The microbial ecology of *Protea repens* (Proteaceae) infructescences and the surrounding environment

by

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Submitted in partial requirements for the degree

Philosophiae Doctor

In the Faculty of Natural & Agricultural Sciences

University of Pretoria

Pretoria

December 2018

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Declaration

I, Zander Rainier Human, declare that the dissertation, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: 

DATE: 10 December 2018

Acknowledgements

First of all, I would like to thank my supervisors, Profs. Mike Wingfield, Wilhelm de Beer and Fanus Venter. I was really privileged to work with you and I really enjoyed all of our engagements. I really hope that we remain friends, and perhaps work together sometime in the future. I would like to specially thank Prof. Mike Wingfield for his financial support as the director of FABI. I am also very grateful to Prof. Fanus Venter for driving the completion of this thesis.

The contribution of other co-authors of the work presented in this thesis is greatly appreciated. I would like to thank prof. Francois Roets for his insightful comments on my manuscripts and for inspiring me to always take a step back and look at the bigger picture. I would like to thank Dr. Casper Crous for his assistance in analyzing data, preparing manuscripts and for his mentorship and friendship.

I would also like to thank past and present labmates, especially Gabrielle Carstensen, Sarah MacRae, Tarren Seale and Solize Vosloo for their friendship, advice and support. I would also like to thank my fellow FABIans for their friendship and support. A special mention to Hardus Hatting, Johan van der Linde, Darryl Herron, Izette Greyling and Marc Bouwer for their friendship, fun fieldtrips and long philosophical discussions.

I would like to thank my late mother Theresa, and father Hugo for all of their support, without which I would not be able to make it this far. Also, thank you to my brothers and best friends Charl and Magnus for all of their support. A special thanks to Braam Viljoen for financial support throughout my studies.

Finally I would like to thank my wife Mia for all of her support, patience and understanding. Thank you for all of your support and understanding through the difficult times.

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Preface

Numerous studies have described the infructescences of serotinous *Protea* trees (Fig. 1) as a unique fungal niche (Lee *et al.*, 2005). This is because, despite it being a protected, moist environment, it is colonized by fairly limited number of highly specialized fungi (Roets *et al*., 2013). To date, studies have focused on specific fungal groups in the infructescences of *Protea* trees (Roets *et al*., 2013), as well as a single study on the actinomycete bacteria in this niche (Human *et al.*, 2016). Here, we attempted to obtain a more complete description of the bacterial and fungal communities in the infructescences of *Protea repens*.

Chapter one of this thesis is a literature review dealing with the microbial communities typically associated with dead or senescent plant material. The review is focused on studies where high-throughput sequencing was used to characterize the bacterial and fungal community composition as these methods were also used during the current study. Furthermore, in the review we attempt to demonstrate that distinct bacterial and fungal communities are present during the different stages of dead wood and litter decomposition. We also explore the importance of the conservation of plant-detritus microbial niches in ecosystem functioning.

In **Chapter two,** we studied the bacterial communities in *P. repens* infructescences by sequencing 16S rRNA amplicons. The bacterial diversity in the infructescences of *Protea* trees have not been considered before, with only a single prior study focused on actinomycetes. A site partially affected by fire was chosen for this study, with the reasoning that the first infructescences formed after fire may be colonized by only the most prominent bacterial associates. Furthermore, in this chapter, we compare the bacterial communities in *P. repens* infructescences with those present in deadwood and litter.

Chapter three was a continuation of a previous study on the actinomycetes in infructescences of *Protea* trees. Although it is known that various *Streptomyces* species colonize the infructescences of *Protea* trees, it is not known during which flowering stage they enter infructescences. Understanding when antifungal actinomycetes colonize *Protea* infructescences will allow a better understanding of the extent of their influence of the fungal communities in this niche. Therefore in this chapter, we sampled the different developmental stages of *P. repens* inflorescences and infructescences to determine whether they have been colonized by actinomycetes.

In **Chapter four** we characterised the fungal diversity in *P. repens* infructescences using high-throughput sequencing of ITS1 amplicons. Current knowledge suggests that *Sporothrix splendens* and *Knoxdaviesia proteae* are the most common fungi inhabiting *P. repens* infructescences. Except for a few studies reporting other ascomycetes from this habitat, very little is known on the total diversity of fungi in *P. repens* infructescences and whether any other fungal species in environment. In this chapter we provide insights into the some of the leaf-inhabiting and litter-degrading fungi present in infructescences.

The aim of **Chapter five** was to identify the bacterial communities that occur in close proximity to the structures of *Knoxdaviesia* and *Sporothrix*. When the bacterial communities in infructescences were studied in Chapter 2, the sample units were large and included material from bracts, seeds and pollen presenters in the same infructescence. These larger sample units most likely include several smaller microbial niches because the effective area for a microbial community is likely to be much smaller than this. Thus, in this chapter, we examined smaller sample units in order to accurately identify bacteria colonizing the areas around the fungi.

The work presented in this thesis attempts to provide the first glimpses into the total bacterial and fungal diversity in *Protea* infructescences. We hope to demonstrate that *P. repens* infructescences is a unique and interesting microbial niche, and an environment where the role of succession and dispersal in dead-plant degrading bacteria and fungi could be further studied.

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Fig. 1. An immature inflorescence from *Protea repens* (left) and an infructescence (right)

Summary

The infructescences of serotinous *Protea* trees is an unusual and unique fungal niche, well-known for its association with specific fungi and their symbiotic mites. However, except for a single study on *Streptomyces* species, little is known about any other microbial groups present in this habitat. In this study, we explored the complete microbial diversity associated with the infructescences of *Protea repens.* Diverse bacterial communities inhabit the infructescences of *P. repens.* Differences were noted between bacterial communities in infructescences in areas where the plants were regrowing after it was destroyed by fire. Depending on the age of vegetation after fire, some bacterial taxa were present in both the litter and infructescences. Actinomycete bacteria were previously reported from *P. repens* and *P. neriifolia* infructescences. In this study, we found two different *Streptomyces* species consistently present in newly-formed and mature infructescences. In a fire-affected site, actinomycetes rarely colonized infructescences, whereas in an unburnt site, the majority of infructescences were colonized by actinomycetes. However, within three months after infructescences had formed, a much larger proportion of infructescences in the burnt site were colonized by these bacteria. *Sporothrix splendens* was the most common fungal OTU in the burnt site, but there was increased competition with an OTU identified as a *Clavulina* sp. in the unburnt site. Although *Knoxdaviesia proteae* was present in all infructescences examined, it did not have a high relative abundance as was expected. This may be due to biases in PCR, library preparation and sequencing, or it may be that the fungus may simply have been consumed by arthropods as was previously suggested. An attempt was made to identify bacteria present in close proximity to fungal structures in *P. repens* infructescences. The bacterial communities in *P. repens* infructescences seemed to be randomly assembled and no consistent bacterial associates of the fungi could be identified. However, it was clear that the presence of the fungi resulted in higher bacterial species richness in the immediate surroundings of the fungi. The fungal communities present in *P. repens* infructescences were similar in composition to bacterial communities of decomposing deadwood and plant litter suggesting that it provides a similar niche as deadwood- and litter, and is possibly a reservoir for decomposer and soil associated fungi in these fire-prone ecosystems.

Chapter 1

Bacterial and fungal diversity in litter and dead wood in the age of high-throughput sequencing technology

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1. Introduction

1.1. High-throughput sequencing in microbial ecology

Microbial ecology encompasses the study of bacterial and fungal diversity, biology and interactions in many diverse environments. Microbial diversity studies have traditionally been culture-based where pure cultures of bacteria and fungi were isolated from specific habitats (Torsvik *et al.*, 1998; Kirk *et al.*, 2004). Bacteria and fungi in pure culture are easily identified through DNA sequencing and species names could then be used to make inferences regarding their ecological functions (Kirk *et al*., 2004). Access to microbial pure cultures has allowed further studies on the functional traits of microorganisms in their environment (i.e. what they do), and allowed the description of novel species of bacteria and fungi (Kirk *et al*., 2004). The major limitation of this approach is that most bacteria are not readily culturable and most cultured bacteria are rarely dominant in the environment (Hugenholtz, 2002). Thousands of fungi are also difficult to culture, including many associated with soil (Martin *et al.*, 2011) and plants (Lewis, 1973). Furthermore, by isolating bacteria and fungi, they are effectively removed from their environment and all subsequent experiments must be performed *in vitro*.

The advent of high-throughput sequencing technologies has revolutionized virtually all fields of biology. In all domains of life, there are well-described barcoding genes that are universal among these groups (Pace *et al.*, 1986; Hebert *et al.*, 2003; Schoch *et al*., 2012). These DNA barcoding genes can be amplified with universal PCR primers and the subsequent sequencing thereof provides an overview of the total diversity of the organism studied (Lane *et al.*, 1985; Hebert *et al.*, 2003; Schoch *et al*., 2012). This approach has made it possible to characterize or profile the total microbial diversity in habitats and has been widely used to study complex microbial communities associated with healthy and diseased subjects (Sogin *et al.*, 2006; Caporaso *et al.*, 2012; Shokralla *et al.*, 2012). This also enables the study of environmental preturbations in different habitats through the manipulation of the physical environment, followed by the complete characterization and comparison of the resulting microbiomes (Shokralla *et al.*, 2012). For a more complete overview of sequencing technologies, their advantages and obstacles, Shokralla *et al.* (2012) provide a detailed account.

High-throughput sequencing provided advances other than just characterizing microbial communities to the field of microbial ecology. Using metagenomic sequencing, a catalogue of the total gene content of an environment can be produced, which provides insights into the functional diversity of the community (Qin *et al*., 2010). The genomes of previously uncultured bacteria can also be assembled from metagenomic sequences (Tyson *et al*., 2004), which provides information on their metabolic potential, ability to survive and adaptations to their respective niches (Hugenholtz and Kyrpides, 2009). Another approach is meta-transcriptomic sequencing, where all the RNA transcripts from an environment are sequenced, which makes it possible to capture information on the active organisms in the environment, the ongoing biological processes and the metabolites produced (Urich *et al*., 2008).

1.2. Why should plant biomass decomposition be studied?

Roughly 90% of the world's terrestrial carbon is contained in land plants and its detritus (Canadell and Raupach, 2008). In forests, approximately 8% and 5% of the carbon is contained in dead wood and litter respectively (Sun et al., 2014). The carbon contained in dead wood and litter, together with the carbon in forest soils, amounts to approximately 35% of the world's carbon (Pan *et al.*, 2011). Recyling this carbon is an important process contributing to health of ecosystems, as it maintains the biodiversity of soil organisms, and sustains the supply of mineral nutrients to plant communities (Hättenschwiler *et al.*, 2005; Bardgett, 2005).

In the world's grasslands and forests, more than 80% of plant biomass forms detritus (Cebrian, 1999). The efficiency of decomposition of plant-derived detritus is closely linked to the nutrient content in the source plants, which then determines decomposition rates (Cebrian, 1999). Thus, the amount of detritus in communities of more palatable plants is lower as a result of higher rates of herbivory and decomposition (Cebrian, 1999). Most nutrients in ecosystems are sustained in this plant-detritus cycle and thus decomposition of dead plant material is essential to ecosystem function (Gessner *et al.*, 2010). Plant-derived detritus supports a diverse community of decomposer organisms, responsible for the remineralization of minerals, which supports plant fitness.

Disturbing the biodiversity, especially the microbial diversity responsible for decomposing litter and dead wood can impact on the rate of detritus decomposition (Gessner, *et al.*, 2010). The loss of cooperative and antagonistic interactions in detritus and soil through environmental preturbations such as drought, may result in an increase in soil plant pathogens (Garbeva *et al.*, 2004). Likewise, the loss of mutualistic fungi and bacteria surrounding the roots of plants may result in reduced growth rates of the associated plants (Raaijmakers *et al.*, 2009). Understanding the effect of disturbances on the biodiversity of communities responsible for the decomposition of plant-detritus is therefore important when attempting to predict how these habitats will react to a continuously changing environment (Wolters *et al.*, 2000; Gessner *et al*., 2010).

In this review, we address the bacterial and fungal community composition of plant litter and dead wood, the two most common plant-detritus habitats. We focus on studies where the microbial communities were characterized using high-throughput sequencing. With high-throughput sequencing, the identity of entire communities of microorganisms are identified leading to a better description of the complete diversity and successional patterns in dead wood and litter. In this review, we also provide a brief overview of the interactions between bacteria and fungi, especially the uncertainty surrounding the role of bacteria in decomposition. Several related reviews are available on this topic. For example, Van der Wal *et al* (2013) provided a view of the fungal communities in decomposing wood. De Boer *et al.* (2005), De Boer and Van der Wal (2008) and Johnston *et al*. (2016) have reviewed the interactions between bacteria and fungi. In this review, we have focused only on studies where high-throughput sequencing of bacteria and fungi were used to study the diversity and interactions of these organisms.

2. Substrates in senescent plant biomass and the degradation thereof

The most abundant substrates present in senescent plant biomass is discussed in **appendix 1**. The microbial groups degrading the various substrates present in dead plant biomass is described in **appendix 2.**

Cellulose and hemicellulose decomposition is widespread among different phylogenetic groups of bacteria especially soil inhabiting species from the actinomycetes, *Pseudomonas* and the *Bacillus* (López-Mondéjar *et al.*, 2016). The same bacterial taxa are also efficient at utilizing hemicellulose,

although it can be utilized by far more bacterial taxa than cellulose (Koeck *et al.*, 2014). Fungal decomposition of cellulose is also known from the white-, brown- and soft-rot fungi, although the brown- and soft-rot fungi both lack at least one of the three cellulases produced by white-rot fungi (Ljungdahl and Eriksson, 1985). The early colonizers of plant detritus such as saprobic ascomycetes and soft-rot fungi are among the most efficient decomposers of hemicellulose (Rajala *et al.*, 2012), but the brown- and white-rot fungi are also very efficient at degrading hemicellulose (Blanchette, 1995; Baldrian, 2008; Eastwood *et al.*, 2011).

Lignin provides structure to plant cell walls, especially in wood and is the most resistant to degradation among plant-derived residues. Bacterial decomposition of lignin is uncommon and has only been reported in the actinomycetes (Kirby, 2005). Fungal lignin degradation is one of the most important processes in the carbon cycle. White-rot fungi are efficient at utilizing lignin, while the brown-rot fungi can only modify the chemical structure of lignin, and are not able to completely metabolize the substrate (Eriksson *et al.*, 2012). Some ectomycorrhizal fungi can also break down lignin, but it is suggested that they oxidize lignin primarily to reach nitrogen (Lindahl *et* al., 2007; Lindahl and Tunlid, 2015). Efficient lignin decomposition is required for releasing hemicellulose and cellulose from lignocellulose complexes, while the modification of lignin by brown-rot fungi may release small amounts of usable sugars.

3. Microbial diversity of dead wood

3.1. Bacterial communities in dead wood

High-throughput sequencing of the 16S rRNA gene has made it possible to determine the total bacterial community in dead wood, although studies on this topic has mostly been limited to forest-tree species in the Northern Hemisphere (Sogin *et al.*, 2006; Caporaso *et al.*, 2012; Baldrian, 2017). This is unfortunate, as some of the most diverse biomes on earth are located in the Southern Hemisphere and the microbial diversity in most of these ecosystems have largely been neglected (Linder, 2001). Nonetheless, the use of high-throughput sequencing in studies on dead wood microbial diversity have already provided a considerably enhanced understanding of the diversity of bacteria inhabiting dead wood.

The bacteria inhabiting dead wood niches are involved in important interactions with fungi. These were known to exist even before the introduction of high-throughput sequencing (De Boer and Van der Wal, 2008; Johnston *et al.*, 2016). Many wood-inhabiting bacterial species are able to fix atmospheric nitrogen, which alleviates a growth-limitation of wood-decay fungi due to a lack of nitrogen (Cowling and Merrill, 1966; Brunner and Kimmins, 2003). Antagonistic interactions between some bacterial groups and fungi also occur in dead wood and are believed to be as a result of bacteria protecting scarce sugar-rich substrates against invading fungal mycelia (De Boer and Van der Wal, 2008). These antagonistic interactions include fungal predation of bacteria, bacterial mycophagy and the secretion of antifungal and antibacterial compounds (De Boer and Van der Wal, 2008). Although the importance of bacteria-fungal interactions in dead wood are well-known, the composition of dead wood bacterial communities and their contribution to wood decomposition remains uncertain (De Boer and Van der Wal, 2008).

Bacteria are always present in dead wood, but their function and contribution to the decomposition process remains unclear (Johnston *et al.*, 2016). Many phylogenetically diverse bacteria have the ability to decompose cellulose (Lynd *et al.*, 2002; López-Mondéjar *et al*., 2016), but the utilization of lignin is limited to a few bacterial groups, the best known of which are the actinomycetes (Kirby, 2005; Brown and Chang, 2014). Lignin is an abundant structural polymer of wood, and an abundant substrate in dead wood. In addition, most cellulose in dead wood is present in matrices with lignin and cannot be accessed without lignin oxidation or through mycelial growth form (De Boer *et al.*, 2005). Therefore, bacterial decomposers of wood depend on fungal lignin decomposition to release cellulose and other sugars to receive a usable substrate.

Bacterial communities in dead wood are affected by pH (Tláskal *et al.*, 2017), with the same effects observed on community composition as in soil with different pH values (Rousk *et al.*, 2010). In decomposing wood, a decrease in pH occurs as a result of fungal cellulose decomposition, which occurs optimally at lower pH ranges (Thompson, 1983; Baldrian, 2008). The low pH in dead wood has a negative effect on many bacterial groups. For example, a decrease in pH results in a decrease in the abundance of Actinobacteria in the later stages of wood decomposition. In the absence of such a decrease , the Actinobacteria are common in dead wood where they are able to efficiently utilize the available substrates (Hoppe *et al.*, 2015).

In the early stages of dead wood decomposition in European boreal and temperate forests, members of the Alphaproteobacteria and Acidobacteria are the dominant (Hoppe *et al.*, 2015; Hoppe *et al.*, 2016; Rinta-Kanto *et al.*, 2016; Tláskal *et al.*, 2017). The Actinobacteria are also abundant during the early stages of dead wood decomposition, but their presence largely differs depending on the tree species and pH (Hoppe *et al.*, 2015). In *Fagus sylvatica* and *Picea abies*, the Burkholderiaceae and Sphingomonadales are the dominant bacterial groups in the early stages of dead wood decomposition (Hoppe *et al.*, 2015). Other common taxa observed during the early stages of decomposition of dead wood that are common to many different tree species belong to *Pseudomonas, Luteibacter* and *Acidisoma* (Sun *et al*., 2014; Tláskal *et al*., 2017). Speices of *Mucalignibacter* and *Burkholderia* are ubiquitous in the early stages of wood-decomposition, and remain present until the very late stages of decomposition. These bacteria are also abundant in the soil surrounding decomposing wood (Sun *et al*, 2014).

The Rhizobiales are the most abundant bacterial group in the intermediate and late stages of wood decomposition. Two studies by Hoppe *et al*. (2015; 2016) have reported that the Rhizobiales account for 25% of all sequences in the late stages of decomposition of *Fagus sylvatica* logs (Hoppe *et al.*, 2015). They have also been observed at high abundances in beech wood blocks (Folman *et al.*, 2008; De Boer and Van der Wal, 2008). The Rhizobiales are apparently important symbionts of wood decay fungi, due to their ability to fix atmospheric nitrogen (Folman *et al.*, 2008; De Boer and Van der Wal, 2008, Hervé *et al.*, 2014; Hoppe *et al.*, 2015, see section 5). The potential of bacteria present in dead wood nitrogen to fix atmospheric nitrogen was assessed by sequencing the *nifH* gene directly from DNA extracted from dead wood (Hoppe *et al.*, 2015). These studies confirmed that the *nifH* copy number is positively correlated with the rate of fungal dead wood decomposition, however this is not a definitive indication of nitrogen fixation (Hoppe *et al.*, 2015).

Methylotrophic bacteria are common during all stages of wood decomposition (Hoppe *et al.*, 2015; Tláskal *et al.*, 2017 and include the genera *Methylovirgula*, *Methylocapsa* and members of the Beijerinckiaceae (De Boer and Van der Wal, 2008; Hoppe *et al.*, 2015; Kielak *et al*., 2016; Morawe *et al*., 2017). Methanol-derived carbon compounds are by-products of fungal lignin decomposition and are abundant in dead wood and provides a suitable substrate for methylotrophs (De Boer and Van der Wal, 2008). Additionally, many of these methylotrophic bacteria are able to fix

atmospheric nitrogen, to the benefit of wood decomposing fungi (Hoppe *et al*., 2015; Kielak *et al.*, 2016).

3.2. Fungal communities in dead wood

Fungi are responsible for larger part of dead wood decomposition as a result of their effective cellulose and lignin-degradation abilities (Van der Wal *et al*, 2013). The fungal communities in dead wood was well understood before the introduction of high-throughput sequencing approaches in microbial ecology (Boddy and Watkinson, 1995; Boddy, 2001). This is because many fungi associated with dead wood can be grown in culture. As a result, their enzymatic systems and genetic relatedness have been studied in detail (Tedersoo *et al.*, 2003; Lindahl and Finlay, 2006).

The tree species of origin have profound effects on the fungal decomposer community and diversity in dead wood (Kubartová *et al.*, 2012; Hoppe *et al.*, 2016; Purahong *et al.*, 2017). The selectivity of fungal colonization of different tree species is due to differences in lignin, nitrogen and phosphorus content and differences in the quality of hemicelluloses (Weedon *et al.*, 2009). Non-structural phenolics and aromatics also result in differences in fungal colonization between different tree species and may be more important than lignin and cellulose content of dead wood (Kögel-Knabner, 2002; Weedon *et al.*, 2009). Xylem structure and wood density may also have profound effects on the rate of desiccation, and thus water content, which results in differences in the preferences of different fungal species for dead wood (Berry and Roderick, 2005; Weedon *et al.*, 2009).

The chemical composition of dead wood changes with progression of wood decay and this affects the fungal community composition. The most important of these changes in the dead wood environment, are the C/N and C/P ratios and the cellulose and lignin content (Purahong *et al.*, 2017). As wood density decreases with decomposition, there is a decrease in the C/N ratio, and an increase in moisture content and the ratio of lignin to cellulose (Rajala *et al.*, 2011; Rajala *et al.*, 2012; Purahong *et al.*, 2017). Higher ratios of lignin:cellulose results in communities dominated by the white-rot fungi because of their specialized lignin-decomposing abilities. Very low C/N ratios result in communities dominated by ectomycorrhizal fungi (Rajala *et al.*, 2015), since these fungi are able to source carbon from plant hosts, and reach dead wood in search of nitrogen (Buée *et al.*, 2007).

The dominant fungi in the early stages of wood decomposition include many species that are present as latent colonists in living trees (Parfitt *et al.*, 2010). But over time, many other fungi colonize dead wood via spores from soil, litter and other pieces of dead wood (Rajala *et al*., 2011). Not all fungi present in tissues of living trees proliferate once the tree dies, and it remains unclear which environmental factors determine the composition of wood decomposer fungal communities in living trees (Parfitt et al., 2010). The variability in early decomposer fungal communities is in part due to antagonistic interactions among early arriving fungi and new colonizing community members (Fukami *et al*., 2010). The outcomes of these interactions shape the fungal communities in early and intermediate dead wood decomposition, based on the sequence of arrival of interacting species (Fukami *et al*., 2010; Dickie *et al.*, 2012). This explains the random distribution of fungi often observed between different pieces of dead wood in the same landscapes (Baldrian *et al*., 2012).

Initially, the fungal communities in individual pieces of dead wood from the same tree species are dominated by only a few fungal species (Parfitt *et al*., 2010). As the decomposition proceeds, the fungal communities in wood pieces in close proximity become more similar, most likely because fungi from the surrounding soil start to colonize the wood (Rajala *et al*., 2011). After some time, the fungal communities in different individual decaying logs in a single landscape converge to have similar fungal communities (Rajala *et al.*, 2011). This accumulation of fungi in dead wood results in increases in species richness, and the highest levels of species richness is thus recorded in the final stages of decomposition (Rajala *et al.*, 2011). Fungal communities follow a successional gradient over the different stages of wood decomposition (Rajala *et al.*, 2011) and peak in wood that is close to being completely decomposed (Rajala *et al.*, 2011).

The decomposition of dead wood in boreal and temperate forests is characterized by a progression from communities dominated by ascomycetes to basidiomycete-dominated communities in the later stages (Rajala *et al.*, 2012). In the intermediate stages, when the brown- and white-rot fungi are abundant, the rate of decay is at its highest (Rajala *et al.*, 2012). Brown-rot fungi are more abundant in the early and intermediate stages of decomposition, which corresponds to the presence of cellulose and hemicellulose and their lack of ability to utilize lignin (Rajala *et al.*, 2015). The white-rot fungi are present during all stages of decomposition, but are especially abundant in the

middle and late stages of decomposition, due to their specialized lignin-decomposing abilities (Rajala *et al.*, 2015).

Ectomycorrhizal fungi outcompete brown- and white-rot fungi in the final stages of wood decomposition. At this stage, all organic substrates have been depleted, and ectomycorrhizal fungi continue to grow because they are able to obtain carbon from their plant symbionts (Baldrian 2009; Rajala *et al.*, 2012). Although most ectomycorrhizal colonization of dead wood occurs through contact with mycorrhizal chords, ectomycorrhizal fungi have been shown to be able to colonize *P. abies* dead wood before it is in direct contact with soil and thus not accessible through mycelial growth. Thus, these ectomycorrhizal fungi, which include species of *Russula* and *Lactarius*, could reach dead wood via spores and exist as facultative saprotrophs in dead wood (Hibbett *et al.*, 2000; Baldrian, 2009; Rajala *et al.*, 2011).

4. Microbial diversity in plant litter

4.1. *Bacterial communities in plant litter*

Bacterial extracellular enzymes are not present among the most abundant proteins present in plant litter (Schneider *et al.*, 2012). As a result, it is assumed that the bacteria inhabiting plant litter do not make a significant contribution to litter decomposition. It is likely that the bacteria in litter utilize low molecular weight compounds as substrates, which are often only available as by-products of fungal litter decomposition (Schneider *et al.*, 2012). Utilizing metabolites produced by other organisms, without contributing to decomposition is known as “cheating”. This cheating behavior is common in litter-inhabiting bacteria (Velicer, 2003). The abundance of bacteria in litter is positively correlated with the concentration of extracellular fungal enzymes, which provide further evidence that most litter-inhabiting bacteria benefit from fungal cellulose and lignin decomposition (Schneider *et al.*, 2012).

Some of the bacteria growing within and on the surfaces of green pre-senescent leaves are among the first colonizers of litter after leaf senescence. However most of these bacteria are completely replaced within a few months after senescence (Tláskal *et al.*, 2016). Soon after leaf senescence, there is a rapid increase in bacterial groups able to utilize low molecular weight carbohydrates and fungal hyphae as a substrate (Brabcová *et al.*, 2016; Tláskal *et al.*, 2016). There is a rapid increase in fungal biomass during the early stages of decomposition and as a result many bacteria with the

ability to utilize fungal hyphae as a substrate also increase in abundance (Tláskal *et al.*, 2016). Bacteria are thus mostly secondary consumers of fungal mycelium and their secreted metabolites and are not directly affected by the quality and chemistry of litter (Štursová *et al.* 2012; Urbanová *et al*., 2015; Brabcová *et al.*, 2016).

Members of the Proteobacteria, Actinobacteria and Bacteroidetes are the dominant bacteria in all stages of litter decomposition in temperate forests (Štursová *et al.*, 2012; Purahong *et al*., 2016a). Species of *Frigoribacterium*, *Sphingomonas*, *Rhizobium*, *Pseudomonas*, *Massilia* and *Methylobacterium* are common in the initial stages of decomposition of litter in many different plant species and are well-known as endophytes or epiphytes (Tláskal *et al.* 2016; Purahong *et al.*, 2016a). Many of these bacteria that are present during the early stages of decomposition are well adapted to persist over all stages of litter decomposition (Purahong *et al.*, 2016a; Tláskal *et* al., 2016).

In the final stages of litter decomposition, various *Streptomyces* species and members of subgroup 1 Acidobacteria are often the most abundant bacteria. However, when the pH in litter is inordinately low, the actinomycetes are not present even though they are among the most efficient litter decomposing bacteria (Lauber *et al.*, 2009; Štursová *et al.*, 2012). *Streptomyces* species are known to be able to utilize cellulose and lignin from plant detritus and oxalic acid released by fungi (De Boer *et al.*, 2005; Purahong *et al*., 2016a). In the litter of both *Quercus petraea* (Sessile Oak) and *P. abies* (Norway spruce), species of the group 1 Acidobacteria, *Burkholderia*, *Bradyrhizobium*, *Mucilaginibacter* were among the dominant bacteria in the late stages of decomposition (Hoppe *et al.*, 2015; Rinta-Kanto *et al.*, 2016; Tláskal *et al.*, 2017;). Most of these bacteria are known to be able to utilize cellulose as substrate, in addition to fungal mycelium (Štursová *et al*., 2012; Tláskal *et al*., 2016).

4.2. Fungal communities in plant litter

Fungi are responsible for the largest contribution to terrestrial plant litter decomposition (Schneider *et al*., 2012). The fungal biomass in litter can be up to three times higher than that of bacteria, although more diverse assemblages of bacteria are present in litter (Baldrian *et al.*, 2012). Fungal species producing large mycelial networks are usually detected at high relative abundance

values in high-throughput sequencing studies because of their considerable biomass (Baldrian *et al.*, 2012). This results in the dominant fungal OTUs comprising as much as 30% of these communities (Baldrian *et al.*, 2012). In contrast, litter-inhabiting bacteria have a more homogenous distribution of several different species (Baldrian *et al.*, 2012). The dominant fungal OTUs in litter are heterogeneous, with few OTUs that are highly abundant in some patches of litter and absent in other patches (Baldrian *et al.*, 2012).

Fungal litter decomposition can be broadly classified into three different successional stages and similar to wood, different fungal species are dominant at different stages of decomposition (Voříšková and Baldrian, 2013). During the early stages, there is rapid decay of litter, with high cellulolytic activity and a high rate of carbon loss. During the intermediate stage, lignin starts to be metabolized by white-rot fungi, while small amounts of cellulose are released from the lignin-cellulose complex. Thus, there is still limited cellulose decomposition during this stage (Voříšková and Baldrian, 2013). During the late stage of litter decomposition, the substrate consists mainly of lignin that is decomposed slowly (Voříšková and Baldrian, 2013).

Phyllosphere endophytic fungi are the most important source of litter decomposer fungi in the early stages of decomposition (Osono, 2006; Žifčáková *et al.*, 2011; Voříšková and Baldrian, 2013; Unterseher *et al*., 2013; Purahong *et al.*, 2016a; Szink *et al.*, 2016). The earliest signs of cellulase activity are detectable right before leaf abscission, and there have been indications that cellulose decomposition starts before leaves reach the ground (Voříšková and Baldrian, 2013). Many leaf-endophytic fungi have efficient saprophytic nutritional modes and are able to utilize the substrates present in litter (Voříšková and Baldrian, 2013). In contrast, most epiphytic fungi are not detected during the early stages of litter decomposition (Peršoh *et al.*, 2013). The fungi shared between the phyllosphere and litter are specific to leaves and are most often not present in the dead wood of the same trees, although the same substrates are present in both these habitats (Unterseher *et al.*, 2013).

The fungal orders most commonly present in the phyllosphere of trees and the resulting leaf litter include to the Capnodiales, Xylariales, Diaporthales and Pleosporales (Unterseher *et al*., 2013). The most common genera present in both the phyllosphere and litter include species of *Cladosporium*, *Epicoccum*, *Alternaria*, *Aspergillus*, *Penicillium*, *Mucor* and *Mortierella* (Szink *et al.*, 2016). Species of *Mycosphaerella*, a genus that includes common endophytes, are often highly

abundant in the early stages of litter decomposition (Voříšková and Baldrian, 2013; Unterseher *et al.*, 2013; Purahong *et al.*, 2016a). Some endophytic fungi are able to persist until the late stages of decomposition (Unterseher *et al.*, 2013), possibly because of the presence of byproducts of fungal lignin-degradation which are easily utilized by these endophytic fungi (Peršoh *et al.*, 2013). It has also been suggested that some endophytic fungi in the leaf litter of beech trees are able to decompose lignin (Osono and Takeda, 1999). These include members of *Xylaria*, *Pestalotiopsis* and *Trichoderma*.

The fungal community composition during the very late stages of litter composition remains stable with extremely low rates of litter decomposition (Schneider *et al.*, 2012; Voříšková and Baldrian, 2013). During this stage, there is an increase in the relative abundance of basidiomycetes, most likely due to the abundance of lignin and the absence of easily utilized sugars. White-rot basidiomycetes are the only group of organisms known to effectively utilize lignin (Osono, 2007). Therefore, the white-rot basidiomycetes are the most common fungi present during the final stages of litter decomposition (Voříšková and Baldrian, 2013). Basidiomycete yeasts are also common fungi during this stage of decomposition, likely because they are are effective at utilizing the very small amounts of remaining cellulose (Štursová *et al*., 2012; Voříšková and Baldrian, 2013).

5. Conflict and cooperation between bacteria and fungi in plant detritus

The first review of interactions between wood decomposing fungi and bacteria was by Blanchette and Shaw (1978). At this time, it was already known that plant-detritus decomposer communities are structured based on interactions between bacteria and fungi. More recently, several reviews on the interactions between bacteria and fungi in decomposing litter and dead wood have been published. De Boer *et al*., (2005) reported the bacteria-fungal interactions in soils containing mainly plant-derived detritus, and how the presence of fungi created novel bacterial niches in soil. De Boer and Van der Wal (2008) and Johnston et al. (2016) reviewed the interactions between bacteria and wood decomposer fungi. Thus, we provide only a brief overview of interactions, focusing on the most recent studies using high-throughput sequencing.

The ability to produce antibiotics is common in litter- and wood-inhabiting bacteria and fungi (De Boer *et al.*, 2005; De Boer and Van der Wal, 2008; Caldeira *et al*., 2008; Popova *et al.*, 2009; De

Boer *et al.*, 2010). It has been hypothesized that non-motile soil bacteria need to protect the surrounding substrates by preventing other organisms from colonizing these valuable resources (Whipps, 2001; Challis and Hopwood, 2003; De Boer et al., 2005). In general, many decomposer organisms are believed to have evolved mechanisms to protect the scarce resources in decomposing wood and litter (Waksman, 1961; Martínez, 2008). However, little evidence is available to suggest that different organism groups are inhibited by antibiotics *in situ* (Murray and Woodward, 2003; Davies, 2006).

One of the best-known functional roles of bacteria in decomposer communities is the provision of nitrogen during wood-decomposition (De Boer and Van der Wal, 2008; Hoppe *et al.*, 2015; Kielak *et al.*, 2016; Purahong *et al.*, 2016b; Tláskal *et al.*, 2017). The growth of fungi on dead wood is often restricted by a lack of nitrogen and nitrogen-fixing bacteria can relieve this shortage (Cowling and Merrill, 1966). Bacteria capable of atmospheric nitrogen fixation are common in dead wood habitats, and often associated with fungi in dead wood (Hoppe et al., 2015; Hoppe et al., 2016). A strong positive correlation exists between the copy number of the nitrogenase encoding *nifH* gene and the rate of wood decomposition (Hoppe *et al.*, 2015). The need for nitrogen is the likely reason for high abundance of members of the Rhizobiales in the intermediate and late stages of wood decomposition (Hoppe *et al.*, 2015; Tláskal *et al.*, 2017).

Fungi are able to restrict bacterial cellulose decomposition and shape the bacterial community by modifying surrounding pH. Most fungal cellulose degradation occurs in acidic environments, with pH levels between 4 and 5 (Thompson, 1983; Baldrian, 2008). Organic acids are released into the substrate during the fungal wood and litter decomposition process, which creates an acidic environment (De Boer *et al*., 2010). This decrease in pH selects for specific bacterial communities capable of growth under low pH conditions (McCarthy, 1987; Bååth, 1996). For example, the Actinobacteria are common in dead wood and litter, but are not detected in these environments when the surrounding pH is low (Lauber *et al.*, 2009). Cellulose degradation in bacteria also requires the substrate to be within neutral pH ranges (McCarthy, 1987). Due to the acidity in the surrounding environment, the Acidobacteria are often abundant in dead wood and litter as they are known to be effective utilizing cellulose at low pH (Lladó *et al.*, 2016). Therefore, the bacterial community composition in dead wood and litter is largely dependent on the fungal communities and their activity in these environments.

Although litter and dead wood do not always provide suitable substrates for most bacteria, the fungi in these habitats provide a substrate for many bacterial species through consumption of fungal mycelium and fungal metabolic by-products such as water soluble sugars and phenolics (De Boer et al., 2005; Folman *et al.*, 2008; Leveau and Preston, 2008; Valášková *et al.*, 2009; Hervé *et* al., 2014; Brabcová et al., 2016; Baldrian et al., 2016). Most bacteria are not able to utilize any of the available recalcitrant plant-derived substrates but are adapted to utilize substrates derived from dead fungal mycelium (Brabcová et al., 2016). The flow of carbon from plant-derived substrates to secondary consumers of fungal biomass has been suggested to form a unique trophic level in plant-based ecosystems, which has been referred to as the sapro-rhizosphere (Ballhausen and De Boer, 2016). Several species of bacteria are frequently detected in close proximity to fungal hyphae and include the genera *Ewingella, Luteibacter rhizovicinus, Pedobacter, Pseudomonas* and many members of the Burkholderiaceae (Noll et al., 2010; Brabcová et al., 2016; Kielak *et al.*, 2016; Tláskal *et al.*, 2017).

6. The importance of understanding of plant-detritus habitats

Disturbances in natural environments are common in biological systems, but their frequency and scale is increasing and this poses a great threat to ecosystem diversity and function (Hansen *et al*., 2001). Disturbances in plant communities can be natural, such as large-scale tree decline due to insect- or disease outbreaks (Dinoor and Eshed, 1984; Ayres and Lombardero, 2000) , or because of anthropogenic factors, such as the harvesting of wood (White and Jentsch, 2001; Garnier *et al.*, 2006). Frequent disturbances in plant communities also affect plant-associated microbial communities (Van der Heijden *et al.*, 2008; Baldrian, 2017). At local scales, the loss of soil and decomposer microorganisms may result in greater vulnerability to invasion by alien plant species (Van der Heijden *et al.*, 2008; Van der Putten, 2010; Philippot *et al.*, 2013) and plant decline due to the loss of beneficial microorganisms (Garbeva *et al.*., 2004; Raaijmakers *et al*., 2009; Berendsen *et al.*, 2012). Plants become more susceptible to pathogens when their native beneficial bacteria and fungi are not present (Garbeva *et al.*, 2004; Raaijmakers *et al*., 2009; Berendsen *et al.*, 2012). Some of these microbial communities require long periods of recovery after disturbance. For example, some ectomycorrhizal fungi can may take up to 90 years to reach pre-disturbance biomass levels in regrowing forests after clearcutting or wildfire (Spake *et al*., 2015).

At global scales, frequent disturbances are slowly affecting the world's carbon balance, while some species are also at risk of extinction due to habitat destruction (Kasischke *et al*., 1995; Saleska *et al.*, 2003).

The effect of disturbances in plant-decomposer microbial communities is well understood in forests where intensive harvesting has significant effects on the surrounding microbial diversity (Toivanen *et al*., 2012; Juutilainen *et al.*, 2014). Some highly specialized wood-inhabiting fungal species are so sensitive to the removal of specific dead-wood niches, that they are now red-listed (Heilmann-Clausen and Christensen, 2003; Ovaskainen *et al.*, 2013). Some of the fungi previously listed as critically threatened include *Omphalina* epichysium, *Camarops tubulina* and *Lepiota boertmannii* (Heilmann-Clausen and Christensen, 2003). It is known that intensive management or harvesting of wood in forests results in an increase in generalist fungal species, while some specialist fungi are lost from the landscape (Nordén *et al*. 2013). Many of these red-listed, specialist fungal species which are readily lost after the recurrent removal of dead wood are the polypores. These are also among the most important decomposers of wood (Boddy and Heilmann-Clausen, 2008; Junninen and Komonen, 2011).

The presence of wood at different stages of decay is important in the development of soil as a fungal habitat, and is an important secondary habitat for some fungal species (Mäkipää *et al.*, 2017). A large number of fungi in boreal forests occur exclusively in dead wood, and the loss of this substrate results in the loss of some wood-specific fungi from the environment (Mäkipää *et al.*, 2017). Some fungi inhabiting dead wood are also present in soil directly under the wood Consequently, fungal communities in soil and wood in close proximity is often more similar than logs farther apart in the same landscape (Mäkipää *et al.*, 2017). The fungal species richness increases until it resembles the community present in the surrounding soil (Mäkipää *et al.*, 2017). During the final stages of decomposition, brown- and white-rot fungi are replaced by soil saprotrophs and ectomycorrhizal fungi such as species of *Piloderma*, *Tylospora* and *Russula* (Mäkipää *et al.*, 2017).

Fire is an important source of disturbance of plant communities. In many ecosystems, frequent fires are required to maintain the biodiversity of plants, and this can also result in the formation of more diverse soil microbial communities. Furthermore, fire plays an important role in remineralization of phosphorus and nitrogen and regulating plant species diversity (Bond and Van

Wilgen, 1996). However, inordinately recurrent fires can diminish soil microbial diversity, and as a result, also decomposer communities (Treseder *et al.*, 2004; Hart *et al.*, 2005; Hamman *et al*, 2007). For example, the rates of litter degradation in Spruce and Aspen forests in the post-fire environment is significantly affected due to changes in soil fungal communities (Holden et al., 2013). As a result of the disturbance, the rates of litter degradation were substantially lower than in sites not affected by fire (Holden et al., 2013).

Fragmentation is common in landscapes where there is degradation or loss of habitat (Nordén *et al.* 2013), and this results in small isolated pieces of healthy habitat patches. Most forms of disturbance, such as fire, invasion by alien plant species, clearcutting or intensive harvesting may result in the fragmenting of habitats. Many fungal species are lost from landscapes when forest habitats become fragmented, most likely because the diversity of niches are reduced and many specialist wood decomposer fungi are not well dispersed (Nordén *et al*., 2013). This effect is not only observed in single forest stands, but also occurs across larger landscapes (Nordén *et al.* 2013). While red-listed fungi disappear, ubiquitous generalist species increase in number because of more available resources and possibly by the removal of competitive fungi (Nordén *et al.* 2013). Similarly, in Mediterranean shrublands, habitat fragmentation results in a decrease in enzymes in decomposing litter (Maltz *et al*., 2017). Along with a loss in specialist decomposer species, habitat fragmentation also results in a loss of interactive species associations across landscapes (Abrego *et al.*, 2017).

7. Relevance of dead wood and litter to the infructescences of *Protea repens*

Leaf litter and dead wood are the most common sources of senescent or dead plant matter in terrestrial ecosystems, although the composition and quantities differ depending on the plant species of origin (Kubartová *et al.*, 2012; Hoppe *et al.*, 2016; Tláskal *et* al., 2016; Purahong *et al.*, 2017). The microbial diversity and the processes in dead plant matter is well understood although they are not always well characterized in different ecosystems. However, by studying unusual senescent or dead plant tissues can help to improve our understanding of processes linked to decomposer communities such as dispersal, succession and competition.

One example of a senescent plant based microbial habitat is the infructescences of serotinous *Protea* species. The mature seeds of serotinous *Protea* species are stored in woody cone-like structures also called infructescences. *Protea* infructescences are formed from inflorescences after

flowering and provide a protective surrounding to seeds while they mature, and thereafter it provides a protective enclosure to seeds until the fire disturbance which is necessary for propagation (Bond, 1985). *Protea* infructescences present a tightly closed, moist surrounding for seeds and which usually remains in this state until fire or they have been completely degraded. These structures can remain closed and intact on *Protea* trees for several years even though it is colonized by various bacteria (Human *et al*. 2016; Human *et al*., 2017) and fungi (Lee *et al.*,, 2005) and often attacked by insects (Coetzee and Giliomee, 1987).

The sclerophyllous leaves of *Protea* trees are more resistant to degradation of *Protea* infructescences than most plant species because of its high lignin content. The outer bracts of *Protea* infructescences are formed from the same leaves and thus are also resistant to decomposition. However, labile carbon compounds are present during in newly-formed infructescence formed after flowering from the large amounts of simple nectar sugars (Nicolson and Van Wyk, 1998). Thereafter, lignin and cellulose are the predominant remaining substrates (Mitchell and Coley, 1987), with the largest proportion being lignin after a year when these structures still remain intact on the trees. Due to these substrates being utilized at different rates by different fungal and bacterial groups, there is the potential for a clear successional path of different microbial groups with different metabolic capabilities.

The major difference between dead wood and litter and *Protea* infructescences is the limited routes of dispersal for decomposer microorganisms into *Protea* infructescences. As previously suggested in this review, many early decomposers are already present in wood and litter, and many of these are the first colonizers. The same is arguably also true for *Protea* infructescences. Then, wind and water dispersed bacterial and fungi may also reach open inflorescences during flowering. However, after flowering, insects and mites are the only route of entry into infructescences (Coetzee and Giliomee, 1987). These characteristics make *Protea* infructescences a unique and interesting microbial habitat, although the complete microbial diversity has not been studied to date.

8. Conclusions

The studies reviewed here suggest that fungi are far more important than bacteria in the decomposition of dead plant matter. Fungal communities have the ability to utilize all the different substrates present in plant tissues, while most bacteria have very limited degradation abilities. However, bacteria appear to be important associates of fungal decomposers, often responsible for the supply of nitrogen essential for decomposition to proceed. The exact roles of bacteria during decomposition, and whether fungi can survive without bacteria present remains unclear.

This review has illustrated that the fungal and bacteria diversity in dead wood and litter in temperate and boreal forests of the Northern Hemisphere is relatively well characterized. It can be argued that forests in the northern hemisphere are important in terms of global carbon cycles, but they are less important in terms of global plant biodiversity. For example, the Mediterranean-type heathland in the southwestern parts of South Africa is a biodiversity hotspot with 9000 different plant species present (Manning and Goldblatt, 2012). Yet, very little is known regarding the bacteria and fungi responsible for carbon and nitrogen cycles in the habitats surrounding any of these plants.

