, within each region, DNA binding sites were also predicted. When compared to the putative bE and bW regions identified in *Malassezia sympodialis*, the regions were found to be similar in structure and length (Gioti *et al*. 2013). For the other species used in this study, the size of the HD binding factors varied in size, however each of these had the required HD region and DNA binding sites. Orientation and number of HD factors remains relatively well conserved, within the exception of *M. miltonrushii*, where the HD1 and HD2 are in reverse order, and has three copies of HD1.

A *C1D1* homolog was identified in the genomes of *Q. eucalypti, T. anomala* and *T. washingtonensis*. The *C1D1* gene, which encodes a putative nuclear regulator, has been commonly reported from the *b* mating locus in smut fungi as a border gene (Heinze 2009) (**Figure 4**). None of the other species used in this study were found to contain this border gene and none were found to contain the second border gene, *nat1*, a putative N-terminal acetyl transferase. These border genes are not required to define the mating loci. This is evident in the MAT2 allele of *Ustilago hordei* and the *b2* allele of *U. maydis*, smut relatives of the Exobasidiomycetes, which does not contain either the *C1D1* or the *nat1* border genes (Que *et al*. 2014; Wang *et al*. 2014).

A *rak1*-like gene was identified and characterised in the genome of *Q. eucalypti*. In fungi Rak1 orthologues are involved in regulation of cell growth and stress responses, including acting as Gβ subunits in the cAMP signalling pathway of *Cryptococcus neoformans* and the model yeast *Saccharomyces cerevisiae* (McLeod *et al.* 2000; Palmer *et al.* 2006; Rothberg *et al.* 2006; Zeller *et al*. 2007; Coyle *et al*. 2008). In the model smut fungus, *U. maydis*, *rak1* was found to a regulator of *rop1* expression, which in turn regulates the pheromone response factor, *prf1*. In deletion experiments, strains in which the the *rak1* gene had been removed showed deficiencies in the formation of conjugation tubes. This deficiency led to a reduction in pathogenicity of these strains. This was due to the reduction in the production of

filaments and appressoria, which are necessary for pathogenic development (Wang *et al*. 2011).

The mating biology has been investigated only in *T. caries* and *T. controversa* and these were shown to follow a bipolar mating system. Five alleles were shown to be present in *T. controversa* and only two in *T. caries* using mating crosses of monosporidial lines of each species (Holton & Kendrick 1957; Hoffmann & Kendrick 1969; Trail & Mills 1990). In the present study we identified at least one of each of these alleles shown in the mating cross study (Holton & Kendrick 1957; Hoffmann & Kendrick 1969; Trail & Mills 1990). Possible linkage has been shown for *Exobasidium vaccinii*, where the *a* and *b* loci occur in very close proximity to each other. Therefore, this may represent a possible MAT locus for this species and may indicate a possible bipolar mating system.

To determine whether the remaining species follow a tetrapolar or bipolar mating strategy, further sequencing of other isolates is required. Many of the genomes for these fungi and available for this study were of poor quality and will need to be re-sequenced in order to assemble high quality scaffolds. The scaffolds can be used to determine how close these two loci (*a* and *b*), are to each other. Sequenced MAT loci could then be used to design primers for rapid screening to identify whether alternative alleles are present in the population and whether recombination is taking place.

This is the first study in which a line of evidence for sexual reproduction in *Q. eucalypti* has been provided. This is also true for various other Exobasidiomycete fungi in which the presence of mating type genes and seemingly intact MAT loci were found. However, to confirm that sexual reproduction takes place in these species, more information on recombination between members of each species, and whether different alleles are present within the species is required. Further sampling of these species and development of gene specific markers will allow for more rapid screening of isolates to determine which alleles are present and if recombination is occurring between them.