Healthy ecosystems require appropriately diverse and functioning microbial decomposer communities (Bardgett and Van der Putten, 2014). These communities are responsible for productive plant communities through the production of soil organic matter, which is essential for plants to grow. Decomposer communities are also abundant in soil and the rhizosphere and can protect plants against infection by pathogens. Therefore, the disturbance of decomposer communities may be to the detriment of whole plant communities. In some ecosystems, where the function and diversity of decomposers are not well understood, the consequences of disturbance may be greater. Therefore, we argue that in the age of high-throughput sequencing in microbial ecology, there needs to be greater emphasis on studying new types of environments. This will lead to a better understanding how widespread specific microbial decomposer groups are and how they respond to disturbance.

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Appendix 1. The substrate content in plant biomass

Cellulose is the most abundant carbon-based polymer on earth (Perez *et al*., 2002), and comprises roughly 45% of dry wood (Perez *et al.*, 2002). Leaf litter from different plant species can consist of 10 to 50 % cellulose (Berg and McClaugherty, 2003). Cellulose consists of D-glucose polymers, joined by β-1,4 linkages to form cellobiose, which in polymerized to form long chains linked by hydrogen bonds (Thompson, 1983).

Hemicellulose is comprised of chains of monosaccharide sugars, and is less abundant in the environment (Berg and McClaugherty, 2003). Even though hemicellulose is incorporated into plant fibers, it does not provide structural support to plant fibers (Berg and McClaugherty, 2003). The ratio of hemicellulose to cellulose could be between 0.7 – 1.2 depending on the plant species (Berg and McClaugherty, 2003). Hemicellulose is the plant-based carbon polymer with the lowest molecular weight (Perez *et al.* 2002). It is formed from polymers of short-chain sugars such as D-xylose, D-glucose, D-arabinose, D-mannose, D-galactose and other less common short chain sugars (Thompson, 1983).

Lignin accounts for 30% of the organic carbon biomass on earth and is the second most abundant natural carbon polymer (Boerjan *et al.*, 2003). The exact composition and quantity of lignin varies dramatically between different plant species but usually, plant litter consists of 15 to 40% lignin. (Berg and McClaugherty, 2003). Lignin content in leaf litter from deciduous trees is usually much lower than that in litter consisting of needles from conifers (Berg and McClaugherty, 2003). Lignin provides structural support to plant cells, and is important in maintaining structural integrity and stiffness in plant stems (Perez *et al*., 2002; Boerjan *et al*., 2003). It is a non-water-soluble polymer, formed by aromatic polymers of phenylpropanoids (Perez *et al*., 2002).

Non-structural nutrient components in plant litter

Studies on the nitrogen and phosphorus in litter are rarely able to track these elements to their sources and therefore the only the total nitrogen and phosphorus content is can be determined (Thompson, 1983; Berg and McClaugherty, 2003). Nitrogen can comprise 0.3 – 3% of plant leaf litter is as low as 0.02% in dead branches. Phosphorus is mainly present in nucleic acids, peptides and phospholipids and comprise between 0.2 – 3% of litter (Thompson, 1983; Berg and

McClaugherty, 2003). Although these compounds are present in living leaves, most plants reabsorb large quantities thereof prior to senescence, which dramatically reduces their concentrations in litter (Staaf and Berg, 1981; Berg and McClaugherty. 2003). For example, the nitrogen content in litter from *Pinus* sp. growing in nutrient poor soils can decrease to about 30% of the original quantity in the time prior to leaf abscission. In nitrogen-rich soils, the resorbtion of nitrogen, phosphorus and soluble sugars is limited and litter may have larger concentrations of nitrogen and phosphorus (Staaf and Berg, 1981). Other compounds comprise a very small portion of carbon-derived substrates, and include chitin, lipids and proteins (Thompson, 1983).

Appendix 2. The degradation of plant biomass by bacteria and fungi

Cellulose

Three enzymes are responsible for cellulose decomposition. The main cellulose degrading enzymes are the endo-β-1,4-endoglucanases, responsible for the random cleavage of β-1,4-glycosidic bonds to produce shorter cellulose chains (Thompson, 1983; Ljungdahl and Eriksson, 1985; Lynd *et al.*, 2002). Exo-β-1,4-endoglucanases can hydrolyze glucose or cellobiose from the ends of cellulose (Ljungdahl and Eriksson, 1985; Thompson, 1983). Finally, β-1,4-glycosidases hydrolyze cellobiose to glucose (Thompson, 1983; Ljungdahl and Eriksson, 1985; Lynd *et al.*, 2002).

Bacterial decomposition of cellulose can occur via aerobic or anaerobic processes (Ljungdahl and Eriksson, 1985; Lynd *et al.*, 2002). Anaerobic cellulose decomposition is mostly present in Gram-positive bacteria, such as members of *Clostridium* and *Rumunicoccus*, usually present in the rumen where they ferment cellulose (Leschine, 1995; Lynd *et al*., 2002). Aerobic cellulose decomposers include members of several actinomycete genera (*Streptomyces*, *Micromonospora* and *Thermobifida*), some members of the Enterobacteriaceae such as *Erwinia* and members of *Bacillus* and *Pseudomonas* (Lynd *et al.*, 2002). These are some of the most well-known cellulose decomposers, however this list could be extended with several more taxa (Thompson, 1983). Many of the bacterial species able to produces cellulases, are also arthro- or endospore formers. Furthermore, many cellulose utilizing bacteria are able to produce growth-inhibitory secondary metabolites, which suggests that the dependence on cellulose as substrate requires further

competitive abilities (Lynd *et al.*, 2002). Aerobic cellulose decomposition occurs via so called non-complexed cellulose systems, which involves the secretion of extracellular cellulases (Lynd *et al.*, 2002).

The three different guilds of decomposer fungi have different cellulolytic abilities (Ljungdahl and Eriksson, 1985; Thompson, 1983). The most complete cellulose decomposition capabilities are present in white-rot and ectomycorrhizal fungi (Thompson, 1983). The brown-rot fungi are known to produce endo-β-1,4-endoglucanases and β-1,4-glycosidases, but lack exo-β-1,4-endoglucanases (Thompson, 1983; Baldrian, 2008). Some soft-rot fungi have complete cellulase systems with all three cellulose-digesting enzymes present, but many lack the exo-β-1,4-endoglucanases required to decompose structurally complex cellulose (Thompson, 1983; Ljungdahl and Eriksson, 1985). Efficient cellulose-decomposing soft-rot fungi include *Trichoderma*, *Penicillium* and *Fusarium* (Thompson, 1983). Complete cellulose degrading enzyme systems are usually present in common litter-decomposing fungi and in ectomycorrhizal fungi (Baldrian, 2008).

Hemicellulose

Hemicellulose is easily degraded and is a preferred substrate in the early stages of litter and wood decomposition. The decomposition yields monomeric sugars and acetic acid (Eriksson *et al*., 2012). The enzymes responsible for the biodegradation of hemicellulose are broadly referred to as hemicellulases, which is further classified according to the monomeric sugar component of the hemicellulose they act on (Eriksson *et al*., 2012). The biodegradation of hemicellulose is widespread in bacteria from the rumen (Dehority, 1973) and termite guts (Schäfer *et al*, 1996) and many different bacteria in soil (López-Mondéjar *et al.*, 2016). Overall, the enzymatic capacity to decompose hemicellulose is more widely distributed among soil bacteria than the ability to decompose cellulose. Some of the bacterial genera well-known for acting on hemicelluloses include *Streptomyces, Bacillus*, *Pseudomonas* and members of the Enterobacteriaceae (Koeck *et al.*, 2014).

Saprobic ascomycetes and soft-rot fungi which are among the first fungi colonizing plant detritus are very efficient decomposers of hemicellulose (Rajala *et al.*, 2012). The brown- and white-rot fungi are also very efficient at degrading hemicellulose, but are also able can continue to utilize other, more recalcitrant substrates when hemicellulose becomes limited in the intermediate stages of decomposition (Blanchette, 1995; Baldrian, 2008; Eastwood *et al.*, 2011). The monomeric

sugars produced as a result of fungal hemicellulose decomposition are often quickly colonized by fast-growing bacteria (Purahong *et al.*, 2016).

Lignin

Bacterial degradation of lignin is uncommon and has been reported only in a few bacterial groups. The actinomycetes are one such group where lignin decomposition has been recorded in genera such as *Streptomyces*, *Nocardia* and *Kitasatospora* (Kirby, 2006). This activity has not been described in many non-actinobacterial taxa, and many authors remain skeptical of the abilities of bacteria to decompose lignin.

Fungal lignin degradation occurs by the production of laccase, lignin peroxidase and manganese peroxidase enzymes (Hatakka, 1994). Effective lignin degradation in fungi is almost exclusively associated with the white-rot basidiomycetes (Hatakka, 1994; Blanchette, 1995). The lignin degradation ability of brown-rot fungi is inferior to even that of actinomycete bacteria, since soft-rot fungi can only modify the chemical structure of lignin, and are not able to completely metabolize the substrate (Eriksson *et al.*, 2012). Some ectomycorrhizal fungi decompose lignin, but they do not have saprophytic phases, and it is suggested that they oxidize lignin, primarily to mobilize nitrogen (Lindahl *et* al., 2007; Lindalh and Tunlid, 2014). Ericoid mycorrhizal fungi are also able to utilize and degrade lignin, and they are known to have saprophytic metabolic lifestyles (Bending and Read, 1997).

Mineral elements required for decomposition

The mineralization and immobilization of nitrogen is an important process during the decomposition of plant residues, and nitrogen concentration in detritus substrates has important effects on the microbial decomposer communities. (Cowling and Merrill, 1966; Barraclough, 1997). The sources of nitrogen in plant residues include the nitrogen present in proteins in physical plant biomass, already mineralized nitrogen and the nitrogen contained in microbial biomass (Mary *et al*., 1996). Previously, it had been assumed that all forms of nitrogen are demineralized and released into the ammonium pool (McNeill and Unkovich, 2007). However, more recent studies have shown that nitrogenous small organic molecules are often absorbed by members of the microbial community (Barraclough, 1997; Gibbs and Barraclough, 1998; McNeill and Unkovich, 2007). As a result, as much as 40% of the entire nitrogen pool in dead plant matter

never reaches the environment as ammonium, and is rather incorporated into new biomass (McNeill and Unkovich, 2007).

The concentration of nitrogen in the dead plant material restricts the rates of decomposition processes (Waring, 2012; Strickland and Rousk, 2010). The effects of excess nitrogen on plant-residue decomposition is different in the early and late stages of decomposition (Berg and Matzner, 1997). In the initial stages of decomposition, when mainly soluble polysaccharides, hemicellulose and cellulose are metabolized, nitrogen is an important requirement for growth, and a lack of nitrogen can restrict decomposition completely. Fungi have lower nutrient requirements than bacteria, and thus when nitrogen and phosphorus levels are low, fungal growth rates are higher than those of bacteria (Strickland and Rousk, 2010).

In the late stages of decomposition, any excess nitrogen has a limiting effect on lignin decomposition by white-rot fungi (Berg and Matzner, 1997). Delignification only starts when a state of nitrogen depletion in the substrate is reached (Fenn and Kirk, 1981; Blanchette, 1991) and if mineral nitrogen is added to the substrate, lignin decomposition is stationary (Blanchette, 1991; Berg and McClaugherty, 2008). If lignin decomposition is stopped during the late stages of decomposition, and the cellulose and hemicellulose in the substrate is depleted, then all decomposition processes cease until the added nitrogen is depleted (Berg and McClaugherty, 2008).

The sources of phosphorus in plant residues include nucleic acids, phospholipids, and moribund microbial biomass (Bünemann and Chondron, 2007). Phosphorus in plant litter accumulates linearly over time as litter mass is lost due to decomposition (Bünemann and Chondron, 2007). However, most phosphorus in decomposed plant material is contained in microbial biomass, especially in bacterial cells (Barsdate *et al.*, 1974; Coleman *et al*., 1977). The microbial uptake of phosphorus is much higher than the rate of mineralization, which means that only small amounts of phosphorus are released into the surrounding environment (Barsdate *et al.*, 1974). Fungal decomposers do not respond significantly to excess phosphate, but bacteria in litter and dead wood have a clear benefit from excess phosphorus (Berg and McClaugherty, 2008; Strickland and Rousk, 2010). An excess of available phosphorus results in bacterial decomposer growth rates similar to those of fungi. When excess nitrogen and phosphate is present, bacterial biomass may exceed that of fungi (Strickland and Rousk, 2010).

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Chapter 2

Inordinantly frequent fires impact negatively on bacterial diversity in a biodiversity hotspot (2018) Zander R. Human, Casparus J. Crous, Francois Roets, Michael J. Wingfield and Z. Wilhelm de Beer and Stephanus N. Venter

Submitted, in review

Inordinantly frequent fires impact negatively on bacterial diversity in a biodiversity hotspot

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Abstract

The bacterial communities in and around plants provide important benefits, such as protection against pathogens and in some cases also sources of nitrogen and phosphorus. The environment surrounding plants is also a habitat for diverse bacterial species, primarily responsible for decomposition of moribund plant biomass. This provides crucial ecosystem services such as mineralizing compounds trapped in wood. In this study, we determined the effect of a fire disturbance on the bacterial communities in a fynbos landscape dominated by *Protea repens* using 16S rRNA amplicon sequencing. Four years after fire, the bacterial diversity in leaf-litter and *P. repens* infructescences had not reached pre-fire levels. In addition, the bacterial community composition in litter, newly-formed infructescences and soil remained different to that in an unburnt site. The bacteria inhabiting *P. repens* infructescences were similar to bacterial taxa well-known from decomposing wood and litter. Several of these dead-wood and litter-inhabiting bacteria are known associates of fungal decomposers and some are known to utilize fungal mycelium as a substrate. The results of this study show that *P. repens* infructescences could present an important source of decomposer organisms in the environment because fynbos landscapes in general have few dead-wood habitats due to the absence of large trees. Consequently, the loss of these bacteria may have consequences for ecosystem functioning. We show that overly frequent fires can remove these potentially important bacteria from landscapes, and that a mosaic of differently-aged vegetation patches could improve the overall microbial diversity.

Introduction

Plants are colonized by a diversity of bacteria, many of which are beneficial to their hosts (Andrews & Harris, 2000; Turner *et al.*, 2013). These benefits vary from protection against pathogens to supplying growth-promoting substances or essential nutrients (Andrews & Harris, 2000; Turner *et al.*, 2013). The composition and diversity of plant-associated bacterial communities differ between different host plant species. However, members of the class Alphaproteobacteria, and the phyla Bacteroidetes and Actinobacteria are common inhabitants of different organs of many different plant species (Müller *et al.*, 2016). These differences are largely due to host-associated factors such as leaf texture and chemical composition (Redford *et al.*, 2010).

The bacteria in and around plant roots are often more diverse and abundant than those on aboveground plant organs (Bulgarelli *et al.*, 2013).

The bacterial community composition varies between different plant organs and tissues. In the phyllosphere, bacteria are exposed to harsher abiotic environments such as large amounts of UV radation and dessication, while in the rhizosphere, nutrients are more abundant in a more preferable environment for bacterial survival (Andrews & Harris, 2000; Lindow & Brandl, 2003; Vorholt, 2012; Turner *et al.*, 2013). Niche differentiation within an individual plant can thus be high and a rapid species turnover is expected when scaling from roots to leaves and other organs (Turner *et al.*, 2013). The microbiomes of different plant organs, even among different aboveground plant organs such as leaves, fruits and flowers can differ significantly (Zarraonaindia *et al.*, 2015). Therefore, the loss of specific plant organs (e.g. drought induced failure of leaf, fruit or flower production) could have important consequences for organ-specific bacterial taxa, and potentially result in the loss of unique bacterial taxa from landscapes.

Protea species are characteristic of Mediterranean-type heathlands (fynbos), a particularly diverse vegetation type that forms part of the Cape Floristic Region in the Western Cape province of South Africa. The Cape Floristic Region is one of 34 biodiversity hotspots known worldwide (Mittermeier *et al.*, 2011), with more than 9000 plant species present (Manning & Goldblatt, 2012). At a microbial level, it is believed that the CFR is a habitat to tremendous fungal diversity (Crous *et al.*, 2006), although most studies have focused on species within *Sporothrix* (Marais & Wingfield, 1994; Wingfield *et al.*, 1999; Roets *et al.*, 2013) and *Knoxdaviesia* (Marais *et al.*, 1998; Roets *et al.*, 2013; Aylward *et al.*, 2015) and fungi causing leaf-spots on Proteaceae (Swart *et al.*, 1998; Crous *et al.*, 2011). Studies on plant-associated bacteria in the Cape Floristic Region have focused on nitrogen-fixing symbionts in roots of various native Fynbos legumes (Beukes *et al.*, 2013; Lemaire *et al.*, 2016). Consequently, there is limited knowledge on the aboveground plant-associated bacterial communities in the CFR.

Some fynbos plants have unique organs not found on other taxa, such as the fire-safe, seed-bearing structures (infructescences) of many *Protea* species. Infructescences are formed from mature inflorescences when the involucral bracts surrounding the florets close to form a cone around seeds (Pretorius, 1985). Senescent (old) infructescences resemble dead plant material, a dead-plant substrate similar to litter, but remain intact on *Protea* plants for several years with only occasional

damage caused by insect borers (Bond, 1985). The infructescences of *Protea* spp. are also known to be inhabited by many different insects (Coetzee & Giliomee, 1987; Roets *et al.*, 2006), mites (Roets *et al.*, 2009; Roets *et al.*, 2011; Theron *et al.*, 2012), fungi (Lee *et al.*, 2005; Roets *et al.*, 2013), and actinomycete bacteria (Human *et al.*, 2016; Human *et al.*, 2018). A substantial base of knowledge has therefore been accumulated on organisms associated with *Protea* infructescences, however, very little is known about their bacterial communities and the interactions of these with other organisms.

Post-fire succession in Fynbos ecosystems follows a distinct pattern, where plants with different fire survival strategies are dominant at different times (Kruger, 1977; Kruger & Bigalke, 1984). The fire frequency, or fire return interval in fynbos landscapes is important in maintaining the diversity and survival of many plant species (Allsopp *et al.*, 2014). The absence of fire for long periods may result in senescence of *Protea* species without successful reseeding and an increase in the abundance of resprouting graminoids (Vlok & Yeaton, 2000; Kraaij *et al.*, 2013; Allsopp *et al.*, 2014). Overly frequent fires in fynbos landscapes is well-known to have negative effects on diversity, and may cause local extinctions of some plant species (Allsopp *et al.*, 2014). Any organisms associated with structures that form only in the mature phase (e.g. older *P. repens* infructescences and litter) may disappear even if fires do not lead to the local extinction of populations of the hosts.

It has previously shown that unburnt *P. repens* populations adjacent to recently burnt populations are an important source for the recolonization of burnt *P. repens* infructescences by *Knoxdaviesia proteae* (Aylward *et al.*, 2015). Most *P. repens* infructescences in a burnt site are not rapidly recolonized by actinomycetes (Human *et al.*, 2018) which are commonly found in *P. repens* infructescences (Human *et al.*, 2016). Even though these studies were focused on specific groups of bacteria and fungi, they clearly showed that fire has important effects on the microbial colonization of *P. repens* infructescences. It is most likely that large parts of the bacterial diversity associated with *P. repens* infructescences and surrounding litter and soil do not successfully return to landscapes after fire when the intervals between successive fires are short.

In this study, we sought to determine the differences in microbial diversity in *P. repens* infructescences and the surrounding litter and soil in a fire affected site, and a neighbouring unaffected site. This was in order to provide a better understanding of the effect of recurrent fires

on bacterial diversity in fire-prone fynbos, and the implications of very high fire-frequencies on the maintenance of this unique ecosystem. We determined whether bacteria that inhabit *P. repens* infructescences are similar to taxa known to decay wood or litter substrates that are particularly rare in recently burned areas. The shared bacterial communities between infructescences and the surrounding litter and soil were compared to assess whether litter and soil are potential sources of inoculum to colonize newly formed infructescences.

Materials and methods

Study area and sample collection

Two adjacent patches of mountain fynbos near Franschhoek in the Western Cape Province of South Africa were selected. One of these patches was burnt in a wildfire in 2009 and the other was unaffected by this fire. Sampling was conducted in 2013, when vegetation in the burnt patch was at the end of the first regeneration phase (4-years- old) and *P. repens* plants were ca. 1m tall. Here, *Protea repens* individuals reached reproductive maturity for the first time after fire. Therefore, *P. repens* individuals produced only their first infructescences and no older infructescences (from the previous flowering season) were present in this population. The vegetation canopy was still dominated by resprouting shrubs (Kruger, 1977; Kruger, 1983) and the sparse leaf litter comprised leaves and twigs from ericoid species. In the unburnt patch, the vegetation was in its mature phase and was dominated by 3m tall *P. repens* trees that were at least 15-years-old (Kruger, 1977; Kruger, 1983). In addition to bearing infructescences from the current flowering season, individuals also carried numerous infructescences from previous flowering seasons. At this site, large amounts of litter, predominantly from *P. repens*, had accumulated on the soil surface.

In the burnt patch, five newly formed (young) infructescences from five different *P. repens* individuals were collected. In the unburnt patch, five newly formed infructescences but also five mature infructescences from the previous flowering seasons from five different *P. repens* trees (i.e. two structures from each tree) were sampled. Four litter samples were collected from each patch in close proximity to the trees from which infructescences were collected. These samples were taken at least 10 meters apart to minimize pseudo replication. In addition, five 60-mm cores were collected from the soil beneath each *P. repens* tree that was sampled for infructescences in both patches and these were bulked for each tree. Collectively, five bulk soil samples were collected from each patch.

Infructescences were separated into florets, involucral bracts and seeds. Three randomly chosen floret pieces (30 mm long), three randomly chosen bract pieces (10-mm^2), and three randomly chosen seeds were selected from each infructescence and used for DNA isolation. For litter samples, small pieces (5 g) were chosen at random per sample for DNA extraction. Collected soil samples were first sieved to remove fine roots, and then 5 g per sample was used for DNA extraction. DNA was extracted from infructescence material and plant litter with a ZymoResearch Bacterial/Fungal DNA extraction kit following the instructions provided. DNA extracted from the three infructescence organs was pooled in equimolar concentrations. DNA extractions from soil samples were conducted using a MoBio PowerMax® Soil DNA Isolation Kit. DNA concentrations and quality were checked using a Nanodrop ND1000 spectrophotometer.

Sequencing of the 16S ribosomal RNA (rRNA)

Pooled DNA extracted from infructescences was submitted to Molecular Research LP (Shallowater, TX, USA) where the V4 region of the 16S rRNA gene was amplified using the universal primers 515f and 816r (Caporaso *et al.*, 2010) and sequenced with a barcode on the forward primer. DNA from soil and litter samples was sent to the sequencing center at the University of Michigan and the V4 region of the 16S rRNA gene region was amplified and sequenced following the the methods described by Kozich *et al.* (Kozich *et al.*, 2013). Because DNA from infructescences and DNA from litter and soil was not sequenced at the same facility, these groups were never compared. Rather, comparisons were always made within the same sample (e.g. samples from burnt and unburnt patches). To account for possible differences due to sequencing errors, the parametric error model estimation of DADA2 (Callahan *et al.*, 2016) and variance stabilizing transformation in the DESeq2 R package (Love *et al.*, 2014) were used.

Raw sequence reads were processed using the DADA2 package (Callahan *et al.*, 2016) in R (R Development Core Team, 2017). Forward reads were trimmed to 240 base pairs and reverse reads trimmed to 180 base pairs. All reads containing ambiguous bases were removed. Sequences were dereplicated and the sequence variants inferred using the parametric error model in DADA2 (Callahan *et al.*, 2016), removing indels and substitutions in sequences. Error corrected forward and reverse reads were merged, and sequences that did not have a perfect overlap were discarded. Chimeric sequences among merged sequences were removed using DADA2 (Callahan *et al.*,

2016). These analyses were performed according to guidelines on the DADA2 Github webpage (http://benjjneb.github.io/dada2/tutorial.html) and in Callahan *et al.* (Callahan *et al.*, 2016).

Because DADA2 infers exact sequence variants through its error correcting algorithm, coupled with merging of error corrected reads, which introduces further reliability, it does not cluster sequences into OTUs, but instead all sequence variants (SVs) are used as representation of sequences in a sample. The use of SVs rather than OTUs provides more precise and reproducible results in amplicon sequencing studies (Callahan *et al.*, 2017; Edgar, 2018). Taxonomic assignment of inferred sequences was done using the RDP Bayesian classifier (Wang *et al.*, 2007) against the SILVA SSU database release 123 (Quast *et al.*, 2012) in DADA2 (Callahan *et al.*, 2016).

Diversity and Statistical Analyses

Species richness (observed SVs) and evenness (Shannon-Weiner index) were determined using the *estimate_species_richness* command in the Phyloseq package (McMurdie & Holmes, 2013) in R (R Development Core Team, 2017). Species diversity estimators were not normally distributed. Therefore, we compared the differences in species richness by performing a generalized linear model (GLM) using the lme4 package (Bates *et al.*, 2015) in R (R Development Core Team, 2017). Rather than rarefying community abundances to an even depth, normalization of counts was performed as suggested by McMurdie and Holmes (McMurdie & Holmes, 2014) using variance stabilizing transformation in the DESeq2 (Love *et al.*, 2014) package in R (R Development Core Team, 2017).

Differences in species assemblage composition between the burnt and unburnt patches for soil, litter and newly formed infructescences and between newly formed and mature infructescences in the unburnt patch were tested using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) by running the *adonis* command in the Vegan package (Oksanen *et al.*, 2017) in R (R Development Core Team, 2017). Distances were calculated based on the Bray-Curtis dissimilarity metric, and species abundances were transformed using variance stabilizing transformation in DESeq2. PERMANOVA was run with 9999 permutations. PERMANOVA was repeated with a version of the OTU table containing only the SVs present in the various core microbiomes of all samples.

Homogeneity of dispersions of communities was tested by performing a permutational analysis of multivariate dispersions on Jaccard's Index distances (PERMDISP (Anderson, 2004)), using the *betadisper* and *permute* commands in the Vegan package (Oksanen *et al.*, 2017) in R (R Development Core Team, 2017). The species assemblage structure was examined by ordination of weighted unifrac distances (Lozupone & Knight, 2005) using principal coordinate analysis (PCoA). This was performed in the Phyloseq package (McMurdie & Holmes, 2013) in R (R Development Core Team, 2017) using the *distance* command to calculate weighted unifrac distances and *plot_ordination* command to create plots of the respective ordination methods. The SVs responsible for significant differences in newly-formed infructescences between the burnt and unburnt patches, and those responsible for the differences between newly-formed and mature infructescences were determined using linear discriminant analysis to determine effect size (LEfSE) using the yingtools package (Ying, 2016) in R (R Development Core Team, 2017).

Sequence variants with a minimum abundance of at least 50 reads per sample and occurring at least once per sample group (total microbiome), and those occurring in all but one sample in a group (core microbiome) were determined using the Microbiome R package (Lahti *et al.*, 2012). The total- and core microbiomes were calculated and subsequently used to determine the number of these taxa shared by different sample groups. A core microbiome was defined as SVs shared between at least 4 of 5 infructescence and soil samples and 3 of 4 litter samples. Total microbiomes were considered taxa that were present in at least one sample in a sample grouping. Subsets of shared SVs were made and heatmaps of these created using the *plot_heatmap* command in Phyloseq (Rajaram & Oono, 2010; McMurdie & Holmes, 2013) in R (R Development Core Team, 2017).

Results

Sequence processing and taxonomic assignment

A total of 2 581 808 raw read pairs were generated. From these, 2 441 698 quality read pairs of 233 bp remained after filtering, sequence inference, merging, and chimera removal. From these, the DADA2 parametric error estimation model produced 5146 sequence variants (SVs) (Table S1).

In litter samples from the burnt and unburnt patches, the majority of sequences were from the Class Alphaproteobactera (31 – 48%), followed by Acidobacteria (17 – 25%), Veruccomicrobia (6 -

7%), Planctomycetes (7 – 8%) and Bacteroidetes (3 – 8%) (Fig. 1). Changes between mean relative abundance in higher taxonomic groups between samples from the burnt and unburnt patches were detected in the Alphaproteobacteria (48% to 31%), Gammaproteobacteria (0.6% to 7%) and Acidobacteria (17% to 25%).

Most sequences from soil samples were from the Alphaproteobacteria (21 – 22%) and Acidobacteria (19%), followed by Planctomycetes (12% – 16%), Verrucomicrobia (8% – 14%) and Actinobacteria (5% – 7%). Members of these taxonomic groups were present at similar relative abundance in the burnt and unburnt patches, except for Verrucomicrobia (8% to 14%) and Planctomycetes (16% to 12%).

In newly-formed infructescences, most sequences were from Alpha- (37 – 42%), Gamma- (12 – 17%) and Betaproteobacteria (8 – 9%), Acidobacteria (5 – 8%), Actinobacteria (5 – 8%), Bacteroidetes (8 – 13%) and Verrucomicrobia (5%). Differences between the burnt and unburnt patches were observed in Alphaproteobacteria (37 to 42%), Gammaproteobacteria (17 to 11%) and Bacteroidetes (12 to 8%).

In mature infructescences, most sequences were from Alphaproteobacteria (34%), followed by Actinobacteria (16%). Acidobacteria, Verrucomicrobia, Bacteroidetes, Gammaproteobacteria, Betaproteobacteria and Deltaproteobacteria were the next most abundant groups, with relative abundances between 5 and 8%. Compared to newly-formed infructescences, there was a decrease in Alphaproteobacteria (41 to 33%) and an increase in Actinobacteria (8 to 15%).

Eight SVs were present among the most abundant SVs in newly-formed infructescences from both the burnt and unburnt patches (Table 1). These were *Luteibacter rhizovicinus*, *Rhizobium*, *Tardiphaga*, *Pseudomonas*, *Mycobacterium*, *Telmatospirillum*, *Rhodopseudomonas* and *Massilia*. In mature infructescences, SVs identified to belong to the Solirubrobacterales, and the genera *Burkholderia*, *Acidibacter*, *Sphingomonas*, *Gryllotalpicola*, *Hyphomicrobium* and one SV from the family Bradyrhizobiaceae all had a mean relative abundance higher than one. In litter samples, *Bryocella*, *Granulicella* and *Telmatobacter* was common in litter from the burnt and unburnt patch. These are all members of the phylum Acidobacteria and known to decompose cellulose in slightly acidic environments. In soil samples, the most abundant SVs were from *Bradyrhizobium* and *Burkholderia*.

Species diversity

The number of observed SVs (Fig. 2) and Shannon's diversity index (Fig. 3) were used to represent total diversity. In litter and young infructescences, the observed number of SVs were significantly lower in the burnt patch than in the unburnt patch. However, no significant differences were detected in the soil samples (Fig. 2). Mature infructescences had significantly more SVs than newly formed infructescences (only unburnt patch considered) (Fig. 2). Shannon's diversity index was significantly higher in the burnt patch in litter and young infructescence samples, while no difference was evident in soil samples (Fig. 3). In mature infructescences, evenness was significantly higher than in newly formed infructescences (Fig. 3).

Shared microbiomes

Newly formed infructescences from the unburnt patch had a core microbiome of 242 SVs (23% of total its microbiome), of which 49 SVs (20%) were unique to the core microbiome of infructescences from the unburnt patch (Table 2). Mature infructescences had a core microbiome consisting of 228 SVs (29%), of which 54 SVs (23%) were unique. Litter in the unburnt patch had a core microbiome of 186 SVs (31% of total microbiome), of which 114 SVs (61%) were unique in this core microbiome. The core microbiome of soil in the unburnt patch consisted of 106 SVs (7% of total diversity), and 61 of these SVs were unique to the core microbiome of soil in the unburnt patch (Table 2).

The core microbiome of newly formed infructescences in the unburnt patch (242 SVs) was larger than that of mature infructescences (228 SVs) (Table 2). The core microbiome of litter from the unburnt patch represented a smaller proportion of the total diversity, however consisted of fewer SVs (n=186, 31%) than that of newly-formed and mature infructescences. Soil from the unburnt patch had a very small core microbiome (n=106, 7%).

In the burnt patch, newly-formed infructescences had a core microbiome of similar size to that in the unburnt patch (n=231, 29%), of which 54 (23%) were unique to this core microbiome (Table 2). Litter from the burnt patch had a very small core microbiome (n=56, 10%) compared to other samples, especially litter from the unburnt patch. Although litter from the burnt patch did not have a large core microbiome, many SVs (n=22, 39%) were unique to this core microbiome. Soil from

the burnt patch had a small core microbiome (84 SVs, 5% of total diversity), but similar in size to soil from the unburnt patch (106 SVs, 7%).

Total microbiomes

Newly-formed infructescences from the unburnt patch had 1040 SVs present when all samples were pooled (hereafter referred to as total microbiome), of which 175 SVs (17%) were unique to these samples (Table 3). Mature infructescences from the unburnt patch had a total microbiome of 1446 SVs, and 609 SVs (42%) were unique. Litter from the unburnt patch had a total microbiome of 598 SVs and about half of these were unique (284 SVs, 47%). In soil from the unburnt patch, the total microbiome consisted of 1419 SVs of which 923 SVs (65%) were unique.

The total microbiome of mature infructescences was the largest, and contained many more SVs than the total microbiome of newly-formed infructescences in the unburnt patch (Table 3). From the substantial number of SVs in mature infructescences, a large number were unique (609 SVs, 42%). The total microbiome of litter in the unburnt patch was much smaller than that of newly-formed and mature infructescences or soil. The total microbiomes of soil in the unburnt site was also large, similar to mature infructescences and many of the SVs detected were unique to these samples (65%).

The total microbiome of newly-formed infructescences from the burnt patch was smaller in size (809 SVs, Table 3) to that in the unburnt patch (1040 SVs), but had a larger portion of unique SVs (n=179, 22%). However, this is most likely because more SVs present in infructescences in the unburnt patch were shared with mature infructescences and litter. When all samples other than those from newly-formed infructescences were excluded (i.e. unique SVs when infructescences from burnt and unburnt sites were compared) then the number of unique SVs in newly-formed infructescences in the unburnt patch more than doubled (n=453, 44%), while in the burnt patch it remained at a similar level (n=222, 42%).

The total microbiome of litter from the burnt patch (n=552) was of similar size to that in the unburnt patch (n=598), but here, more SVs were unique to these samples (n=310, 56%). Similar to newly-formed infructescences, many SVs from litter in the unburnt patch were shared with other sampled groups and thus when litter was considered in isolation, the proportion of unique SVs in the unburnt patch was substantially higher (n=426, 71%). This was also true for litter in the burnt

patch, where unique SVs increased (380 SVs, 69%) when litter was considered in isolation. Soil from the burnt patch had the largest number of SVs (n=1658) of all sample groups, and a large number of these were unique to this sample group (n=69%).

Altogether, there were 1871 SVs unique to all infructescences, suggesting that if *P. repens* is lost from a landscape there is a large potential for loss of bacterial biodiversity (Fig. 4). Furthermore, there were 1068 SVs from fire-prone material (newly-formed and mature infructescences and litter in the unburnt site) in the unburnt patch which had not colonized the same samples in the burnt patch.

Newly-formed infructescences from the burnt and unburnt patches shared 161 core microbiome SVs (Table 4, Fig 5, Fig 6), many of which are likely to be host-associated and possibly endophytic taxa. A total of 587 SVs occurred at least once in newly-formed infructescences from the burnt and unburnt patch. Newly-formed infructescences from the unburnt patch shared 141 core SVs with mature infructescences from the unburnt patch, while newly-formed infructescences from the burnt patch shared 123 core SVs with mature infructescences from the unburnt patch. Newly-formed infructescences shared 32 core taxa with litter from the unburnt patch, while 129 SVs occurred at least once in both these sample groups, an indication that there is occasional movement of bacteria between litter and infructescences. In the burnt patch, only 8 core SVs from newly-formed infructescences was also present in litter, while 21 SVs occurred once in each sample group (Table 4). This lower number compared to the unburnt patch is likely due to a lack of *P. repens* derived litter in the burnt site.

Differences in community assemblages

Bacterial communities in litter, soil and newly formed infructescences were significantly different between samples from the burnt and unburnt patch (Table 5). When the core microbiomes were considered, the communities in the litter, soil and newly formed infructescences were significantly different between the burnt and unburnt patch, but with larger effect sizes and smaller p-values than when the total microbiome was considered.

The Jaccard's Index of distance of litter samples from the burnt and unburnt patches were heterogeneously dispersed according to PERMDISP analysis. The Jaccard's index distances of bacterial communities in newly-formed infructescences and soil from the burnt and unburnt

patches were homogeneously dispersed (Table 6). When considering the core microbiomes in the litter, soil and newly-formed infructescence samples from the burnt and unburnt patch the dispersion of Jaccard's Index distances was not significantly different. Fifteen SVs were significantly associated with newly-formed infructescences from the burnt patch, and a further 12 significantly differentiated newly-formed infructescences form the unburnt patch (Table S2) according to linear discriminant analysis of effects size (LEfSE).

The bacterial community assemblages in newly formed infructescences and mature infructescences in the unburnt patch were also significantly different (Table 5). When the core microbiomes of newly-formed and mature infructescences from the unburnt patch were compared, significant differences were also detected, with larger effect sizes and smaller p-values than total microbiomes (Table 7). Furthermore, the Jaccard's index distances of newly-formed and mature infructescences from the unburnt patch were also homogeneously dispersed. This was also the case when comparing core microbiomes from newly-formed and mature infructescences from the unburnt patch. Fifty-two SVs differentiated mature infructescences from newly-formed infructescences and 50 SVs differentiated newly-formed infructescences from mature infructescences (Table S3) according to LEfSE analysis.

Samples from infructescences, litter and soil were distinctly separated in the PCoA (Fig 7). Samples from burnt and unburnt litter partially overlapped in PCoA, and so too in soil, although samples from soil in the burnt patch had a smaller distribution. Newly formed infructescences from the burnt patch formed a small cluster while those from the unburnt patch had a larger distribution. Mature infructescences formed a small cluster in the PCoA while newly formed infructescences from the unburnt patch had a distribution spread between newly formed infructescence from the burnt patch to mature infructescences.

Discussion

The results of this study describe the bacterial diversity in the infructescences of *P. repens*, a habitat in which only fungi and actinomycetes have previously been considered (Human *et al.*, 2016; Human *et al.*, 2018). Many of these bacteria were unique to infructescences, suggesting that this is a unique, specialized bacterial habitat in fynbos landscapes. The results also showed that the bacterial communities in newly-formed infructescences, litter and soil were different in a patch reaching reproductive maturity for the first time after fire and another patch unaffected by the fire.

Overall, the data suggest that the bacterial communities in mountain fynbos landscapes are slow to recover after fire.

The time required for bacteria to recolonize fynbos landscapes after fire was shown to be even longer than the period required for host plants to reach reproductive maturity. Local extinctions of serotinous Proteaceae occur when fires recur before new seeds and infructescences have formed (Kruger, 1977; Bond *et al.*, 1984). One possible impact of inordinantly frequent fires may be the loss of unique bacteria due to the removal of *Protea* infructescences and the surrounding litter. More than 44% of the bacterial taxa present in infructescences from the unburnt patch were absent in the burnt patch. This suggests that there are many *P. repens*- inhabiting bacteria that had not recolonized infructescences in the burnt patch.

Most of the SVs that were dominant in newly formed infructescences are not commonly associated with living above ground plant tissues. In contrast, the dominant bacteria found in newly-formed infructescences, are known from decaying wood (Kielak *et al.*, 2016), the structures of white-rot fungi on decaying wood (Hervé *et al.*, 2014) and freshly cut pine wood chips (Noll *et al.*, 2010). These newly formed infructescences, which are considered woody structures, represent a unique bacterial habitat where decomposition occurs. However, the outside of this plant organ remains tightly closed, protected from the outside environment, and can remain intact for several years, likely because of its high lignin content (Mitchell & Coley, 1987). This also suggests that *P. repens* infructescences may be an important reservoir for decomposer bacteria in the environment and that may be lost due to inappropriate fire management.

Some of the dominant bacteria in mature infructescences were from genera such as *Bradyrhizobium* and *Burkholderia* that are common in the late stages of litter of wood decomposition (Voříšková & Baldrian, 2013; Tláskal *et al.*, 2016). The change in bacterial community assemblages between newly-formed infructescences and mature infructescences was due to changes in the abundance of SVs belonging to known groups of soil bacteria. These included 10 families of Actinobacteria and five families of both Myxococcales and Rhizobiales, which are known from the late stages of wood decomposition and soil (Table S2). The high concentration of recalcitrant plant polymers in infructescence- bracts (Mitchell & Coley, 1987), and possibly dead microbial biomass in litter-type environments are the most likely reasons for the high relative abundance of these taxa present in mature infructescences.

Many of the SVs detected in newly-formed infructescences were also present in litter from the unburnt patch, but not in soil (Table 3; Fig. 4; Fig. 5). This suggests that they are common in the *P. repens* environment. Some of the SVs shared between newly-formed infructescences and litter from the unburnt patch such as *Massilia, Pseudomonas and Sphingomonas* are known from both the phyllosphere (Yashiro & McManus, 2012) and litter of different plants (Tláskal *et al.*, 2016). Other shared taxa included the Subdivision 1 Acidobacteria, which are known for their ability to decompose cellulose (Stursova *et al.*, 2012; Lladó *et al.*, 2016). The taxa shared between infructescences and litter may be common in living *P. repens* leaves and inflorescences and later accumulate in litter.

Newly-formed Infructescences from the burnt and unburnt patches shared numerous SVs, many of which never occurred in litter or soil. In addition to these presumably host-associated bacteria, many of the remaining SVs are expected to be introduced from the surrounding environment. The most common routes of dispersal of environmental bacteria would be through wind and rainfall and -splash (Lindemann & Upper, 1985; Butterworth & McCartney, 1991; Brodie *et al.*, 2007) onto open inflorescences or through the numerous arthropods that enter the closed *P. repens* infructescences (Roets *et al.*, 2011; Theron *et al.*, 2012; Hubert *et al.*, 2016).

Older infructescences, where the greatest species diversity was observed, represent a common habitat for mites and insects that move from desiccating mature infructescences to newly formed infructescences (Coetzee & Giliomee, 1987; Roets *et al.*, 2009; Theron *et al.*, 2012). Some insects that have been found in infructescences belong to groups commonly associated with litter (Coetzee & Giliomee, 1987; Roets *et al.*, 2006), and these could disperse different bacteria from litter into infructescences. As is the case with the common *Sporothrix* and *Knoxdaviesia* fungi in *Protea* infructescences (Roets *et al.*, 2009; Roets *et al.*, 2011), arthropods are the most likely vectors of many of the bacteria into newly-formed infructescences.

Species richness and composition was significantly different in litter between samples from the burnt and unburnt sites. In litter, bacterial diversity and communities were significantly different, possibly because of the differences in above ground plant species composition (Kruger, 1977; Kruger & Bigalke, 1984), which is the main contributor to litter. The plant communities in burnt and unburnt patches are very different in the first years after fire (Kruger, 1977; Kruger, 1983). The burnt site was rich in annual plants, whereas the unburnt site was dominated by larger

Proteaceae (Kruger, 1977; Kruger, 1983). The differences in plant species composition results in very different substrates available to be colonized by bacteria (Pérez *et al.*, 2003).

The species richness in soil samples from the burnt and unburnt site did not differ, but community assemblages were significantly different. Soil in mountain fynbos ecosystems are not completely sterilized by fires (Kruger & Bigalke, 1984), and at depths of lower than 10 mm, the effect of fire is minimal (Kruger & Bigalke, 1984). Therefore, the differences between the burnt and unburnt patches in these communities in the post-fire environment was small. The low nutrient levels, especially low nitrogen and phosphorus of soils that characterize fynbos vegetation (Specht & Moll, 1983; Witkowski & Mitchell, 1987) most likely shapes the soil bacterial community composition. Taxa such as *Bradyrhizobium* and *Burkholderia* were among the most abundant bacteria in soil samples, and have an ability to form associations with plants in nitrogen-poor environments (Garau *et al.*, 2009; Bontemps *et al.*, 2010).

The presence of many different bacteria known for their ability to fix atmospheric nitrogen in soil, wood and litter is noteworthy. In other environments, N_2 fixing bacteria often occur in association with fungal decomposers of plant based matter. In fact, in some environments, the copy number of N_2 fixing genes are correlated with fungal biomass. Proteoid litter is of very low nutrient quality and consists predominantly of lignin, smaller amounts of cellulose and very little nitrogen and phosphorus (Mitchell & Coley, 1987). Furthermore, it has been reported that there is a substantial increase in nitrogen and phosphorus content in floral tissues of *P. repens*, especially in seeds after infructescences have closed (Esler *et al.*, 1989). This is a surprising coincidence and further research should examine the copy number of N_2-fixing genes in the *P. repens* environment, and its role in fungal cellulose decomposition.

Several of the most common SVs detected in newly-formed infructescences have been reported from other environments where the fungi in the genus *Sporothrix* are known to occur (Wingfield, 1987; Six & Paine, 1999; Suh *et al.*, 2013). *Burkholderia*, *Pseudomonas*, *Ewingella* and *L. rhizovicinus* and *Rhizobium* have been reported from the pine-wood nematode, its host, the Japanese Sawyer Beetle (Xie & Zhao, 2008; Kwon *et al.*, 2010; Proença *et al.*, 2010; Zhu *et al.*, 2012) and from bark beetles from the genus *Dendroctonus* (Dohet *et al.*, 2016). Interestingly, all of these ecosystems have *Sporothrix* spp. in common with *P. repens* infructescences. All these reports where these bacteria co-occur with *Sporothrix* on a host are from the Northern Hemisphere.

Our discovery of this association also for *P. repens* in the southern hemisphere seems unlikely to be coincidental. Consequently, we suggest further investigation into the possible symbiotic associations between *Sporothrix* and their co-occurring bacteria.

Previous studies have found that the occurrence of actinomycetes (Human *et al.*, 2018), fungi (Roets *et al.*, 2005) and insects (Roets *et al.*, 2006) can vary temporally in *P. repens* infructescenses. In the present study, we did not sample across the biogeographic range of *P. repens*, neither at different time points. However, the results provide valuable insights into the bacterial community composition of *P. repens* infructescences and the surrounding environment. Strikingly, four years after fire, the bacterial community in soil, litter and newly-formed *P. repens* infructescences remained different. This result highlights the possible negative effect that inordinantly frequent fires can have on bacterial diversity.

The younger *P. repens* population also had unique bacteria, and thus we suggest that landscapes with a mosaic of plants having different developmental stages would support the highest bacterial diversity. The same is true for birds (Chalmandrier *et al.*, 2013) and for insects (Usher & Jefferson, 1991). Frequent fires, and fires over large areas in landscapes, mostly due to anthropogenic impacts are responsible for the homogenization of these landscapes and no doubt results in the loss of unique microbial biodiversity. Clearly, further studies on the effect of fire in fynbos ecosystems should strongly consider plant and insect associated microorganisms as these may provide important ecosystems services such as the provision of nitrogen and phosphorus to their environment.

Acknowlegements

We thank the National Research Foundation (NRF) and the Department of Science and Technology (DST)/NRF Centre for Tree Health Biotechnology (CTHB) for financial support. We also thank the Cape Nature Conservation Board for providing collection permits.

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Table 1. The 15 most abundant sequence variants (SVs) with their best taxonomic identification from all respective sample categories with the average and the range of relative abundance values

		Relative abundance (%)				Relative abundance (%)	
	Taxon ID	Ave	Range	Taxon ID	Ave	Range	
Litter Burnt	Rhizobiales (1174-901-12)	5.79	4.09 - 7.16	*Bryocella*	4.22	2.57 - 5.67	Litter Unburnt
	Sphingomonas	4.55	0 - 7.22	*Stenotrophomonas*	4.20	0.63 - 10.35	
	Acidiphilium	3.08	0.43 - 5.61	*Terriglobus*	3.02	2.41 - 3.68	
	Bryocella	2.68	1.6 - 3.14	*Bryocella*	2.51	1.25 - 3.97	
	Acidiphilium	2.13	1.09 - 3.95	*Flavobacterium*	2.21	0.62 - 4.35	
	Delftia	1.81	0.36 - 3.44	*Sphingomonas*	2.05	1.56 - 2.35	
	Amnibacterium	1.75	0.54 - 2.70	*Flavobacterium*	1.90	1.0 - 3.16	
	Bryocella	1.46	0.87 - 1.77	*Granulicella*	1.61	1.28 - 2.01	
	Beijerinckiaceae	1.46	0.75 - 2.13	*Pseudomonas*	1.56	0.98 - 2.22	
	Sphingomonas	1.4	0.34 - 2.19	*Granulicella paludicola*	1.35	0.55 -1.92	
	Terriglobus	1.39	1.01 - 1.64	*Burkholderia*	1.33	1.21 - 1.54	
	Granulicella	1.29	0.51 - 1.94	*Acidiphilium*	1.32	0.6 - 1.79	
	Acetobacteraceae	1.18	0 - 2.13	*Acidiphilium*	1.31	0.91 - 1.95	
	Rhizobiales (1174-901-12)	1.17	0 - 1.99	*Bryocella*	1.15	0.53 - 1.6	
	Acidobacteriaceae Gp 1	1.14	0 - 2.46	Acidobacteriaceae Gp1	1.12	0.71 - 1.4	
Soil Burnt	*Bradyrhizobiaceae*	2.00	1.03 - 3.36	*Burkholderia*	2.04	1.17 - 2.48	Soil Unburnt
	Bradyrhizobium	1.74	1.01 - 3.24	Bradyrhizobiaceae	2.01	1.62 - 2.9	
	Rhodospirillales (DA111)	1.04	0.86 - 1.21	Acidobacteriaceae Gp1	1.95	1.36 - 2.52	
	Bryobacter	1.03	0 - 2.13	*Chthoniobacter*	1.29	1.07 - 1.44	
	Burkholderia	0.98	0.55 - 1.45	*Variibacter*	1.24	0.78 - 1.49	
	Acidobacteriaceae Gp1	0.85	0.37 - 1.2	Rhodospirillales	1.08	0.72 - 1.59	
	Sphingomonas	0.83	0.48 - 1.41	*Endobacter*	1.08	0.31 - 1.84	
	Sphingomonas	0.75	0.52 - 1.07	*Acidobacterium*	1.01	0.67 - 1.12	
	Chthoniobacterales (DA101)	0.72	0.41 - 1.27	*Burkholderia*	0.99	0 - 1.47	
	Chthoniobacter	0.72	0.31 - 1.19	*Bryobacter*	0.84	0 - 1.44	
	Phycisphaerae (WD2101)	0.71	0.51 - 1.45	Rhodospirillales	0.74	0.28 - 1.22	
	Acidibacter	0.70	0.67 - 1.42	*Bryobacter*	0.70	0 - 1.85	
	Chthoniobacterales (DA101)	0.70	0 - 1.43	*Candidatus* Methylacidiphilum	0.69	0 - 0.98	
	Mycobacterium	0.69	0.38 - 1.45	*Rhizomicrobium*	0.69	0.5 - 0.95	
	Solirubrobacterales	0.69	0 - 1.04	*Granulicella*	0.68	0.43 - 0.87	
NF Infructescences Burnt	*Luteibacter rhizovicinus*	5.34	2.22 - 9.45	*Telmatospirillum*	4.12	0.14 - 10.1	NF Infructescences
	Rhizobium	4.44	3.12 - 6.04	*Rhizobium*	3.95	0.21 - 6.89	
	Tardiphaga	3.55	0.81 - 6.43	*Pseudomonas*	3.02	0.09 - 13.04	
	Ewingella	2.76	1.59 - 6.29	*Luteibacter rhizovicinus*	1.90	0.13 - 7.13	
	Pseudomonas	2.61	1.25 - 6.68	*Massilia*	1.54	0.03 - 4.57	
	Simkania	2.42	0.44 - 4.6	*Rhodopseudomonas*	1.42	0.11 -3.24	
	Methylophilaceae	2.39	1.12 - 4.54	*Duganella*	1.31	0.02 - 6.32	
	Mycobacterium	2.02	0.22 - 4.11	*Rhizobium*	1.28	0.07 - 3.8	
	Telmatospirillum	1.98	0.12 - 6.54	*Mycobacterium*	1.18	0.26 - 2.76	
	Rhodopseudomonas	1.97	0.1 - 6.62	*Telmatobacter*	1.08	0.19 - 1.63	
	Janthinobacterium	1.67	0.29 - 5.99	*Granulicella*	1.07	0.08 - 3.39	
	Telmatospirillum	1.49	0.11 - 4.50	*Tardiphaga*	1.00	0.14 - 2.41	
	Massilia	1.48	0.11 - 2.92	*Methylorosula*	0.95	0.18 - 2.28	
	Telmatospirillum	1.33	0 - 5.11	*Acidibacter*	0.94	0.04 - 2.61	
	Sphingomonas	1.18	0.76 - 1.58	*Acidisoma*	0.91	0.04 - 3.79	
Mature Infructescences Unburnt	Solirubrobacterales (480-2)	2.57	0.25 - 7.0				
	Burkholderia	1.88	0.43 - 3.23				
	Acidibacter	1.41	0.26 - 1.68				
	Sphingomonas	1.28	0.02 - 5.98				
	Gryllotalpicola	1.27	0 - 5.47				
	Hyphomicrobium	1.24	0.34 - 1.82				
	Bradyrhizobiaceae	1.22	0.38 - 3.51				
	Bacillus	1.00	0 - 4.91				
	Rhodanobacter	0.99	0 - 4.88				
	Haliangium	0.98	0.3 - 1.23				
	Bradyrhizobium	0.93	0.44 - 1.3				
	Micromonosporaceae	0.91	0.03 - 2.45				
	Acetobacteraceae	0.82	0.04 - 3.47				
	Sandaracinaceae	0.82	0.01 - 3.01				
	Acidibacter	0.79	0.07 - 2.46				

Table 2. A summary of core SVs for each of the sample groups separated into burnt and unburnt patches, including the number of unique SVs in each core microbiome.

Samples	Patch	Core SVs	%Core SVs	Core SVs Unique	% Core SVs Unique	Unique in sample site	% Unique in sample site	Total SVs in sample	Total SVs in sample group
Newly formed infructescences	Unburnt	242	23%	49	20%	81	33%	1040	1849
Newly formed infructescences	Burnt	231	29%	54	23%	70	30%	809	
Mature infructescences	Unburnt	228	16%	69	30%	69	30%	1446	1446
Litter	Unburnt	186	31%	114	61%	152	82%	598	1149
Litter	Burnt	56	10%	22	39%	22	39%	552	
Soil	Unburnt	106	7%	61	58%	66	62%	1419	3077
Soil	Burnt	84	5%	40	48%	44	52%	1658	

1. Unique sequence variants in a sample group when samples from the burnt and unburnt sites were compared in isolation to exclude SVs shared between sample groups from the same site.

Table 3. A summary of the total bacterial SV including the number of unique SVs from the total bacteria microbiomes

Samples	Patch	Total SVs in sample	Unique SVs	% Unique SVs	Unique SV in sampling site[1]	% Unique SVs in sampling site[1]	SVs shared group	Unique to sample group	Total SVs in sample group
Newly formed infructescences	Unburnt	1040	175	17%	453	44%	1040	1262	1849
Newly formed infructescences	Burnt	809	179	22%	222	27%	809		
Mature infructescences	Unburnt	1446	609	42%	609	42%	1446	609	1446
Litter	Unburnt	598	284	47%	426	71%	598	683	1149
Litter	Burnt	552	310	56%	380	69%	552		
Soil	Unburnt	1419	923	65%	979	69%	1419	2462	3077
Soil	Burnt	1658	1141	69%	1218	73%	1658		

1. Unique sequence variants in a sample group when samples from the burnt and unburnt sites were compared in isolation to exclude SVs shared between sample groups from the same site.

Table 4. Shared core SVs (Table 2) amongst different sample groups

Samples compared	Core SVs shared[1]	SV shared[2]
Burnt and unburnt newly formed infructescences	161	587
Unburnt-Newly formed infructescences and unburnt-mature infructescences	141	715
Burnt newly formed infructescences and unburnt-mature infructescences	123	491
All newly formed infructescences and unburnt mature infructescences	112	460
Unburnt litter and unburnt newly formed infructescences	32	129
Unburnt litter and burnt newly formed infructescences	34	56
Burnt litter and burnt newly formed infructescences	8	21
Burnt and unburnt litter	34	172
Unburnt soil and unburnt newly formed infructescences	11	24
Burnt soil and burnt newly formed infructescences	8	21
Unburnt litter and unburnt soil	7	38
Burnt litter and burnt soil	2	9

1. Sequence variants present in 4/5 samples for soil and infructescences, and 3/4 from litter samples
2. Sequence variants present in at least one sample in each sample category considered

Table 5. Results of PERMANOVA using the Bray-Curtis distances comparing the total microbiomes of litter, soil and newly-formed infructescences from burnt vs unburnt patches and newly-formed and mature infructescences from the unburnt patch

Sample	**Pseudo F**	**R^2**	**p-value**
Burnt against Unburnt			
Newly formed infructescences	1.72	0.18	0.027
Litter	5.23	0.47	0.029
Soil	2.55	0.24	0.016
Newly formed against mature infructescences			
Unburnt – NF v mature Infr	2.28	0.22	0.023

Table 6. Results of PERMDISP using the Jaccard's Index similarity measure comparing samples from burnt vs unburnt patches and newly-formed and mature infructescences from the unburnt patch

Sample	F	p-value
Burnt against Unburnt		
Litter	10.01	0.0001
Soil	1.27	0.27
Newly formed infructescences	0.16	0.70
Newly formed against mature infructescences		
Unburnt Newly formed against Mature Infructescences	0.88	0.42

Table 7. Results of PERMANOVA using the Bray-Curtis distances comparing the core microbiomes from the burnt and unburnt patch and newly-formed infructescences and mature infructescences from the unburnt patch

Sample	Pseudo F	R^2	p-value
Burnt against Unburnt			
Newly formed infructescences	13.64	0.63	0.008
Litter	67.88	0.92	0.028
Soil	21.47	0.73	0.009
Newly formed against mature infructescences			
Unburnt – NF v mature Infr	10.97	0.58	0.008

Figures

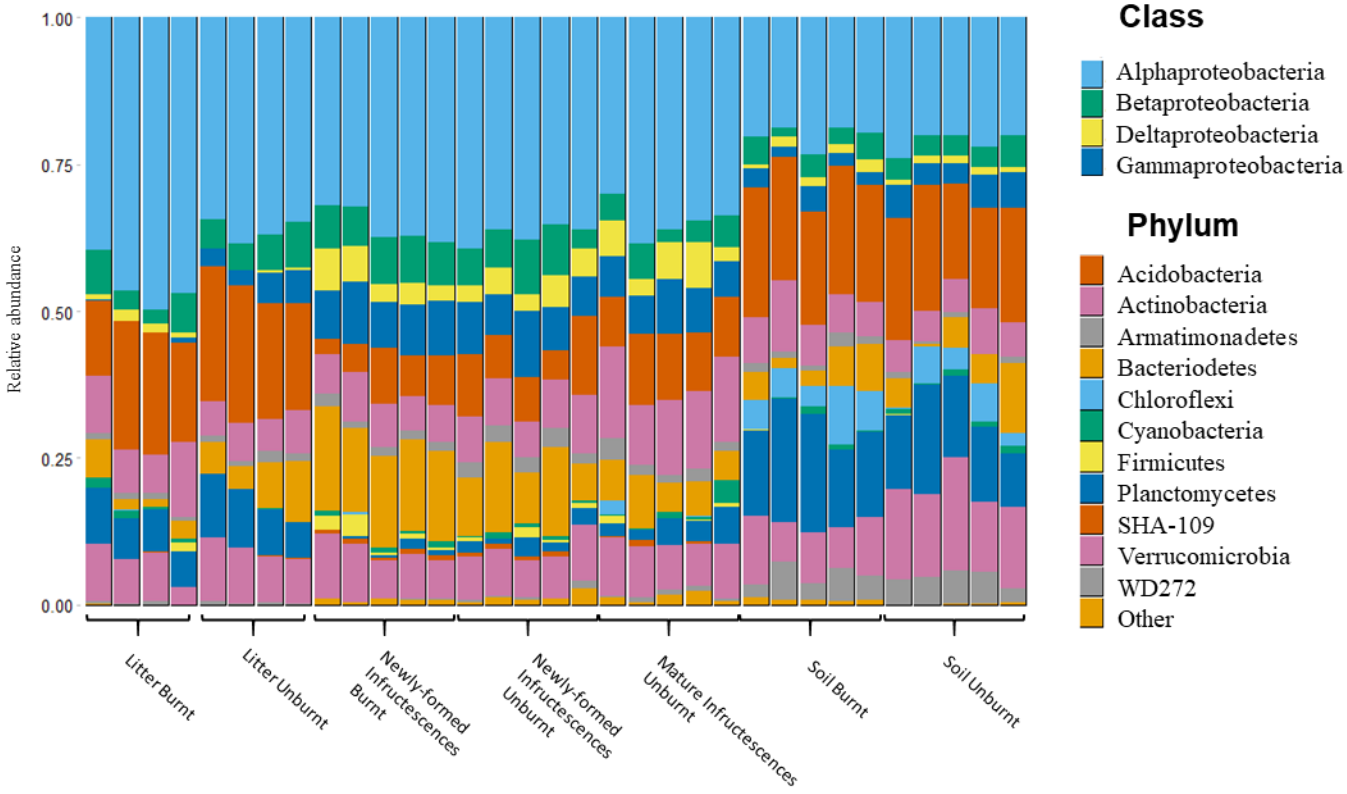

Fig 1. Stacked bar graph showing the taxonomic composition and relative abundance of all samples across their various sample categories

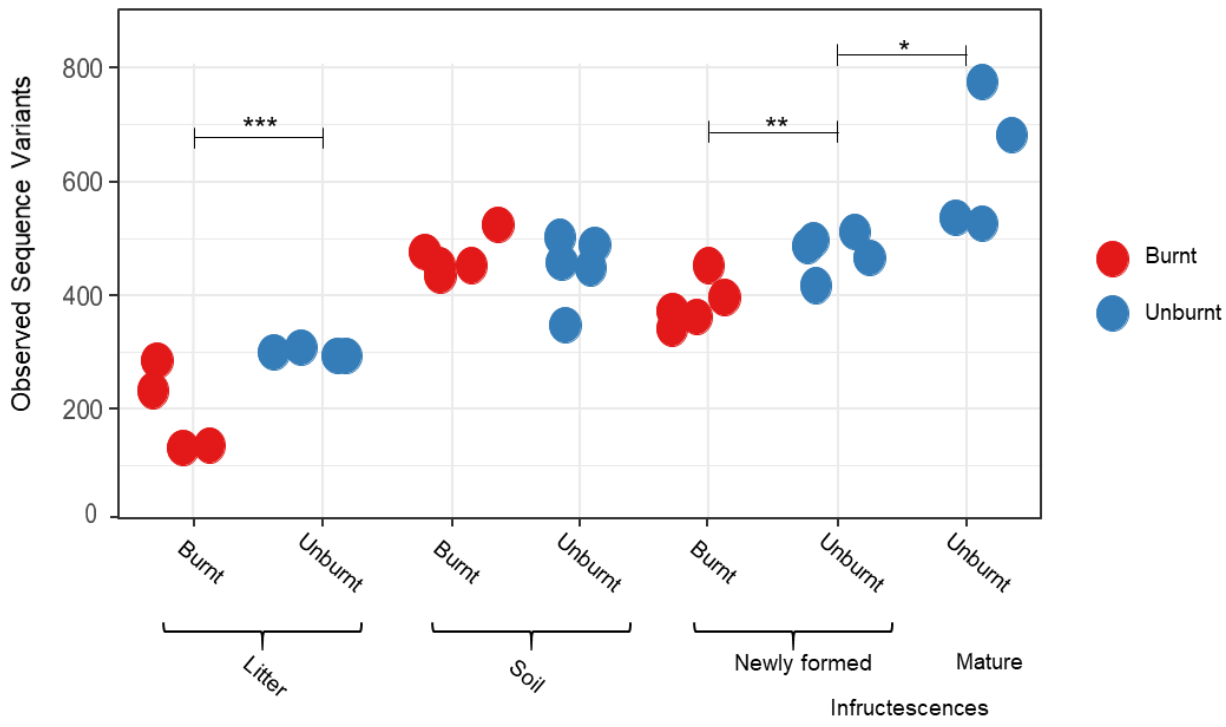

Fig 2. Species richness according to the observed species metric for all respective sample categories

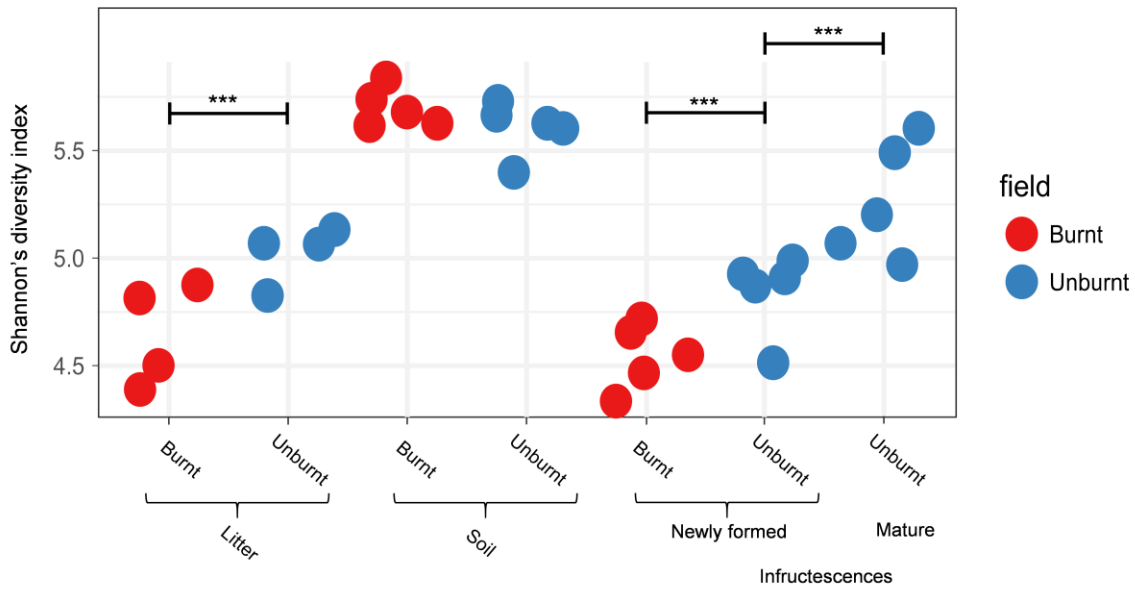

Fig 3. Species diversity (richness and evenness) according to Shannon's diversity index for all respective sample categories

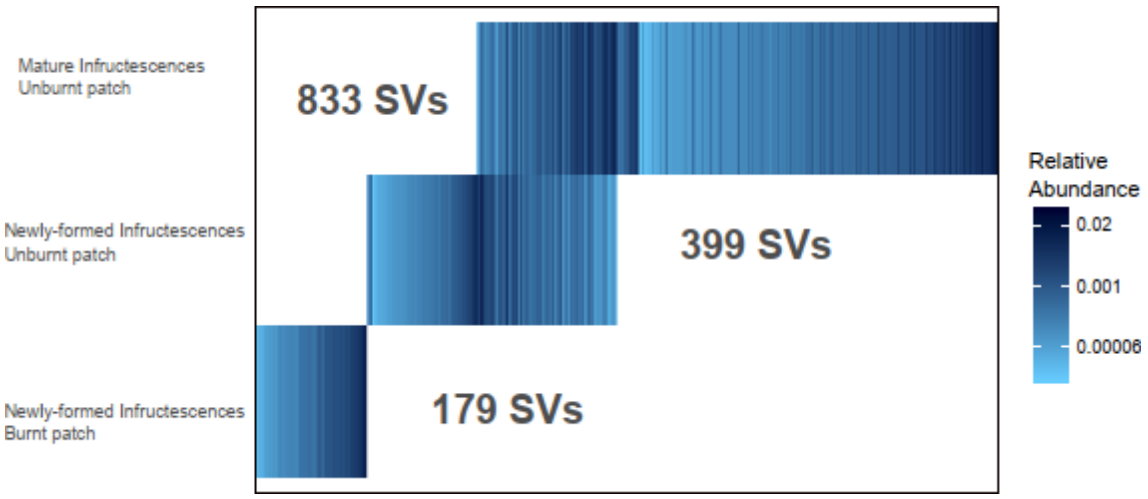

Fig 4. Heatmap showing the SVs that were unique to newly-formed and mature infructescences and to either the burnt or unburnt patches

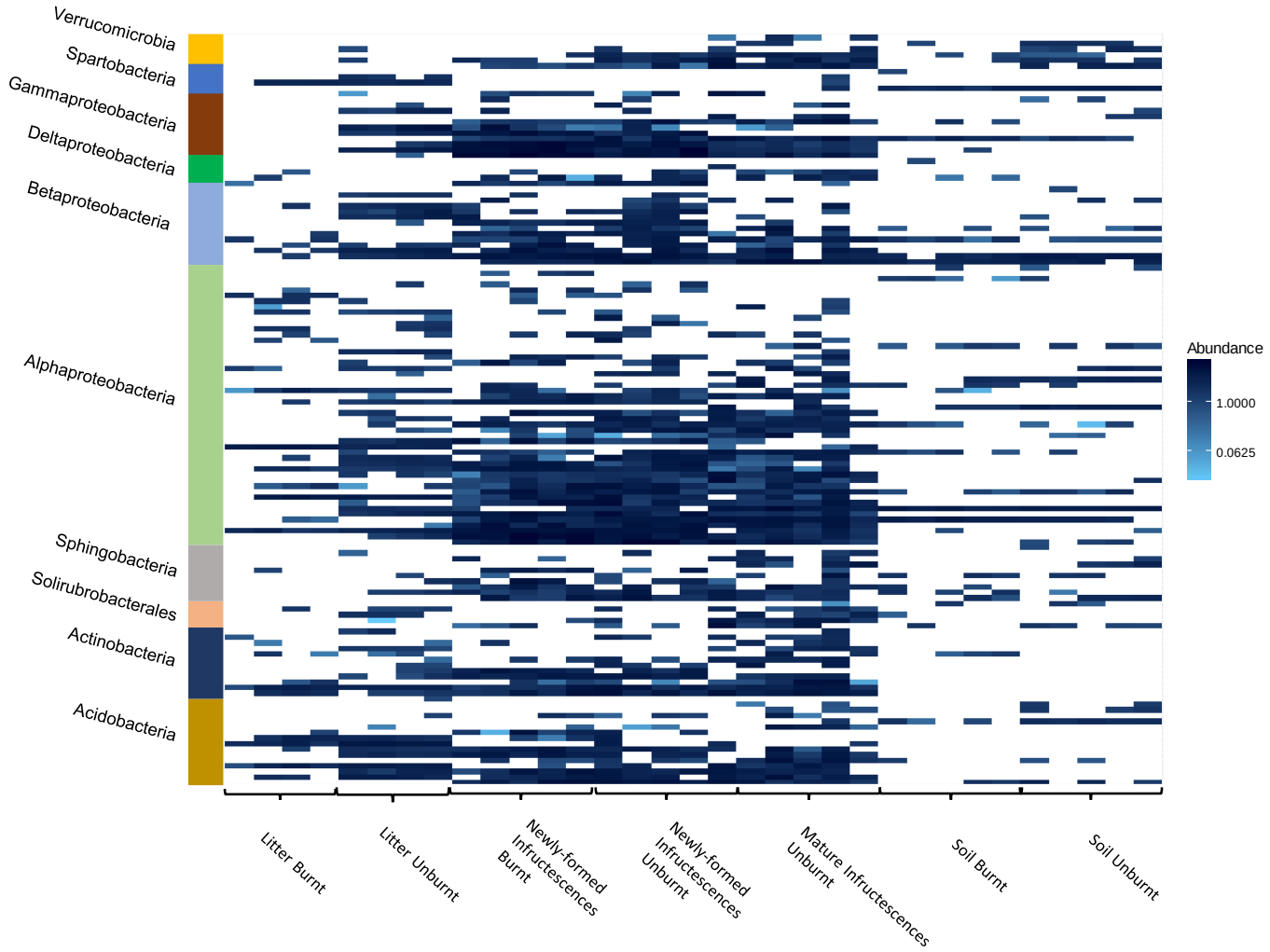

Fig 5. Heatmap showing sequence variants present in at least 75% of samples from two sample categories

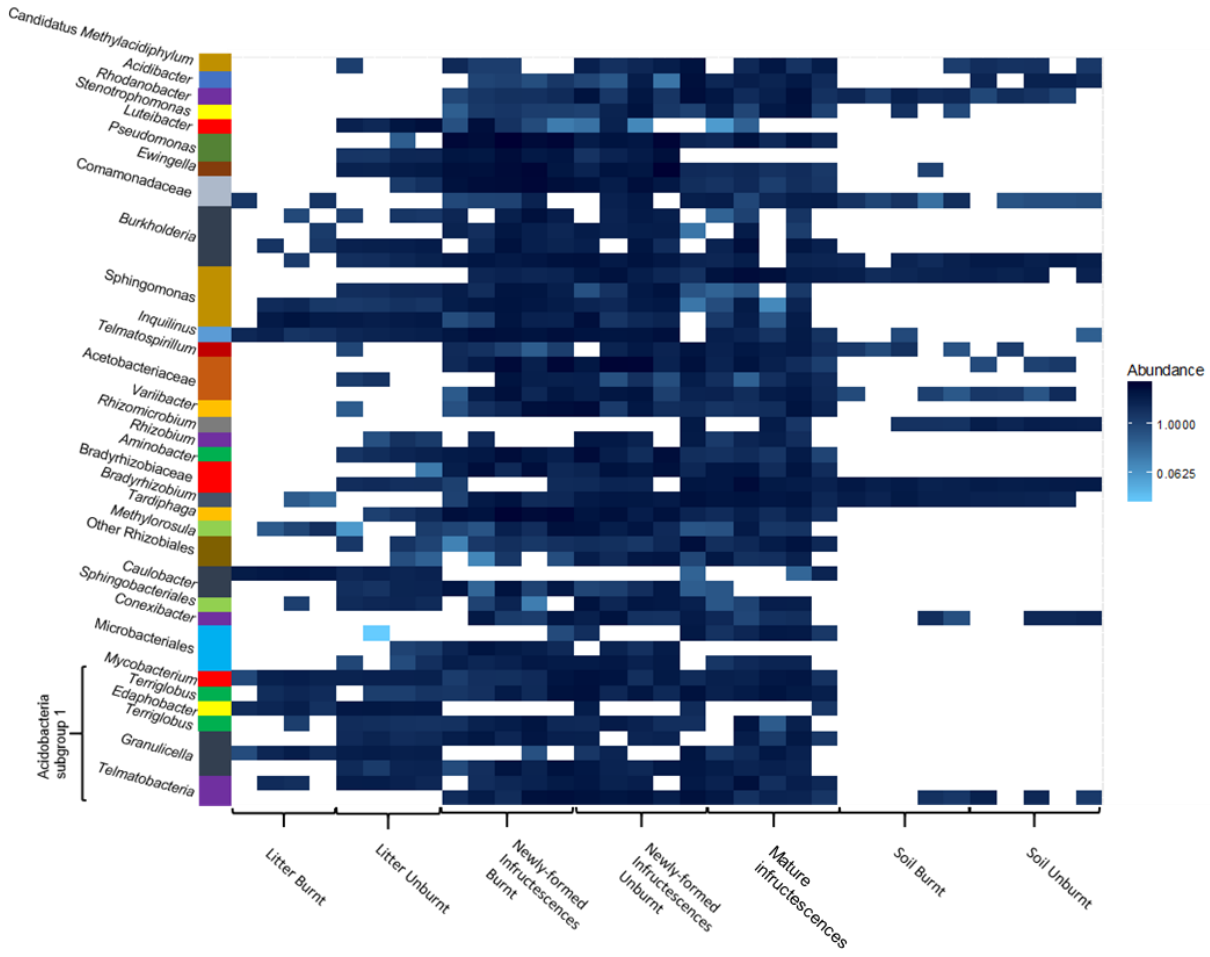

Fig 6. Heatmap showing the 50 most common SVs among those shared between infructescences, litter and soil. The best taxonomic identification for each SV is indicated. Abundances have been transformed to account for differences in sequence depth

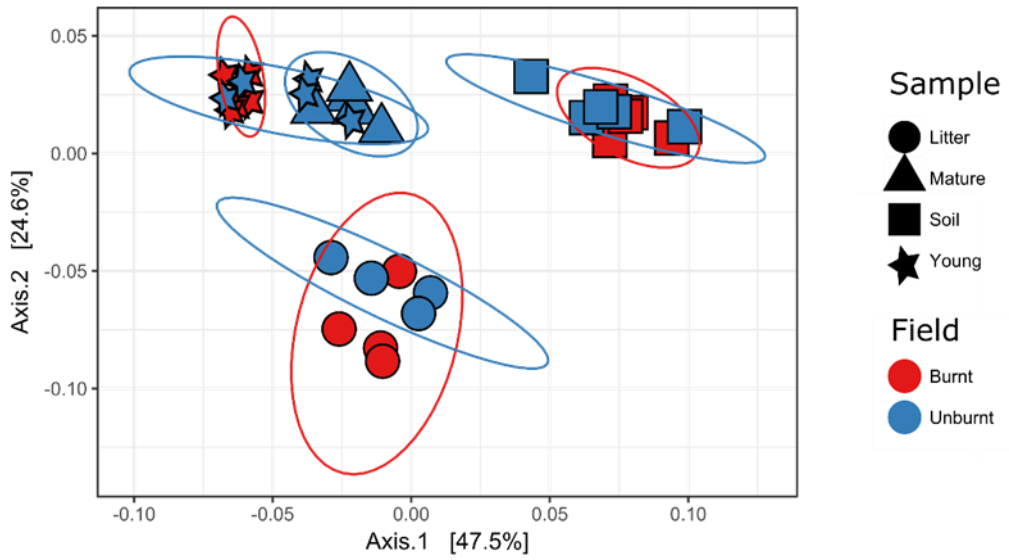

Fig. 7. Comparison of the weighted-UNIFRAC distances of species assemblages through PCoA. Ellipses represent 95% confidence intervals

Table S1 Summary of sequences for all samples

Sample	Sample type	Burnt/Unburnt	Raw reads	Filtered reads	Unique SVs
Pro1	Newly-formed	Unburnt	147 914	132 091	484
Pro2	Mature	Unburnt	27 458	24 347	285
Pro3	Newly-formed	Burnt	228 090	205 539	451
Pro4	Newly-formed	Unburnt	157 645	141 117	495
Pro5	Newly-formed	Unburnt	184 875	166 610	466
Pro6	Newly-formed	Burnt	161 714	145 523	372
Pro7	Newly-formed	Burnt	145 861	130 424	369
Pro8	Newly-formed	Unburnt	142 361	126 533	416
Pro9	Newly-formed	Burnt	159 846	143 224	361
Pro10	Newly-formed	Burnt	161 642	145 325	396
Pro11	Newly-formed	Unburnt	160 403	144 957	511
Pro12	Mature	Unburnt	147 960	131 885	681
Pro13	Mature	Unburnt	192 521	170 907	534
Pro14	Mature	Unburnt	149 441	132 554	522
Pro15	Mature	Unburnt	171 956	152 972	773
Lit0.1	Litter	Burnt	4 529	3 979	136
Lit0.2	Litter	Burnt	11 468	10 268	233
Lit0.3	Litter	Burnt	25 251	23 121	285
Lit0.4	Litter	Burnt	5758	5 114	132
Lit1.1	Litter	Unburnt	20 883	18 901	309
Lit1.2	Litter	Unburnt	21 146	19 138	294
Lit1.3	Litter	Unburnt	20 236	18 398	300
Lit1.4	Litter	Unburnt	24 439	22 228	295
Soil0.1	Soil	Burnt	23 026	20 450	452
Soil0.2	Soil	Burnt	26 952	23 611	435
Soil0.3	Soil	Burnt	20 750	18 403	454
Soil0.4	Soil	Burnt	29 207	25 533	524
Soil0.5	Soil	Burnt	23 928	20 964	477
Soil1.1	Soil	Unburnt	30 007	26 716	489
Soil1.2	Soil	Unburnt	26 654	23 292	449
Soil1.3	Soil	Unburnt	28 725	25 609	503
Soil1.4	Soil	Unburnt	26 092	23 229	458
Soil1.5	Soil	Unburnt	21 030	18 736	349

Table S2. Significantly different sequence variants (SVs) in newly-formed infructescences in the burnt and unburnt sites according to linear discriminant analysis effect size (LEFSE)

Phylum	Class	Order	Family	Genus	Species	Site	lda	p
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconobacter*		Burnt	3.28	0.01
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Cytophaga*		Burnt	3.30	0.01
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*		Burnt	3.43	0.05
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Spirosoma*		Burnt	3.35	0.02
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Mucilaginibacter*	*boryungensis*	Burnt	3.42	0.01
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Asaia*		Burnt	2.87	0.02
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconobacter*		Burnt	3.23	0.01
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Zymomonas*		Burnt	2.93	0.03
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Janthinobacterium*		Burnt	3.26	0.03
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae			Burnt	3.22	0.02
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae			Burnt	3.22	0.02
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Ewingella*		Burnt	3.25	0.01
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteibacter*	*rhizovicinus*	Burnt	3.34	0.02
Proteobacteria	Alphaproteobacteria	Rhizobiales				Burnt	4.05	0.01
Actinobacteria	Thermoleophilia	Solirubrobacterales	480_2			Unburnt	3.33	0.05
Armatimonadetes	Armatimonadia	Armatimonadales				Unburnt	3.58	0.01
Armatimonadetes	Chthonomonadetes	Chthonomonadales	Chthonomonadaceae	*Chthonomonas*		Unburnt	3.23	0.05
Planctomycetes	Phycisphaerae	WD2101_soil_group				Unburnt	3.52	0.05
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae			Unburnt	3.84	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales				Unburnt	3.40	0.05
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae			Unburnt	3.54	0.03
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Inquilinus*		Unburnt	3.17	0.02
Proteobacteria	Gammaproteobacteria	Xanthomonadales				Unburnt	3.07	0.02
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Nevskiaceae	*Nevskia*		Unburnt	3.39	0.02
Verrucomicrobia	OPB35_soil_group					Unburnt	3.53	0.03

Table S3. Significantly different SVs between newly-formed and mature infructescences according to linear discriminant analysis effect size (LEfSE)

Phylum	Class	Order	Family	Genus	Species	Samples	lda	p
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales	*Bauldia*		Mat	3.30	0.00
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae SG 1	*Acidicapsa*		Mat	3.36	0.04
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae SG 1	*Acidobacterium*		Mat	3.83	0.02
Acidobacteria	Acidobacteria	Subgroup_3	Unknown_Family	*Bryobacter*		Mat	3.53	0.00
Acidobacteria	Acidobacteria	Subgroup_3	Unknown_Family	*Ca.* Solibacter		Mat	3.21	0.00
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiaceae			Mat	2.72	0.04
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Acidimicrobiales_	*Ca.* Microthrix		Mat	2.40	0.04
Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae	*Gordonia*		Mat	3.04	0.05
Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	*Acidothermus*		Mat	3.14	0.00
Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Frankia*		Mat	2.75	0.03
Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	*Jatrophihabitans*		Mat	3.68	0.04
Actinobacteria	Actinobacteria	Frankiales				Mat	3.47	0.00
Actinobacteria	Actinobacteria	Kineosporiales	Kineosporiaceae			Mat	2.45	0.04
Actinobacteria	Actinobacteria	Micrococcales	Cellulomonadaceae	*Actinotalea*		Mat	2.97	0.04
Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae	*Ornithinimicrobium*		Mat	2.62	0.04
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Gryllotalpicola*		Mat	3.50	0.00
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Humibacter*	*albus*	Mat	3.08	0.01
Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Actinomycetospora*		Mat	3.00	0.04
Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptacidiphilus*		Mat	2.74	0.04
Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	*Streptomyces*		Mat	3.23	0.01
Actinobacteria	Thermoleophilia	Solirubrobacterales	480_2			Mat	3.70	0.00
Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	*Conexibacter*		Mat	3.44	0.01
Actinobacteria	Thermoleophilia	Solirubrobacterales	TM146			Mat	2.42	0.04

Phylum	Class	Order	Family	Genus	Species	Samples	lda	p
Actinobacteria	Thermoleophilia	Solirubrobacterales	YNPFFP1			Mat	2.72	0.04
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae			Mat	3.19	0.00
Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Planctomyces*		Mat	3.04	0.00
Proteobacteria	Alphaproteobacteria	Rhizobiales	alphaI_cluster			Mat	3.39	0.02
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Devosia*		Mat	3.30	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*		Mat	3.37	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Rhodoplanes*		Mat	2.78	0.04
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae			Mat	2.92	0.04
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*		Mat	3.17	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_	*Bauldia*		Mat	3.30	0.00
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_	*Rhizomicrobium*		Mat	4.02	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Pseudolabrys*		Mat	3.40	0.02
Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Variibacter*		Mat	3.05	0.02
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae			Mat	3.79	0.03
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Inquilinus*		Mat	3.24	0.01
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillales_	*Reyranella*		Mat	3.20	0.04
Proteobacteria	Alphaproteobacteria	Rickettsiales	bac2nit3			Mat	2.92	0.01
Proteobacteria	Alphaproteobacteria	Sphingomonadales	7B_8			Mat	2.41	0.04
Proteobacteria	Betaproteobacteria	TRA3_20				Mat	2.78	0.04
Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	*Haliangium*		Mat	3.66	0.00
Proteobacteria	Deltaproteobacteria	Myxococcales	mle1_27			Mat	2.68	0.02
Proteobacteria	Deltaproteobacteria	Myxococcales	Myxococcaceae	*Corallococcus*		Mat	2.84	0.04
Proteobacteria	Deltaproteobacteria	Myxococcales	P3OB_42			Mat	2.77	0.02
Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	*Byssovorax*		Mat	3.16	0.02
Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	*Labilithrix*		Mat	3.00	0.01
Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	*Aquicella*		Mat	3.45	0.03

Phylum	Class	Order	Family	Genus	Species	Samples	lda	p
Proteobacteria	Gammaproteobacteria	NKB5				Mat	2.96	0.01
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadales	*Acidibacter*		Mat	3.70	0.01
Verrucomicrobia	OPB35_soil_group	Unknown_Order	Unknown_Family	*Pedosphaera*		Mat	2.74	0.04
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Herbiconiux*		NF	3.02	0.02
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Lysinimonas*		NF	3.36	0.01
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae			NF	3.35	0.01
Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	*Aeromicrobium*		NF	3.26	0.00
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Cytophaga*		NF	3.16	0.04
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*	*hamtensis*	NF	3.20	0.01
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Spirosoma*		NF	3.37	0.00
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Epilithonimonas*		NF	3.18	0.01
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Chitinophaga*	*oryziterrae*	NF	2.86	0.00
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Ferruginibacter*		NF	3.37	0.00
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Hydrotalea*		NF	3.40	0.01
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae			NF	3.83	0.00
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Taibaiella*		NF	2.92	0.03
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	env_OPS_17			NF	3.32	0.04
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	KD3_93			NF	3.07	0.04
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Mucilaginibacter*	*boryungensis*	NF	3.08	0.03
Chlamydiae	Chlamydiae	Chlamydiales	Simkaniaceae	*Simkania*		NF	3.40	0.00
Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae			NF	3.08	0.02
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Caulobacter*	*henricii*	NF	3.20	0.00
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Caulobacter*		NF	3.43	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Rhodopseudomonas*	*rhenobacensis*	NF	3.33	0.00
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Tardiphaga*		NF	3.43	0.01
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*		NF	3.74	0.01

Phylum	Class	Order	Family	Genus	Species	Samples	lda	p
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Acetobacter*		NF	3.12	0.05
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Acidocella*		NF	3.37	0.03
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconobacter*		NF	2.97	0.02
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Tanticharoenia*	*sakaeratensis*	NF	2.92	0.04
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Telmatospirillum*		NF	3.95	0.00
Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12			NF	3.08	0.04
Proteobacteria	Alphaproteobacteria	Sphingomonadales				NF	3.45	0.00
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae			NF	3.00	0.02
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium*		NF	3.44	0.02
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	*glacialis*	NF	3.07	0.01
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Zymomonas*		NF	2.63	0.02
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae			NF	3.40	0.01
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Xylophilus*		NF	2.99	0.04
Proteobacteria	Betaproteobacteria	Burkholderiales				NF	3.18	0.00
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Duganella*		NF	3.19	0.01
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Janthinobacterium*		NF	3.21	0.01
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*		NF	3.49	0.00
Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae			NF	3.38	0.00
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Ewingella*		NF	3.37	0.00
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*		NF	3.86	0.00
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Nevskiaceae	*Hydrocarboniphaga*		NF	3.10	0.04
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Dyella*		NF	3.30	0.03
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteibacter*	*rhizovicinus*	NF	3.18	0.04
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*		NF	2.89	0.05
Verrucomicrobia	Spartobacteria	Chthoniobacterales	Xiphinematobacteraceae	*Ca.* Xiphinematobacter		NF	3.53	0.03
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Haloferula*		NF	3.36	0.04

Phylum	Class	Order	Family	Genus	Species	Samples	lda	p
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae			NF	3.17	0.02

Chapter 3

Published as:

Human ZR, Crous CJ, Roets F, Venter SN, Wingfield MJ and De Beer, ZW (2018) Biodiversity and ecology of flower-associated actinomycetes in different flowering stages of *Protea repens*. Antonie van Leeuwenhoek 111: 209-226.

Antonie van Leeuwenhoek (2018) 111:209–226
https://doi.org/10.1007/s10482-017-0942-3

ORIGINAL PAPER

Biodiversity and ecology of flower-associated actinomycetes in different flowering stages of *Protea repens*

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Received: 14 July 2017 / Accepted: 6 September 2017 / Published online: 21 September 2017

Abstract Actinomycete bacteria have previously been reported from reproductive structures (infructescences) of *Protea* (sugarbush/suikerbos) species, a niche dominated by fungi in the genera *Knoxdaviesia* and *Sporothrix*. It is probable that these taxa have symbiotic interactions, but a lack of knowledge regarding their diversity and general ecology precludes their study. We determined the diversity of actinomycetes within *Protea repens* inflorescence buds, open inflorescences, young and mature infructescences, and leaf litter surrounding these trees. Since the *P. repens* habitat is fire-prone, we also considered the potential of these bacteria to recolonise infructescences after fire. Actinomycetes were largely absent from flower buds and inflorescences but were consistently present in young and mature infructescences. Two *Streptomyces* spp. were the most consistent taxa recovered, one of which was also routinely isolated from leaf litter. Lower colonisation rates were evident in samples from a recently burnt site. One of the most consistent taxa isolated from older trees in the unburnt site was absent from this site. Our findings show that *P. repens* has a distinct community of actinomycetes dominated by a few species. These communities change over time and infructescence developmental stage, season and the age of the host population. Mature infructescences appear to be important sources of inoculum for some of the actinomycetes, seemingly disrupted by fire. Increased fire frequency limiting maturation of *P. repens* infructescences could thus impact future actinomycete colonisation in the landscape. *Streptomyces* spp. are likely to share this niche with the ophiostomatoid fungi, which merits further study regarding their interactions and mode of transfer.

Electronic supplementary material The online version of this article (doi:10.1007/s10482-017-0942-3) contains supplementary material, which is available to authorized users.