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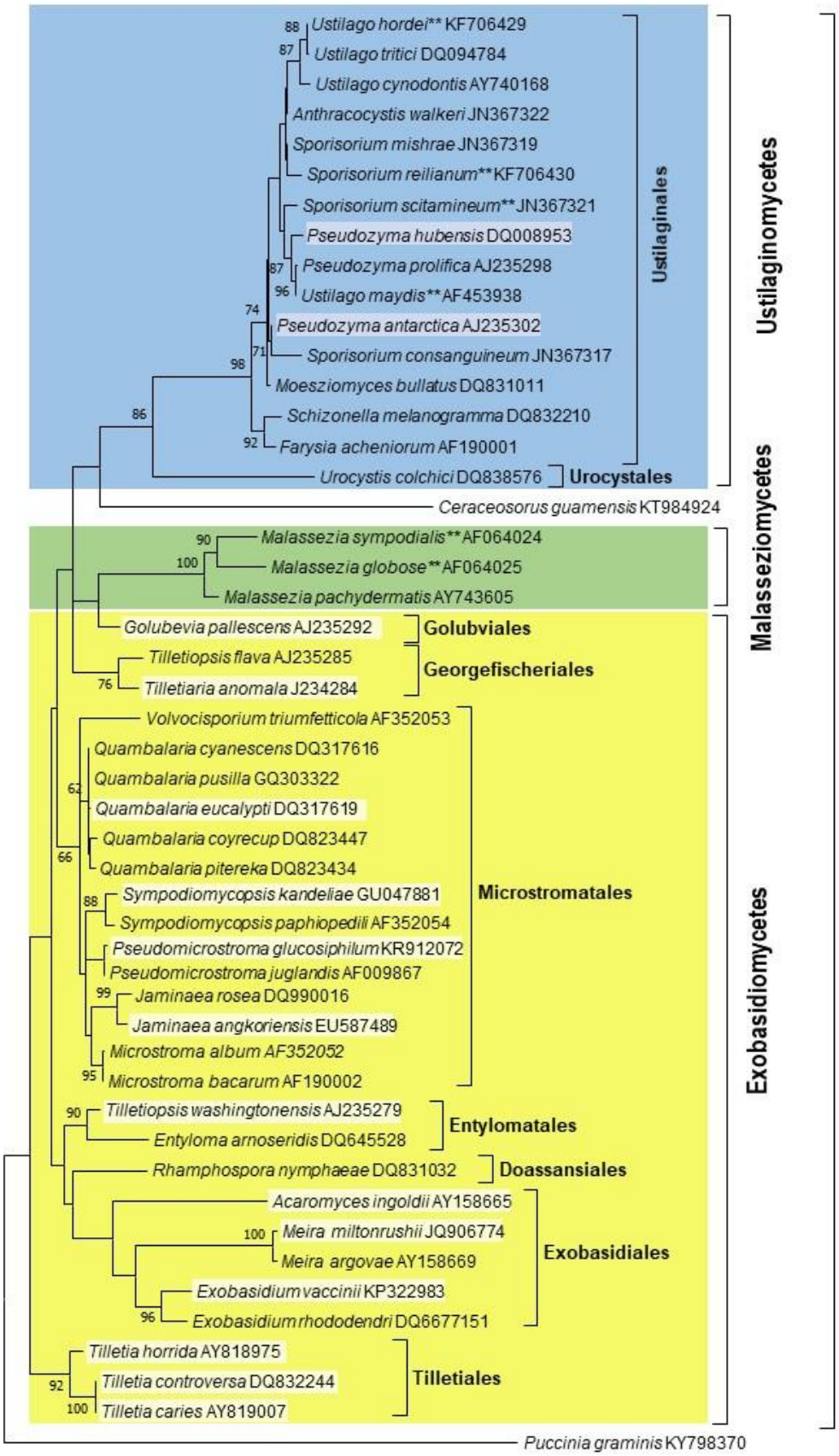
## Figures

**Figure 1.** Phylogram resulting from ML analyses using RaxML of LSU sequences of selected species of each family within the Ustilaginomycotina. Support values at branches resulted from 1000 bootstraps and only values above 60% are indicated. Species in highlighted boxes had genome sequences analysed during this study. ** indicates species within known mating type genes.