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Keywords Proteaceae · *Streptomyces* · Cape Floristic Kingdom · *Sporothrix* · *Knoxdaviesia*

Introduction

The actinomycetes are filamentous, Gram-positive and often spore-forming bacteria that belong to the order Actinomycetales in the class Actinobacteria (Gao and Gupta 2012; Lechevalier and Lechevalier 1967). Actinomycetes have classically been considered as mainly soil-associated organisms, where they can be present in their millions per gram of soil (Goodfellow and Williams 1983). However, they are also prevalent in marine sediments, as well as in or on various terrestrial and marine invertebrates and plants (Seipke et al. 2012). These bacteria are unique in their ability to produce a large number of antibiotics (Bérdy 2005). For example, the most commonly studied actinomycetes, members of the genus *Streptomyces*, are estimated to produce close to 100,000 different antibiotics (Watve et al. 2001). This feature, and their ability to proliferate in low nutrient environments, make actinomycetes attractive symbionts of insects and plants (Kaltenpoth 2009; Seipke et al. 2012).

Actinomycetes have evolved to form mutualistic associations with numerous organisms including arthropods and fungi (Kaltenpoth 2009; Seipke et al. 2012). *Streptomyces* species in particular are important components in actinomycete-insect-fungus symbioses globally (Kaltenpoth 2009; Seipke et al. 2012). For example, the symbiotic relationships between fungi and leaf cutter ants are protected from an *Escovopsis* fungal pathogen infection by a co-evolved *Pseudonocardia* sp. (Actinomycetales: Pseudonocardiaceae) (Cafaro and Currie 2005; Currie 2001; Currie et al. 1999). However, due to their general attractiveness as symbionts, this fungus and leaf cutter ant association is further sustained by antibiotic-producing *Streptomyces* and *Amycolatopsis* spp. acquired from the surrounding soil (Barke et al. 2010; Cafaro and Currie 2005; Haeder et al. 2009). The protection of insect-fungus mutualisms by actinomycetes also extends to bark-beetle systems where various *Streptomyces* spp. protect the fungal gardens of beetles against antagonistic fungal species (Scott et al. 2008). In these bark beetle systems, associated fungal symbionts gain protection from competitor fungi by being tolerant to antibiotics produced by the bacteria (Kaltenpoth 2009; Scott et al. 2008; Seipke et al. 2012).

Streptomyces spp. have recently been isolated from the infructescences of *Protea repens* and *Protea neriifolia* in the hyper diverse fynbos biome (Human et al. 2016). The fynbos biome in the southwestern Cape of South Africa is well known for its Mediterranean-type climate (cold and wet Winters, dry and hot Summers) and distinctive flora, dominated by sclerophyllous shrubs (Manning and Goldblatt 2012). Importantly, these fynbos ecosystems are fire-prone, burning on average every 15 years (van Wilgen 1987). Fynbos plants have evolved seed serotiny as a strategy to cope with cyclical fire disturbance (Bond 1985). Serotinous *Protea* species, which are considered keystone fynbos species, are killed during these fires. However, they regenerate by releasing the seeds stored within their fruiting structures (infructescences) in the post-fire environment (Bond 1985). After successful regeneration, these species will flower within three to 8 years after fire (3 years for *P. repens*) (Le Maitre 1987) after which the infructescences form in about 3 months (Le Maitre and Midgley 1992). Seed serotiny is thus particularly important to sustain populations of non-sprouting *Protea* species after fires. At another trophic level, fire disturbance in the fynbos, and for these *Protea* infructescences in particular, may act as a biotope 'cleanser' in that many biotic components are locally incinerated, or at least reduced in numbers over vast areas (Kruger 1983). For example, by using population genetic techniques, Aylward et al. (2015) demonstrated that *Knoxdaviesia proteae* recolonise infructescences of *P. repens* over vast distances from unburnt areas as soon as these structures form again after fire. Yet very little is known about the post-fire colonisation dynamics of serotinous *Protea* associated actinomycete communities.

To better understand the biodiversity and ecology of actinomycetes associated with a plant host in an ephemeral landscape, we determined (1) the identity of the actinomycetes associated with *P. repens* developmental stages (flower bud, inflorescence, young infructescence and mature infructescence) and plant litter, both across collection season (Winter and Spring) and host population age (time after most recent fire); (2) which stage(s) in the development of buds into mature inflorescences and subsequently infructescences, is specifically associated with high actinomycete colonisation; and (3) whether variation

in actinomycete community assemblages can be predicted by fire disturbance, sampling season, or their interaction.

Materials and methods

Sampling sites and protocol

This study was conducted in mountain fynbos vegetation in the Franschhoek pass, Western Cape Province, South Africa (Burnt site—S 33.926.21 E 19.158.40, Unburnt site—S 33.925.32 E 19.159.71). Aylward et al. (2015) used the same study area to investigate the post-fire recolonisation patterns of *K. proteae* associated with *P. repens*. Some of the vegetation at this site burnt during 2009. *P. repens* individuals in the burnt site flowered for the first time in 2012 (3 years after the fire). The area adjacent to the burnt site was not affected by this fire due to the presence of a tarred road that acted as a firebreak (Fig. 1) and individuals here were more than 15-years-old.

For the purposes of this study the following definitions were used to characterise the developmental stages of inflorescences and infructescences of *P. repens*. A 'bud' contained immature flowers still enclosed by the involucral bracts (Fig. 2a). An 'inflorescence' had multiple open florets visible after the opening of the involucral bracts. At this stage, the florets are accessible to pollinators and other flower visitors (Fig. 2b). 'Infructescences' are the fruit-bearing structures formed once florets in the inflorescences are pollinated. 'Young infructescences' (c.a. 3 months after initially opening) were those where the involucral bracts had just closed around the florets, and started to turn brown and woody (Pretorius 1985) (Fig. 2c). 'Mature infructescences' were those formed during the previous flowering season (ca. 1-year-old) that were drier and woodier than young infructescences, and sometimes degraded or damaged by insects (Fig. 2d). All of these structures are hereafter collectively referred to as developmental stages.

In August 2013 (late Winter), five of each of the following samples were collected (Fig. 3): inflorescence buds, inflorescences, young infructescences and mature infructescences from five separate trees from the unburnt site (n = 25 per developmental stage). We collected 25 flower buds, inflorescences and young infructescences from 25 trees in the burnt site (each tree had only one development stage present, i.e. we collected from 75 young trees). Mature infructescences could not be collected from the burnt site because these structures had not yet formed and are found only in the second year of flowering. During November 2013 (late spring) 25 young and 25 mature infructescences were collected for a second time from the unburnt site (five of each structure from five different trees) and 25 young infructescences (from 25

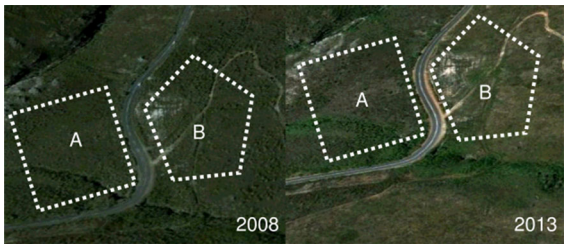

Fig. 1 Aerial image of sampling site in 2008 before fire occurred, and in 2013 after the fire. In 2008 larger trees can be seen on both sides of the road whereas in 2013 larger trees are only visible on the left side of the tar road. (A) Depicts the unburnt site and (B) the burnt site

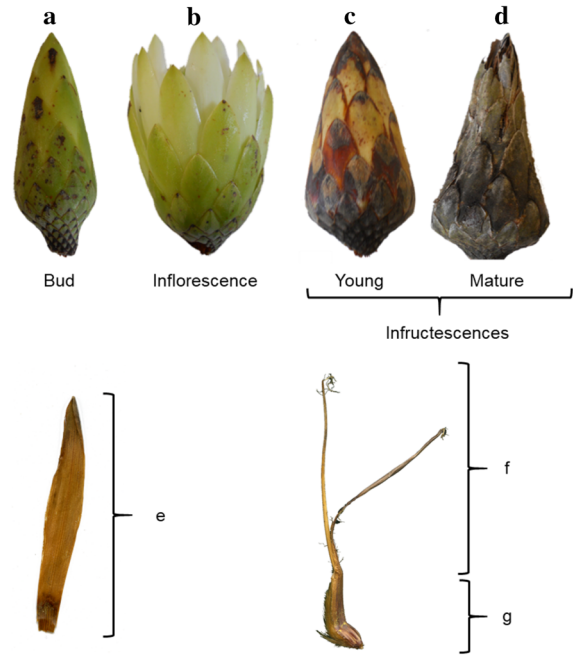

Fig. 2 Different *P. repens* flowering stages (**a**–**d**) and parts **e** involucral bracts, **f** florets and **g** seeds

young trees) from the burnt site, since an a posteriori finding suggested that actinomycetes were only consistently associated with infructescences. During this time, litter was also collected from around the bases of *P. repens* plants. Five bulk samples (ca. 1 dm^3) of litter were collected from the same five trees where infructescences had been collected in the unburnt site, and five bulk samples were collected in areas between the 25 trees sampled in the burnt site in order to acquire a representative sample for each of these two sites. Bulk litter samples were maintained in paper bags at 4 °C until isolations were performed.

Isolation and identification of actinomycetes

For isolations, whole developmental stage structures were separated into three pieces, which we refer to as floral organs. The actinomycetes from the inner involucral bracts, florets and the surface of seeds (Fig. 2e–g), within the different reproductive stages were isolated by aseptically removing these parts and placing them onto water agar (Sardi et al. 1992) and glycerol aspargine agar (Shirling and Gottlieb 1966). Water agar has been used extensively in the isolation of endophytic and plant-associated actinomycetes

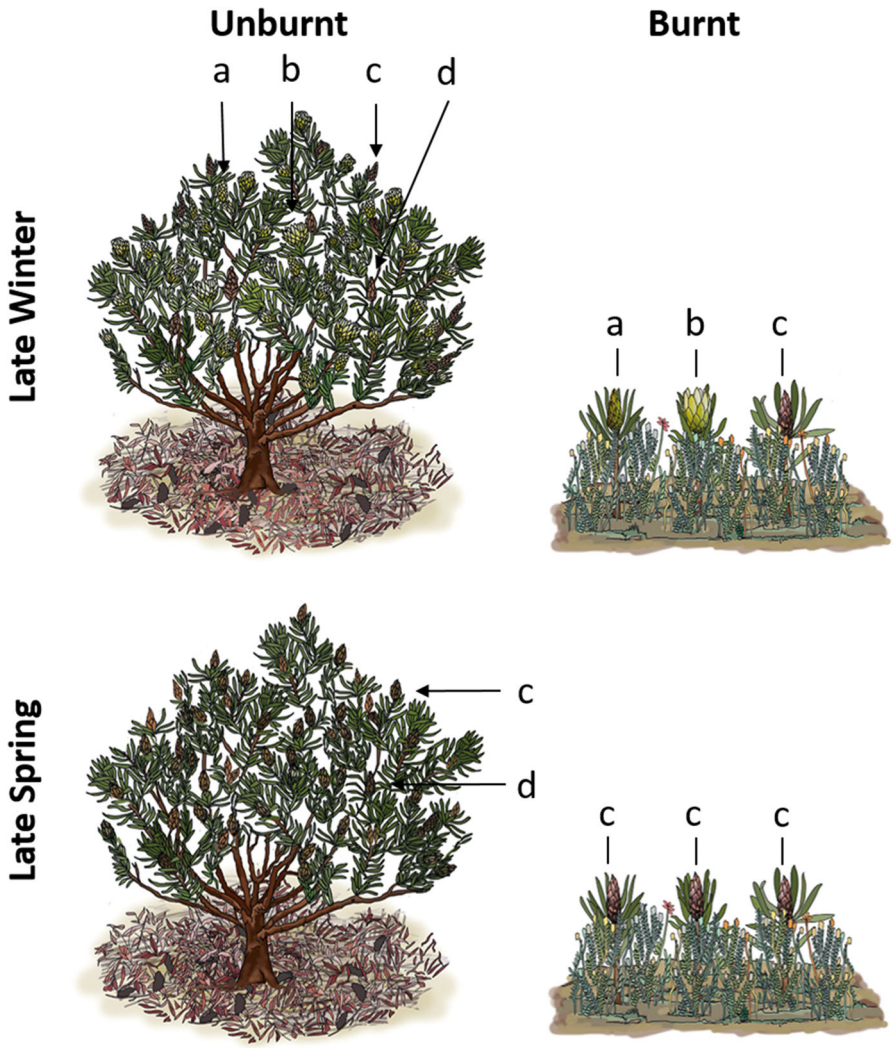

Fig. 3 Graphic representation of *P. repens* trees sampled. In late winter (August), trees from the unburnt site (ca. 15 yo, >3 m high) had buds (a), inflorescences (b), newly formed infructescences (c), and mature infructescences (d) on a single tree. Young *P. repens* in the burnt site (ca. 4 yo, 1 m high) were flowering for the first time and each tree had either a bud (a), inflorescence (b), or newly formed infructescence (c). In late spring (November), the same *P. repens* trees in the unburnt site had only newly formed (c) and mature (d) infructescences, while trees in the burnt site only had newly formed infructescences (c)

(Sardi et al. 1992; Castillo et al. 2002), while glycerol asparagine agar was also used to avoid possible isolation bias. Three pieces of the florets (ca. 30 mm), three whole seeds separated from florets, and three ca. 10 mm^2 pieces of involucral bract were placed on two replicate plates for each media type. For each of the five litter samples from the burnt and unburnt sites, five random pieces per sample (ca. 10 mm^2) were also placed onto plates of water agar and glycerol asparagine agar. Each litter sample was plated (replicated) five times. Both media types were supplemented with nystatin (0.05 g l^{-1}) and cycloheximide (0.05 g l^{-1}) to inhibit the growth of fungi. Plates were incubated at 28 °C in the dark and monitored regularly for the development of actinomycete colonies. These were recognised by their unique appearance due to the formation of aerial hyphae. Putative actinomycete colonies were re-inoculated onto yeast malt extract agar (YMEA) (Cafaro and Currie 2005) with cycloheximide (0.05 g l^{-1}) and nystatin (0.05 g l^{-1}) to obtain pure cultures. At least one re-isolation was made from each cluster of actinomycete colonies on material during primary isolation to ensure an adequate representation of the different taxa. Taxa could often be distinguished based on colour or morphology and care was taken to select at least one of each growth form per plate. Pure cultures of all isolates were grouped based on colony colour, spore colour and the colour of substrate pigments produced (Antony-Babu et al. 2010). Any isolates that showed even slight deviations in any of these characters were considered unique. Representative isolates from each of these unique groups were identified by sequencing their 16S rRNA genes; sequences have been deposited in Genbank under accession numbers KY952658–KY952673.

For sequence-based identification of isolates, genomic DNA was extracted from single pure colonies using a Prepman Ultra extraction kit (Applied Biosystems) following the manufacturer's protocol. PCR reactions consisted of 25 mM of each dNTP, 0.1 µM of primers 27f and 1294r (Frank et al. 2008), 2.5 mM $MgCl_2$ and 2.5 units Super-Therm *taq* polymerase (Southern Cross Biotechnology, Cape Town, South Africa). Reaction cycles had an initial denaturation step (94 °C for 10 min) followed by another 30 cycles of denaturation (94 °C for 1 min), annealing (58 °C for 1 min), extension (72 °C for 1 min) and a final extension step (72 °C for 5 min). Successful amplification of the 16S rRNA was confirmed using agarose gel electrophoresis. DNA amplicons were stained with GelRed™ (Biotium, Hayward, CA) and visualised with a Gel Doc EZ Imager (Bio-Rad, Hercules, CA) using the Image lab software (Bio-Rad, Hercules, CA). Amplicons were purified with *E. coli* exonuclease I and alkaline phosphatase (Werle et al. 1994). Partial 16S rRNA amplicons were sequenced in reactions containing 0.5 µl ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 2.5 µl sequencing buffer, 4 µl purified template DNA and 2.5 µM of the 27f primer (Frank et al. 2008). Sequencing products were cleaned by sodium acetate precipitation (BigDye® Terminator v3.1 Cycle Sequencing Kit Manufacturers Protocol) (Maxam and Gilbert 1977) and analyzed on an ABI 3130xl sequencer (Applied Biosystems).

Sequence reads were visually confirmed and sequence ends were manually trimmed using BioEdit (Hall 1999). Sequences were assigned to operational taxonomic units (OTUs) through calculation of pairwise distances between them and clustered at 99% similarity (Davelos et al. 2004) in the Mothur sequence processing pipeline (Schloss et al. 2009). The best-matching type strains to representative sequences for each OTU were identified using the EZTaxon server (Kim et al. 2012) (http://www.ezbiocloud.net/eztaxon). The best-matching environmental isolates were identified by comparing amplified 16S rRNA sequences to all sequences in the NCBI GenBank database using the BlastN search function (Benson et al. 2013).

A representative sequence for each OTU was aligned to selected type strains and highly similar published plant or insect associated sequences from GenBank (Table 1) with MAFFT version 7 (Katoh et al. 2005). Sequence alignments were used to construct a neighbor-joining phylogenetic tree in MEGA 6 with 1000 bootstrap replications (Tamura et al. 2013).

Actinomycete colonisation frequency and species richness on different *P. repens* developmental stages

Species richness of actinomycetes associated with different stages in the development of mature *P. repens* infructescences was estimated using the incidence coverage-based estimator (ICE) (Chazdon et al.

Table 1 Best matches achieved for representative sequence for each OTU from EZTaxon and GenBank

Isolate	Samples	Isolates	Strain name	Similarity (%)	Strain	Ref	GenBank accession
OTU1	NI, OI, L	322	*Streptomyces misionensis*	99.6	T		AB184285
			Streptomyces sp. WW1-3c	100	E	Mason et al. (2014)	KJ143662
OTU2	NI, OI	118	*Streptomyces costaricanus*	100	T	Esnard et al. (1995)	AB249939
			Streptomyces sp. NRRL 30562	100	E	Castillo et al. (2002)	AY127079
OTU3	L	8	*Streptomyces puniceus*	100	T		AB184163
OTU4	L	2	*Streptomyces hundungensis*	99.6	T	Nimaichand et al. (2013)	JN560157
OTU5	INF, OI	13	*Streptomyces anulatus*	100	T		DQ026637
OTU6	NI	2	*Streptomyces scopuliridis*	99.8	T	Farris et al. (2011)	JOEI01000056
			Streptomyces sp. WW4-5s	99	E	Mason et al. (2014)	KJ143672
OTU7	OI	1	*Streptomyces alni*	98.6	T	Liu et al. (2009)	DQ460470
			Streptomyces sp. Wi3-3s	99	E	Mason et al. (2014)	KJ143664
OTU8	NI, L	3	*Streptomyces stramineus*	98.4	T		AB184720
			Streptomyces sp. GW4-Bs	99	E	Mason et al. (2014)	KJ143670
OTU9	L	2	*Amycolatopsis equina*	99.2	T	Everest and Meyers (2011)	HQ021204
OTU10	NI, OI	4	*Streptomyces althioticus*	98.6	T		AY999791
			Streptomyces sp. 36bB	99	E	Takasuka et al. (2013)	HM235469
OTU11	L	2	*Streptomyces yunnanensis*	99.8	T	Zhang et al. (2003)	AF346818
OTU12	L	4	*Streptomyces subrutilus*	100	T		DQ460470
			Streptomyces sp. SAUK6063	100	E	Zhao et al. (2012)	GU143055
OTU13	OI, L	7	*Streptomyces graminifolii*	100	T	Lee and Whang (2014)	HQ267984
			Streptomyces sp. SAP837.1	100	E	Álvarez-Pérez and Herrera (2013)	JX067713
			Streptomyces sp. SW4-2s	100	E	Mason et al. (2014)	KJ143667
OTU14	NI, L	3	*Streptomyces cavourensis*	99.8	T	Skarbek and Brady (1978)	AB184264
OTU15	OI	1	*Amycolatopsis saalfeldensis*	98.9	T	Carlsohn et al. (2007)	HQ021204
OTU16	B, OI	6	*Streptomyces maoxianensis*	97.7	T	Guan et al. (2015)	KF887908
			Streptomyces sp. AcH505	99	E	Riedlinger et al. (2006)	DQ231567

Samples are abbreviated *B* bud, *INF* inflorescence, *NI* new infructescence, *OI* mature infructescence, *L* litter. Strains are indicated as *T* type strain or *E* environmental strain

1998) and the Chao2 estimator (Chao 1987) in EstimateS (Colwell 2005) because we had a set of incidence data (Gotelli and Colwell 2011). The effect of different categorical predictors on the estimated species richness and the observed number of species was tested for significance by fitting a generalised linear mixed effects model (GLMM) using a Poisson distribution (Bolker et al. 2009) with the *glmer* function in the *lme4* package (Bates et al. 2014) using R (R Developmental Core Team 2015). Fixed effects included in the model were site (burnt/unburnt) and developmental stage (litter, buds, inflorescences, young infructescences and mature infructescences), while season (Winter/Spring) was used as random effect. Over-dispersion was tested for by using the *dispersion_glmer* function in the *blmeco* R package (Korner-Nievergelt et al. 2015) and the selected model was found not to be overdispersed. The best fit model was richness estimator (Sobs/ICE/Chao2) ~ "Developmental stage" + (1|Season), where the richness estimators used were the response variable, sample type the fixed effect, and season the random effect. A post hoc analysis of the levels within sample type was performed with the *glht* function in the *multcomp*

package (Hothorn et al. 2008) using the Tukey–Kramer Honestly Significant Difference (HSD) contrasts.

The frequency of isolation for each individual OTU isolated was calculated by dividing the total number of samples that contained actinomycetes by the total sample size for each respective sample category (n = 25 for all flowerheads; n = 5 for litter samples). The relative abundance of each OTU was calculated by dividing the total number of occurrences of an OTU in a specific sample category by the total number of isolates of all OTUs in that sample category.

To test the hypothesis that frequency of occupancy of actinomycetes is dependent on developmental stage of the reproductive structures (bud, inflorescence, young and mature infructescences) and site (burnt/unburnt), a binary logistic regression analysis was performed because our binary response variable follows a binomial distribution (McDonald 2009). To minimise the effect of season on results, and because flower buds and inflorescences were available only during Winter, data from structures collected in Spring were not included in these analyses. Binomial logistic regression models were constructed using presence or absence of actinomycetes as a binary response variable and site, developmental stage and their interaction as explanatory variables. Complete separation was common because this dataset contained some variables consisting of mostly zeros (actinomycetes absent). To account for this, we used the *logistf* (Heinze et al. 2013) package in R (R Developmental Core Team 2015), applying Firth's bias reduction (Heinze and Schemper 2002). The best-fit model was selected by comparing the Akaike information criterion (AIC) values of models constructed in a stepwise manner, adding an explanatory variable at each step. The final model contained field (burnt/unburnt) and developmental stage (bud/inforescence/young infructescence/mature infructescence) as well as their interaction (field × developmental stage) as explanatory variables and presence or absence of any actinomycetes as a response variable.

Odds ratios (Szumilas 2010) were calculated for all levels of the variables included in the models. In this study, a factor having an odds ratio of 1 can be interpreted as having an equal chance of having or not having actinomycetes. Odds ratios larger than one indicate that they were more likely than not to have actinomycetes. The larger the magnitude of the odds ratios when larger than one provides further confidence that actinomycetes are likely to be present. The opposite is true for magnitudes of odds ratios smaller than one.

Frequency of occurrence and association of common OTUs in *P. repens* infructescences

Frequency of colonisation data were presence/absence. We therefore used binary logistic regression models in R (using the *logistf* package) (Heinze et al. 2013) to test for differences in colonisation patterns of actinomycetes in infructescences collected under different sampling conditions. Three categorical explanatory variables were included in model selection. These were season sampled (Winter/Spring), site sampled (burnt and unburnt sites) and age of infructescence (young/mature infructescences) and these were tested for explanatory power for all actinomycetes encountered and for the main OTUs (OTU1 and OTU2) separately. For all actinomycetes, the best fit model contained season, site and the interaction of season and site as significant effects, for OTU1 season, site and the interaction of season and site was the best model and for OTU2 the best model contained season, site and age of infructescence. Odds ratios (Szumilas 2010) were calculated for all levels of the variables and interpreted as described above.

Actinomycete community assemblage across site, sampling season and infructescence age

To assess whether there were differences in actinomycete species assemblage composition between young and mature infructescences, burnt and unburnt sites and between the different seasons (Winter vs. Spring), we used permutational multivariate analyses of variance (PERMANOVA) (Anderson 2001) in PRIMER 6 (Anderson et al. 2008; Clarke and Warwick 2005). For this analysis, the presence data for the five infructescences from a single tree were added to obtain a count for each OTU. In the burnt site, the OTU presence data for individual *P. repens* from five clusters of trees were added to obtain equivalent sampling size. To determine F and p values for assemblage composition we used 9999 permutations of between-sample Bray–Curtis similarity measures on fourth-root-transformed data (to reduce the effect of common taxa) (Anderson 2001). Actinomycete

assemblage composition for significant factors was visually explored by performing principal component analysis using the *ggbiplot* package (Vu 2011) in R (R Developmental Core Team 2015).

Results

Actinomycetes from floral developmental stages and surrounding plant litter

In total, 624 putative actinomycete isolates were collected from a total of 830 different floral organs from 250 different developmental stages and 10 litter samples. Of these, 176 separate floral organs (bracts, seeds and florets) from *P. repens* and eight litter samples contained actinomycetes. These floral organs were from three buds, three inflorescences, 43 young infructescences, and 37 mature infructescences. Phylogenetic analyses (below) identified the actinomycetes as belonging to 16 OTUs. In total three isolates (all OTU 16) originated from three different buds, three isolates (all belonging to OTU 5) from three different inflorescences, 172 from young infructescences, and 281 from mature infructescences. A total of 67 isolates were from litter from the burnt and unburnt sites. Actinomycetes were isolated from all litter samples collected from the unburnt site (n = 5), whereas only two litter samples collected from the burnt site contained actinomycetes (n = 5).

Of the 16 OTUs detected from buds, inflorescences, infructescences and litter, 14 were identified as *Streptomyces* spp. (Fig. 4; Table 1) and two were identified as *Amycolatopsis* spp. (Fig. 4; Table 1). The two most common OTUs, OTU1 and OTU2 were of the same two *Streptomyces* spp. detected in a previous study on *P. repens* and *P. neriifolia*, and have known antifungal properties (Human et al. 2016). For several isolates, there were matches with 100% similarity in GenBank but were reported only when a valid reference was available (Table 1). OTU1, an isolate previously reported to have antifungal activity against a wide spectrum of fungi, was also very similar to a previously reported isolate from Cranberry bogs (Mason et al. 2014). OTU2 was very similar to an isolate from *Kennedia nigriscans* that produces a range of novel wide-spectrum antibiotics known as Munumbicins (Castillo et al. 2002). Three OTUs (OTU7, 8 and 13) had very strong similarity (>99%) to isolates from Sphagnum bogs (Mason et al. 2014). Two OTUs (OTU7 and OTU12) were very similar (>99%) to *Streptomyces* isolates from the rhizosphere of trees (Liu et al. 2009; Zhao et al. 2012). Partial 16S rRNA of OTU3 was 100% similar to an isolate from a *Crematogaster* ant from Cameroon (Hanshew et al. 2014). OTU13 had a high level of similarity to an isolate previously reported from floral nectar of Mediterranean plants (Álvarez-Pérez and Herrera 2013) and OTU16 was similar to an isolate known to be a mutualist of *Amanita muscaria* (Riedlinger et al. 2006).

Actinomycete colonisation frequency and species richness in different inflorescence organs

Actinomycetes were isolated from bracts, seeds and florets (Table S1). There were no significant differences in species richness or frequency of colonisation of the different floral parts (See Supplementary methods; Table S2). The entire developmental stage structure (i.e. buds, inflorescences or infructescences) from which these isolates were obtained are therefore treated as sample units in the following sections.

Actinomycete colonisation frequency and species richness on different *P. repens* developmental stages

Estimates of species richness for all samples are listed in Table 2. Similar results were obtained when comparing observed species richness and both measures of estimated species richness between samples (Table 2). No differences were found for species richness between buds and inflorescences. The numbers of species in buds and inflorescences were significantly lower than those for both young and mature infructescences (Table 3). Species richness in young infructescences was significantly lower than that from mature infructescences (Table 3).

Only 2% of all buds (n = 50, burnt and unburnt combined) and 4% of flowers (n = 50, burnt and unburnt combined) contained actinomycetes. In contrast, 68% of young infructescences in the unburnt field contained actinomycetes, but this was for the Winter sampling only (Table 4). The frequency of isolation of actinomycetes varied amongst infructescences sampled, ranging from 8% (n = 25) in young infructescences from the burnt site sampled in Winter,

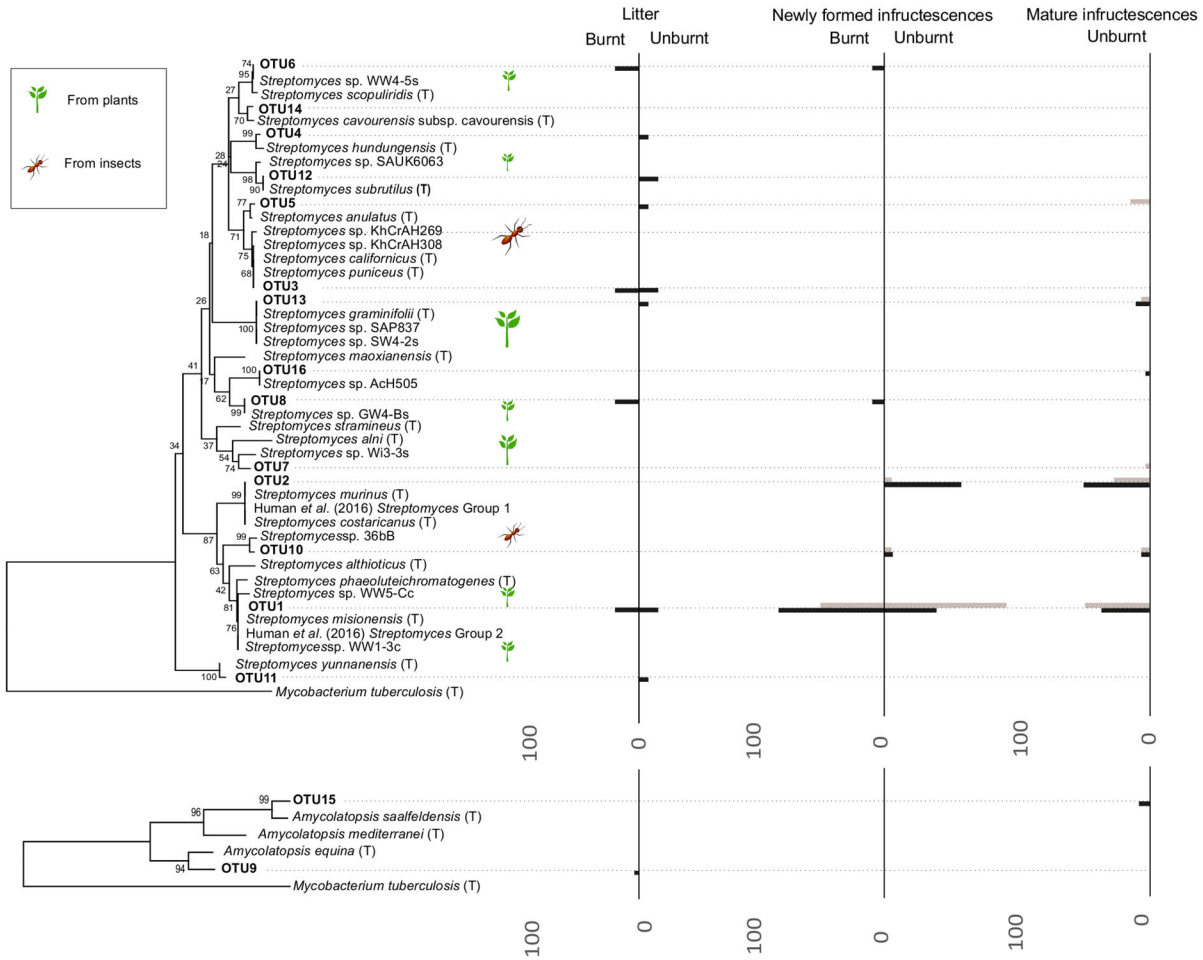

Fig. 4 Neighbour joining tree representing a 16S rRNA phylogeny of our isolates (bold type), closest matching type-strains (T) and relevant reference sequences from literature. Sources of references strains are labelled when associated with plants or insects. Relative abundances for winter (black) and spring (grey) are indicated for respective sample categories

to 76 and 72% in mature infructescences from the unburnt site in Winter and Spring respectively (Table 4). There was a substantial increase in the colonisation of infructescences by actinomycetes in the burnt site from Winter to Spring. Only two young infructescences (8%) from the burnt site contained actinomycetes in Winter, which increased to 11 (44%) in Spring. Newly formed infructescences from the unburnt site had a frequency of isolation of 68% in Winter and 52% in Spring.

Results of regression analyses showed that the presence of actinomycetes was significantly influenced by developmental stage ($p < 0.001$; Fig. 5). Actinomycetes were likely to occur in both young and mature infructescences while buds and inflorescences were structures unlikely to contain actinomycetes (Fig. 5). Infructescences from the unburnt site (old host population) were found to have a high likelihood of containing actinomycetes while structures from the burnt field (young host population) seldom contained actinomycetes (Fig. 5). The best fit model suggested that the interaction between developmental stage and site was significant ($p < 0.001$). The association of actinomycetes with young infructescences from the unburnt site was strong and with mature infructescences from the unburnt site even stronger, i.e. these more mature structures in the unburnt site were most likely to contain actinomycetes. Buds and inflorescences from the burnt and unburnt sites and young infructescences from the burnt site were unlikely to have a strong association with actinomycetes.

Table 2 Alpha diversity estimates for all sample categories from this study

Month	Field	Sample type	S_{obs}	ICE (±SD)[a]	Chao2 (±SD)	Isolates	Unique OTUs
Nov	Total	Litter	11	40.05 (±0.84)	32.05 (±2.88)	40	5
Nov	Burnt	Litter	4	8.8	8.8 (±4.78)	14	1
Nov	Unburnt	Litter	8	16.87 (±0.19)	10.67 (±8.39)	26	3
Aug	Burnt	Bud	0	0	0	0	0
Aug	Unburnt	Bud	1	1	1	3	0
Aug	Burnt	Inflorescence	1	1	1	2	0
Aug	Unburnt	Inflorescence	1	1	1	1	0
Total	Total	Infructescence	10	25.9	25.9 (±3.32)	453	5
Aug	Burnt	Young infructescence	2	2.96	2.96 (±0.62)	3	0
Nov	Burnt	Young infructescence	3	3.96	3.96 (±0.96)	36	0
Aug	Unburnt	Young infructescence	3	3.96	3.96 (±0.5)	90	0
Nov	Unburnt	Young infructescence	3	3	3 (±0.3)	43	0
Aug	Unburnt	Mature infructescence	6	6.75 (±0.07)	6.24 (±0.78)	151	2
Nov	Unburnt	Mature infructescence	5	7.88	7.88 (±1.4)	130	1

[a]Absent ICE SD values are 0

Table 3 Comparisons of species observed, ICE and Chao2 values between different developmental stages using Tukey's HSD contrasts

Response variable	Fixed effect	Random effect	Post-hoc tests	*p* value
Species observed	Sample-type	Season	Inflorescence = bud	0.39
			Young infructescence > bud	<0.001
			Mature infructescence > bud	<0.001
			Young infructescence > inflorescence	0.002
			Mature infructescence > inflorescence	<0.001
			Mature infructescence > young infructescence	<0.001
ICE	Sample-type	Season	Inflorescence = bud	0.39
			Young infructescence > bud	<0.001
			Mature infructescence > bud	<0.001
			Young Infructescence > inflorescence	<0.001
			Mature infructescence > inflorescence	<0.001
			Mature infructescence > young infructescence	<0.001
Chao2	Sample-type	Season	Inflorescence = bud	0.42
			Young infructescence > bud	<0.001
			Mature infructescence > bud	<0.001
			Young infructescence > inflorescence	<0.001
			Mature infructescence > inflorescence	<0.001
			Mature infructescence > young infructescence	<0.001

Frequency of occurrence and association of common isolates in infructescences

Regarding the dominant OTUs, one of the two isolates collected during Winter from the young infructescences in the burnt site was OTU1, while in Spring 92% of the 36 isolates were represented by OTU1 (Table 2; Fig. 4). OTU2 was never collected from the burnt site. In young infructescences collected in the unburnt site in Winter, OTU1 had a relative

Table 4 Relative abundance and frequency of isolation for all actinomycetes, OTU1 and OTU2 in different sample categories

Month	Field	Sample type	Isolates	Relative abundance			Frequency of isolation		
				OTU1 (%)	OTU2 (%)	Other OTUs (%)	Total[a]	OTU1 (%)	OTU2 (%)
Nov	Burnt	Litter	14	25	0	75	40	4	0
Nov	Unburnt	Litter	26	18	0	82	100	8	0
Aug	Burnt	Young infructescence	2	5	0	50	8	4	0
Nov	Burnt	Young infructescence	36	92	0	8	44	40	0
Aug	Unburnt	Young infructescence	90	94	4	3	68	68	4
Nov	Unburnt	Young infructescence	43	44	52	4	52	24	36
Aug	Unburnt	Mature infructescence	151	62	18	20	76	68	36
Nov	Unburnt	Mature infructescence	130	39	52	9	72	44	60

[a]Frequency of samples containing any actinomycete

abundance of 94% (90 isolates), while this decreased to 44% in Spring (43 isolates) (Table 2; Fig. 4). At the same time, OTU2 increased from 4% in Winter to 52% in Spring in these young infructescences. In Winter, in mature infructescences from the unburnt site, OTU1 had a relative abundance of 62% and OTU2 18%. In spring, this changed to 39% for OTU1 and 52% for OTU2. In litter, OTU1 had a relative abundance of 25% in the burnt field and 18% in the unburnt field. OTU2 was absent from litter. The relative abundance of other OTUs in infructescences was generally low, ranging from 4 to 20% (Table 2; Fig. 4).

A strong association was detected between total actinomycetes and infructescences from the unburnt site in both Winter and Spring (Fig. S1). OTU1 was also strongly associated with infructescences from the unburnt site in Winter and Spring and in the burnt site only in Spring. OTU2 had a strong association with infructescences from the unburnt site. OTU2 was also more likely to occur in Spring.

Actinomycete community assemblage across site, sampling season and infructescence age

Season ($p = 0.008$), site (burnt/unburnt) ($p = 0.004$) and their interaction ($p < 0.001$) had significant effects on actinomycete assemblage within infructescences (Table 5). The age of infructescences had a marginally significant effect on assemblage composition ($p = 0.05$) and the interaction of season and age of infructescences was not significant ($p = 0.115$). Principal component analysis indicated that actinomycete assemblages from infructescences could be separated into clusters according to site and season (Fig. 6) and that OTU1 and OTU2 had a large effect in differentiating these assemblages (Fig. 6).

Discussion

We were able to demonstrate that two *Streptomyces* spp. are common associates of the closed infructescences of *P. repens* and that the early floral developmental stages, such as buds and open inflorescences, are unlikely to be an actinomycete niche. We isolated two of the antifungal *Streptomyces* spp. previously reported by Human et al. (2016) and thus suggest that these are common symbionts of *P. repens*. We further report 16 different OTUs from *P. repens* developmental stages and litter, many similar to actinomycetes previously reported from various unrelated plants and insects. As expected, the frequency of colonisation of infructescences in the burnt site was much lower than that in an adjacent unburnt site. This fire-cleansing effect is consistent with findings for fungi within *P. repens* infructescences (Aylward et al. 2015). A strong seasonal species turnover effect was also detected, where the dominant infructescence inhabiting *Streptomyces* OTU was replaced by another as the season progressed. Overall, these results contribute to the relatively few reports of *Streptomyces* on above ground plant tissues compared with

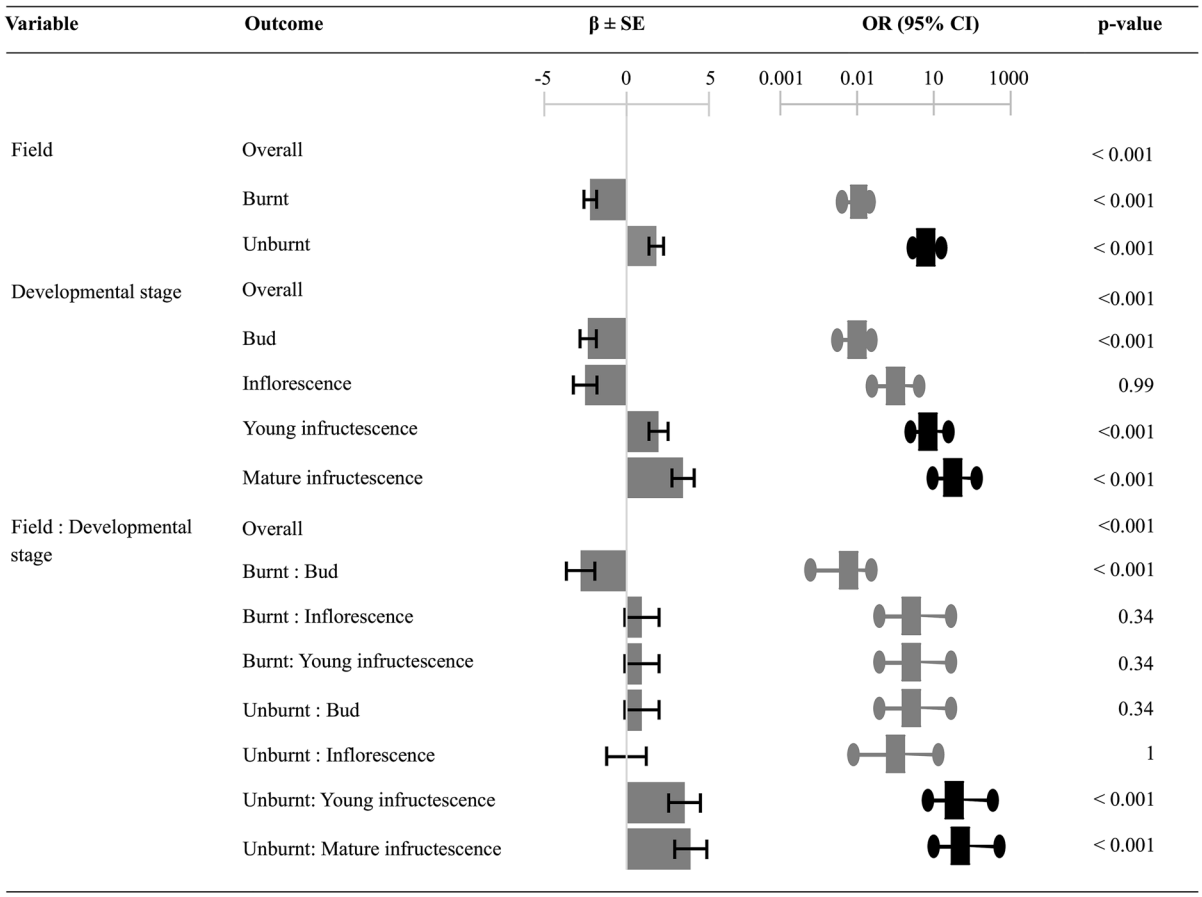

Fig. 5 Regression coefficients (β), odds ratios (OR) and p-values from binary logistic regression of actinomycete presence in different *P. repens* inflorescence developmental stages and age of the host population. *Note* Positive regression coefficients, and odds ratios larger than one (in black) with significant p-values are considered to represent a likely association. Odds ratios larger than one, indicative of associations, are in black

those on roots and in the rhizosphere (Passari et al. 2015; Sarria-Guzmán et al. 2016; Taechowisan et al. 2003).

The closed, woody infructescences of *P. repens* appear to be the floral stage most commonly associated with actinomycetes. Actinomycetes, specifically *Streptomyces* spp., are regarded as facultative oligotrophs (Hodgson 2000) and often found only in nutrient-poor environments such as soil, litter and sediments inaccessible to many other bacteria (Kämpfer 2006). In contrast, *P. repens* bud and inflorescence stages, where actinomycetes were rarely isolated, are nutrient rich environments containing nectar consisting almost entirely of glucose and fructose (Van Wyk and Nicolson 1995). Actinomycetes may be outcompeted by bacteria that have faster growth rates on such simple sugars (van Dissel et al. 2014). Furthermore, glucose is known to inhibit antibiotic production in many species of *Streptomyces*, further disabling them from being competitive (Sánchez et al. 2010). Nonetheless, that actinomycetes are clearly specific to the closed infructescences of *P. repens* suggests that their establishment requires the presence of specific vectors that are able enter these tight, access-restricted parts of the plant.

Wind and water are common pathways of actinomycete dispersal (Pearce et al. 2009). However, if this was true for *P. repens,* we would have expected more frequent isolations of actinomycetes on the more open, wind and water exposed parts of the plants. Instead, actinomycetes are associated with the more closed-off infructescences. Because of the physical barriers that such tightly closed infructescences present, arthropods are more likely responsible for their dispersal. Both

Table 5 Results of PERMONOVA analysis testing the effect of season, site, age of infructescence and their respective interactions on community composition

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Season	1	1785.3	1785.3	4.687	0.0078	9952	0.0084
Site	1	2776.7	2776.7	7.290	0.0004	9955	0.0008
Age	1	1074.4	1074.4	2.820	0.05	9959	0.0601
Season × site	1	4039.2	4039.2	10.60	0.0001	9956	0.0001
Season × age	1	810.61	810.61	2.128	0.1149	9962	0.1189
Res	24	9141.4	380.89				
Total	29	23,123					

insects and mites have been shown to be effective vectors of actinomycetes, specifically *Streptomyces* spp. (Kaltenpoth 2009; Ruddick and Williams 1972; Seipke et al. 2012). These animals are also well-known to colonise the closed infructescences of serotinous *Protea* spp.

There are numerous mites that are known to inhabit *P. repens* infructescences, some of which are mutualists of *K. proteae* and *Sporothrix splendens* (Roets et al. 2007, 2009, 2011; Theron et al. 2012). These mites vector their mutualist fungi to newly formed infructescences from older desiccating infructescences (Roets et al. 2009, 2011). In addition, several soil inhabiting mites have been found in *P. repens* infructescences (Theron et al. 2012). Dispersal of microorganisms by beetles and their phoretic mites is also regarded as a key mechanism by which juvenile or younger individuals of *P. repens* are re-colonised by fungi after fire (Aylward et al. 2014, 2015; Roets et al. 2009). Actinomycete dispersal is thus likely also by mites and infructescence-boring insects, since they are the only known routes of entry and exit in infructescences.

The lower observed frequency of bacterial colonisation of infructescenses at the burnt site could be attributed to the fact that borers infesting infructescences are absent from *P. repens* populations after fire (Wright and Samways 1999). The borers gradually return once the *Protea* spp. reach reproductive maturity and may thus occur in very low abundance for a few seasons (Wright and Samways 1999). These infructescence-inhabiting insects, in the orders Coleoptera, Hemiptera and Psocoptera, are known vectors of the ophiostomatoid fungi and possibly actinomycetes into uncolonised *P. repens* infructescences, either independently or via transmission by mites for which they are also vectors (Aylward et al. 2015; Roets et al. 2009, 2011).

There was a consistently higher frequency of isolation of the two most common *Streptomyces* spp. in older infructescences. Older infructescences are thus important sources of actinomycetes in fynbos ecosystems. Indeed, one of the most common OTUs isolated was never detected in litter and another was less abundant in litter than in older infructescences. This provides further evidence that mature infructescences are an important source actinomycetes. Older infructescences are also known as a source of ophiostomatoid fungi and mites, mutually dispersing to newly formed infructescences when mature infructescences begin to desiccate (Aylward et al. 2014, 2015; Roets et al. 2009, 2011). This provides additional evidence that mites probably vector actinomycetes from older infructescences in the same way they do fungi. The absence of older infructescences after removal by fire (and thus absence of an actinomycete source population) is thus also the most probable explanation for the slower rate of colonisation of OTU1 and the absence of OTU2 in infructescences from the burnt site.

Streptomyces species produce numerous antibiotics and they are important components in many fungus-actinomycete mutualisms or antagonisms (Kaltenpoth 2009; Seipke et al. 2012). We found that the peak in occurrence of isolates belonging to OTU1 coincided with the peak of two *P. repens* associated fungi, *Sporothrix* and *Knoxdaviesia* (Roets et al. 2005). Although OTU1 produces the antifungal antibiotic fungichromin in low amounts (Human et al. 2016), the fungi and this actinomycete may be able to co-exist. However, isolates that were the same species as OTU2, and were dominant and abundant in late Spring, produced higher levels of fungichromin (Human et al. 2016). OTU2 also replaced OTU1 at this later sampling stage. Interestingly, *Sporothrix* and *Knoxdaviesia* were shown to be absent in the

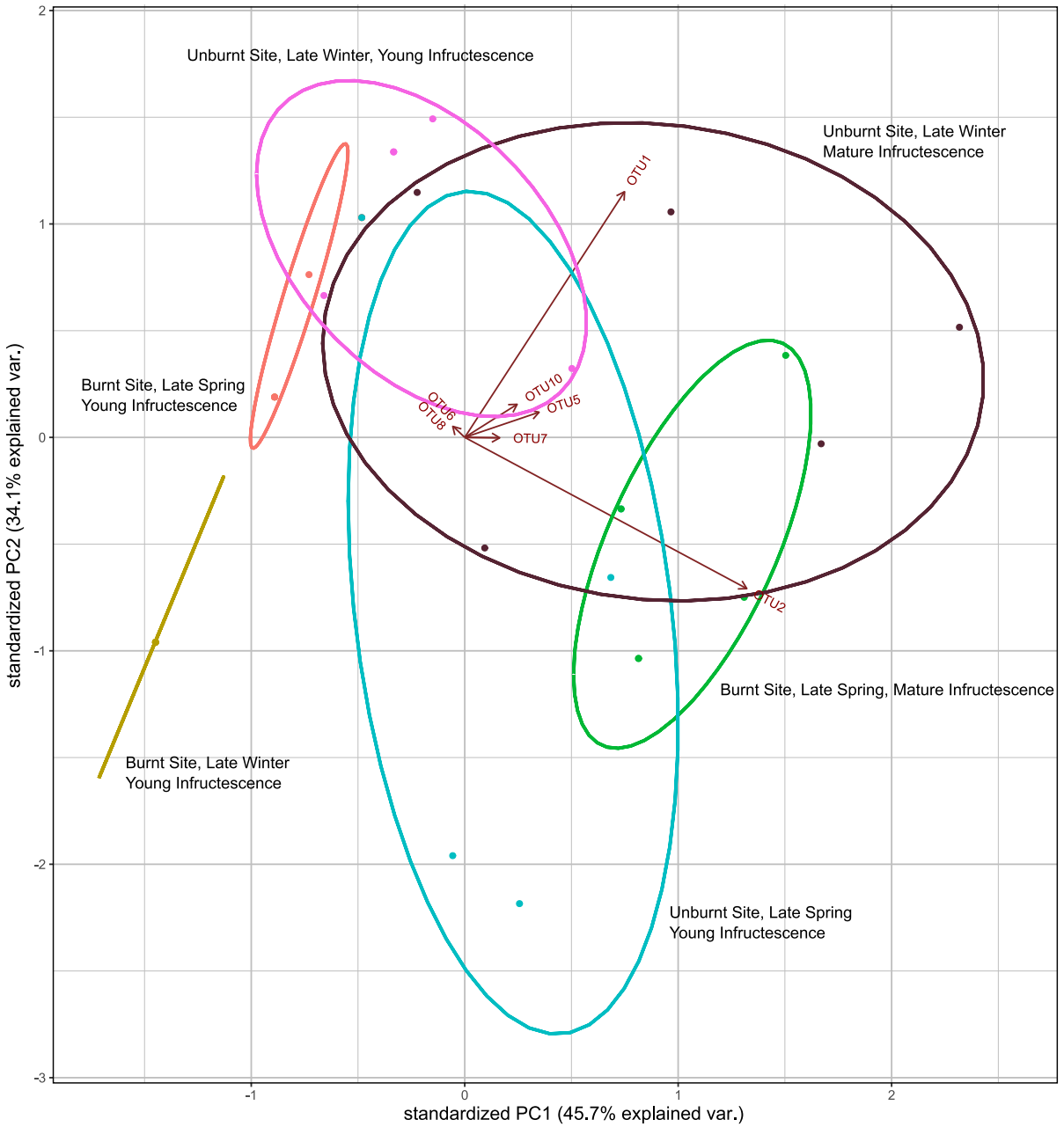

Fig. 6 Principal component analysis of square root transformed assemblage data. Assemblages are grouped by sample site (burnt/unburnt) and season (winter/spring) and age of infructescences

infructescences of *P. repens* in the late Spring and Summer months (Roets et al. 2005). It consequently appears that the presence of OTU1 provides an opportunity for *Sporothrix* and *Knoxdaviesia* to momentarily utilise the infructescence niche.

The factors responsible for the pattern of actinomycete assembly in *P. repens* infructescences remain unknown. It is possible that antibiotics are already present within *P. repens* infructescences when *Streptomyces* species are introduced. These bacteria are resistant to many different antibiotics (Davies and Davies 2010), which enable them to colonise environments containing antibiotics more effectively than many other saprotrophic bacteria and fungi. It is also

possible that changes in the available nutrients in infructescences result in shifts in the dominant microbiota. For example, the exhaustion of easily utilisable sugars may cause shifts in the dominant bacterial population towards taxa better adapted to utilise the remaining lignin (Mitchell and Coley 1987). Further research should determine how the *Streptomyces* spp. discovered in this study interact, and how efficiently they utilise different carbon compounds. This may reveal how the actinomycete community in *Protea* infructescences are shaped.

Conclusions

This study provides the first analysis of actinomycete diversity associated with *P. repens* in a fynbos ecosystem. Actinomycetes were most consistently found in the infructescences of *P. repens* where they they may influence the diversity of saprotrophic bacteria and fungi through the many antibiotics they are known to produce. The accumulation of actinomycetes in mature *P. repens* infructescences provides excellent opportunities for antibiotic bioprospecting. We have also shown that future sampling efforts should consider the timing of sampling and fire frequency which significantly influence actinomycete presence and diversity estimations. Mature infructescences were the most important sources of actinomycetes. This implies that any premature disturbance, such as increased fire frequency in fire-prone fynbos habitats, could potentially impact their local re-colonisation ability.

Acknowledgements We thank the National Research Foundation (NRF) and the Department of Science and Technology (DST)/NRF Centre for Tree Health Biotechnology (CTHB) for financial support. We also thank the Cape Nature Conservation Board for providing collection permits. We further wish to thank Glenda Brits from the Department of Education Innovation, University of Pretoria, for producing Fig. 3.

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary Methods

Actinomycete colonization frequency and species richness on different *P. repens* flower organs

Species richness estimators were calculated for each flower organ (bracts, seeds and stylets), for each reproductive stage evaluated (buds, inflorescences, young infructescences and mature infructescences), for both collection seasons (Winter and Spring) and at both sites (burnt and unburnt) according to the Incidence-based coverage (ICE) (Chazdon et al. 1998) and Chao2 (Chao 1987) estimators in EstimateS version 9.10 (Colwell 2005). These two species richness estimators were used because the OTU data represented a set of replicated incidence data (presence/absence data) (Gotelli and Colwell 2011). To test whether species richness varied among flower organs, a GLMM using the Poisson distribution was constructed, using observed species richness as response variable, organ type as fixed effect and season, site and developmental stage as random effects.

To test whether the frequency of detection (i.e. frequency of colonization/occupation) of actinomycetes varied for different flower organs, a binary logistic regression model was constructed with presence/absence of actinomycetes as response variable, organ type as fixed effect and season, field and developmental stage as random effects. Species richness is count data and followed a Poisson distribution (Bolker et al. 2009), while presence/absence data were binary and followed a binomial distribution (McDonald 2009). Both these models were constructed using the *glmer* function in the *lme4* package (Bates et al. 2014) in the R statistical software suite (team 2015). The GLMM for detecting differences in actinomycete species richness between different organs was tested for over-dispersion using the *dispersion_glmer* function in the *blmeco* R package and found not to be overdispersed (Korner-Nievergelt et al. 2015).

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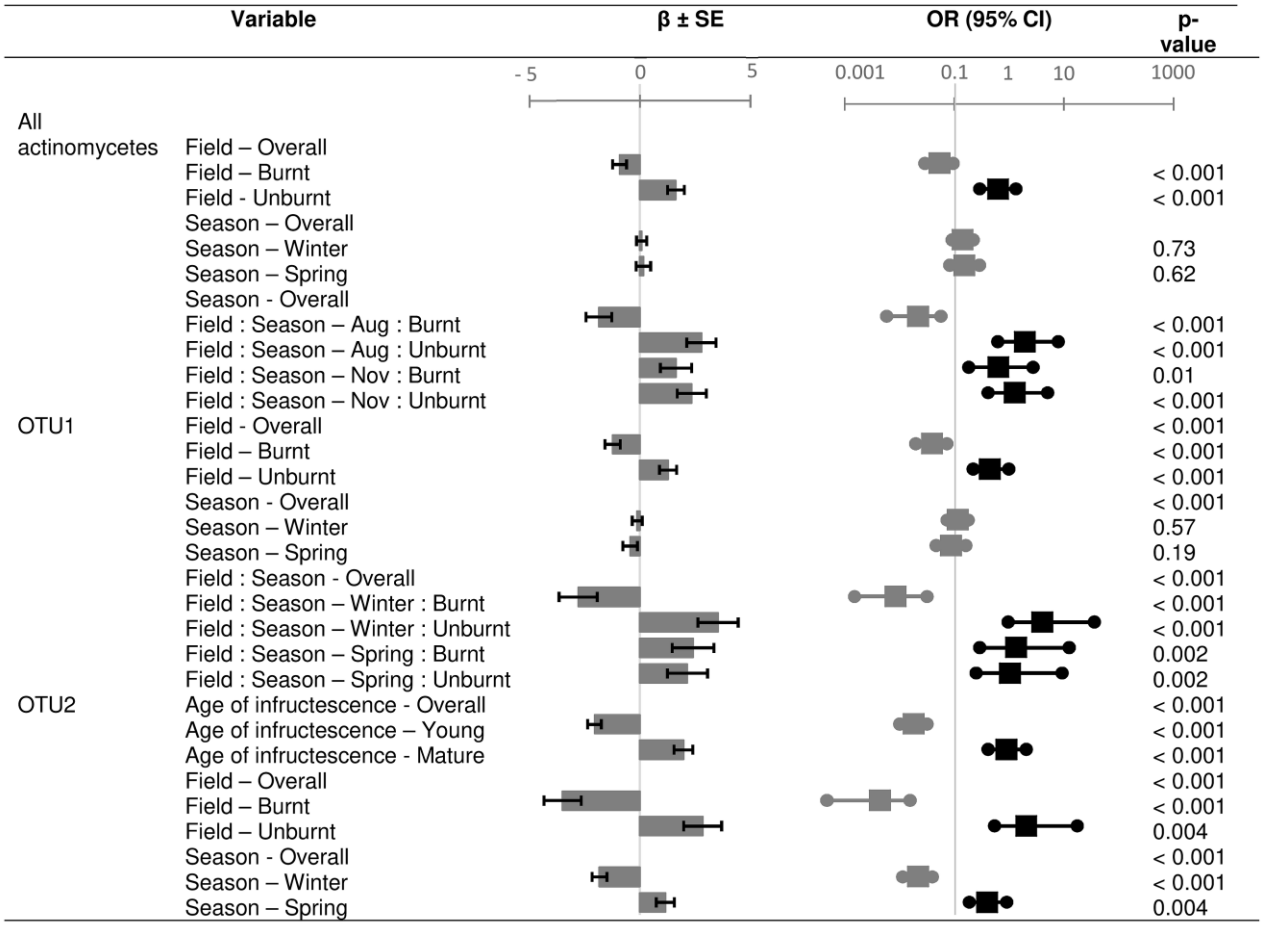
Supplementary figure

Fig S1. Regression coefficients (β), odds ratios (OR) and p-values from binary logistic regression of presence or absence of all actinomycetes, OTU1 and OTU2 in infructescences



Note: Positive regression coefficients, and odds ratios larger than one (in black) with significant p-values are considered to represent a likely association. Odds ratios larger than one, indicative of associations, are in black.

Supplementary tables

Table S1. Species richness estimators and colonization frequencies for all sample categories in this study.

Field	Month	Organ	Developmental stage	Sobs	ICE	Chao2	Chao2 SD	% colonized	% OTU1	% OTU2
Unburnt	Nov	Bract	young infructescence	3	3.99	3	0.48	40%	20%	24%
Burnt	Nov	Bract	young infructescence	1	1	1	0	44%	44%	0%
Unburnt	Aug	Bract	young infructescence	1	1	1	0.01	40%	40%	0%
Unburnt	Aug	Bract	mature infructescence	6	7.42	7.92	3.6	60%	56%	16%
Unburnt	Nov	Bract	mature infructescence	5	7.88	7.88	4.22	64%	36%	36%
Unburnt	Aug	Stylets	mature infructescence	5	6.82	5.96	2.12	60%	44%	16%
Unburnt	Nov	Stylets	mature infructescence	4	5	4.48	1.28	56%	24%	48%
Unburnt	Aug	Stylets	young infructescence	3	3.96	3.96	2.04	56%	56%	4%
Burnt	Nov	Stylets	young infructescence	1	1	1	0.13	16%	16%	0%
Unburnt	Nov	Stylets	young infructescence	2	2	2	0.16	32%	16%	20%
Unburnt	Aug	Seeds	young infructescence	1	1	1	0.02	32%	32%	0%
Burnt	Nov	Seeds	young infructescence	3	3.96	3.96	2.04	36%	32%	4%
Unburnt	Nov	Seeds	young infructescence	2	3	2.48	1.28	12%	4%	8%
Unburnt	Nov	Seeds	mature infructescence	3	3	3	0.48	48%	28%	32%
Unburnt	Aug	Seeds	mature infructescence	4	4.5	4	0.56	60%	52%	12%
Burnt	Aug	Seeds	young infructescence	2	2.96	2.96	1.97	8%	4%	0%
Unburnt	Aug	Bract	bud	1	1	1	0.48	4%	0%	0%
Burnt	Aug	Seeds	inflorescence	1	1	1	0.48	4%	0%	0%
Unburnt	Aug	Seeds	inflorescence	1	1	1	0.48	4%	0%	0%
Unburnt	Aug	Bract	bud	0	0	0	0	0%	0%	0%
Unburnt	Aug	Stylets	bud	0	0	0	0	0%	0%	0%
Burnt	Aug	Stylets	bud	0	0	0	0	0%	0%	0%
Burnt	Aug	Seeds	bud	0	0	0	0	0%	0%	0%
Burnt	Aug	Seeds	bud	0	0	0	0	0%	0%	0%
Burnt	Aug	Stylets	inflorescence	0	0	0	0	0%	0%	0%
Unburnt	Aug	Stylets	inflorescence	0	0	0	0	0%	0%	0%
Burnt	Aug	Bract	inflorescence	0	0	0	0	0%	0%	0%
Unburnt	Aug	Bract	inflorescence	0	0	0	0	0%	0%	0%
Burnt	Aug	Stylets	young infructescence	0	0	0	0	0%	0%	0%
Burnt	Aug	Bract	young infructescence	0	0	0	0	0%	0%	0%

Table S2. Results of mixed effects models testing the effect of inflorescence organ on species richness and presence of actinomycetes.

Response variable	Fixed effects	Random effects	Type	Fixed effect levels	p-value
a. Species observed (Sobs)	Inflorescence organ	Field, Month, Developmental stage	Poisson	Bracts	0.991
				Florets	0.734
				Seeds	0.639
b. Presence of actinomycetes	Inflorescence organ	Field, Month, Developmental stage	Binomial	Bracts	0.06
				Florets	0.313
				Seeds	0.33

Chapter 4

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Fungi and fire: *Protea* infructescences as reservoirs for fungal biodiversity in fire-prone environments

Zander R. Human, Casparus J. Crous, Michael J. Wingfield, Francois Roets, Stephanus N. Venter, Z. Wilhelm de Beer

Abstract

The infructescences of *Protea repens* have been described as an unique fungal niche due to a specific and intriguing association with a group of so-called ophiostomatoid fungi. However, the diversity of other fungi in this habitat remains poorly understood. We used Illumina sequencing of ITS1 amplicons to determine the fungal diversity in newly-formed and mature *P. repens* infructescences and surrounding litter and soil in a mountain fynbos landscape recently affected by fire. *Sporothrix splendens* was the most abundant fungal OTU in newly-formed infructescences from the site recently affected by fire. In the unburnt site, *S. splendens* was also very common but was replaced by a *Clavulina* sp. in some samples. We identified several wood- and litter decomposing fungi, especially in older infructescences where simple sugars had presumably been consumed and more recalcitrant lignocellulose remained as the main substrate. Several fungi previously reported from various members of the Proteaceae were also detected in *P. repens* infructescences and the surrounding litter. This study suggests that the complete fungal diversity of *P. repens* infructescences deserves more attention as it may be important in the conservation of microbial diversity and in plant and ecosystem fitness.

Introduction

The Mediterranean-type heathlands (fynbos) of the Core Cape Subregion (CCR) in the south-western parts of South Africa is a biodiversity hotspot known for its extremely high plant diversity and endemicity (Manning and Goldblatt, 2012). *Protea* is arguably the most well-known genus of fynbos shrubs due to the showy inflorescences produced by many members (Coetzee *et al*., 2007). As keystone members of this unique vegetation type, *Protea* spp. support a myriad of other organisms including birds (Rebelo *et al*., 1984), rodents (Wiens *et al*., 1983), arthropods (Coetzee and Giliomee, 1985) and microorganisms (Lee *et al*., 2005; Human *et al*., 2016). Because *Protea* spp. are cultivated worldwide for the floriculture industry, numerous studies have focused on

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damage-causing arthropods and microbes associated with the roots and leaves (Knox-Davies *et al*., 1985; Crous et al. 2011a).

The protective nature and humid environment (Roets *et al*. 2012) has resulted in the infructescences of *Protea* spp. providing habitats for numerous organisms including insects (Coetzee and Giliomee, 1985; Roets *et al.*, 2006a; Coetzee and Giliomee, 1987), mites (Roets *et al.,* 2007; Roets *et al.*, 2009; Roets *et al.,* 2009; Theron *et al.*, 2012), actinomycete bacteria (Human *et al*., 2016; Human *et al.*, 2017) and fungi (Marais and Wingfield, 1994; Lee *et al.*, 2005; Roets *et al*., 2013). The first fungi reported from this niche were the so-called ophiostomatoid fungi (Wingfield et al. 1994) *Knoxdaviesia proteae* (Wingfield *et al.,* 1988) and *Sporothrix splendens* (Marais and Wingfield, 1994), known for their unique appearance and association with insects. Subsequent to this discovery, the number of species of ophiostomatoid fungi known from *Protea* infructescenses has grown to three *Knoxdaviesia* spp. and eleven *Sporothrix* spp. (Marais and Wingfield, 1997; Marais *et al.*, 1998; Roets *et al*., 2006b; Roets *et al*., 2008; Roets *et al.*, 2013; De Beer *et al.*, 2016; Ngubane *et al*., 2018). This ecologically interesting group of fungi is well known for symbiotic associations with beetles and mites that act as spore dispersers (Roets *et al.*, 2007; 2009; 2011). In addition, the interaction between mites and *Sporothrix* from this niche is often mutualistic as the mites feed and reproduce on their fungal partners (Roets *et al*., 2007; Theron de-Bruin *et al.*, 2018).

Few non-ophiostomatoid fungi are known from *Protea* infructescences despite the fynbos ecosystems being biologically extremely diverse, where they might be expected to have a rich fungal diversity (Crous *et al.*, 2006). The most likely reason for this apparent dominance of ophiostomatoid fungi (Roets *et al*., 2005) is that the ophiostomatoid fungi may have a competitive advantage over other fungal taxa (Roets *et al*., 2012). This may be through more effective use of available substrates, better tolerance to the physical environment or due to tolerance to antimicrobial compounds present in the surrounding environment. Although this has not been tested, this apparent dominance may be due to inherent stronger competitiveness, association with antibiotic-producing actinomycete bacteria (Human *et al.,* 2016) or due to early colonization of the inflorescences due to spore-dispersal via *Protea* pollinators (Roets *et al*. 2007; 2009; Theron-de Bruin *et al*. 2018).

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It is also possible that *Protea* infructescenses have a substantial fungal diversity that has yet to be discovered, having been overlooked because previous studies have focused on the abundant and clearly visible structures belonging to *Sporothrix* and *Knoxdaviesia*. For example, Marais and Wingfield (1994) reported ten species of *Penicillium*, five species of *Cladosporium*, five species of *Acremonium*, *Alternaria alternata* and *Fusarium anthophilum* from *Protea* infructescences. Subsequent studies by Lee *et al.* (2003, 2005) and Visagie (2012) added several other fungi to this list, although ophiostomatoid fungi were consistently the dominant taxa. In addition, several fungal species have been reported from litter associated with *Protea* trees (Lee *et al.*, 2005, Visagie, 2012) although this niche has also clearly been underexplored.

Recurring summer fires represent the largest form of natural disturbance in fynbos ecosystems and generally consume all aboveground plant biomass every ca. 15 years (Van Wilgen, 1987). An interesting adaptation to fire-survival is found in many members of *Protea*. Involucral bracts of mature inflorescences close around developing fruit to form fire-retardant cone-like structures (infructescences) that remain on trees for several years (Bond, 1985). These *Protea* spp. release their seeds from the protected infructescences after parental plant death after fires, which enhances the possibility of successful recruitment of offspring in the post fire environment (Bond, 1985). *Protea* spp. reach reproductive maturity for the first time again in ca. 4 years after fire (Le Maitre, 1987).

Recolonization of infloresences and infructescences of young plants after fire by ophiostomatoid fungi seems to mainly originate from patches of adjacent unburnt populations (Aylward *et al*., 2015). This is easily explained by the association of the fungus-carrying mites with *Protea*-pollinating beetles (Roets *et al.*, 2009). However, Aylward *et al.* (2015) also hypothesized that long-distance dispersal of *K. proteae*, presumably through *Protea*-pollinating birds and this was more recently confirmed by Theron-De Bruin *et al.* (2018). The mites and the mutualistic fungi carried by these agents of long-distance dispersal can therefore reach inflorescences in *Protea* populations as soon as the first flowers appear after a fire event (Aylward *et al*., 2015). Other microbes may also find their way into *Protea* inflorescences in this way, with wind, or may arrive via infructescence-colonizing arthropods that return to *Protea* populations after fire (Wright and Samways, 1999). A succession of different fungal species is therefore expected over time as increasing numbers of fungal species recolonize these structures.

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The aim of this study was first to verify the apparent dominance of *S. splendens* and *K. proteae* on *P. repens* infructescences three months after flowering in areas recently affected by fire, in comparision to adjacent unaffected areas. We used modern metabarcoding techniques for this purpose because they overcome many constraints posed on fungal diversity studies when only using culture-based methods (Martin *et al*., 2011). We then set out to determine the identity and relative abundance of other fungal inhabitants of *P. repens* infructescences (young and mature) and of those associated with the surrounding litter and soil and compared to previous records obtained by culturing. The overall aim was to provide much needed data on the diversity and ecology of fungi associated with one of the keystone plant groups of the exceptionally biodiverse CCR. This could furthermore, provide useful information on best practices to reduce inoculum levels of pathogenic taxa in plantations for the floriculture industry.

Materials and methods

Site description and sampling

The study site was on the Franschhoek pass in the Western Cape Province of South Africa. This was the same site used in previous studies on the population genetic structure of *K. proteae* (Aylward *et al.*, 2015) and the colonization of actinomycete bacteria (Human *et al.*, 2017) in the inflorescences and infructescences of *P. repens* after a fire event. This site contains an area where a fire destroyed all aboveground plant parts and litter in 2009 (S 33.926.21 E 19.158.40) and an adjacent area (S 33.925.32 E 19.159.71) which was not affected by any fire for at least 15 years. In the unburnt site, the vegetation was in the mature phase with numerous plant individuals (including *Protea*) starting to become senescent. The over story is dominated by *P. repens* and *P. neriifolia* and the soil is covered by litter consisting mainly of *Protea* leaves, twigs and old parts of infructescences (Kruger, 1977; Kruger, 1983). Material from other plant taxa was also fairly abundant. Infructescences at this site varied from 3-months-old (those formed during the current flowering season) to ca. 7 years old. When collections took place in 2013, the burnt site was in its fourth year after fire, which is considered the youth phase of mountain fynbos (Kurger, 1977; Kruger, 1983). A few *P. repens* individuals in this site flowered for the first time in 2012 (3 years after the fire), but the greater majority flowered for the first time during June/July 2013. Therefore, most infructescences of this species at the burnt site were only ca. 3-months-old (i.e. the very first post-fire cohort). The involucral bracts for these infructescences had closed around the florets after

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flowering, and started to turn brown and woody (Pretorius, 1985). This site has a higher diversity of other plant species including short-lived graminoids and shrubs while the *Protea* spp. only begin to emerge from the canopy. Due to the vegetation being so young, litter was sparse and consisted of leaf and flower material from *Erica* spp. and other small shrubs (Kruger, 1977; Kruger, 1983). Very little litter at this site originated from *Protea*.

All material used in this study was collected during November 2013. In the burnt site, we collected five newly-formed infructescences (ca. 3-months-old), each from a different tree and at least 20 meters apart. In the unburnt site, we collected five newly-formed infructescences and five infructescences formed during the previous flowering season (ca. 1-year-old), hereafter referred to as mature infructescences) also from different individual trees. Since the burnt site was in its first flowering season after fire, mature infructescences could not be collected from this site. From each of the burnt and unburnt sites we collected four litter samples between sampled trees and five 50 mm soil cores for five different sites from the areas surrounding sampled *P. repens* trees.

DNA extraction and sequencing

From infructescences, we aseptically removed three 30-mm long pieces of florets, three 10-mm^2 pieces of involucral bracts and three whole fruit (nut-like structures) and extracted DNA from the separate tissues using a ZymoResearch Bacterial/Fungal DNA extraction kit. The extracted DNA from different infructescence parts were pooled in equimolar amounts for each infructescence prior to sequencing to save on sequencing costs. For each litter sample, DNA was extracted from 5 g of material using a ZymoResearch Bacterial/Fungal DNA extraction kit. DNA was extracted from 5 g of each soil sample using a MoBio PowerMax® Soil DNA Isolation Kit. The four litter samples from each of the burnt and the unburnt sites were pooled in equimolar amounts per site to yield a single DNA sample for the burnt and unburnt site respectively. The same was done for soil samples, except that five separate samples were pooled per site. The ITS1 region was selected for high-throughput amplicon sequencing due to the availability of conserved primers for most fungal phyla, and because sequence databases contain the most complete information for this marker. The ITS1 region was amplified using the primers ITS1F (Gardes and Bruns, 1993) and ITS2 (White *et al*., 1990) and sequenced on the Illumina Miseq platform at an external sequencing facility (Molecular Research LP, Shallowater, TX).

Sequence processing and diversity analyses

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Demultiplexed sequences with primers removed were processed using the DADA2 package (Callahan *et al*., 2016) in R (R Developmental Core Team, 2017). All sequences containing ambiguous bases were removed, after which forward reads were trimmed to 240 base pairs and reverse reads to 180 base pairs, in the DADA2 package (Callahan *et al*., 2016) in R (R Developmental Core Team, 2017). All forward and reverse reads were dereplicated before the DADA2 parametric error correction model was applied to sequences to infer accurate sequences (Callahan *et al*., 2016). Subsequently, forward and reverse reads with identical overlapping regions were joined, and chimeras removed using the chimera removal tool in DADA2 (Callahan *et al*., 2016). Fungal ITS1 sequences were extracted from these merged sequences using the ITSx Perl-based software tool (Bengtsson-Palme *et al.*, 2013). The ITSx software was also used to confirm that all sequences were in the same orientation and identified any chimeras that may have remained in the dataset (Bengtsson-Palme *et al.*, 2013). Operational taxonomic unites (OTUs) were clustered at 97% similarity using USEARCH (Edgar, 2010) implemented in the QIIME suite of sequence analysis tools (Caporaso *et al.*, 2010). Taxonomic assignment of the representative sequences for all OTUs were made by performing a BLAST search (Altschul *et al.*, 1990) against the UNITE database (Kõljalg *et al*., 2013) in the QIIME sequence analysis pipeline (Caporaso *et al.*, 2010).

Diversity analyses

The number of observed OTUs was used as a measure of species richness. Species diversity was calculated according to Shannon's Diversity Index. Both these diversity indices were determined using the *estimate_richness* function in Phyloseq (McMurdie and Holmes, 2013) in R (R Developmental Core Team, 2017). The group means of different sample types were compared by performing analysis of variance (ANOVA) because these data were normally distributed and had equal group variances.

Identification of focal fungal groups and ecological guilds

To test whether any novel species of *Sporothrix* were detected in this study, the sequences identified as *Sporothrix* spp. were compared to all currently described species of *Sporothrix* (De Beer *et al.*, 2016; Ngubane *et al*., 2018). These sequences were aligned with MAFFT (Katoh *et al*., 2002), and a maximum-likelihood phylogeny constructed in MEGA v. 7 (Kumar *et al.*, 2016) with 1000 bootstrap replicates.

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Abundance values for all OTUs were converted to compositional values, i.e. relative abundances of all OTUs per sample for taxon abundance analyses. Modified rank-abundance plots were constructed using the obtained relative abundance values for the top 25 most abundant OTUs in each sample category using ggplot2 (Wickham, 2011). The combined average abundances in each sample group was used to determine the order of the taxa shown in the rank-abundance plots. The ecological guilds of common fungal OTUs and the core OTUs shared amongst all infructescences was determined using the FUNguild web-based tool (Nguyen *et al*., 2016). The output from this analysis provides information on the ecological guilds, the confidence of the assignment to guilds, additional information on morphological categories and a citation.

Representative sequences from all OTUs recovered in the present study were compared to a custom database containing the ITS1 sequences from fungi isolated from *Protea* and other Proteaceae that are maintained in the culture collection at the Westerdijk Fungal Biodiversity Centre (Utrecht, The Netherlands) (Table S1). This custom database contained a total of 286 fungal taxa for which the ITS sequences were retrieved from the NCBI Genbank database. The ITS1 region was extracted using the ITSx Perl Script (Bengtsson-Palme *et al.*, 2013), and an id-to-taxonomy file required for taxonomic assignments in QIIME was created using the *entrez_qiime.py* python script (Baker, 2014). The representative sequences for all OTUs from this study were compared against the custom *Protea* sequence database using the UCLUST taxonomic classifier with a 97% similarity threshold implemented in the Qiime sequence processing software (Caporaso *et al.*, 2010). All sequences that produced a match against the *Protea* database were selected and a heatmap created using the *plot_heatmap* function in the Phyloseq package (McMurdie and Holmes, 2013) in R (R Developmental Core Team, 2017).

Core and combined microbiomes

The core microbiomes of newly-formed infructescences from the burnt and unburnt sites, and mature infructescences from the unburnt site were determined using the Microbiome package (Lahti *et al.*, 2017) in R (R Developmental Core Team, 2017). Only OTUs with less than 20 reads were discarded for Core OTU calculations in an effort to exclude less common fungi present in the surrounding environment. Core OTUs required more than 20 sequences present in at least 4 of the 5 sampled infructescences. Only a single pooled sample is available for soil and litter from the burnt and unburnt sites, and therefore all OTUs present at more than 20 reads were used for

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comparing core microbiomes. Then, the sum of all OTUs present in all replicates of a single sample group were determined and considered as the 'combined microbiome'.

The OTUs shared between any two sample groups in the core- and combined microbiomes were determined. The number of OTUs that were unique to each core microbiome and combined microbiome were also determined. OTUs that were unique to a sample group at one site, and absent in the same sample group at the opposite site (i.e. present in infructescences from the burnt site and absent in infructescences from the unburnt site) were also determined.

Community composition

For community analyses, absolute OTU abundances were normalized using variance-stabilizing transformation in the DESeq2 (Love *et al.*, 2014) R Package. This approach was used to normalize sequence library sizes, rather than rarefaction, as suggested by McMurdie and Holmes (2014). Bray-Curtis distances of communities were calculated using the *distance* function in the Phyloseq package in R (McMurdie and Holmes, 2013). Species assemblage composition of newly-formed infructescences from the burnt and unburnt sites were compared by performing Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2005) with the *adonis* function in the Vegan package (Oksanen *et al.*, 2017) in R (R Developmental Core Team, 2017). The same PERMANOVA procedure was used to compare the community composition of OTUs between newly-formed and mature infructescences from the unburnt site. Homogeneity of group dispersions of these same communities was tested with Permutational Analysis of Dispersions (PERMDISP; Anderson, 2004) using the *betadisper* and *permute* functions in the Vegan package (Oksanen *et al.*, 2017) in R (R Developmental Core Team, 2017) on a Jaccard's Index distance matrix constructed from presence/absence data. The same analyses were repeated for a comparison of the fungal communities between newly-formed infructescences and mature infructescences from the unburnt site. Similarities between communities in all newly-formed and mature infructescences were visually compared by performing nonparametric multidimensional scaling (NMDS) on Bray-Curtis distances of the normalized OTU table using the *ordinate* and *plot_ordination* functions in the Phyloseq (McMurdie and Holmers, 2013) package in R (R Developmental Core Team, 2017).

Results

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DNA Sequencing and diversity analyses

A total of 8 442 700 forward and reverse reads were obtained by Illumina Miseq Sequencing. From these, 2190 dereplicated sequence variants were inferred from 1 893 501 error-corrected sequences, eventually clustering into 1470 OTUs at 97% sequence similarity. Species richness according to the number of observed OTUs (Fig 1a) was not significantly different between newly formed infructescences from the burnt and unburnt site ($F = 0.009$, $P = 0.9$) or between newly formed and mature infructescences from the unburnt site ($F = 0.019$, $P = 0.089$). Species diversity according the Shannon's Diversity Index (Fig 1b) was also not different between newly formed infructescences from the burnt and unburnt site ($F = 0.61$, $P = 0.49$) or between newly formed and mature infructescences from the unburnt site ($F = 0.017$, $P = 0.9$). Because the litter and soil from both the burnt and unburnt sites were pooled prior to sequencing, they were not compared to infructescences. However, when the sequences from all sample groups were pooled (Fig 1c), the number of OTUs from infructescences were similar to litter in the burnt site, while soil samples and litter from the unburnt site had fewer OTUs present than the same samples from the burnt site. Species diversity according to Shannon's Index was lower in infructescences. Furthermore, the Shannon's Index was higher in litter and soil samples from the burnt site, than in the same sample types from the unburnt site (Fig 1d). Because these were only single values per sample group, they were not statistically compared.

Identification of focal fungal groups

Ophiostomatoid fungi

Sporothrix splendens, was the OTU with the highest relative abundance in four of the five newly formed infructescences from the burnt site (Fig. 2a) and in one of the five newly formed infructescences from the unburnt site (Fig. 2b). The relative abundance of *S. splendens* in newly-formed infructescences ranged from 24 – 28% (n = 5) in the burnt site and 3 – 46% (n = 5) in the unburnt site. *Sporothrix splendens* was present in all mature infructescences (Fig. 2c) (relative abundance 0.2 – 13%, n =5), as well as all litter (burnt – 0.2%, unburnt – 0.8%, both n = 1) and soil samples (burnt – 0.3%, unburnt – 0.4%, both n = 1).

Knoxdaviesia proteae was detected in four newly-formed infructescences from the burnt site, all newly-formed infructescences in the unburnt site and three mature infructescences. Furthermore,

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K. proteae was not present in soil or litter in the burnt site, but it was detected in soil and litter in the unburnt site. In the 14 newly-formed and mature infructescences where *K. proteae* was detected, it had less than 50 reads in 11 samples and more than 500 reads in only three infructescence samples.

Based on our phylogenetic analysis of taxa in the Ophiostomatales, two OTUs (denovo644 and denovo5) were identified as *S. splendens* (Fig. 3). Another OTU (denovo378) formed a sister group within the *S. stenoceras* complex, but was separate from *S. splendens* and the subgroup containing *S. protearum, S. africana and S. zambiensis* with 92% bootstrap support. Another OTU (denovo403) grouped was similar to *S. bragantina* (from soil in Brazil; Pfenning and Oberwinkler, 1993), *S. thermara* (isolated from a beetle on *Euphorbia ingens* in South Africa; Van der Linde *et al.*, 2016) and *S. smangaliso* recently described from *Protea gaguedii* (Ngubane *et al.*, 2018). However the ITS1 sequence from this OTU clustered separately from all of these described species. A lack of overlapping sequence data precludes more accurate description of the relatedness of these sequences, especially between sequences from this study and those from Ngubane *et al.* (2018).

Other common taxa

In one newly-formed infructescence from the burnt site not dominated by *S. splendens*, an OTU identified as *Clavulina* (Basidiomycetes) had the highest relative abundance. This OTU (*Clavulina*) sp. was very common in newly-formed infructescences in samples from both the burnt (0.1 – 32%, n = 5) and unburnt (0.1 – 38%, n = 5) sites. The sequence of this OTU was very similar to a *Clavulina* sp. from Pine needle litter in Sweden, an air sample in a North American forest and the phylloplane of bryophytes (Kauserud *et al.*, 2008; Boberg *et al.*, 2011; Huffman *et al.*, 2013). In some newly-formed infructescences from the unburnt site another Basidiomycetes OTU, identified only as a member of the Corticiaceae, also had higher relative abundance than *S. splendens*. This OTU and another of the Corticiaceae were also common in newly-formed infructescences from the burnt site. The Corticiaceae includes many white- and brown rot fungi and almost all members are usually associated with dead wood (Hjortstam *et al.*, 1987). Other common OTUs present at high relative abundances in newly-formed infructescences from the burnt and unburnt sites were identified as members of the Helotiales, *Wickerhamomyces rabaulensis, Cladosporium delicatulum*, *Phialocephala, Epicoccum nigrum, Alternaria alternata,*

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Cladophialophora, an *Aureobasidium* sp. and two other OTUs that could be identified only as members of the Basidiomycota (Fig 2a and 2b, Table S2). These taxa include endophytes, saprophytes, and some have been reported as parasites or decomposers of other fungi (Table S3), which are common in freshly fallen leaves and decomposing leaf litter (Jensen, 1974).

Some OTUs that were present in newly-formed infructescences also had high relative abundance in mature infructescences. These included members of the genus *Phialocephala* (relative abundance = 0.2 – 48%, n = 5), *Cladophialophora* (0.6 – 21%, n = 5) and a member of the Helotiales (0.2 – 21%, n = 5). *Phialocephala* spp. are dark-septate endophytic fungi from the Helotiales, and especially prevalent in the roots of trees in temperate forests (Stroheker *et al.*, 2016). Interestingly, the sequence of this *Phialocephala* OTU was 99% similar to fungi isolated from the roots of *Erica caffra* and *E. demissa* in South Africa (Bizabani, 2015). This genus has been reported as endophytes of branches and roots of coniferous trees and also in decaying leaf litter (Kowalski and Kehr, 1995; Korkama-Rajala *et al.*, 2008). Other OTUs with high relative abundance in mature infructescences included *Punctularia strigosozonata*, a wood-decaying yeast capable of lignin decomposition (Cullen, 2014), members of the Sebacinales (well-known ericoid-mycorrhiza and mycorrhizae of orchids), numerous endophytic fungi and a few saprobic species (Weiss *et al*., 2011).

A *Corticium* sp. was the most abundant OTU in litter from the burnt site, followed by a member of the Helotiales and another OTU from the Corticales (Fig S1). *Epicoccum nigrum* was the most common OTU in litter from the unburnt site by a large margin (Fig S1), followed by a *Teratosphaericola* sp. *Epicoccum* has been reported from many terrestrial habitats, including leaves, twigs and roots of trees (Arenal *et al.*, 1999), and *Teratosphaericola* was described as a pathogen on *Eucalyptus* leaves in Zambia (Quaedvlieg *et al.*, 2014). One OTU identified as a member of the Corticiaceae was reported as an arbuscular mycrorrhiza from grass in North America (Hawkes *et al*., 2006). OTUs that were from the Teratosphaeriaceae were common in the litter from both the burnt and unburnt sites. In the burnt site, these included *Xenoteratosphaeria jonkershoekensis, Parateratosphaeria altensteinii, Devriesia shelburniensis, D. tardicrescens, Catenulostroma hermanusense, Xenophacidiella pseudocatenata* and another from the genus *Parateratosphaeria*. In the unburnt site, these included *Parateratosphaeria altensteinii, Teratosphaeria parva* and other OTUs identified only as the genera *Teratosphaericola*,

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Teratosphaeria and *Xenophacidiella*. These fungi have been associated with several different plant species (Quaedvlieg *et al.*, 2014), although in one study, they were present on a single *Phaenocoma prolifera* (Asteraceae; Crous and Groenewald, 2011).

In soil samples from the burnt site, OTUs identified as a *Phialocephala* sp. and *Neofabraea* sp. were the most abundant, while OTUs from the Hypocreales and Dermataceae had the highest relative abundance in the unburnt site. There were also several *Penicillium*, *Mortierella* and *Archeaorhizomyces* OTUs present in soil samples from both the burnt and unburnt sites (Fig. S2).

Fungi previously reported from Proteaceae

In addition to the ophiostomatoid fungi, 41 OTU's identified in this study were identified as taxa previously reported from Proteaceae in our custom database (Fig. 4). Nine species were omnipresent including two *Penicillium* species, which are easily dispersed by air and water. Other fungi that were present in all samples include possible endophytic and plant pathogenic fungi such as *Verrucoconiothyrium nitidae* and *Aureobasidium proteae*. An OTU identified as a *Glonium* sp. was also present in all samples. This fungus seems to be common in dead *Protea* infructescences and the surrounding litter. Many of these fungi were identified from *P. repens* for the first time in this study and some are known as pathogens on other *Protea* species (Crous *et al*., 2011). Many of the *Protea*-associated fungi identified from litter and soil in this study have previously been reported only from other *Protea* host species as possible pathogens. For example, *Aureobasidium proteae*, present in all samples from this study, was first identified from *P. cynaroides* where it caused leaf-spots (Crous *et al*., 2011). It was subsequently also collected from leaf-spots on an unidentified *Protea* sp. intercepted in The Netherlands (Crous *et al.*, 2011). *Ilyonectria leucospermi*, a fungus which causes black foot rot disease of *Protea* cuttings in nurseries (Lombard *et al.*, 2012), was also found in soil in this study.

Core and combined microbiomes

The number of OTUs in the core fungal communities in newly-formed infructescences from the burnt and unburnt and mature infructescences were very similar (79 and 75 OTUs respectively; Table 1). In addition, the number of core OTUs in mature infructescences was also very similar (75). There were no unique core OTUs in any of the newly-formed and mature infructescences, (Table 1). In the combined microbiomes, more OTUs colonized newly-formed infructescences in

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the burnt site than the unburnt site (22% and 14% respectively, Table 1). However, when the combined microbiomes of infructescences from the burnt and unburnt site were compared directly, 31% of the OTUs in each combined microbiome was present in corresponding samples from the compared site (burnt or unburnt). A larger number of OTUs in newly-formed infructescences in the unburnt site were present in the soil, litter and the mature infructescences. In contrast, many OTUs present only in the newly-formed infructescences in the burnt site were found only in the newly-formed infructescences

Litter and soil samples represented a single pooled sample for each sample from the burnt and unburnt site. The microbiomes of litter and soil samples, where low-abundance OTUs (<20 reads) had been removed, were largely similar, although litter from the burnt site contained many more unique OTUs. There were more unique OTUs in litter from the burnt site compared to litter from the unburnt site. This was apparent when all samples were compared, and when the same sample groups were directly compared between the burnt and unburnt sites. However, when the combined microbiomes were considered, the number of unique OTUs were more similar between litter samples. When low abundance reads were considered, soil from the unburnt site contained more unique OTUs than soil from the burnt site.

The number of core fungal OTUs shared between newly-formed infructescences from the burnt and unburnt site, and between infructescences and litter and soil were always very similar (62 - 66 OTUs; Table 2). Fewer core OTUs were shared between the microbiomes of newly-formed infructescences and soil from the unburnt site than soil from the burnt site. Overall, litter shared more core OTUs with infructescences than soil. This pattern was consistent in the combined microbiomes shared between all infructescences, soil and litter (Table 2).