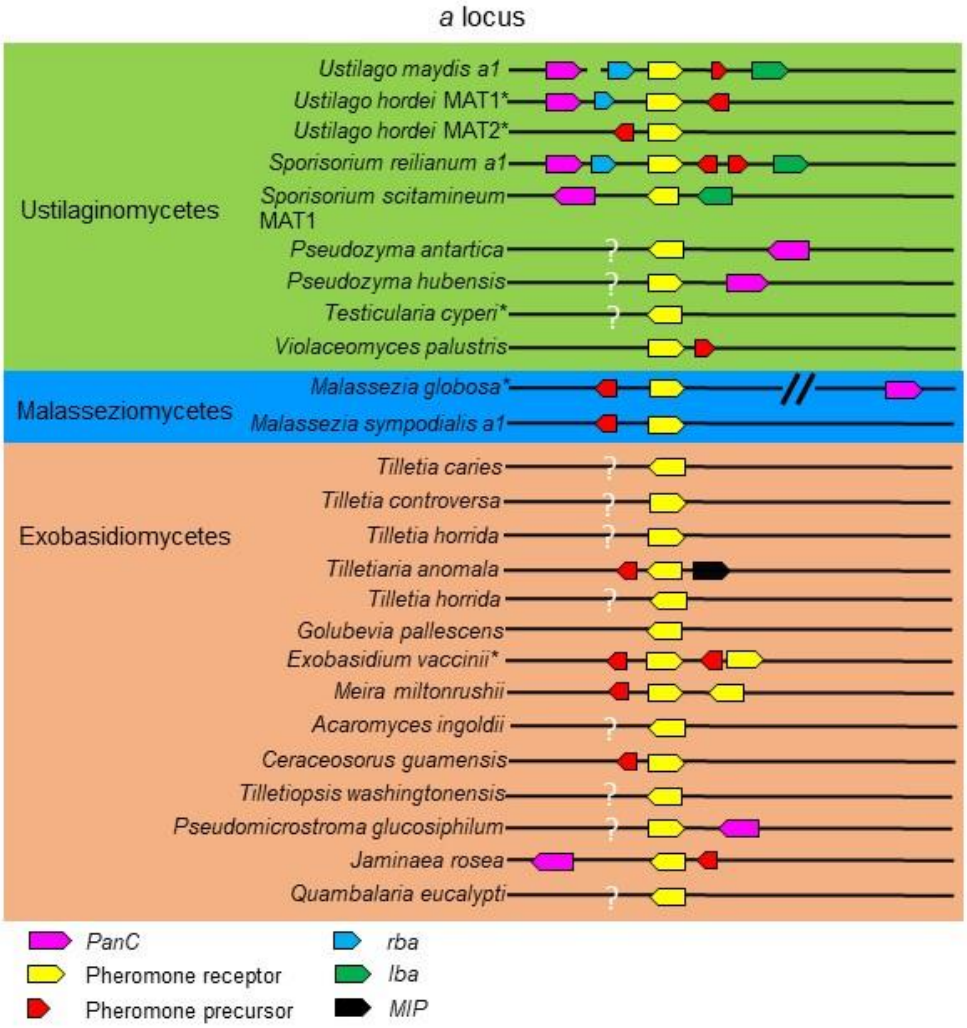



**Figure 2.** the *a* mating locus. This figure does not take into account the length of the locus, which varies between species or the size of the genes. The question mark indicates a missing pheromone precursor gene.
**a* and *b* loci are linked in these species; *Exobasidium vaccinii* less than 10kb apart, *Testicularia cyperi* less than 127kb apart, *Malassezia globosa* less than 135kb apart, *Ustilago hordei* less than 527kb apart.