Ecological guilds of core- and common taxa

Among the 25 most abundant OTUs in newly-formed infructescences form the burnt site, 7 were yeasts (Table S3). Sixteen OTUs were classified as saprotrophs, of which at least 7 had previously been found on wood, and 1 in soil. Nine OTUs were classified as possible plant pathogens, 6 as endophytes and 1 as a fungal parasite (Table S3). Three OTUs were from genera known as white-rot fungi, and 1 OTU as soft-rot (Table S3). In newly-formed infructescences from the unburnt site, 5 OTUs were from genera known to be yeasts (Table S4). Twenty one OTUs were classified as saprotrophs, of which 8 were wood saprotrophs and 1 OTU was classified as a soil saprotroph

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(Table S4). Nine OTUs were classified as putative plant pathogens, 9 others as endophytes, 1 OTU as a fungal parasite, and 2 OTUs as entomopathogens. Four OTUs were of genera known to be capable of white-rot and two others were soft-rot fungi (Table S4). In mature infructescences, among the 25 five most abundant OTUs, 3 were yeasts, while 15 were saprotrophs (Table S5). Of these, at least 7 were wood saprotrophs and 4 were plant saprotrophs. Thirteen OTUs were classified as endophytes, 1 as an epiphyte, 3 as possible plant pathogens and 3 as taxa known as ericoid mycorrhiza, endophytes, or saprophytes. Two OTUs are from taxa known to be white-rot fungi, and another 2 soft-rot fungi. There were also 8 OTUs identified as being dark septate endophytes, often present in plant roots (Newsham, 2011; Weiss *et al.*, 2011).

Of the 70 core OTUs that were shared between all newly-formed infructescences and mature infructescences, the majority (n=43) were classified as saprotrophs (Table S6), of which 11 OTUs are known wood saprotrophs. Another 17 OTUs were classified as possible endophytes and 4 as epiphytes. A further 21 OTUs were classified as putative plant pathogens, 8 as entomopathogenic fungi, 7 OTUs are fungal parasites and 6 OTUs were from genera that are known to have ectomycorrhizal and saprophytic trophic modes. Among the core OTUs, 6 are known as white-rot fungi, and another 5 as soft-rot fungi (Table S6).

Community composition

The fungal communities in newly-formed infructescences from the burnt and unburnt sites were homogeneously dispersed ($F = 0.05$, $P = 0.9$) according to PERMDISP. Fungal communities in newly-formed and mature infructescences from the unburnt site were also homogenously dispersed ($F = 0.01$, $P = 0.99$). Fungal community assemblages in newly-formed infructescences from the burnt and unburnt sites were not significantly different according to PERMANOVA ($F = 1.19$, $R^2 = 0.13$, $P = 0.059$). However, the community assemblages between newly-formed and mature infructescences in the unburnt patch were significantly different ($F = 1.78$, $R^{2\,=}\ 0.18$, $P = 0.028$; Fig. 5).

Discussion

The results of this study confirm that *S. splendens* is an early colonist of *P. repens* infructescences and that it is the dominant fungal taxon in newly-formed structures (Roets *et al*. 2005). It is also one of the first fungi to colonize the infructescences after fire, confirming prevalence of long-

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distance, vector-mediated transport (Roets *et al.*, 2006c; 2009; 2011; Theron-De Bruin *et al.*, 2018). Interestingly, two additional *Sporothrix* OTUs were identified in this study that likely represent novel species.

Contrary to previous views, *Knoxdaviesia proteae* was present at lower relative abundance than expected, but it was nevertheless detected in nearly all infructescences, also indicating its capacity for long-distance dispersal (Roets *et al.,* 2009; 2011; Aylward *et al.*, 2015; Theron-De Bruin *et al*., 2018). Previous studies have suggested that the structures of *K. proteae* are abundant in autumn months, but that they virtually disappear during summer months, most likely due to arthropod grazing (Roets *et al.*, 2005). A further reason for the low abundance of *K. proteae* could be due to biases in the primers or biases in the Illumina sequencing technology. The ITS1f/ITS2 primer set used in this study is known to be robust to the presence of plant-derived ITS1 copies, but has known biases against some fungal groups (Bellemain *et al.*, 2010). It is therefore uncertain whether the low abundance of *K. proteae* was due to a biological effect, or if the results were biased by the methods used.

Although *Knoxdaviesia proteae* and *S. splendens* are considered predominant *Protea*-associated taxa as indicated by Roets *et al*. (2005), an additional ca. 70 fungal taxa (OTUs) were identified that form part of a reasonably consistent core microbiome within newly-formed infructescences. This was quite surprising given the finite nutrient and moisture resources within these structures, the young age of this habitat (ca. 3 months) and the aerial position of these structures on plants. The age of the host population after fire had little effect on species diversity and community composition, suggesting similarities in the colonization of newly formed infructescences in both young and old vegetation. As time progressed, community composition within infructescences changed and wood- and litter inhabiting fungi became much more abundant. All of these taxa may survive recurrent fires within this fire-retardant niche. Infructescences therefore likely play an important role in post-fire recolonization of landscapes by fungi.

As expected, *S. splendens* was the dominant fungal OTU in newly-formed infructescences (Roets et al. 2005) and confirmed the dominance of this fungus the first infructescences that appear after fire. It has recently been established that the dominant *S. phasma* in *P. nerifolia* infructescences is dispersed between infructescences by mites carried by *Protea*-pollinating birds (Theron-De Bruin et al. 2018). However, the same birds responsible for transporting the fungus-carrying mites

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between inflorescences of *P. neriifolia* are also known to visit the inflorescences of *P. repens* (Theron-De Bruin *et al*., 2018). Furthermore, the same mites implicated in the transport of *S. phasma* are known to also inhabit *P. repens* infructescences (Roets *et al*., 2009a). The absence of *S. phasma* from *P. repens* infructescences and the dominance of *S. splendens* in these structures is intriguing. This may be due to a combination of factors including flower visitor consistency by birds or pollinating insects on particular *Protea* species or nutritional/ chemical differences between the *Protea* spp.

The two putative novel *Sporothrix* species detetected in this study were both present in only a single but different infructescence. One of these grouped in the *S. stenoceras* complex that includes four closely related species from *Protea* hosts (De Beer *et al.*, 2016). The other species grouped in the *S. bragantina* complex that includes only one described *Protea*-associated *Sporothrix* (*S. smangaliso*), associated with grassland *Protea* species more than 500 km from the closest populations of *P. repens* (Ngubane *et al*., 2018). *Sporothrix smangaliso* is very rarely encountered *Protea* species leading Ngubani *et al*. (2018) to speculate that it may not be a preferred host. This also because other species in the Sporothrix complex are found on very distantly related host plants as well as in other niches (Crous *et al.*, 2015; De Beer *et al.*, 2016; Osorio *et al*., 2016).

In some newly formed infructescences from the unburnt site, *Sporothrix splendens* was replaced by a *Clavulina* species, which was the second most dominant fungal taxon on average. Species in this genus are generally accepted as ectomycorrhizal (Dickie and Moyersoen, 2008), but saprophytic modes are also known for this genus (Gebauer and Taylor, 1999; Hou *et al.*, 2012). Saprophytic habitats of members of this genus include leaf litter (Boberg *et al.*, 2011), the phylloplane of bryophytes (Kauserud *et al*., 2008) and it has also been detected in air samples collected in a North American forest (Huffman *et al.*, 2013). Fungi in this genus are not able to utilize lignin, and they have a preference for cellulose and simple soluble sugars as a substrate (Boberg *et al.*, 2011) which are present in *Protea* nectar (Nicolson and Van Wyk, 1998). The *Clavulina* sp. likely colonizes inflorescences at an early stage via air-borne spores (Huffman *et al.*, 2013) where it may compete with *S. splendens* for resources. In the late developmental stages of infructescences it almost disappears from the detected fungal communities when lignin and small amounts of cellulose are presumably the only available substrates (Mitchell and Coley, 1987).

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Results of this study suggest a succession toward fungi more efficient at utilizing plant polysaccharides in older infructescences. Sclerophyllous *Protea* tissues predominantly consist of lignin (Mitchell and Coley, 1987), while most cellulose is also contained in lignin-complexes (Blanchette, 1995). Thus, when soluble sugars become limited and with little available cellulose, known wood-decomposer saprophytes that are more efficient at utilizing the remaining lignocellulosic substrates (Mitchell *et al.*, 1986; Mitchell and Coley, 1987) replace *S. splendens* and *Clavulina*. This is evident in the high abundance of two aggressive wood-decomposers in mature infructescences, such as the lignicolous yeast, *Punctularia strigosozonata* and a *Phialocephala* sp. (Floudas *et al.*, 2012). Lignin is abundant in the sclerophyllous tissues of *Protea* trees and is usually the most abundant substrate in older sclerophyllous *Protea* tissues (Mitchell *et al.*, 1986; Mitchell and Coley, 1987). In environments, such as those created in the second year of litter decomposition, microbial decomposition proceeds very slowly with a few specialized fungal decomposers being active (Voříšková and Baldrian, 2013).

The presence of many wood- and litter decomposer fungi in mature infructescences suggest that fungi may be important in the pre-fire release of *P. repens* seed. The woody bracts that enclose the infructescences of serotinous *Protea* species protect seeds against predation (Bond, 1985; Lamont *et al*., 1991), but ultimately seeds are released within one to six years after flowering, depending on the degree of serotiny in different *Protea* species (Bond, 1985; Lamont *et al.*, 1991). Although the lignin in infructescence bracts provide a barrier to degradation (Read and Mitchell, 1983), white-rot fungi, such as those detected in mature infructescences are able to decompose this substrate (Blanchette, 1984; Baldrian, 2008). Decomposition of lignin may take several years, but together with photo-degradation it may be the most important explanation for the eventual breakdown of infructescences and release of seeds. This may be an important mechanism in the poorly understood controls on *Protea* reproduction and dispersal.

Protea infructescences may serve as a microbial seed bank for post fire recolonization of fire-affected topsoil. Many of the fungi detected in this study are not only effective decomposers, but also known from soil and plant roots, such as the ericoid mycorrhizae and the dark septate endophytes (Kowalski and Kehr, 1995; Korkama-Rajala *et al*., 2008; Bizabani, 2015; Stroheker *et al*., 2016). It has been shown that fires in Mediterranean landscapes often decimate fungal communities, while bacteria are better adapted to survive these fires and become the dominant

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community thereafter (Bárcenas-Moreno *et al*., 2011). Considering that a suitable niche and means for dispersal exist in the post-fire environment (Bond, 1985; Lamont *et al.*, 1991), *Protea* infructescence in these fire affected landscapes may have an important source-sink relationship with the surrounding soil, which could be important in the post-fire colonization of fire-affected landscapes. This hypothesis is also supported by the number of core fungal OTUs shared between infructescences and soil. Diverse soil fungal communities present several different functional roles, and are important in protecting slow growing fynbos plant species against invaders and are important belowground symbionts of the first successional plants such as graminoid and ericoid species (Van der Heijden *et al*., 2008).

Several putative pathogenic fungi known to occur on the different Proteaceae were detected in the infructescences and surrounding litter. Many of these taxa are reported from *P. repens* for the first time here. Infructescences and the surrounding litter may thus be reservoirs of pathogens, although the detection of their DNA does not necessarily indicate that these organisms have the potential to be dispersed and cause disease (Carini *et al*., 2016). Recurrent fires in fynbos landscapes may therefore not only promote recruitment of fynbos plants (Kruger, 1977; Kruger, 1984), but also help lower numbers of infective propagules of plant associated fungi (Hardison, 1976). Importantly, we have shown that even pathogens that are normally associated with other plant structures such as leaves may be contained within *Protea* infructescences. This is not only of ecological relevance, but may also be useful in *Protea* production. For example, in order to lower inoculum levels of leaf pathogens it may be helpful to reduce leaf litter in plantations and to remove all infructescences from plants.

Fungal communities in mature infructescences were different to those from newly-formed infructescences. These also contained many unique OTUs not present in either newly-formed infructescences, litter or soil. Older infructescences therefore represent an important component of overall fungal biodiversity in the landscape, and when these structures are lost as is expected when fires are inordinately frequent (Kruger, 1977; Kruger, 1983), many rare taxa might disappear. Fire is consequently an important consideration not only to maintain plant biodiversity in mountain fynbos landscapes (Kruger, 1977; Kruger, 1983), but also for microorganisms. Fynbos landscapes that represent a mosaic of vegetation developmental stages give rise to a considerable diversity of

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insects, birds and bacteria (Chapter 2; Usher and Jefferson, 1991; Chalmandrier *et al*., 2013). The same level of diversity might be expected for their associated fungi.

In this study, we confirmed the presence of *Sporothrix* and *Knoxdaviesia* in the first *P. repens* infructescences formed after fire. We also showed that *S. splendens* is often the dominant fungus in *P. repens* infructescences. Two possible novel species of *Sporothrix* were detected along with numerous fungi known for their abilities to decay wood. The results illustrate the possibility that decomposer fungi present in the mature infructescences may be important in the decomposition and therefore destruction of infructescences. This would contribute to the dispersal of *Protea* seeds. Decomposer fungi, many which are also known from soil, could also be important pioneer colonizers of fynbos soil in the post fire environment. Overall, it is clear that *P. repens* infructescences represent a unique habitat for pathogenic and decomposer fungi, which are potentially important in shaping the vegetation in mountain fynbos ecosystems.

Acknowledgements

We thank the DST/NRF Centre for Excellence in Tree Health Biotechnology (CTHB), the National Research Foundation (NRF) of South Africa, and the University of Pretoria for financial support.

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Table 1. The number of (a) core OTUs (at least 20 reads in 4 out of 5 samples) and total OTUs in each sample category, (b) the number of unique core OTUs in each sample group, and (c) the combined number of unique OTUs in each sample group

	(a)			(b) Core microbiome - Unique OTUs				(c) Combined microbiome - Unique OTUs			
	Core OTUs	Combined Microbiome	% Core OTUs	Unique OTUs	% Unique OTUs	Unique OTUs (site)	% Unique (Site)**	Unique OTUs	% Unique OTUs	Unique OTUs (site)	% Unique (Site)**
NFI - Burnt	79	414	31%	0	0	5	6%	104	22%	148	31%
NFI - Unburnt	75	426	29%	0	0	9	12%	68	14%	146	31%
Mature - Unburnt	75	423	27%	0	0	-	-	111	22%	111	22%
Litter - Burnt	354	454	78%*	120	33%	226	74%	171	35%	347	76%
Litter - Unburnt	229	355	65%*	39	17%	127	46%	117	27%	248	70%
Soil - Burnt	220	347	63%*	51	23%	142	53%	124	29%	108	31%
Soil - Unburnt	205	314	65%*	59	29%	105	43%	137	34%	141	44%

*sample groups consisted of a single sample and thus all OTUs with more than 20 reads present were considered as part of the core community.

** OTUs which were not present in the same sample type from the other site (i.e. these OTUs were present in newly-formed infructescences from the burnt site and not present in any newly-formed infructescences from the unburnt site).

Table 2. The number of OTUs shared between the core- and combined microbiomes of different sample categories

	Core Microbiomes						Combined Microbiomes					
	NFI - Burnt	NFI – Unburnt	Mature - Unburnt	Litter - Burnt	Litter - Unburnt	Soil - Burnt	NFI - Burnt	NFI – Unburnt	Mature - Unburnt	Litter - Burnt	Litter - Unburnt	Soil - Burnt
NFI - Burnt												
NFI - Unburnt	70						322					
Mature - Unburnt	61	60					284	295				
Litter - Burnt	68	67	66				216	225	284			
Litter - Unburnt	62	63	66	131			210	228	220	228		
Soil - Burnt	50	50	55	94	77		175	175	175	185	166	
Soil - Unburnt	42	44	46	67	67	112	141	154	157	138	162	205

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Figures

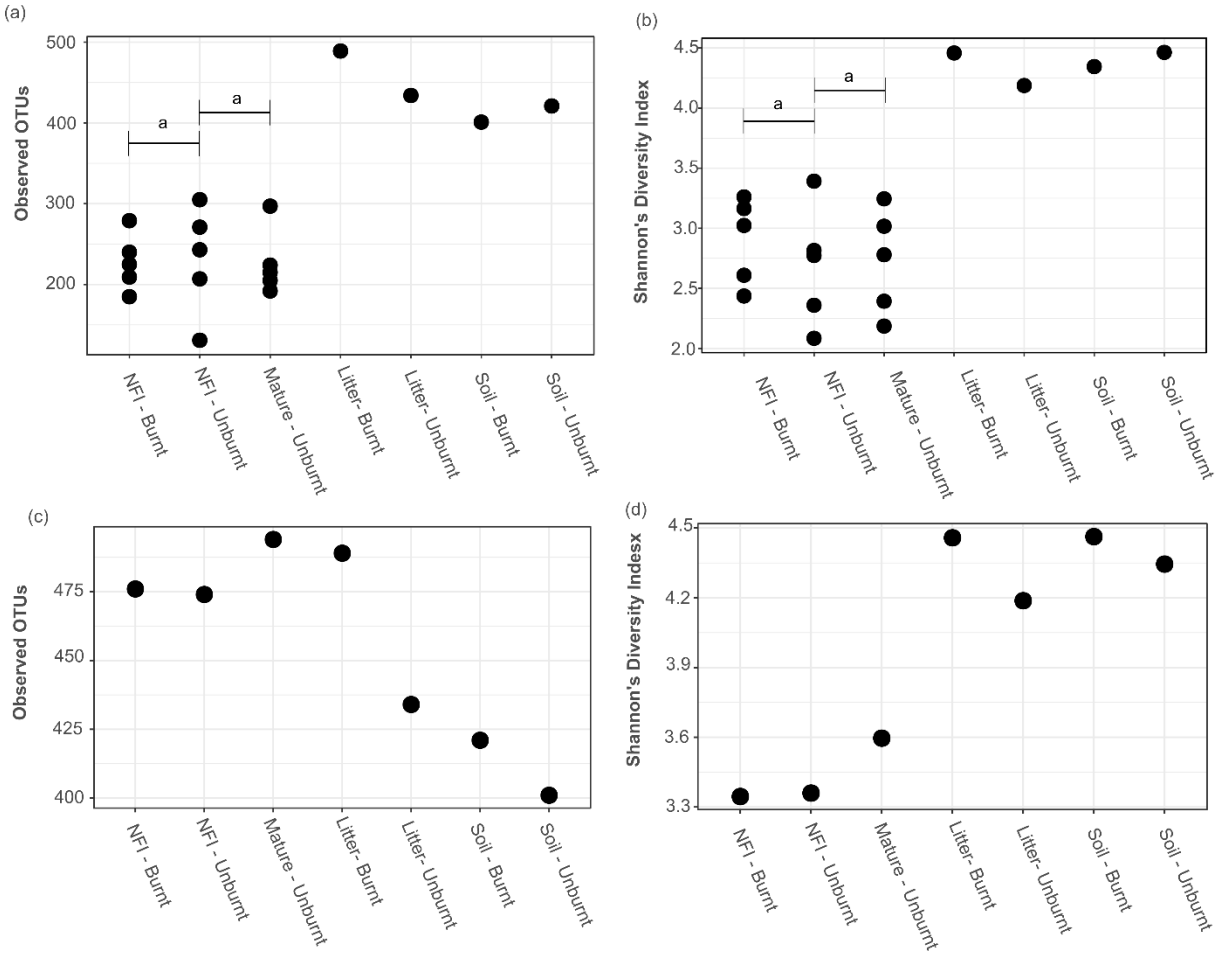

Fig. 1: Measures of species diversity in newly-formed infructescences (NFI), mature infructescences (mature), litter and soil were (a) observed OTUs, (b) Shannon's Diversity Index, (c) observed OTUs from individual sample groups pooled, (d) Shannon's Diversity Index from individual samples pooled

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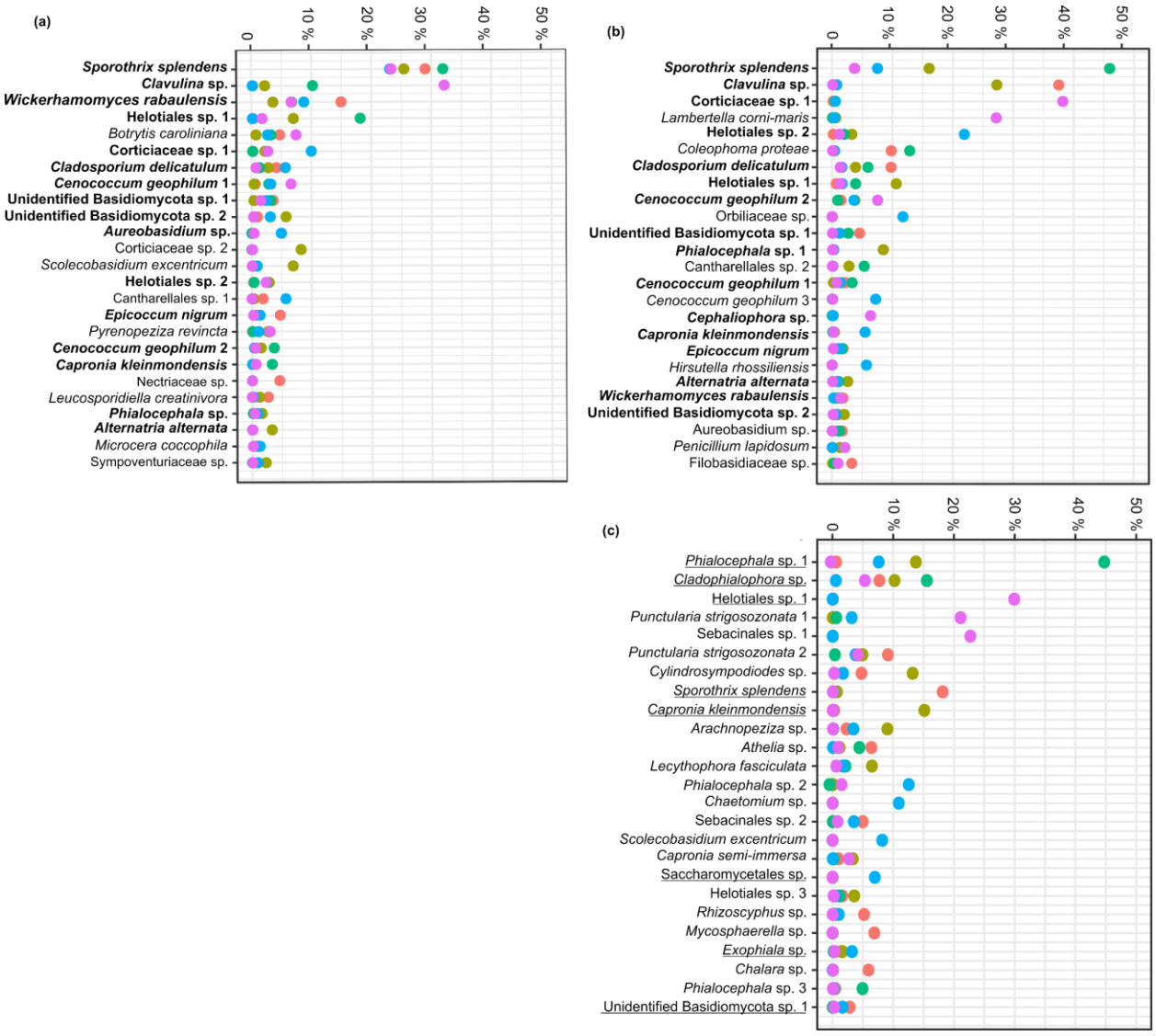

Fig. 2: Relative abundances of the most common OTUs in (a) newly-formed infructescences in the burnt site, (b) newly-formed infructescences in the unburnt site and (c) mature infructescences in the unburnt site. The order of OTUs on the y-axis was determined by their mean relative abundance in their respective sample groups. Taxonomic information is provided in Table S2. Names of OTUs in bold-type were shared between the burnt and unburnt sites and underline OTU names in (c) were OTUs also present in newly-formed infructescences in the unburnt site. Each colour refers to a different infructescence sample

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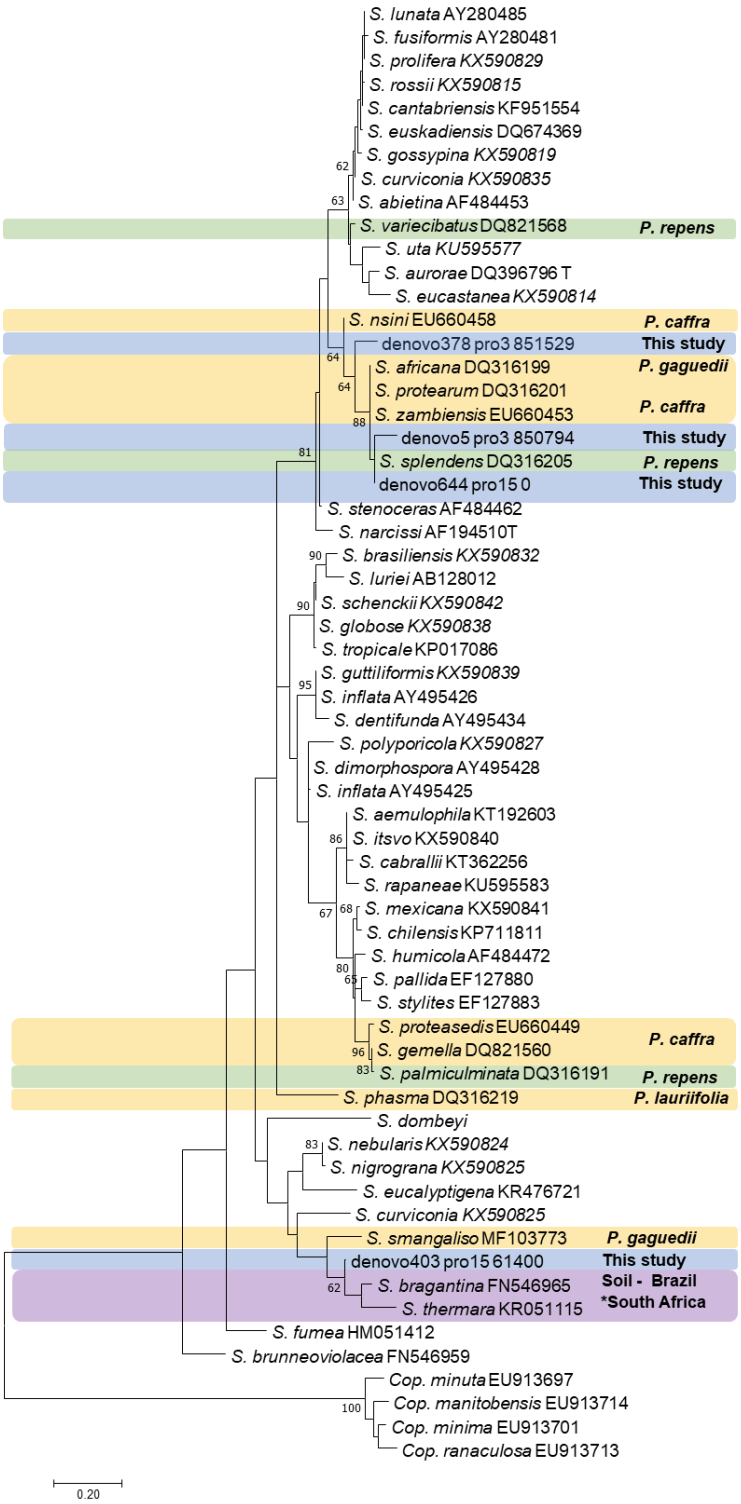

Fig. 3: A maximum-likelihood phylogeny of sequences identified as *Sporothrix* in this study and all *Sporothrix* type strains, including *S. smangaliso* and *S. nsini* with limited overlapping nucleotides. Sequences from this study are in bold type and highlighted in blue, while type

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strains reported from *P. repens* previously are highlighted in green. Type strains from other *Protea* species are in yellow

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Fig 4. A heatmap depicting all strains that were identified in this study from a custom-made database containing all strains isolated from various Proteaceae available in the Westerdijk Fungal Biodiversity Centre culture collection (Table S2). Sample labels are on the x-axis and taxa names on the y-axis

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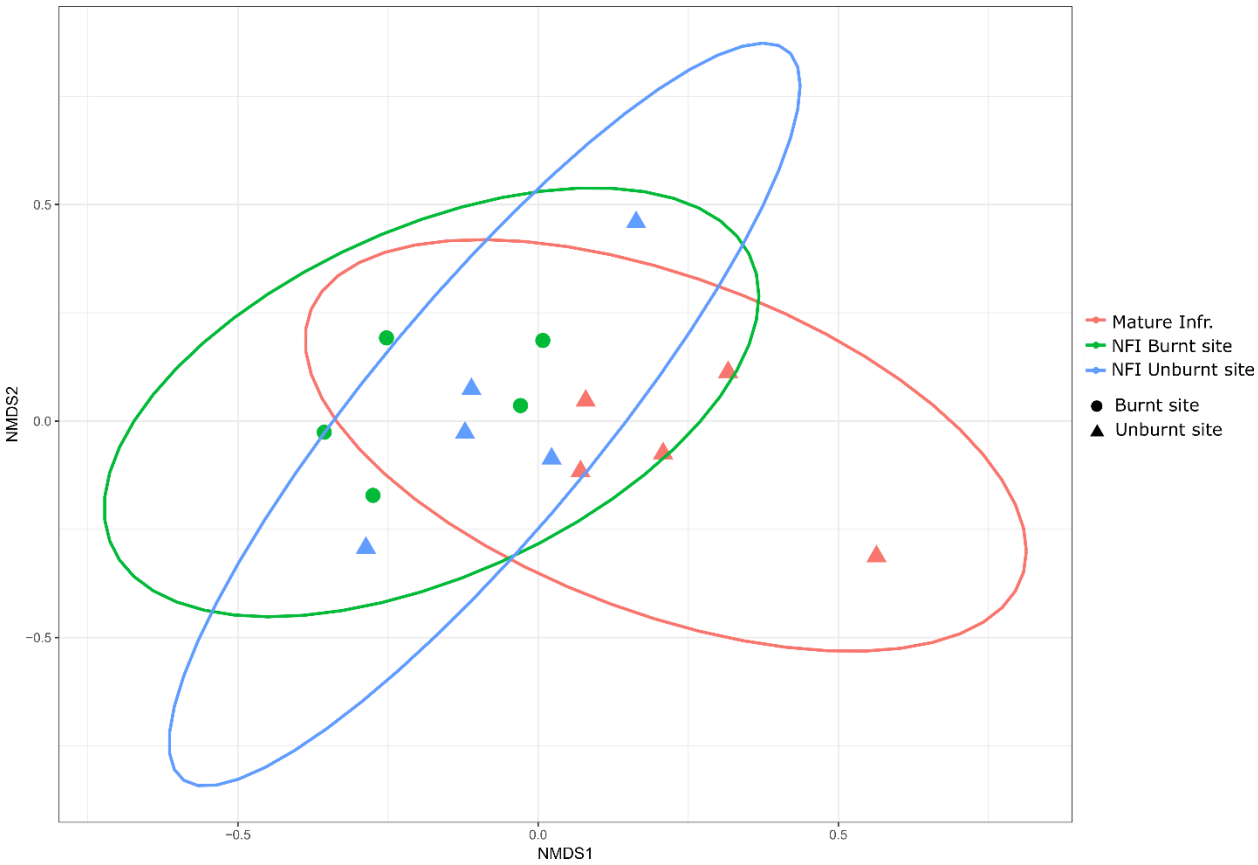

Fig 5. An NMDS plot showing the community structure of newly-formed infructescences (NFI) from the burnt and unburnt site, and mature infructescences from the unburnt site. Ellipses indicate 95% confidence intervals for each sample group

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Supplementary tables

Table S1. All fungi available in the culture collection of the Westerdijk Fungal Biodiversity Institute that were previously reported from various Proteaceae

CBS number	Taxon name	Country
CBS 134212	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa
CBS 134301	*Colletotrichum proteae* F. Liu, U. Damm, L. Cai & P.W. Crous, Fungal Diversity 61: 100 (2013) [MB#802498]	South Africa
CBS 134302	Colletotrichum proteae F. Liu, U. Damm, L. Cai & P.W. Crous, Fungal Diversity 61: 100 (2013) [MB#802498]	South Africa
CBS 134555	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa
CBS 134556	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa
CBS 135125	*Penicillium annulatum* Visagie & K. Jacobs, Mycological Progress 14 (10/96): 14 (2015) [MB#809817]	South Africa
CBS 135126	*Penicillium annulatum* Visagie & K. Jacobs, Mycological Progress 14 (10/96): 14 (2015) [MB#809817]	South Africa
CBS 135670	*Penicillium ortum* Visagie & K. Jacobs, Mycological Progress 14 (10/96): 18 (2015) [MB#809820]	South Africa
CBS 135883	*Penicillium riverlandense* C.M. Visagie & K. Jacobs, Persoonia 36: 149 (2016) [MB#808269]	South Africa
CBS 135884	*Penicillium riverlandense* C.M. Visagie & K. Jacobs, Persoonia 36: 149 (2016) [MB#808269]	South Africa
CBS 135885	*Penicillium riverlandense* C.M. Visagie & K. Jacobs, Persoonia 36: 149 (2016) [MB#808269]	South Africa
CBS 135886	*Penicillium riverlandense* C.M. Visagie & K. Jacobs, Persoonia 36: 149 (2016) [MB#808269]	South Africa
CBS 135899	*Penicillium* Link, Magazin der Gesellschaft Naturforschenden Freunde Berlin 3 (1): 16 (1809) [MB#9257], Penicillium austricola C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa

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CBS number	Taxon name	Country
CBS 135900	*Penicillium* Link, Magazin der Gesellschaft Naturforschenden Freunde Berlin 3 (1): 16 (1809) [MB#9257], Penicillium austricola C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 135901	Penicillium Link, Magazin der Gesellschaft Naturforschenden Freunde Berlin 3 (1): 16 (1809) [MB#9257], Penicillium austricola C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 135902	*Penicillium* Link, Magazin der Gesellschaft Naturforschenden Freunde Berlin 3 (1): 16 (1809) [MB#9257], Penicillium austricola C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 135903	*Penicillium* Link, Magazin der Gesellschaft Naturforschenden Freunde Berlin 3 (1): 16 (1809) [MB#9257], Penicillium austricola C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 135904	*Penicillium austricola* C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 136253	*Penicillium austricola* C.M. Visagie & K. Jacobs, Persoonia 36: 139 (2013) [MB#805184]	South Africa
CBS 136426	*Harknessia proteae* Crous, Persoonia 31: 227 (2013) [MB#805840]	South Africa
CBS 13650	*Metschnikowia drakensbergensis* C de Vega, B Guzmán, M.A. Lachance, International Journal of Systematic and Evolutionary Microbiology (2014) [MB#809688]	South Africa
CBS 13651	*Metschnikowia caudata* de Vega C.; Guzmán, B.; Lachance, M.A., International Journal of Systematic and Evolutionary Microbiology (2014) [MB#809689]	South Africa
CBS 13729	*Metschnikowia caudata* de Vega C.; Guzmán, B.; Lachance, M.A., International Journal of Systematic and Evolutionary Microbiology (2014) [MB#809689]	South Africa
CBS 137379	*Talaromyces crassus* C. M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810900]	South Africa
CBS 137380	*Talaromyces crassus* C. M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810900]	South Africa
CBS 137381	*Talaromyces crassus* C. M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810900]	South Africa
CBS 137384	Talaromyces wortmannii (Klöcker) C.R. Benj., Mycologia 47: 683 (1955) [MB#344294]	South Africa

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CBS number	Taxon name	Country
CBS 137385	*Talaromyces infraolivaceus* C.M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 52 (2015) [MB#810901]	South Africa
CBS 137386	*Talaromyces acaricola* C.M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810899]	South Africa
CBS 137387	*Talaromyces acaricola* C.M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810899]	South Africa
CBS 137388	*Talaromyces acaricola* C.M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810899]	South Africa
CBS 137390	*Talaromyces acaricola* C.M. Visagie, N. Yilmaz & K. Jacobs, Persoonia 36: 49 (2015) [MB#810899]	South Africa
CBS 137744	*Penicillium malmesburiense* Visagie, Houbraken & K. Jacobs, Studies in Mycology 78: 429 (2014) [MB#809969]	South Africa
CBS 137747	*Penicillium infra-aurantiacum* Visagie, Houbraken & K. Jacobs, Studies in Mycology 78: 426 (2014) [MB#809966]	South Africa
CBS 139.52	*Aspergillus awamori* Nakaz., Rep. Gov. Res. Inst. Formosa: 1 (1907) [MB#119955]	Japan
CBS 139140	*Penicillium citreosulfuratum* Biourge, La Cellule 33: 285 (1923) [MB#260947]	South Africa
CBS 139157	*Penicillium momoii* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 99 (2016) [MB#811007]	South Africa
CBS 139158	*Penicillium citreosulfuratum* Biourge, La Cellule 33: 285 (1923) [MB#260947]	South Africa
CBS 139162	*Penicillium citreosulfuratum* Biourge, La Cellule 33: 285 (1923) [MB#260947]	South Africa
CBS 139164	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139165	*Penicillium citreosulfuratum* Biourge, La Cellule 33: 285 (1923) [MB#260947]	South Africa
CBS 139167	*Penicillium pagulum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 102 (2016) [MB#811005]	South Africa
CBS 139168	*Penicillium melinii* Thom, The Penicillia: 273 (1930) [MB#270876]	South Africa
CBS 139169	*Penicillium melinii* Thom, The Penicillia: 273 (1930) [MB#270876]	South Africa

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CBS number	Taxon name	Country
CBS 139172	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139173	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139174	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139175	*Penicillium repensicola* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 102 (2016) [MB#811006]	South Africa
CBS 139176	*Penicillium repensicola* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 102 (2016) [MB#811006]	South Africa
CBS 139177	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139178	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 139179	*Penicillium xanthomelinii* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 105 (2016) [MB#811009]	South Africa
CBS 139180	*Penicillium xanthomelinii* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 105 (2016) [MB#811009]	South Africa
CBS 139181	*Penicillium xanthomelinii* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 105 (2016) [MB#811009]	South Africa
CBS 139182	*Penicillium atrolazulinum* C.M. Visagie & K. Jacobs, IMA Fungus 7 (1): 91 (2016) [MB#811001]	South Africa
CBS 140644	*Knoxdaviesia capensis* M.J. Wingf. & P.S. van Wyk, Mycological Research 97: 710 (1993) [MB#359765]	South Africa
CBS 183.62	*Conidiobolus nanodes* Drechsler, American Journal of Botany 42: 439 (1955) [MB#295477]	India
CBS 221.30	*Penicillium roqueforti* Thom, U.S.D.A. Bureau of Animal Industry Bulletin 82: 35 (1906) [MB#213525]	USA
CBS 246.84	*Phanerochaete chrysosporium* Burds., Mycotaxon 1 (2): 124 (1974) [MB#319705]	Sweden

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CBS number	Taxon name	Country
CBS 338.62	*Rhizopus microsporus* var. *oligosporus* (Saito) Schipper & Stalpers, Studies in Mycology 25: 31 (1984) [MB#116951]	Indonesia
CBS 433.92	*Gondwanamyces capensis* (M.J. Wingf. & P.S. van Wyk) G.J. Marais & M.J. Wingf., Mycologia 90: 140 (1998) [MB#442580]	South Africa
CBS 452.64	*Neofabraea alba* (E.J. Guthrie) Verkley, Studies in Mycology 44: 125 (1999) [MB#482723]	England
CBS 475.90	*Alternaria proteae* (E.G. Simmons) Woudenberg & Crous, Studies in Mycology 75: 193 (2013) [MB#803707]	Western Australia
CBS 482.82	*Colletotrichum nymphaeae* (Pass.) Aa, Netherlands Journal of Plant Pathology 84: 110 (1978) [MB#311502]	Victoria
CBS 486.88	*Gondwanamyces proteae* (M.J. Wingf., P.S. van Wyk & Marasas) G.J. Marais & M.J. Wingf., Mycologia 90: 139 (1998) [MB#442579]	South Africa
CBS 487.88	*Gondwanamyces proteae* (M.J. Wingf., P.S. van Wyk & Marasas) G.J. Marais & M.J. Wingf., Mycologia 90: 139 (1998) [MB#442579]	South Africa
CBS 488.88	*Gondwanamyces proteae* (M.J. Wingf., P.S. van Wyk & Marasas) G.J. Marais & M.J. Wingf., Mycologia 90: 139 (1998) [MB#442579]	South Africa
CBS 513.92	*Melanoporia castanea* (Imazeki) T. Hatt. & Ryvarden, Mycotaxon 50: 29 (1994) [MB#357949]	Unknown
CBS 532.72	*Phaeoisaria curvata* de Hoog & Papendorf, Persoonia 8 (4): 412 (1976) [MB#319582]	South Africa
CBS 543.72	*Chaetomium strumarium* (J.N. Rai, J.P. Tewari & Mukerji) P.F. Cannon, Transactions of the British Mycological Society 87: 64 (1986) [MB#103141]	South Africa
CBS 548.88	*Gondwanamyces proteae* (M.J. Wingf., P.S. van Wyk & Marasas) G.J. Marais & M.J. Wingf., Mycologia 90: 139 (1998) [MB#442579]	South Africa
CBS 557.92	*Sporothrix splendens* G.J. Marais & M.J. Wingf., Mycological Research 98: 373 (1994) [MB#359978]	South Africa
CBS 693.77	*Arthrographis kalrae* (R.P. Tewari & Macph.) Sigler & J.W. Carmich., Mycotaxon 4 (2): 360 (1976) [MB#309023]	India
CBS 778.97	*Septoria protearum* Viljoen & Crous, South African Journal of Botany 64 (2): 144 (1998) [MB#445448]	South Africa

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CBS number	Taxon name	Country
CBS 779.69	*Acremonium chrysogenum* (Thirum. & Sukapure) W. Gams, Cephalosporium-artige Schimmelpilze: 109 (1971) [MB#308135]	Italy
CBS 7793	*Myxozyma vanderwaltii* Spaaij, G. Weber & M.T. Sm., Antonie van Leeuwenhoek 63 (1): 18 (1993) [MB#361500]	South Africa
CBS 819.72	*Aspergillus oryzae* var. *oryzae* (1884) [MB#418420]	Japan
CBS 8803	*Saccharomyces cerevisiae* Meyen ex E.C. Hansen, Medd. Carlsberg Lab.: 29 (1883) [MB#163963]	Unknown
CBS 895.71B	*Sarocladium kiliense* (Grütz) Summerbell, Studies in Mycology 68: 158 (2011) [MB#519592]	Unknown
CBS 101951	*Neocatenulostroma microsporum* (Joanne E. Taylor & Crous) Quaedvlieg & Crous, Persoonia 33: 27 (2014) [MB#807808]	South Africa
CBS 101953	*Mycosphaerella stromatosa* Joanne E. Taylor & Crous, Mycological Research 104 (5): 625 (2000) [MB#464473]	South Africa
CBS 101954	*Pseudopassalora gouriqua* Crous, Persoonia 27: 41 (2011) [MB#560571]	South Africa
CBS 101955	*Phloeosporella protearum* Joanne E. Taylor & Crous, Mycological Research 104 (5): 627 (2000) [MB#464801]	Zimbabwe
CBS 102187	*Catenulostroma elginense* (Joanne E. Taylor & Crous) Crous & U. Braun, Studies in Mycology 58: 16 (2007) [MB#504506]	South Africa
CBS 110127	*Anthostomella proteae* S.J. Lee & Crous, Mycological Research 107 (3): 366 (2003) [MB#462817]	South Africa
CBS 110696	*Batcheloromyces proteae* Marasas, P.S. van Wyk & Knox-Dav., South African Journal of Botany 41 (1): 43 (1975) [MB#121215]	South Africa
CBS 110756	*Teratosphaeria macowanii* (Sacc.) Crous, Persoonia 23: 115 (2009) [MB#509735]	South Africa
CBS 110890	*Neocatenulostroma microsporum* (Joanne E. Taylor & Crous) Quaedvlieg & Crous, Persoonia 33: 27 (2014) [MB#807808]	South Africa
CBS 111029	*Teratosphaeria macowanii* (Sacc.) Crous, Persoonia 23: 115 (2009) [MB#509735]	South Africa

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CBS 111031	*Neocatenulostroma microsporum* (Joanne E. Taylor & Crous) Quaedvlieg & Crous, Persoonia 33: 27 (2014) [MB#807808]	South Africa
CBS 111302	*Verrucoconiothyrium nitidae* (Crous & Denman) Crous, Sydowia 67: 110 (2015) [MB#812550]	Unknown
CBS 111303	*Microsphaeropsis proteae* (Crous & Denman) Crous & Denman, Persoonia 27: 32 (2011) [MB#560560]	Unknown
CBS 111319	*Microsphaeropsis proteae* (Crous & Denman) Crous & Denman, Persoonia 27: 32 (2011) [MB#560560]	Unknown
CBS 111320	*Microsphaeropsis proteae* (Crous & Denman) Crous & Denman, Persoonia 27: 32 (2011) [MB#560560]	Unknown
CBS 111321	*Verrucoconiothyrium nitidae* (Crous & Denman) Crous, Sydowia 67: 110 (2015) [MB#812550]	Unknown
CBS 111322	*Verrucoconiothyrium nitidae* (Crous & Denman) Crous, Sydowia 67: 110 (2015) [MB#812550]	Unknown
CBS 111380	*Verrucoconiothyrium nitidae* (Crous & Denman) Crous, Sydowia 67: 110 (2015) [MB#812550]	Unknown
CBS 111494	*Neopestalotiopsis surinamensis* Maharachch., K.D. Hyde & Crous, Studies in Mycology 79: 149 (2014) [MB#809781]	Zimbabwe
CBS 111501	*Apiognomonia errabunda* (Roberge) Höhn., Annales Mycologici 16 (1-2): 51 (1918) [MB#264192]	South Africa
CBS 111503	*Pestalotiopsis australis* Maharachch., K.D. Hyde & Crous, Studies in Mycology 79: 155 (2014) [MB#809731]	South Africa
CBS 111524	*Neofusicoccum ribis* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, Studies in Mycology 55: 249 (2006) [MB#500881]	USA
CBS 111531	*Colletotrichum simmondsii* R.G. Shivas & Y.P. Tan, Fungal Diversity 39: 119 (2010) [MB#515420]	USA
CBS 111577	*Batcheloromyces leucadendri* P.S. van Wyk, Marasas & Knox-Dav., South African Journal of Botany 51: 344 (1985) [MB#104043]	South Africa
CBS 111667	*Cladophialophora proteae* Viljoen & Crous, South African Journal of Botany 64 (2): 137 (1998) [MB#444776]	South Africa
CBS 111697	*Neocatenulostroma microsporum* (Joanne E. Taylor & Crous) Quaedvlieg & Crous, Persoonia 33: 27 (2014) [MB#807808]	South Africa

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CBS 111699	*Parateratosphaeria bellula* (Crous & M.J. Wingf.) Quaedvlieg & Crous, Persoonia 33: 28 (2014) [MB#807820]	South Africa
CBS 111700	*Parateratosphaeria bellula* (Crous & M.J. Wingf.) Quaedvlieg & Crous, Persoonia 33: 28 (2014) [MB#807820]	South Africa
CBS 111702	*Curreya grandicipis* (Joanne E. Taylor & Crous) Joanne E. Taylor & Crous, Persoonia 27: 32 (2011) [MB#560559]	South Africa
CBS 111703	*Coccomyces proteae* Marincowitz, M.J. Wingf. & Crous, Microfungi occurring on the Proteaceae in the fynbos: 32 (2008) [MB#506204]	South Africa
CBS 111704	*Coccomyces proteae* Marincowitz, M.J. Wingf. & Crous, Microfungi occurring on the Proteaceae in the fynbos: 32 (2008) [MB#506204]	South Africa
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CBS 112163	*Catenulostroma wingfieldii* Crous, Persoonia 20: 67 (2008) [MB#506592]	South Africa
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CBS 112982	*Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib., Mycoscience 44 (1): 48 (2003) [MB#372362]	Zimbabwe
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CBS 113001	*Colletotrichum alienum* B.S. Weir & P.R. Johnston, Studies in Mycology 73: 139 (2012) [MB#563591]	South Africa
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CBS 113640	*Everhartia hymenuloides* Sacc. & Ellis, Michelia 2 (8): 580 (1882) [MB#210871]	South Africa
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CBS 115178	*Pyrenophora leucospermi* Crous & L. Swart, Persoonia 27: 26 (2011) [MB#560564]	Spain
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CBS 115206	*Saccharata proteae* (Wakef.) Denman & Crous, Cultivation and diseases of Proteaceae: *Leucadendron*, *Leucospermum* and *Protea*: 104 (2004) [MB#370531]	Australia
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CBS 119333	*Anthostomella conorum* (Fuckel) Sacc., Sylloge Fungorum 1: 283 (1882) [MB#175931]	South Africa
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CBS 124909	*Sporothrix protea-sedis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf., Studies in Mycology 83: 179 (2016) [MB#817585]	Zambia
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CBS 124912	*Sporothrix zambiensis* (Roets, M.J. Wingf. & Z.W. de Beer) Z.W. de Beer, T.A. Duong & M.J. Wingf., Studies in Mycology 83: 180 (2016) [MB#817588]	Zambia
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CBS 130595	*Brunneosphaerella nitidae* Crous, Persoonia 27: 29 (2011) [MB#560558]	South Africa
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CBS 130602	*Teratosphaeria capensis* Crous, Persoonia 27: 38 (2011) [MB#560567]	South Africa
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CBS 132812	*Ilyonectria protearum* L. Lombard & Crous, Australasian Plant Pathology 42: 345 (2012) [MB#800709]	South Africa

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CBS 132813	*Ilyonectria vredenhoekensis* L. Lombard & Crous, Australasian Plant Pathology 42: 346 (2012) [MB#800710]	South Africa
CBS 132814	*Ilyonectria vredenhoekensis* L. Lombard & Crous, Australasian Plant Pathology 42: 346 (2012) [MB#800710]	South Africa
CBS 132815	*Ilyonectria capensis* L. Lombard & Crous, Australasian Plant Pathology 42: 342 (2013) [MB#800707]	South Africa
CBS 132816	*Ilyonectria capensis* L. Lombard & Crous, Australasian Plant Pathology 42: 342 (2013) [MB#800707]	South Africa
CBS 132882	*Colletotrichum proteae* F. Liu, U. Damm, L. Cai & P.W. Crous, Fungal Diversity 61: 100 (2013) [MB#802498]	South Africa
CBS 133.52	*Aspergillus oryzae* var. *oryzae* (1884) [MB#418420]	Unknown
CBS 133575	Penidiella drakensbergensis Crous, Persoonia 29: 161 (2012) [MB#801770]	South Africa
CBS 133930	*Colletotrichum alienum* B.S. Weir & P.R. Johnston, Studies in Mycology 73: 139 (2012) [MB#563591]	Portugal
CBS 134.52	*Aspergillus sojae* Sakag. & K. Yamada ex Murak., Rep. Res. Inst. Brewing: 8 (1971) [MB#292860]	Unknown
CBS 134209	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa
CBS 134210	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa
CBS 134211	*Penicillium amaliae* C.M. Visagie, J. Houbraken & K. Jacobs, Persoonia 31: 52 (2013) [MB#803784]	South Africa

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Table S2. Taxonomic information of the most abundant fungi in newly-formed and mature infructescences. These relative abundances of these taxa are plotted in Fig. 2

Phylum	Class	Order	Family	Genus	ID/Species	NFI* - Burnt	NFI* - Unburnt	Mat**
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria alternata*	x	x	
Ascomycota	Leotiomycetes	Helotiales	Arachnopezizaceae	*Arachnopeziza*	*Arachnopeziza* sp.			x
Basidiomycota	Agaricomycetes	Atheliales	Atheliaceae	*Athelia*	*Athelia* sp.			x
Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Aureobasidium*	*Aureobasidium* sp.	x	x	
Basidiomycota					Basidiomycota sp. 1	x	x	x
Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	*Botrytis*	*Botrytis caroliniana*	x		
Basidiomycota	Agaricomycetes	Cantharellales			Cantharellales sp. 1	x	x	
Basidiomycota	Agaricomycetes	Cantharallales			Cantharallales sp. 2			
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Capronia*	*Capronia kleinmondensis*	x	x	x
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Capronia*	*Capronia semi-immersa*			x
Ascomycota	Dothideomycetes	Hysteriales	Gloniaceae	*Cenococcum*	*Cenococcum geophilum*	x	x	x
Ascomycota	Pezizomycetes	Pezizales	Pezizales inc. sed.	*Cephaliophora*	*Cephaliophora* sp.		x	
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*	*Chaetomium* sp.			x
Ascomycota	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	*Chalara*	*Chalara* sp.			x
Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium*	*Cladosporium delicatulum*	x	x	
Basidiomycota	Agaricomycetes	Cantharellales	Clavulinaceae	*Clavulina*	*Clavulina* sp.	x	x	
Ascomycota	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	*Coleophoma*	*Coleophoma proteae*		x	
Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae		Corticiaceae sp. 1	x	x	
Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae		Corticiaceae sp. 2	x		
Ascomycota	Dothideomycetes	Venturiales	Venturiaceae	*Cylindrosympodiodes*	*Cylindrosympodioides* sp.			x
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Epicoccum*	*Epicoccum nigrum*	x	x	
Basidiomycota	Tremellomycetes	Tremellomycetidae	Filobasidiaceae		Filobasidiaceae sp.		x	
Ascomycota	Leotiomycetes	Helotiales			Helotiales sp. 1	x	x	x
Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	*Hirsutella*	*Hirsutella rhossiliensis*		x	
Ascomycota	Leotiomycetes	Helotiales	Rutstroemiaceae	*Lambertella*	*Lambertella corni-maris*		x	
Ascomycota	Sordariomycetes	Coniochaetales	Coniochaetaceae	*Lecythophora*	*Lecythophora fasciculata*			x

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Phylum	Class	Order	Family	Genus	ID/Species	NFI* - Burnt	NFI* - Unburnt	Mat**
Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	*Leucosporidiella*	*Leucosporidiella creatinivora*	x		
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Microcera*	*Microcera coccophila*	x		
Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Mycosphaerella*	*Mycosphaerella* sp.			x
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae		Nectriaceae sp	x		
Ascomycota	Orbiliomycetes	Orbiliales	Orbilliaceae		Orbiliaceae sp.		x	
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	*Penicillium lapidosum*		x	
Ascomycota	Leotiomycetes	Helotiales	Vibrisseaceae	*Phialocephala*	*Phialocephala* sp	x	x	x
Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	*Punctularia*	*Punctularia strigosozonata*			x
Ascomycota	Leotiomycetes	Helotiales	Helotiales fam Inc. sed.	*Pyrenopeziza*	*Pyrenopeziza revincta*	x		
Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	*Rhizoscyphus*	Rhizoscyphus sp. 1			x
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolales Inc. sed.	*Rhodotorula*	*Rhodotorula nothofagi*	x		
Ascomycota	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	Pezizomycotina Inc. sed.	*Scolecobasidium*	*Scolecobasidium excentricum*	x		x
Basidiomycota	Agaricomycetes	Sebacinales			Sebacinales sp. 1			x
Basidiomycota	Agaricomycetes	Sebacinales			Sebacinales sp. 2			x
Ascomycota	Sordariomycetes	Ophiostomatales	Ophiostomataceae	*Sporothrix*	*Sporothrix splendens*	x	x	x
Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales Inc. sed.	*Wickerhamomyces*	*Wickerhamomyces rabaulensis*	x	x	

*NFI – Newly-formed infructescence

**Mat – Mature infructescence

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Table S3. Ecological guilds of top 25 OTUs in newly-formed infructescences from the burnt site

Taxon_ID	ID used for guild	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Alternaria alternata	*Alternaria*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Plant Pathogen, Wood Saprotroph	Possible	Microfungus	Soft Rot	Duncan & Eslyn, 1966; Seehann et al., 1975; Tedersoo et al. 2014
Aureobasidium sp.	*Aureobasidium*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Epiphyte, Plant Pathogen, Saprotroph	Possible	Facultative Yeast	-	Costa et al., 2012; Tedersoo et al., 2014; Wachowska & Glowacka, 2014; Irinyia et al. 2016
Botrytis caroliniana	*Botrytis*	Pathotroph, Saprotroph	-	Probable	Facultative Yeast, Microfungus	-	Kurtzman, 2011
Cantharellales sp. 1	Cantharellales	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Ectomycorrhizal, Saprotroph, Plant pathogen	Possible	-	White Rot	Veldre et al., 2013
Capronia kleinmondensis	*Capronia*	Symbiotroph	Endophyte	Highly Probable	Facultative Yeast	Dark Septate Endophyte, known from plant roots.	Tedersoo et al., 2014
Cladosporium delicatulum	*Cladosporium*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Lichen Parasite, Plant Pathogen, Wood Saprotroph	Possible	Microfungus	-	Seehann et al., 1975; Lawrey & Diederich, 2016
Clavulina sp.	*Clavulina*	Symbiotroph	Ectomycorrhizal/Saprotroph	Highly Probable	Clavarioid	-	Tedersoo et al., 2014
Corticiaceae sp. 1	Corticiaceae	Pathotroph, Saprotroph	Lichen Parasite, Plant Pathogen, Wood Saprotroph	Possible	Corticioid, Microfungus	Saprobes or pathogens on a wide range of mainly woody substrata; a few species are lichenicolous and some are grass pathogens (Cannon & Kirk 2007)	Cannon & Kirk, 2007
Corticiaceae sp. 2	Corticiaceae	Pathotroph, Saprotroph	Lichen Parasite, Plant Pathogen, Wood Saprotroph	Possible	Corticioid, Microfungus	White Rot; Saprobes or pathogens on a wide range of mainly woody substrata; a few species are lichenicolous and some are grass pathogens (Cannon & Kirk 2007)	Cannon & Kirk, 2007
Epicoccum nigrum	*Epicoccum*	Pathotroph	Fungal Parasite, Plant Pathogen	Probable	-	-	Tedersoo et al., 2014

Table S3. Continued

Taxon_ID	ID used for guild	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotrophs	Possible	-	Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Leucosporidiella creatinivora	*Leucosporidiella*	Saprotroph	Wood Saprotroph	Highly Probable	Yeast	Dead wood and soil yeast	Middelhoven, 2006
Microcera coccophila	*Microcera*	Pathotroph	Entomopathogen	Possible	-	Unknown	Miyabe & Sawada, 1912
Nectriaceae sp	Nectriaceae	Pathotroph, Saprotroph	Plant Pathogen, Saprotroph	Possible	-	Unknown	
Phialocephala sp	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable	-	Dark Septate Endophyte, known from plant roots.	Newsham, 2011
Pyrenopeziza revincta	*Pyrenopeziza*	Pathotroph	Plant Pathogen	Probable	-	-	
Rhodotorula nothofagi	*Rhodotorula*	Saprotroph	Saprotroph	Probable	Yeast	Advanced stages of wood decomposition	Ramirez & Gonzalez, 1985
Scolecobasidium excentricum	*Scolecobasidium*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Sporothrix splendens	*Sporothrix*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Plant Saprotroph, Soil Saprotroph	Probable	-		Dik et al., 1998; Rodrigues et al.,, 2016
Basidiomycota sp. 1	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	
Wickerhamomyces rabaulensis	*Wickerhamomyces*	Saprotroph	Saprotroph	Probable	Yeast	-	Kurtzman et al., 2008
Cladophialophora sp. R43C3	*Cladophialophora*	Saprotroph	Saprotroph	Probable	Facultative Yeast	-	James et al., 2006
Capronia/Exophiala	*Capronia/Exophiala*	Saprotroph	Endophyte, Saprotroph	Possible	,	From wood, roots and leaves	Yuan et al., 2010; Yamamoto et al., 2014
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotrophs	Possible	-	Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Saccharomycetales sp.	Saccharomycetales sp.	Saprotroph	Saprotroph	Possible	-	-	

Table S4. Ecological guilds of top 25 OTUs in newly-formed infructescences from the unburnt site

Taxon_ID	ID used for guild	Trophic Mode	Guild	Confidence R	Growth Morphology	Notes	Citation/Source
Alternaria alternata	*Alternaria*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Plant Pathc	Possible	Microfungus	Soft Rot	Duncan & Eslyn, 1966; Seehann et al., 1975; Tedersoo et al., 2014
Aureobasidium sp.	*Aureobasidium*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Epiphyte, P	Possible	Facultative Yeast	-	Costa et al., 2012; Tedersoo et al., 2014; Wachowska & Glowacka, 2014; Irinyia et al. 2016
Cantharellales sp. 1	Cantharellales	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Ectomycorr	Possible	-	White Rot	Veldre et al., 2013
Capronia kleinmondensis	*Capronia*	Symbiotroph	Endophyte	Highly Probable	Facultative Yeast	Dark Septate Endophyte, known from plant roots.	Tedersoo et al., 2014
Cephaliophora sp.	*Cephaliophora*	Saprotroph	Saprotroph	Probable	-	From dead leaves and dung	Mayoral et al., 1964
Cladosporium delicatulum	*Cladosporium*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endc	Possible	Microfungus	-	
Clavulina sp.	*Clavulina*	Symbiotroph	Ectomycorrhizal/Saprot	Highly Probable	Clavarioid	White Rot	Tedersoo et al., 2014
Coleophoma proteae	*Coleophoma*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Corticiaceae sp. 1	Corticiaceae	Pathotroph, Saprotroph	Lichen Parasite, Plant F	Possible	Corticioid, Microfungus	White Rot; Saprobes or pathogens on a wide range of mainly woody substrata; a few species are lichenicolous and some are grass pathogens (Cannon & Kirk 2007)	Cannon & Kirk, 2007
Epicoccum nigrum	*Epicoccum*	Pathotroph	Fungal Parasite, Plant F	Probable	-	-	Tedersoo et al., 2014
Filobasidiaceae sp.	Filobasidiaceae	Saprotroph	Saprotroph	Possible	Yeast	Basidiomycete yeast	Kwon-Chung, 1998
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotropl	Possible	-	Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Hirsutella rhossiliensis	*Hirsutella*	Pathotroph	Animal Pathogen	Probable	-	-	Tedersoo et al., 2014
Lambertella corni-maris	*Lambertella*	Saprotroph	Saprotroph	Probable	Microfungus	-	Tedersoo et al., 2014
Orbiliaceae sp.	Orbiliaceae	Saprotroph	Wood Saprotroph	Probable	Microfungus	Saprobic on wood; Many species are nematophagous low-nitrogen environments	Cannon & Kirk, 2007
Penicillium lapidosum	*Penicillium*	Saprotroph	Dung Saprotroph, Sapr	Highly Probable	-	Mold, Soft Rot	Duncan & Eslyn, 1966; Tedersoo et al., 2014
Phialocephala sp	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable	-	Dark Septate Endophyte, known from plant roots.	Newsham, 2011

Table S4 Continued

Taxon_ID	ID used for guild	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Sporothrix splendens	*Sporothrix*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endc	Probable	-	-	Dik et al., 1998; Rodrigues et al., 2016
Basidiomycota sp. 1	Unknown	Unknown	-	-	-	-	-
Wickerhamomyces rabaulensis	*Wickerhamomyces*	Saprotroph	Saprotroph	Probable	Yeast	-	Kurtzman et al., 2008
Cladophialophora sp.	*Cladophialophora*	Saprotroph	Saprotroph	Probable	Facultative Yeast	-	James et al., TY, et al., 2006
Capronia/Exophiala	*Capronia/Exophiala*	Saprotroph	Endophyte, Saprotroph	Possible		From wood, roots and leaves	Yuan et al., 2010; Yamamoto et al., 2014
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotropl	Possible	-	Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Cantharellales sp. 1	Cantharellales	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Ectomycorr	Possible	-	White Rot	Veldre et al., 2013
Basidiomycota sp. 1	Unknown	Unknown	Unknown	Unknown	Unknown	-	

Table S5. Ecological guilds of top 25 OTUs in mature infructescences from the unburnt site

Taxon ID	ID used for guild	Trophic Mode	Guild	Confidence	Growth Morphology	Notes	Citation/Source
Arachnopeziza sp.	*Arachnopeziza*	Saprotroph	Saprotroph	Probable	Microfungus		Tedersoo et al., 2014
Athelia sp.	*Athelia*	Pathotroph, Saprotroph, Symbiotroph	Epiphyte, Leaf Saprotroph, Lichen Parasite, Lichenized, Plant Pathogen, Wood Saprotroph	Possible	Corticioid, Thallus	Lichenized and non-lichenized taxa	Adams & Kropp. 1996; Carisse et al., 2000; Esslinger, 2014
Capronia semi-immersa	*Capronia*	Symbiotroph	Endophyte	Highly Probable	Facultative Yeast	Dark Septate Endophyte, known from plant roots.	Tedersoo et al., 2014
Capronia kleinmondensis	*Capronia*	Symbiotroph	Endophyte	Highly Probable	Facultative Yeast	Dark Septate Endophyte, known from plant roots.	Tedersoo et al., 2014
Cylindrosympodioides sp.	*Cylindrosympodioides*	Saprotroph	Saprotroph , Opportunistic pathogen	Probable		Leaf spots on Brabejum sp.	Crous et al. 2016
Saccharomycetales	Saccharomycetales	Saprotroph	Saprotroph	Possible			
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotrophs	Possible		Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Chaetomium sp.	*Chaetomium*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Dung Saprotroph, Endophyte, Epiphyte, Plant Saprotroph, Wood Saprotroph	Possible	Microfungus	Soft Rot; Saprobic, acting as key decay organisms of plant materials and plant-derived commodities (Cannon & Kirk 2007)	Seehann et al., 1975; Cannon & Kirk, 2007; Bills et al., 2013; Tedersoo et al., 2014; Irinyi et al., 2015; Massimo et al. 2015; Busby et al. 2016; David et al., 2016
Chalara sp.	*Chalara*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Plant Pathogen, Wood Saprotroph	Probable		Soft Rot	Duncan & Eslyn, 1966; Tedersoo et al., 2014
Lecythophora fasciculata	*Lecythophora*	Symbiotroph	Endophyte	Highly Probable		Dark Septate Endophyte, known from plant roots.	Taylor et al., 2014
Mycosphaerella sp.	*Mycosphaerella*	Pathotroph	Plant Pathogen	Probable	Microfungus	-	Tedersoo et al., 2014
Phialocephala sp.	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable		Dark Septate Endophyte, known from plant roots.	Newsham, 2011
Punctularia strigosozonata	*Punctularia*	Saprotroph	Saprotroph	Probable	Corticioid	White Rot	Tedersoo et al., 2014
Rhizoscyphus sp. 1	*Rhizoscyphus*	Pathotroph, Saprotroph, Symbiotroph	Bryophyte Parasite, Ectomycorrhizal, Ericoid Mycorrhizal, Saprotroph	Possible	Microfungus; Dark Septate Microfungus	Syn.: Hymenoscyphus among other (see e.g., Pezoloma ericae)	Baral & Krieglsteiner 2006; Huhtinen et al. 2010; Bent et al. 2011; Tedersoo et al., 2014
Scolecobasidium excentricum	*Scolecobasidium*	Saprotroph	Saprotroph	Probable			Tedersoo et al., 2014

Table S5. Continued

Taxon ID	ID used for guild	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Sebacinales sp. 1	Sebacinales	Saprotroph, Symbiotroph	Endophyte, Ectomycorrhizal, Ericoid Mycorrhizal, Saprophyte	Possible		Dark Septate Endophyte, known from plant roots.	Weiá et al. 2011
Sebacinales sp. 2	Sebacinales	Saprotroph, Symbiotroph	Endophyte, Ectomycorrhizal, Ericoid Mycorrhizal, Saprophyte	Possible		Dark Septate Endophyte, known from plant roots.	Weiá et al. 2011
Sporothrix splendens	*Sporothrix*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Plant Saprotroph, Soil Saprotroph	Probable			Dik et al., 1998; Rodrigues et al., 2016
Basidiomycota sp. 1	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	
Cladophialophora sp.	*Cladophialophora*	Saprotroph	Saprotroph	Probable	Facultative Yeast	-	James et al., 2006
Capronia/Exophiala	*Capronia/Exophiala*	Saprotroph	Endophyte, Saprotroph	Possible		From wood, roots and leaves	Yuan et al., 2010; Yamamoto et al., 2014
Helotiales sp. 1	Helotiales	Symbiotroph, Saprotroph	Endophytes, Saprotrophs	Possible		Endophytes - Well-known root endophytes in Ericaceae roots	Zijlstra et al., 2005
Punctularia strigosozonata	*Punctularia*	Saprotroph	Saprotroph	Probable	Corticioid	White Rot	Tedersoo et al., 2014
Phialocephala sp.	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable		Dark Septate Endophyte, known from plant roots.	Newsham, 2011
Phialocephala sp.	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable		Dark Septate Endophyte, known from plant roots.	Newsham, 2011

Table S6. Ecological guilds of core OTUs shared between newly-formed infructescences and mature infructescences

Taxon_ID	Taxon for guilds	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Candida parapsilosis	*Candida*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endosymbiont, Saprotroph	Probable	Yeast	Polyphyletic; Some species function very differently depend on what they are	Manolakaki et al., 2010
Filobasidium wieringae	*Filobasidium*	Saprotroph	Saprotroph	Highly Probable	Facultative Yeast	-	Kwon-Chung, 1998; Kurtzman et al., 2011
Mycosphaerella marksii	*Mycosphaerella*	Pathotroph	Plant Pathogen	Probable	Microfungus	-	Tedersoo et al., 2014
Penicillium spinulosum	*Penicillium*	Saprotroph	Dung Saprotroph, Saprotroph, Wood Saprotroph	Highly Probable	-	Mold, Soft Rot	Duncan & Eslyn, 1966; Seehann et al., 1975; Costa et al., 2012; Bills et al., 2013; Tedersoo et al., 2014
Gibberella sp. FLS-2010	*Gibberella*	Pathotroph	Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Sistotrema sp. 5 OG-2012	*Sistotrema*	Saprotroph, Symbiotroph	Ectomycorrhizal**, Wood Saprotroph	Possible	Corticioid	White Rot	Gilbertson & Ryvarden, 1987; Rinaldi et al. 2008; Tedersoo et al., 2010
Arachnopeziza delicatula	*Arachnopeziza*	Saprotroph	Saprotroph	Probable	Microfungus	-	Tedersoo et al., 2014
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014
Capronia kleinmondensis	*Capronia*	Symbiotroph	Endophyte	Highly Probable	Facultative Yeast	Dark Septate Endophyte, known from plant roots.	Tedersoo et al., 2014
Sarocladium kiliense	*Sarocladium*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Lambertella corni-maris	*Lambertella*	Saprotroph	Saprotroph	Probable	Microfungus	-	Tedersoo et al., 2014
Candida odintsovae	*Candida*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endosymbiont, Saprotroph	Probable	Yeast	Polyphyletic; Some species function very differently depend on what they are	Manolakaki et al., 2010
Phoma sp. PHY-32	*Phoma*	Saprotroph , Pathotroph	Plant Pathogen, Wood Saprotroph	Possible	-	Soft Rot	Seehann et al. 1975; Costa et al., 2012; Tedersoo et al. 2014
Mollisia sp. ZJLQ160	*Mollisia*	Pathotroph, Symbiotroph	Endophyte, Plant Pathogen	Probable	Microfungus	Dark Septate Endophyte, known from plant roots.	James et al., 2006
Cladosporium herbarum	*Cladosporium*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Lichen Parasite, Plant Pathogen, Wood Saprotroph	Possible	Microfungus	-	Seehann et al. 1975; Lawrey & Diederich , 2016
Arachnopeziza delicatula	*Arachnopeziza*	Saprotroph	Saprotroph	Probable	Microfungus	-	Tedersoo et al., 2014
Chalara sp. PRM915970	*Chalara (Hymenoscyphus*	Pathotroph, Saprotroph, Symbiotroph	Endophyte, Plant Pathogen, Wood Saprotroph	Probable	-	Soft Rot; Capable of lignin degradation	Duncan & Eslyn, 1966; Tedersoo et al., 2014
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014

Table S6 Continued

Taxon_ID	Taxon for guilds	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Nigrospora sp.	*Nigrospora*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Aureobasidium pullulans	*Aureobasidium*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Epiphyte, Plant Pathogen, Saprotroph	Possible	Facultative Yeast	-	Costa et al., 2012; Tedersoo et al., 2014; Wachowska & Glowacka, 2014; Irinyia et al., 2016
Arthrographis sp.	*Arthrographis*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Cosmospora viridescens	*Cosmospora*	Pathotroph	Fungal Parasite	Highly Probable	-	-	John Plischke III, Samuels, 2007
Alternaria alternata	*Alternaria*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Plant Pathogen, Wood Saprotroph	Possible	Microfungus	Soft Rot	Duncan & Eslyn, 1966; Seehann et al., 1975; Tedersoo et al., 2014
Taphrina caerulescens	*Taphrina*	Pathotroph	Plant Pathogen	Probable	-	-	Mix, 1949
Punctularia subhepatica	*Punctularia*	Saprotroph	Saprotroph	Probable	Corticioid	White Rot	Tedersoo et al., 2014
Phaeosphaeria sp. MUT2127	*Phaeosphaeria*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Phialocephala nodosa	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable	-	Dark Septate Endophyte, known from plant roots.	Newsham, 2011
Filobasidium magnum	*Filobasidium*	Saprotroph	Saprotroph	Highly Probable	Facultative Yeast	-	Kwon-Chung, 1998; Kurtzman et al., 2011
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014
Paraphaeosphaeria sp. PHY-31	*Paraphaeosphaeria*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Dermea sp. SB9154a	*Dermea*	Pathotroph	Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Epicoccum nigrum	*Epicoccum*	Pathotroph	Fungal Parasite, Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Coleophoma sp. c36	*Coleophoma*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Cladophialophora sp. R43C3	*Cladophialophora*	Saprotroph	Saprotroph	Probable	Facultative Yeast	-	James et al., 2006
Punctularia sp. XL-A10	*Punctularia*	Saprotroph	Saprotroph	Probable	Corticioid	White Rot	Tedersoo et al., 2014
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014
Epicoccum nigrum	*Epicoccum*	Pathotroph	Fungal Parasite, Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Uwebraunia dekkeri	*Uwebraunia*	Saprotroph	Saprotroph	Probable	-	-	Tedersoo et al., 2014
Devriesia sp.	*Devriesia*	Pathotroph	Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Epicoccum sorghinum	*Epicoccum*	Pathotroph	Fungal Parasite, Plant Pathogen	Probable	-	-	Tedersoo et al., 2014

Table S6 Continued

Taxon_ID	Taxon for guilds	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Cryptococcus sp.	*Cryptococcus*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Epiphyte, Saprotroph	Possible	Yeast	-	Andrews et al., 1983; Kurtzman et al., 2011
Otidea brunneoparva	*Otidea*	Symbiotroph	Ectomycorrhizal/Saprotroph**	Highly Probable	-	-	Rinaldi et al., 2008
Phialocephala catenospora	*Phialocephala*	Symbiotroph	Endophyte	Highly Probable	-	Dark Septate Endophyte, known from plant roots.	Newsham, 2011
Pseudocercospora colombiensis	*Pseudocercospora*	Pathotroph	Plant Pathogen	Probable	Microfungus	-	Tedersoo et al., 2014
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014
Bryochiton monascus	*Bryochiton*	Pathotroph	Plant Pathogen	Probable	-	-	Tedersoo et al., 2014
Hirsutella gigantea	*Hirsutella*	Pathotroph	Animal Pathogen	Probable	-	-	Tedersoo et al., 2014
Arachnopeziza delicatula	*Arachnopeziza*	Saprotroph	Saprotroph	Probable	Microfungus	-	Tedersoo et al., 2014
Sporothrix splendens	*Sporothrix*	Saprotroph , Symbiotroph	Endophyte, Plant Saprotroph, Soil Saprotroph	Probable	-		Dik et al., 1998; Rodrigues et al., 2016
Botrytis cinerea	*Botrytis*	Saprotroph , Pathotroph	Plant Pathogen , Saprotroph	Probable	Facultative Yeast-Micr	-	Kurtzman et al., 2011
Penicillium corylophilum	*Penicillium*	Saprotroph	Dung Saprotroph, Saprotroph, Wood Saprotroph	Highly Probable	-	Mold, Soft Rot	Duncan & Eslyn, 1966; Seehann et al., 1975; Costa et al., 2012; Bills et al., 2013; Tedersoo et al., 2014
Mycosphaerella sp. GX5-3C	*Mycosphaerella*	Pathotroph	Plant Pathogen	Probable	Microfungus	-	Tedersoo et al., 2014
Aureobasidium pullulans	*Aureobasidium*	Saprotroph, Symbiotroph	Endophyte, Epiphyte, Plant Pathogen, Saprotroph	Possible	Facultative Yeast	-	Costa et al. 2012; Tedersoo et al., 2014; Wachowska & Glowacka, 2014; Irinyia et al., 2016
Cladosporium cladosporioides	*Cladosporium*	Saprotroph, Symbiotroph	Endophyte, Lichen Parasite, Plant Pathogen, Wood Saprotroph	Possible	Microfungus	-	Seehann et al., 1975; Lawrey & Diederich, 2016
Austroafricana parva	*Austroafricana*	Pathotroph, Symbiotroph	Endophyte, Saprotroph	Probable	Microfungus	On dead and living leaves (Quaedvlie et al. 2014)	Quaedvlieg et al., 2014
Abrothallus parmeliarum	*Abrothallus*	Pathotroph	Lichen Parasite	Possible	Microfungus	-	Esslinger, 2014
Cryptococcus sp. T11-10-1	*Cryptococcus*	Pathotroph, Saprotroph, Symbiotroph	Animal Pathogen, Endophyte, Epiphyte, Saprotroph	Possible	Yeast	-	Andrews et al., 1983; Kurtzman et al., 2011
Capronia/Exophiala	*Capronia/Exophiala*	Saprotroph	Endophyte, Saprotroph	Possible	-	From wood, roots and leaves	Yuan et al., 2010; Yamamoto et al., 2014

Table S6 Continued

Taxon_ID	Taxon for guilds	Trophic Mode	Guild	Confidence Ranking	Growth Morphology	Notes	Citation/Source
Cephaliophora sp.	*Cephaliophora*	Saprotroph	Saprotroph	Probable	-	From dead leaves and dung	Mayoral et al., 1964
Clavulina/Sistotrema	*Clavulina/Sistotrema*	Saprotroph , Symbiotroph	Ectomycorrhizal**, Wood Saprotroph	Possible	Corticioid	White Rot	Gilbertson & Ryvarden, 1987; Rinaldi et al., 2008; Tedersoo et al., 2010
Cystofilobasidium capitatum	*Cystofilobasidium*	Saprotroph , Pathotroph	Fungal Parasite, Saprotroph	Probable	Yeast	Isolated from Stinkhorn sporocarp	Sampaio, 2010
Udeniomyces pyricola	*Udeniomyces*	Saprotroph , Pathotroph	Fungal Parasite, Saprotroph	Probable	Yeast	Isolated from leaves	Niwata et al., 2002
Sebacinales sp. 5173	Sebacinales sp.	Saprotroph , Symbiotroph	Endophyte, Ectomycorrhizal, Ericoid Mycorrhizal, Saprophyte	Possible	-	Unassigned	Weiß et al. 2011
Cadophora	*Cadophora*	Saprotroph , Symbiotroph	Ectomycorrhizal, Saprotroph, Endophyte	Probable	-	Dead wood/ Root endophyte	Linnakoski et al., 2018 Walsh et al., 2018
Erythrobasidium yunnanense	*Erythrobasidium*	Saprotroph	Saprothroph	Probable	Yeast		Hamamoto et al., 2011
Hyphodiscus sp.	*Hyphodiscus*	Saprotroph	Saprotroph, Fungal parasite	Possible		Isolated from dead wood, and dead fungal mycelia/fruiting bodies	Pärtel & Põldmaa, 2011
Symmetrospora	*Symmetrospora*	Saprotroph, Pathotroph	Saprotroph, Plant pathogen	Probable	Yeast	(=Sporobolomyces)	Wang and Bai, 2004
Fusicladium proteae	*Fusicladium*	Pathotroph	Plant pathogen	Probable	-	Causes leaf-spots on Protea	Crous et al., 2011
Clavulina	*Clavulina*	Saprotroph , Symbiotroph	Ectomycorrhizal**, Wood Saprotroph	Possible	Corticioid	White Rot	Tedersoo et al., 2014
Clavulina	*Clavulina*	Saprotroph , Symbiotroph	Ectomycorrhizal**, Wood Saprotroph	Possible	Corticioid	White Rot	Tedersoo et al., 2014

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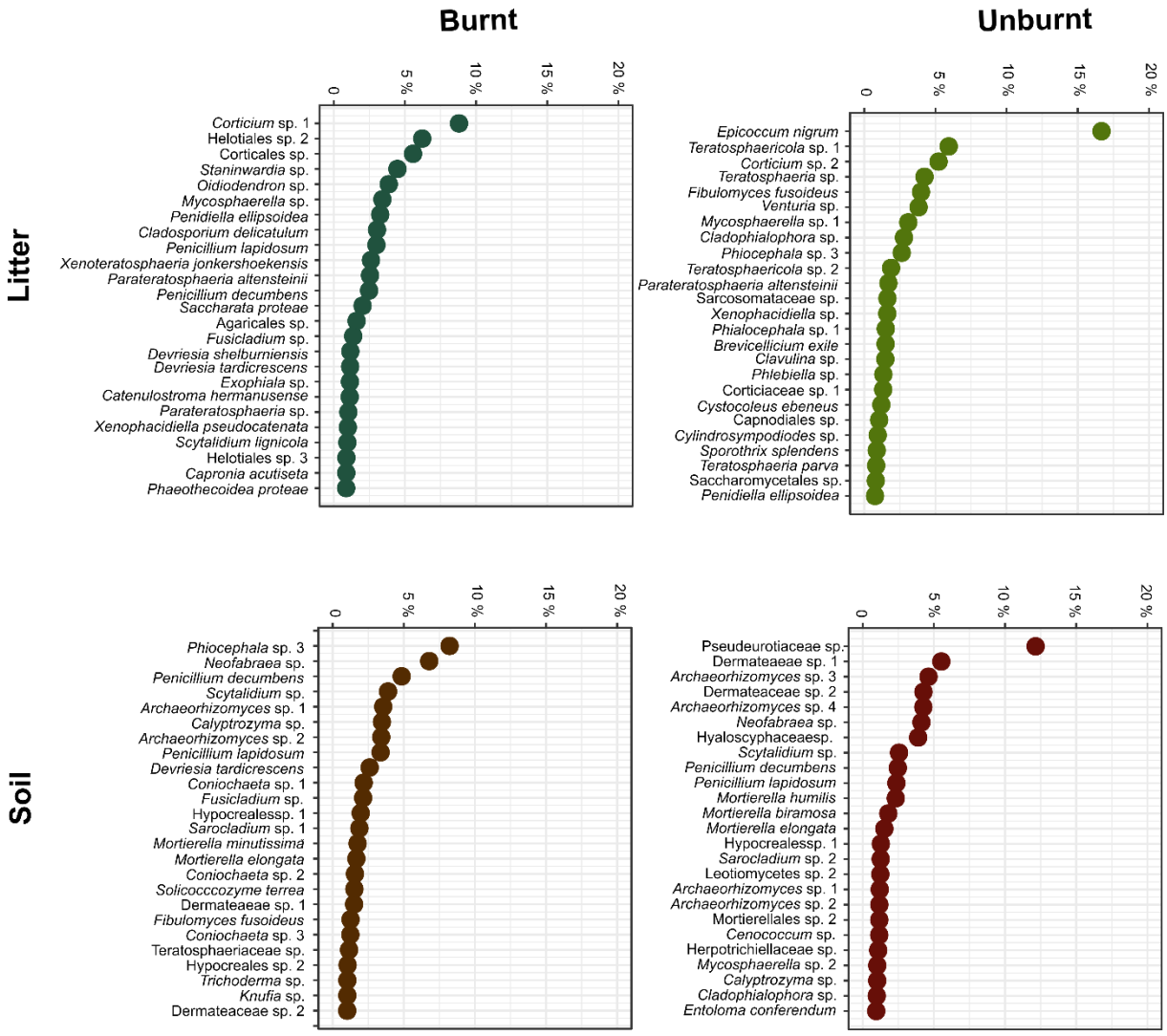

Fig. S1: Rank-abundance plots of taxa with the highest relative abundance in litter and soil samples are on the y-axis and their relative abundance on the x-axis.

Chapter 5

Diverse but inconsistent bacterial communities accompany ophiostomatoid fungi in *Protea repens* infructescences

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Abstract

The seed-storage organs of *Protea* spp., known as infructescences, present an unusual plant surface colonized by numerous bacteria and fungi. These structures are well known niches for highly dominant arthropod-associated *Knoxdaviesia* and *Sporothrix* fungi that appear to have have strong symbiotic associations with certain bacteria. Although the bacterial communities in *P. repens* infructescences have been characterized, the possible bacterial associates of fungi in this niche is unknown. In an effort to identify possible taxa involved in such symbiotic interactions, we identified the bacterial communities in areas colonized by *Knoxdaviesia* and *Sporothrix* within *Protea repens* infructescences. These were compared to bacterial communities in areas without fungal colonization using culture independent methods. We sequenced 16S rRNA amplicons from total DNA extracted from plant fragments that either contained fungi or fragments that were devoid of the fungi. Bacterial species richness was significantly higher in areas colonized by these fungi. This suggests that the fungi either provide a niche for many bacterial species, or that some bacteria are introduced into infructescences alongside the arthropod-vectored fungal spores. Community structure of bacterial communities were strongly inconsistent between samples taken from different infructescences, irrespective of fungal presence, indicating that *P. repens* infructescences contain largely randomly assembled bacterial communities. The bacterial taxa most strongly associated with samples containing fungal structures included *Pseudomonas* spp., which are well known to produce antifungal compounds *in situ*. Future studies should therefore investigate members of this genus as possible mutualists of *Knoxdaviesia* and *Sporothrix* fungi in this intriguing environment.

Introduction

The ophiostomatoid fungi, more specifically *Sporothrix* (Ophiostomatales, Ophiostomataceae) and *Knoxdaviesia* (Microascales, Gondwanamycetaceae) are well-known colonists of inflorescences and infructescences of *Protea* (Proteaceae) trees in Africa (Roets *et al.*, 2013). This

association is best studied in the Core Cape Subregion (CCR), a biodiversity hotspot in the south-western parts of South Africa (Manning and Goldblatt, 2012; Roets *et al.*, 2013) where 11 *Sporothrix* species and three *Knoxdaviesia* species have been described from various *Protea* species (Marais and Wingfield, 1994; Marais *et al.*, 1998; Roets *et al.*, 2006; Roets *et al*., 2008; Roets *et al.*, 2013; Ngubane *et al*. 2018). These saprobic species dominate the fungal communities in this niche (Roets et al. 2005) despite an abundance of nutrient resources in the form of nectar sugars and decomposing floral parts (Mitchell and Coley, 1987; Nicolson and Van Wyk, 1998; Aylward *et al*. 2017).

Sporothrix splendens was shown to be one of the most common fungi associated with *P. repens* infructescences in several studies (Marais and Wingfield, 1994; Roets *et al.*, 2005), and was the dominant fungus in an amplicon-sequencing study on the same habitat (Chapter 4). *Sporothrix splendens* colonize young *Protea* inflorescences (Theron-De Bruin *et al*., 2018) and remain the most abundant fungi in infructescences for at least three months after flowering (Chapter 4). *Knoxdavesia proteae* is also present in most newly-formed *P. repens* infructescences, but at much lower abundances than *S. splendens* (Chapter 4). Although it seems that *K. proteae* is common for shorter periods of time (Roets *et al*., 2005), it is clear that *S. splendens* is able to occupy *P. repens* infructescences and outcompete other fungi over longertime scales. The reasons for the dominance of this fungal group in *Protea* infructescences remains unclear.

Common saprophytic fungi other than *Sporothrix* and *Knoxdaviesia* are rarely observed in serotinous *Protea* infructescences, even though the abiotic environment appears suitable for fungal colonization (Roets *et al.*, 2012). Intriguingly, even a different *Protea*-associated fungus (*S. phasma*), which is common in nearby *P. neriifolia* infructescences do not colonize *P. repens* (Roets *et al.*, 2012). This is in spite of its vector mites being present in *P. repens* infructescences in large numbers (Theron-De Bruin *et al.*, 2018). The ophiostomatoid fungi such as those found in *Protea* infructescences are also the most abundant fungal group in the galleries of bark- and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae), particularly those infesting conifers in the northern hemisphere (Klebzig and Six, 2004). This is a comparatively similar niche to *Protea* infructescences in terms of moisture and protection (Klepzig and Six, 2004). Host tree defenses (Klepzig and Six, 2004) have been suggested as possible explanation, but an alternative hypothesis that has received more attention is that actinomycete bacteria, moved by the same vectors as the

ophiostomatoid fungi, inhibit the growth of competing fungi (Scott *et al.*, 2008; Aanen *et al.*, 2009).

In the *Protea* system, three different species of actinomycetes with antifungal activity have been identified (Human *et al*., 2016). These are commonly detected in newly formed infructescences (Human *et al.*, 2018). Although, *Streptomyces* species are common in *Protea* infructescences, the association between the actinomycetes and infructescences was not stable in a new population of *P. repens* plants flowering for the first time (Human *et al.*, 2018). The antifungal compounds produced by these bacteria and also detected in infructescence material was shown to be a potent inhibitor of the ophiostomatoid fungi. This suggests that the presence of these compounds may be to the detriment of the *Knoxdaviesia* and *Sporothrix* species in infructescences (Human *et al*., 2016). The actinomycetes appear rather to benefit from the multiple arthropod vectors available, a protected dead-wood niche and the absence of sugars and the copiotrophic bacteria using them.

Some bacterial species other than actinomycetes may have beneficial associations with the ophiostomatoid fungi eg. common endo- and epiphytic bacteria. Genera such as *Pseudomonas* and *Burkholderia* were amongst the most common bacteria detected in infructescences using amplicon sequencing on all the flower organs inside *P. repens* infructescences (Chapter 2). Species in these genera that include many epi- and endophytes (Balandreau *et* al., 2007; Suárez-Moreno *et al*., 2012) produce a variety of antibiotics and are known symbionts of many different fungal species (Ligon *et al.*, 2000; Vandamme *et al.*, 2007; Depoorter *et al.*, 2016). They are effective inhibitors of fungal growth on plants to such an extent that many have been developed as commercial antifungal biocontrol agents (Raaijmakers *et al.*, 2002). When these bacteria were first detected in *P. repens* infructescences, samples containing both fungal-colonized and uncolonized areas of pollen presenters, bracts and seed coats were combined (Chapter 2). It was therefore not possible to identify the bacterial communities that are consistently associated with the ophiostomatoid fungi.

Apart from the natural bacterial population associated with the plant, another potential source of bacteria in *P. repens* infructescences could be the numerous insects (Coetzee and Giliomee, 1985; Roets *et al*., 2006), birds (Theron-De Bruin *et al*., 2018) and mites (Roets *et al*., 2009; 2011; Theron *et al.*, 2012; Theron-De Bruin *et al.*, 2018) that visit inflorescences and infructescences throughout its different developmental stages. These include pollinator insects and birds and their

phoretic mites, which visit the open inflorescences, or the beetles and mites that usually enter mature infructescences at a later stage (Roets *et al.*, 2011; Aylward *et al.*, 2015). Among these fungus-vectors are many with the potential to introduce bacteria into the various *P. repens* floral structures. Most of the beetle- and bird-associated mites and the insects themselves are capable of vectoring diverse assemblages of bacteria on their exoskeletons such as members of the Bacteroidetes, Actinobacteria and Firmicutes and members of the Gammaproteobacteria such as the Enterobacteriaceae and *Pseudomonas* (Jones *et al*., 2013). It was also suggested that mites and insects are the most likely vectors of the actinomycetes, which accumulate over time in older mature infructescences (Human *et al.*, 2018). However, in order to identify the bacteria introduced into infructescences by the vectors of the fungi, a detailed knowledge of the bacteria that consistently co-occur with these fungi would be required.