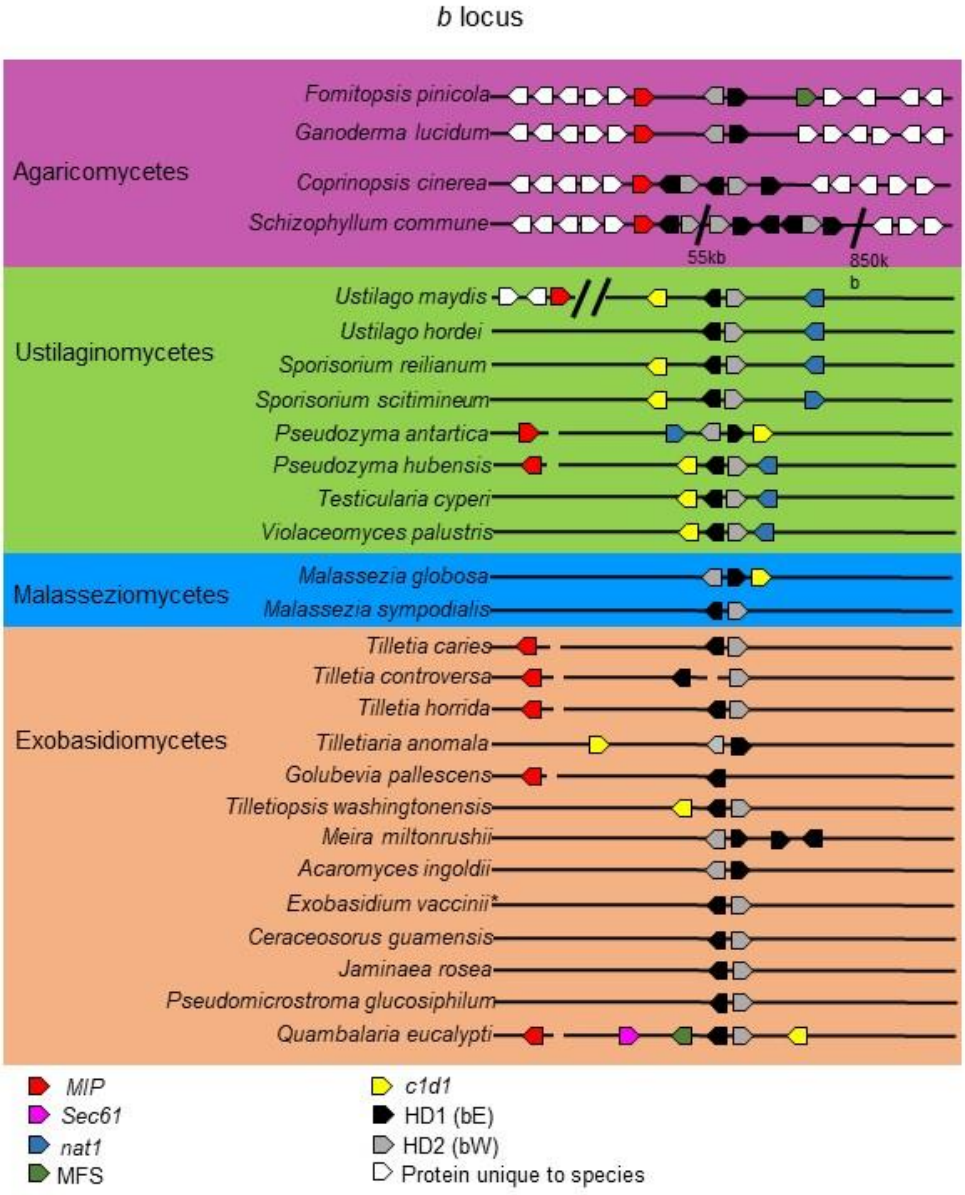



**Figure 3.** the *b* mating locus. This figure does not take into account the length of the locus, which varies between species or the size of the genes.
*a and *b* loci are linked in these species; *Exobasidium vaccinii* less than 10kb apart, *Testicularia cyperi* less than 127kb apart, *Malassezia globosa* less than 135kb apart, *Ustilago hordei* less than 527kb apart.