In this study, we attempted to characterize the bacterial communities in the immediate areas surrounding the fruiting structures of *Sporothrix splendens* and *Knoxdaviesia proteae* within *P. repens* infructescences using non culture-based techniques. These were compared with samples from areas within the same infructescences without fungal colonization in an attempt to identify taxa that are consistently associated with these fungi. The overall justification for this comparison was that these taxa could be important for the survival and fitness of the ophiostomatoid fungi and might even depend on the same vector organisms as the fungi.

Materials and methods

Sample collection and DNA extraction

Infructescences were collected from *P. repens* trees in the Jonkershoek Nature Reserve near Stellenbosch in the Western Cape Province of South Africa (33°58'42.8"S 18°56'50.4"E). *Protea repens* trees growing in this area were at least 10-years-old and had already flowered for multiple seasons. In February 2015, 21 mature infructescences (ca. 7 months after flowering) were collected, each from a different *P. repens* individual. Infructescences not containing mature ascomata (sexual fruiting structures) of either *Knoxdaviesia proteae* or *Sporothrix splendens* were ignored. From each infructescence, a 5-mm segment of a pollen presenter containing ascomata of *K. proteae* and *S. splendens*, and a similar section in the same infructescence devoid of these fungal structures, were excised under sterile conditions. The fragments were individually placed into Eppendorf tubes containing genomic lysis buffer (Zymo Research Bacterial Fungal DNA

extraction kit, Orange, CA, USA), 3 mm Tungsten Carbide beads (Qiagen, Hilden, Germany) and shaken using a Qiagen TissueLyser II (Qiagen, Hilden, Germany) for 5 minutes. Hereafter, DNA extractions were performed using a Zymo Research Bacterial/Fungal DNA extraction kit (Orange, CA, USA) following the manufacturer's protocols.

16S rRNA Sequencing

The V4 region of the 16S ribosomal RNA (rRNA) was sequenced at the University of Michigan sequencing facility (Ann Arbor, Michigan) using primers 515F and 806R (Caporaso *et al*., 2011) and following to the protocols described by Kozich *et al.* (2013). Forward and reverse reads were processed using the DADA2 package (Callahan *et al.*, 2016) in R (R Developmental Core Team, 2017). Forward reads were trimmed to the first 240 bases, while reverse reads were trimmed to 160 bases. All sequences containing ambiguous bases were removed. Error rates in forward and reverse reads were estimated using the nonparametric error estimating algorithm in DADA2 (Callahan *et al.*, 2016). Thereafter, sequences were dereplicated and sequence variants (SVs) determined with the sequence variant inference algorithm in DADA2. Forward and reverse sequences were merged to yield exact sequence variants. These sequence variants were screened for chimeras in DADA2 (Callahan *et al.*, 2016) in R and taxonomic assignments were made against the SILVA v128 database (Quast *et al.*, 2013) with the implementation of the naïve Bayesian Classifier (Wang *et al.*, 2007) available in DADA2. Species designations were added to taxonomic assignments using the *addspecies* function in DADA2 (Callahan *et al.*, 2016).

Data analysis

Sequence variants determined using the DADA2 package in R were further analyzed using the Phyloseq package (McMurdie and Holmes, 2013) in R (R Developmental Core Team, 2017). Species richness according to the observed species metric was determined using the *estimate_richness* function in the Phyloseq package. The number of observed species was not normally distributed and, because pairs of samples were collected from the same infructescences, these were compared in a generalized linear model. The identity of infructescences where samples were collected was used as a random effect, and the presence or absence of ophiostomatoid fungal structures was used as fixed effect. Observed species richness was the response variable used in the model with a negative binomial distribution in the lme4 package (Bates, 2010) in R (R Core Team, 2017).

Read counts between different samples were normalized to eliminate the effect of different read counts by variance stabilizing transformation (McMurdie and Holmes, 2014) using the DESeq2 package (Love *et al.*, 2014) in R. Weighted Unifrac distances (Lozupone *et al.*, 2011) of the normalized SV abundances was calculated using the *distance* command in the Phyloseq package (McMurdie and Holmes, 2013) in R (R Core Team, 2017) and a dendrogram of these distances was plotted using average linkage clustering using the built-in *hclust* function in R (R Core Team, 2017).

To test whether there were significant differences in the bacterial community composition between samples with fungal structures and those with no visible fungi, we performed permutation multivariate analysis of variance (PERMANOVA; Anderson, 2001)) using the *adonis* function in the vegan package (Oksanen *et al.*, 2017) in R (R Core Team, 2017). To test the homogeneity of multivariate dispersions between the two communities, we performed permutation analysis of multivariate dispersions (PERMDISP; Anderson, 2004). In addition, to determine whether a core microbiome exists around fungal structures or in areas without fungal structures, the bacterial taxa present in 90%, 70% and 50% and 30% of samples were determined with the *core_microbiome* command in the "microbiome" package (Lahti *et al*., 2012) in R (R Core Team, 2017).

Taxa significantly associated with either samples where fungal structures were present, or those where structures were absent were identified using the *indval* function in the "indicspecies" package (De Cáceres and Jansen, 2012) in R. A heatmap of taxa significantly associated with either samples with fungal structures or without them, was created using the *plot_heatmap* function in the Phyloseq package (McMurdie and Holmes, 2013), which implements the method available in Neatmap (Rajaram and Oono, 2010) in R to display the SVs significantly associated with either sample type. In this heatmap, abundances were shown as relative abundance, calculated by the *transform* function in the 'microbiome' (Lahti *et al*., 2012) package in R.

Sequences from previous studies where bacterial communities were studied in environments containing ophiostomatoid fungi were retrieved and merged into a single sequence database. These included the samples from the mycangia or exoskeletons of the bark and ambrosia beetles *Dendroctonus frontalis, Xyleborus affinis, Xb. bispinatus-ferrugineus, Xb. glabratus, Xylosandrus crassiusculus* and *Xs. germanus* from Hulcr *et al*. (2012). Sequences from the pine-wood nematode (PWN), *Bursaphelenchus xylophilus* from Xiang *et al*. (2015) and Proença *et al.* (2010) were also

included in the analysis. Thereafter, all sequences were classified using the naïve Bayesian Classifier (Wang *et al.*, 2007) in DADA2 (Callahan *et al.*, 2016). All SVs present in at least three *P. repens* samples where the fungi were present, and also present in at least one beetle or PWN sample were selected. The 100 most abundant sequences were selected, identified to genus level and plotted in a heatmap using the *plot_heatmap* function in the Phyloseq package in R.

Results

A total of 39 individual samples were successfully amplified and sequenced. These included 21 samples from plant fragments where fungal structures were visible, and 18 from fragments without any visible fungal structures. Some samples where no fungal structures were present failed to produce sequencing libraries. Among the successfully amplified and sequenced samples, 15 were from paired samples within individual infructescences. In all 39 samples, a total of 1235 different sequence variants were identified. The richness of sequence variants (SVs) varied between 14 and 199, with large variation among samples (n = 39, mean = 76.67, SE = 7.26). A higher number of SVs were present in samples where fungal structures were present (n = 21, mean = 91.71, SE = 11.68) compared to those without the structures (n = 18, mean = 59.1, SE = 6.3). In the samples where fungal structures were visible there were significantly higher numbers of SVs (Table 1) than samples from the same infructescences where no structures were present (Fig 1).

The most abundant sequence variants were from *Paraburkholderia* followed by *Acidisoma, Streptomyces* and *Sphingomonas* (Table S1). In many cases, a single genus dominated the entire bacterial community (Fig. 2). Furthermore, no SVs were present in all samples, while the only SV present in at least 70% of all samples was identified as *Paraburkholderia*. Eleven SVs were present in 50% of samples, which included three different SVs identified as *Paraburkholderia* and also *Acidisoma*, *Terriglobus*, *Bradyrhizobium, Methylobacterium*, two SVs from the Beijerinckiaceae and one from Rhizobiales. Core bacterial communities were inconsistent, and no taxa were present in more than 90% of samples in samples with or without fungal structures (Table 2). When a core taxon was considered to be present in 70% of samples, there were five SVs in samples with fungi present, and two in samples without fungi. In samples where fungal structures were present, there were 17 and 46 SVs present when in 50% and 30% of samples respectively. In samples where no fungal structures were visible, there were seven and 32 SVs present in 50% and 30% of samples (Table 2). PERMANOVA analysis showed that there were no differences between communities

surrounding fungal structures and communities from samples where no fungal structures were present (Table 3). Group dispersions for communities associated with visible fungal structures and those without fungal structures were homogeneous according to PERMDISP (Table 4). The bacterial communities surrounding fungal structures did not have a consistent structure, which could be used to discern them from samples without visible fungal structures (Fig. 2a). The bacterial communities from four different infructescences (i.e. eight different samples) had very similar community composition, irrespective of the presence of visible fungal structures. In the remainder of samples, pairs of samples taken from the same infructescences had different bacterial community structures, although there was no clear pattern between samples where fungal structures were present, and those without structures.

According to indicator species analysis that tests for taxa significantly associated with sample groups with or without fungal structures, sequence variants identified as *Pseudomonas*, *Caulobacter*, a member of the Acetobacteriaceae and another from the Obscuribacterales were significantly associated with samples where fungal structures were visible (Fig. 3). One SV identified as *Gemmata* was associated with sample fragments where no fungal structures were visible.

All sequences from this study were compared to sequences from other environments where members of the Ophiostomatales and Microascales are common. These included the pine wood nematode, and several different bark- and ambrosia beetles. Thirty six different bacterial genera were present in at least three samples from *P. repens* infructescences and at least one beetle species, or the PWN (Fig. 4). Among these, the bacterial taxa common to all these environments were *Pseudomonas, Paraburkholderia, Mycobacterium, Rhizobium, Bradyrhizobium* and *Sphingomonas*. *Pseudomonas* was associated with samples with fungal structures and absent in most samples where no fungal structures were visible. This genus was also present in all beetle- and PWN-samples.

Discussion

In this study we identified the bacterial communities co-inhabiting surfaces within *P. repens* infructescences with ophiostomatoid fungi. Using high-throughput sequencing of 16S rRNA amplicons, the bacterial communities in small patches of tissue on pollen presenters in *P. repens* infructescences were shown to be heterogeneous. The consistency of bacterial communities

surrounding fungal structures between replicates precluded the identification of consistent bacterial associates of the fungi. However, areas surrounding the ophiostomatoid fungi had higher bacterial species richness, suggesting that some bacterial taxa may be closely associated with the fungi and that these organisms may interact. Some of these bacteria are introduced into infructescences by the birds, insects and mites that vector ophostomatoid fungi in this habitat (Roets *et al.*, 2009; 2011; Theron-De Bruin *et al.*, 2018). Species of *Pseudomonas* and *Paraburkholderia* were especially prevalent and abundant in areas surrounding the fungal structures.

Although we were not able to identify consistent bacterial symbionts of fungal structures, bacterial species richness was consistently higher when fungal structures were visible. In this regard, the presence of fungi can establish bacterial niches, especially in environments with very low concentrations of easily accessible sugars (De Boer *et* al., 2005) such as the late stages of *Protea* infructescences (Mitchell and Coley, 1987). Numerous phylogenetically distinct bacteria colonize the surface of fungal mycelia (Hoffman and Arnold, 2010; Arendt *et al*., 2016), while as many as seven different phyla have been reported from within fungal hyphae (Shaffer *et al.*, 2016). Many bacterial species utilize the metabolic by-products of fungal lignocellulose decomposition, whereas the bacteria themselves are not able to (Rudnick *et al*., 2015; Brabcová *et al.*, 2016). For this reason, the areas in *Protea* infructescences associated with fungal activity may be rich in easily-utilized sugars, supporting a more diverse bacterial community. This would be supported by the presence of several copies of cellulose-decomposing genes in the genome of *K. proteae* (Aylward *et al.*, 2017). Some of the most common bacterial groups reported from fungal mycelia were also abundant in this study and these include members of Burkholderiaceae, Rhizobiales and Enterobacteriaceae (Shaffer *et al*., 2016; Araldi-Brondolo *et al.*, 2017).

The most common bacterial SV in this study, irrespective of the presence of fungal structures was *Paraburkholderia*. Species in this genus, formerly *Burkholderia*, were among the most common bacteria in newly-formed and mature *P. repens* infructescences and the surrounding soil in a previous study (Chapter 2). These bacteria are very likely in close contact with the dominant fungi in this niche since they are highly abundant on most surfaces studied. *Burkholderia/Paraburkholderia* are common associates of white-rot fungi on decomposing wood (Lim *et al.*, 2003), fungal plant pathogens (Partida-Martinez *et al*., 2007), and can also utilize

fungal mycelia to move in soil (Yang *et al.*, 2017). The source of these bacteria in *P. repens* infructescences remains unclear. Although Paraburkholderia *spp*. are associated with many different insects such, honeybees (Martinson *et al*., 2011), and bark- and ambrosia beetles (Cardoza *et al.*, 2009; Adams *et al*., 2013), they are also present as an endophytes in many plants (Suárez-Moreno *et al*., 2012), and in nectar (Álvarez-Pérez *et al.*, 2012). The presence of the group in most *Protea* infructescens samples with no fungal structures suggest their colonization independent of the fungi.

Pseudomonas, was common in samples where fungal structures were present, and often absent in samples without fungal structures. Furthermore, species of *Pseudomonas* were also present in all samples from the exoskeletons of bark- and ambrosia beetles, and the pine wood nematode. This ecologically versatile genus of bacteria have the ability to utilize many different carbon substrates (Silby *et al*., 2011) and produce antimicrobial compounds in soil and on plant surfaces (Dowling and O'Gara, 1994; Ligon *et al.*, 2000). *Pseudomonas spp*. are commonly used as biocontrol agents, especially on plant crops against several plant diseases (Raaijmakers *et al*., 2002; Weller, 2007). Given their commonality in the *Protea* infructescence niche and their close proximity to the sporulating structures of *Knoxdaviesia* and *Sporothrix*, they likely interact with the fungi at some level. The absence of this SV in samples without fungal structures suggests that they could also share vectors with the fungi. Such an insect-vector hypothesis would also be supported by the fact that *Pseudomonas* spp. are also common in bark- and ambrosia beetle galleries (Adams *et al*., 2009; Adams *et al*., 2013).

The heterogeneous microbial communities in *P. repens* infructescences found in this study is in contrast with a previous study where the bacterial community structure in whole infructescences was stable and homogenous between sample replicates (Chapter 2). Microbial communities often exist as a heterogeneous landscapes of different communities (Franklin and Mills, 2003), structured according to changes in physicochemical parameters, such as nutrient content, pH and moisture content (Becker *et al.*, 2006). When smaller sample sizes are considered, communities often differ substantially. This is in contrast to larger sample sizes such as those in a previous study on bacteria in *P. repens* infructescences (Chapter 2) where different replicates were very similar. Large sample sizes often contain many smaller microbial communities in a variety of different

niches, and the sum of all these communities would appear to be homogenous (Franklin and Mills, 2003).

In this study, we found that the inside surfaces of *P. repens* infructescences is colonized by heterogeneous and diverse bacterial communities. However, no clear bacterial symbionts of the ophiostomatoid fungi could be identified which suggests bacterial symbionts are not the likely cause of the apparent dominance of these fungi in this habitat. Species of *Paraburkholderia* were the most common in all samples, while *Pseudomonas* spp. were common in areas surrounding fungal structures. However, since members of these two taxa can typically be cultured, their interactions *in vitro* with the ophiostomatoid fungi on different substrates should provide insights into whether they provide a benefit to these fungi. It is clear that the presence of *Knoxdaviesia* and *Sporothrix* in *P. repens* infructescences provides a benefit to the bacteria in this niche. Future studies characterizing both bacteria and fungi, and sampling a larger number of timepoints throughout inflorescence and infructescences development should provide clear evidence of any beneficial symbionts associated with the fungi.

Acknowledgements

We thank the National Research Foundation (NRF) and the Department of Science and Technology (DST)/NRF Centre for Tree Health Biotechnology (CTHB) for financial support. We also thank the Cape Nature Conservation Board for providing collection permits.

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Tables

Table 1. Results of a mixed-effects model testing the differences in observed sequence variants (SVs) in different infructescences

Fixed effect					
	df	**Estimate**	**Std. Error**	**z value**	**P(>|z|)**
Intercept	20	4.34	0.097	44.85	< 2e-16

Random effects			
	Variance	**Std. Dev.**	**Corr**
Intercept	0.0019	0.045	
	0.062	0.25	-1.00

Table 2. Comparison of the number of sequence variants (SVs) in core microbiomes across 30%, 50%, 70% and 90% of samples where fungal structures were visible and others where no fungal structures were visible

	Number of SVs in core microbiomes	
Percentage of samples	**Fungal structures present**	**Fungal structures absent**
90%	0 SVs	0 SVs
70%	5 SVs	2 SVs
50%	17 SVs	7 SVs
30%	46 SVs	32 SVs

Table 3. Results of PERMANOVA comparing communities where fungal structures were visible against those where no fungal structures were visible

	Df	Sums_of_Sqs	Mean_Sqs	F.Model	R2	Pr (>F)
Exp	1	0.29	0.29	0.84	0.022	0.77
Residuals	37	12.52	0.34		0.98	
Total	38	12.81			1	

Table 4. Results of PERMDISP comparing communities surrounding fungal structures against those with no visible fungal structures

	Df	Sum Sq	Mean Sqs	F	N. Perm	P (>F)
Groups	1	0.0027	0.0027	1.83	999	0.18
Residuals	37	0.054	0.0015			

Figures

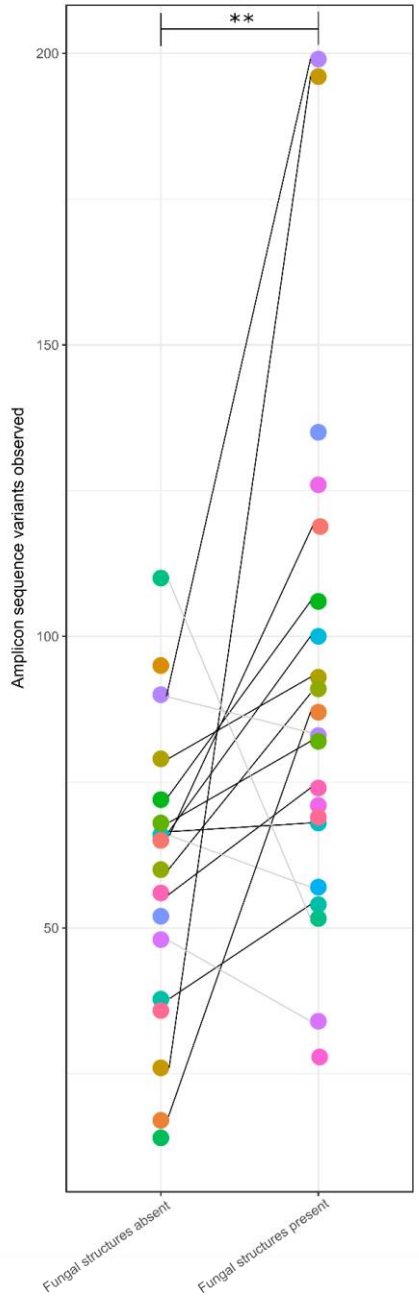

Fig 1. Observed number of sequence variants (SVs). Different colours represents material collected from different infructescences. Black connecting lines represent higher richness in areas that contained fungal structures in sample pairs from within the same infructescence, and grey lines represent lower richness in areas that contained fungal structures in sample pairs from within the same infructescence

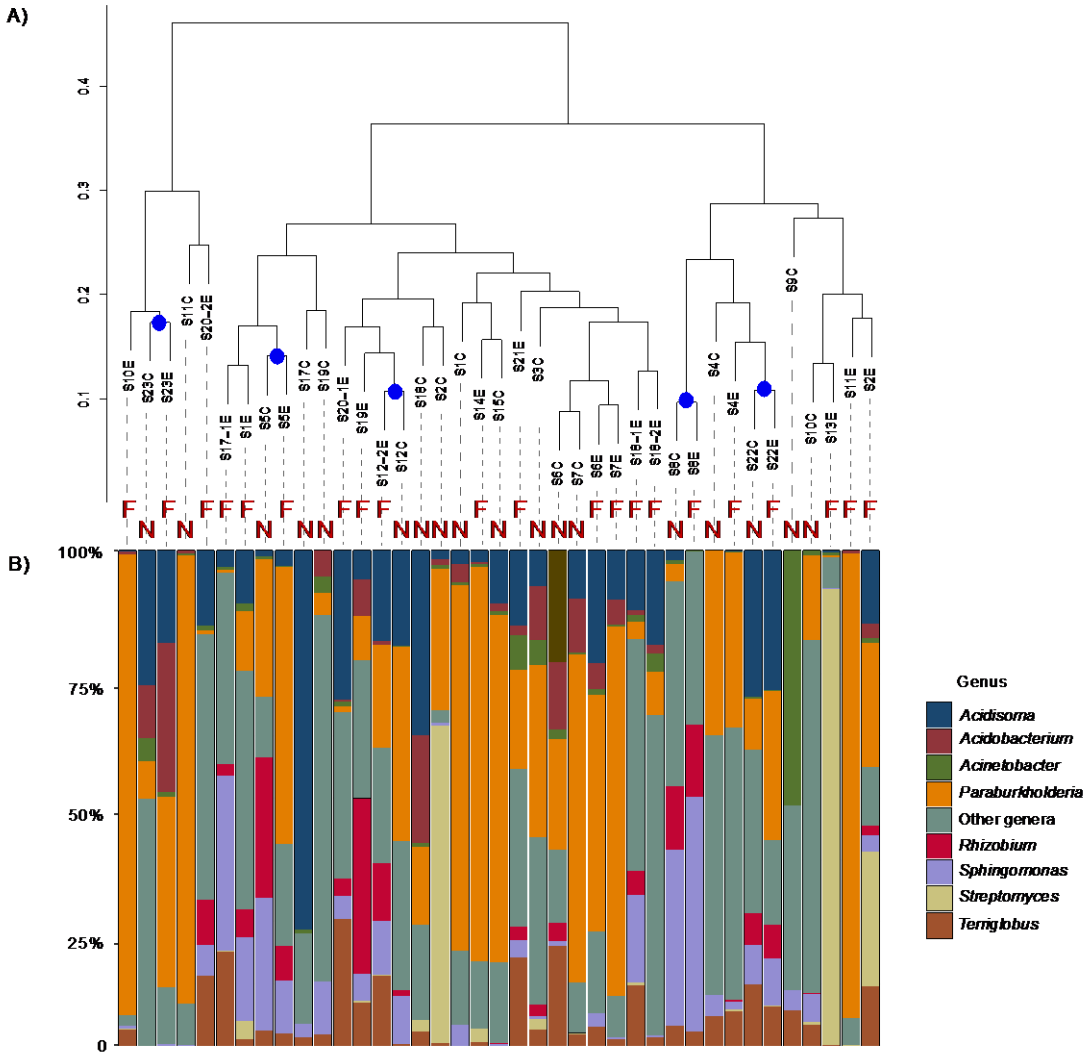

Fig 2. (A) A cluster dendrogram depicting community structure of samples from this study based on the weighted Unifrac distance metric (top). Samples with visible fungal structures (F) and those that had no visible fungal structures (N) are indicated. Sample pairs that were very similar (>95%) are shown with blue dots. (B) Stacked bar chart (bottom) of relative abundances of different bacterial taxonomic groups corresponding with each sample in (A)

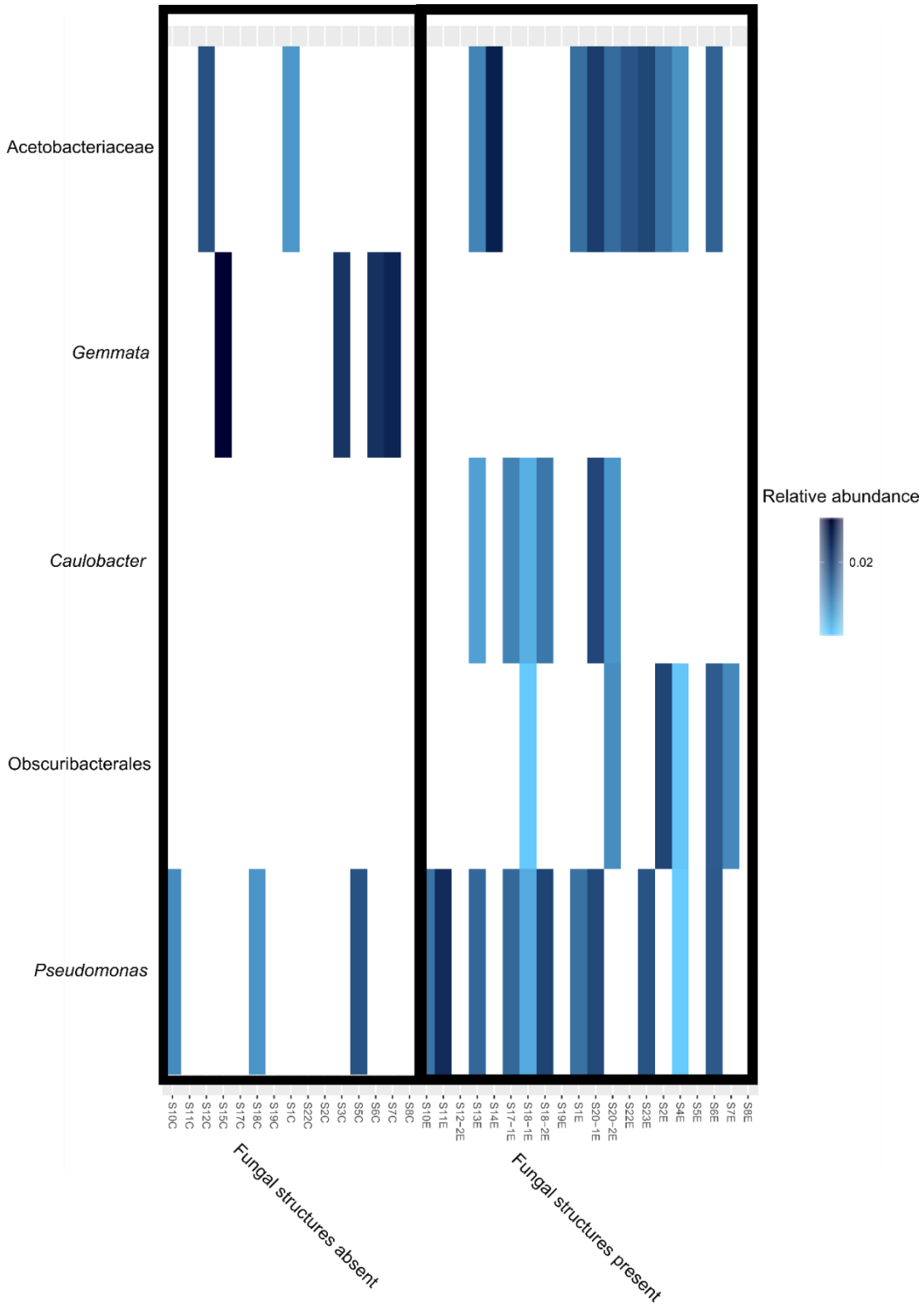

Fig 3. Heatmap of indicator species analysis indicating taxa that were significantly different between sample groups with and without fungal structures present

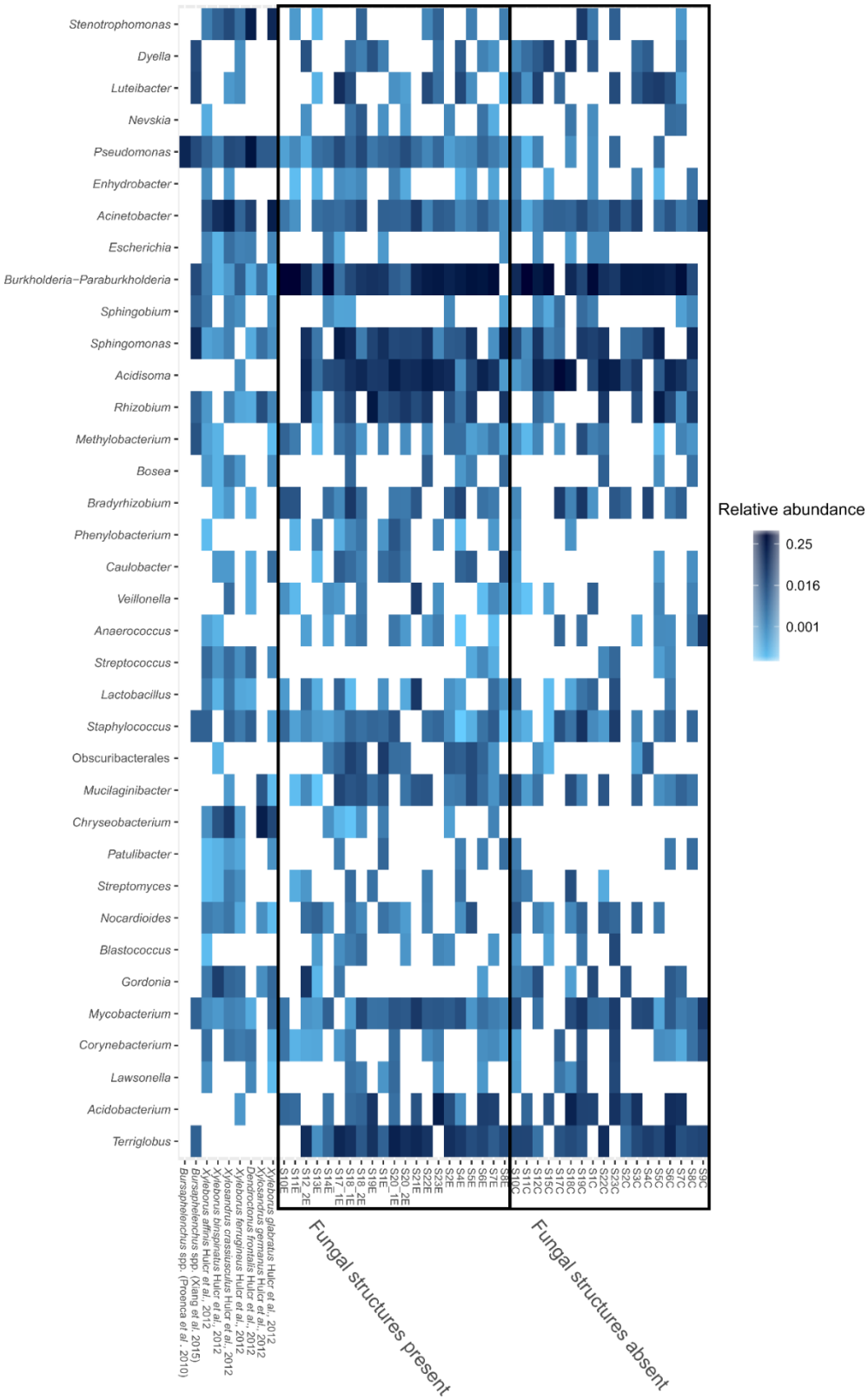

Fig 4. Heatmap of the relative abundance of taxa present three or more *P. repens* infructescences with visible fungal structures and in at one or more bark beetle or pine-wood nematode sample

Table S1. The 15 most abundant sequence variants in each sample identified to the highest taxonomic rank.

Sample names ending with E indicates samples where fungal structures were present while sample names with C are from samples where no fungal structures were visible. The respective numbers represent different infructescences.

S1E	**S1C**	**S2E**
Rhizobiales	*Burkholderia-Paraburkholderia*	*Streptomyces*
Microbacteriaceae	*Burkholderia-Paraburkholderia*	*Burkholderia-Paraburkholderia*
Acidisoma	*Acidobacterium*	*Acidisoma*
Obscuribacterales	*Gordonia*	*Terriglobus*
Acidocella	*Acidisoma*	Burkholderiales
Burkholderia-Paraburkholderia	*Sphingomonas*	*Terriglobus*
Sphingomonas	Beijerinckiaceae	Beijerinckiaceae
Sphingomonas	*Dyella*	Acetobacteraceae
Beijerinckiaceae	*Edaphobacter*	*Burkholderia-Paraburkholderia*
Kaistia	*Granulicella*	*Burkholderia-Paraburkholderia*
Streptacidiphilus	*Sphingomonas*	Beijerinckiaceae
Hydrocarboniphaga	*Lactobacillus*	*Acidobacterium*
Sphingomonas	*Sphingomonas*	*Rhizobium*
Candidatus _Xiphinematobacter	*Singulisphaera*	*Acidisoma*

S2C	**S3C**	**S4E**
Streptomyces	*Burkholderia-Paraburkholderia*	*Burkholderia-Paraburkholderia*
Burkholderia-Paraburkholderia	*Acidobacterium*	*Burkholderia-Paraburkholderia*
Burkholderia-Paraburkholderia	*Acidisoma*	*Hyphomicrobium*
Streptomyces	Solirubrobacterales	*Chitinophaga*
Acidisoma	*Acinetobacter*	*Terriglobus*
Gordonia	*Gemmata*	Beijerinckiaceae
Acidobacterium	*Amnibacterium*	*Mesorhizobium*
Beijerinckiaceae	*Corallococcus*	*Paucimonas*
Acinetobacter	Microbacteriaceae	Microbacteriaceae
Bradyrhizobium	*Granulicella*	*Bradyrhizobium*
Sphingomonas	Gammaproteobacteria	*Luteibacter*
Terriglobus	*Jatrophihabitans*	*Burkholderia-Paraburkholderia*
Acetobacteraceae	*Mycobacterium*	*Tardiphaga*
Sphingomonadales	*Streptomyces*	*Dyella*

S4C
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Beijerinckiaceae
Hyphomicrobium
Chitinophaga
Mesorhizobium
Enterobacteriaceae
Terriglobus
Microbacteriaceae
Luteibacter
Sphingomonas
Bradyrhizobium
Paucimonas
Mycobacterium

S5E
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Sphingomonas
Amnibacterium
Rhizobium
Microbacteriaceae
Obscuribacterales
Mucilaginibacter
Acidisoma
Terriglobus
Tardiphaga
Tepidisphaeraceae
Nocardioides

S5C
Rhizobium
Sphingomonas
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Luteibacter
Sphingomonas
Burkholderia-Paraburkholderia
Acidobacteriaceae_(Subgroup_1)
Terriglobus
Granulicella
Methylobacteriaceae
Sandaracinobacter
Terriglobus
Aeromicrobium

S6E
Burkholderia-Paraburkholderia
Acidisoma
Burkholderia-Paraburkholderia
Acidobacterium
Beijerinckiaceae
Terriglobus
Singulisphaera
Burkholderia-Paraburkholderia
Sphingomonas
Acidisoma
Caulobacteraceae
Caulobacteraceae
Solirubrobacterales
Granulicella

S6C
Acidisoma
Burkholderia-Paraburkholderia
Terriglobus
Acidobacterium
Beijerinckiaceae
Beijerinckiaceae
Rhizobium
Beijerinckiaceae
Solirubrobacterales
Burkholderiales
Caulobacteraceae
Luteibacter
Acinetobacter
Mycobacterium

S7E
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Acidisoma
Caulobacteraceae
Beijerinckiaceae
Acidobacterium
Solirubrobacterales
Burkholderia-Paraburkholderia
NA
Staphylococcus
Terriglobus
Rhizobiales 1174-901-12
Singulisphaera
Granulicella

S7C
Burkholderia-Paraburkholderia
Acidobacterium
Acidisoma
Solirubrobacterales
Burkholderia-Paraburkholderia
Beijerinckiaceae
Terriglobus
Chitinophagaceae
Beijerinckiaceae
Gemmata
Gluconacetobacter
Burkholderia-Paraburkholderia
Singulisphaera
Caulobacteraceae

S8E
Sphingomonas
Sphingomonas
Sphingomonas
Rhizobium
Burkholderiales
Chitinophaga
Tardiphaga
Lysinimonas
Rhizobium
Rhizobiales
Sphingomonas
Enterobacteriaceae
Terriglobus
Caulobacter

S8C
Sphingomonas
Burkholderiales
Rhodospirillaceae
Lysinimonas
Chitinophaga
Sphingomonas
Sphingomonas
Rhizobiales
Terriglobus
Marmoricola
Burkholderia-Paraburkholderia
Rhizobium
Rhizobiales 1174-901-12
Rhizobium

S9C
Acinetobacter
Caulobacteraceae
Acetobacteraceae
Mycobacterium
Anaerococcus
NA
Solirubrobacterales
Rhodospirillaceae
Terriglobus
Corynebacterium_1
Sphingomonas
Capnocytophaga
Blastocatellaceae_(Subgroup_4)
Sphingomonas

S10E
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Terriglobus
Rhizobiales 1174-901-12
Terriglobus
Acidobacterium
Curtobacterium
Dyella
Sphingomonas
Amnibacterium
Rhizobiales 1174-901-12
Gordonia
Acetobacteraceae
Streptomyces

S10C
Lysinimonas
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Rhizobiales 1174-901-12
Herbiconiux
Leifsonia
Microbacteriaceae
Rhizobiales 1174-901-12
Acetobacteraceae
Mycobacterium
Sphingomonas
Tardiphaga
Curtobacterium
Burkholderiales

S11E
Burkholderia-Paraburkholderia
Rhizobiales 1174-901-12
Bryocella
Bradyrhizobium
Bradyrhizobium
Acetobacteraceae
Beijerinckiaceae
Caulobacteraceae
Methylovirgula
Rhizobiales
Acidobacterium
Bryocella
Beijerinckia
Acidibacter

S11C
Burkholderia-Paraburkholderia
Bradyrhizobium
Bryocella
Jatrophihabitans
Rhizobiales 1174-901-12
Methylobacterium
Staphylococcus
Acidobacterium
Leifsonia
Rhizobiales 1174-901-12
Acidibacter
Corynebacterium_1
Mycobacterium
Acinetobacter

S12-2E
Burkholderia-Paraburkholderia
Terriglobus
Rhizobium
Acidisoma
Gordonia
Acidisoma
Sphingomonas
Beijerinckiaceae
Beijerinckiaceae
Microbacteriaceae
Burkholderiales
Dyella
Sphingomonas
Edaphobacter

S12C
Burkholderia-Paraburkholderia
Acidisoma
Luteibacter
Sphingomonas
Burkholderiales
Luteibacter
Singulisphaera
Burkholderia-Paraburkholderia
Acidisoma
Acidobacteriaceae_(Subgroup_1)
Gordonia
Beijerinckiaceae
Microbacteriaceae
Reyranella

S13E
Streptacidiphilus
Streptomyces
Beijerinckiaceae
Edaphobacter
Rhizobiales 1174-901-12
Solirubrobacterales
Beijerinckiaceae
Acidobacteriaceae_(Subgroup_1)
Methylocystaceae
Nocardioides
Acetobacteraceae
Lysinimonas
Burkholderiales
Burkholderia-Paraburkholderia

S14E
Burkholderia-Paraburkholderia
Burkholderia-Paraburkholderia
Edaphobacter
Acidisoma
Bryocella
Beijerinckiaceae
Beijerinckiaceae
Streptacidiphilus
Burkholderia-Paraburkholderia
Streptomyces
Singulisphaera
Mycobacterium
Terriglobus
Paracoccus

S15C
Burkholderia-Paraburkholderia
Edaphobacter
Acidisoma
Dyella
Acidisoma
Gemmata
Beijerinckiaceae
Acidisoma
Acidobacterium
Jatrophihabitans
Acinetobacter
Solirubrobacterales
Rhizobiales
Acidicapsa

S17-1E
Sphingomonas
Terriglobus
Luteibacter
Beijerinckiaceae
Gluconacetobacter
Acidisoma
Mucilaginibacter
Rhizobiales
Pseudomonas
Singulisphaera
Burkholderiales
Granulicella
Rhizobium
Mucilaginibacter

S17C
Acidisoma
Bradyrhizobium
Sphingomonas
Rhodospirillaceae
Staphylococcus
Terriglobus
Archangium
Rhizobiales 1174-901-12
Corynebacterium_1
Acinetobacter
Enterobacteriaceae
Alcaligenaceae
Rubellimicrobium
Luteibacter

S18-1E
Terriglobus
Bradyrhizobium
Beijerinckiaceae
Enterococcus
Acidisoma
Sphingomonas
Chitinophagaceae
Acidisoma
Klebsiella
Microbacteriaceae
Sphingomonas
Rhizobium
Obscuribacterales
Luteibacter

S18-2E
Klebsiella
Beijerinckiaceae
Acidisoma
Acidisoma
Burkholderia-Paraburkholderia
Acetobacteraceae
Mycobacterium
Mucilaginibacter
Acinetobacter
Edaphobacter
Acetobacteraceae
Obscuribacterales
Pseudomonas
Telmatobacter

S18C
Acidisoma
Acidobacterium
Burkholderia-Paraburkholderia
Dyella
Burkholderia-Paraburkholderia
Acidisoma
Beijerinckiaceae
Streptomyces
Acidobacteriaceae_(Subgroup_1)
Mucilaginibacter
Acetobacteraceae
Terriglobus
Edaphobacter
Mycobacterium

S19E
Rhizobium
Granulicella
Terriglobus
Burkholderia-Paraburkholderia
Granulicella
Luteibacter
Acidisoma
Acidobacterium
Acidobacterium
Sphingomonas
Sphingomonas
Dyella
Burkholderia-Paraburkholderia
Acidisoma

S19C
Caulobacteraceae
Staphylococcus
Bradyrhizobium
Granulicella
Stenotrophomonas
Sphingomonas
Mycobacterium
Acidobacterium
Acetobacteraceae
Burkholderia-Paraburkholderia
Mycobacterium
Methylobacterium
Methylobacteriaceae
Lawsonella

S20-1E
Acidisoma
Terriglobus
Acidicapsa
Chitinophagaceae
Telmatospirillum
Rhizobium
Rhodospirillaceae
Terriglobus
Hyphomicrobium
Acidisoma
Terriglobus
Sphingomonas
Staphylococcus
Terriglobus

S20-2E
Beijerinckiaceae
Acidisoma
Chitinophagaceae
Rhizobium
Terriglobus
Tardiphaga
Tardiphaga
Telmatospirillum
*Candidatus*_ Methylacidiphilum
Dongia
Terriglobus
Pseudomonas
Singulisphaera
Sphingomonas

S21E
Burkholderia-Paraburkholderia
Terriglobus
Acidisoma
Mycobacterium
Acetobacteraceae
Veillonella
Acetobacteraceae
Lactobacillus
Acidisoma
Acinetobacter
NA
Acinetobacter
Lactobacillus
Rhizobium

S22E
Burkholderia-Paraburkholderia
Acidisoma
Sphingomonas
Rhizobium
Terriglobus
Luteibacter
Mesorhizobium
Sphingomonas
Mucilaginibacter
Mycobacterium
Rhizobiales 1174-901-12
Edaphobacter
Bosea
Mucilaginibacter

S22C
Acidisoma
Mesorhizobium
Burkholderia-Paraburkholderia
Terriglobus
Sphingomonas
Legionella
Rhizobium
Terriglobus
Rhizobiales 1174-901-12
Rhizobium
Candidatus _Methylacidiphilum
Singulisphaera
Hansschlegelia
Tardiphaga

S23E
Burkholderia-Paraburkholderia
Acidisoma
Acidobacterium
Acidobacterium
Burkholderia-Paraburkholderia
Caulobacteraceae
Granulicella
Beijerinckiaceae
Granulicella
Mycobacterium
Acetobacteraceae
Beijerinckiaceae
Acidobacterium
Stenotrophomonas

S23C
Acidisoma
Acidobacterium
Burkholderia-Paraburkholderia
Beijerinckiaceae
Mycobacterium
Staphylococcus
Acetobacteraceae
Phenylobacterium
Acinetobacter
Corynebacterium_1
Blastococcus
Lawsonella
Solirubrobacterales
Curtobacterium

Conclusions

The infructescences of *Protea repens* is an unique habitat for insects, mites, fungi and bacteria, but to date most studies have only focused on very specific groups of bacteria and fungi. It is likely that these approaches neglected large amounts of microbial diversity which may be important in the ecology of *Protea* plants. In general, the research presented in this thesis attempt to characterize the bacterial and fungal diversity in the infructescences of *P. repens* for the first time, making use of high-throughput sequencing. The studies on the microbial communities in *P. repens* infructescences was done on a fire-affected site, with the aim to identify more important and stable inhabitants of these structures, and also how fire, an essential part of the ecology of this plant genus, affects its microbial associates.

The bacterial diversity and community composition in *P. repens* infructescences was significantly different between infructescences and litter sampled from the burnt and unburnt sites. Our results further suggest that several litter associated bacterial and fungal taxa are shared between litter and infructescences, however these associations were mainly limited to the unburnt site. Fungal communities seemed to be more easily dispersed as there were no real differences in community composition and diversity between samples from the burnt and unburnt site. *Sporothrix splendens* was the dominant fungal OTU in most cases, more so in the burnt site. An OTU identified as *Clavulina* sp. competed with *S. splendens* as the most abundant fungal OTU, especially in the unburnt site. Overall, both bacterial and fungal communities resembled those from dead wood and the late stages of litter decomposition, likely because the sclerophyllous *Protea* tissues contain large amounts of lignin.

Previous reports on actinomycetes in *Protea* infructescences were also further investigated, and it was shown that the members of this group consistently colonize infructescences. Early flowering stages were also studied, where we found that no actinomycetes colonize inflorescences before they become senescent. Over time, mature infructescences are colonized by a diverse set of *Streptomyces* species which suggests that *Protea* infructescences are an consistent habitat for actinomycetes with suitable substrates and routes of entry. Finally, we considered whether there are consistent bacterial communities surrounding the structures formed by *Sporothrix* and *Knoxdaviesia* species. Although we always detected higher diversity associated with the presence of fungal structures, these communities were inconsistent between replicates and therefore no symbionts of the fungi identified.

The work presented in this thesis suggests that *P. repens* infructescences to be a unique dead-plant habitat. Due to its tightly-closed structure, it may be interesting to study the microbial colonization and the arthropod associations involved in dispersal of bacteria and fungi into these structures. Furthermore, it is suggested that the substrate content and concentrations changes several times, which provide an ideal environment to study how microbial communities respond to changes in the available substrate in a reasonably closed system. Considering the limited available nutrients and the fact that infructescences are closed and protected, then these structures could also be considered as an ideal study system for microbial competition and interaction.