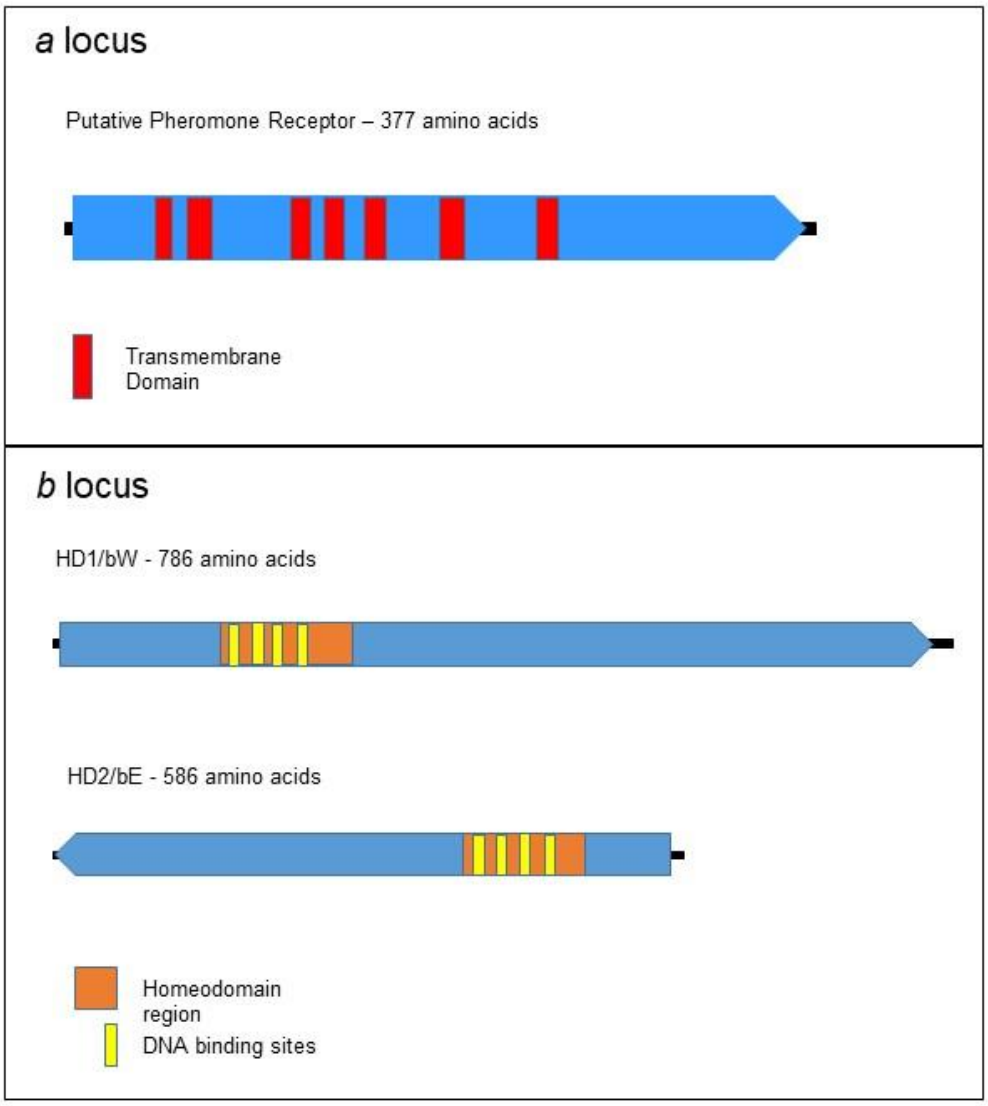



**Figure 4.** Putative mating loci of *Quambalaria eucalypti*. **A**. the partial *a* locus, with the pheromone receptor gene displaying the typical transmembrane domains. **B**. the *b* locus contains the homeodomain regions along with the DNA binding sites within the region. Analysis done using InterProScan.

**Summary**

The genus *Quambalaria* (Microstromatales, Exobasidiomycetes) to date included six species, five of which are pathogens of *Eucalyptus* and *Corymbia* species. The only exception is *Q. cyanescens*, which has been shown to be non-pathogenic to plants, but can be an opportunistic human pathogen. The symptoms on trees include shoot and leaf blight, which is characteristic of *Q. eucalypti* and *Q. pitereka* and stem cankers caused by *Q. coyrecup.* The complete body of research on *Quambalaria* was reviewed, focusing on the taxonomy, pathogenicity, host range and geographical distribution. The review revealed a lack of knowledge on basic aspects of its biology, especially in terms of its mating behaviour and sexual reproduction. The premise of this dissertation was the discovery of a *Quambalaria*-like species infecting *Eucalyptus* seed capsules in South Africa. DNA sequence analyses revealed that this represented a novel species, described here as *Q. erniae* nom. prov. The latter fungus is reported for the first time from South Africa, co-occurring with *Q. erniae* in seed capsules. The whole genome sequence of *Q. eucalypti* was generated and assembled. and explored for the presence of mating type genes. This genome, together with available genomes of other species of the Exobasidiomycetes, were explored for the presence of mating type loci. Several mating type genes and seemingly intact MAT loci were identified in *Q. eucalypti* as well as the majority of the other species. These results are the first evidence for possible sexual reproduction in *Q. eucalypti* and several other Exobasidiomycete spp.