The diversity and ecology of actinomycetes associated with environments dominated by ophiostomatoid fungi

BY

ZANDER RAINIER HUMAN

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Supervisor: Prof. S.N. Venter

Co-Supervisors: Prof. M.J. Wingfield

Prof. B. Slippers

Dr. Z.W. de Beer

DECLARATION

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

Zander Rainier Human

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I dedicate this dissertation to the memory of my late mother, Theresa and aunt, Riekie.

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Summary

Ophiostomatoid fungi have been observed to be present and even common in specific niches from which other saprophytic fungi are rarely isolated. The galleries of several bark beetle species are representative of such a niche and have been subject to a number of studies. The infructescences of *Protea* spp. are another niche dominated by ophiostomatoid fungi. In both examples other saprophytes are very rarely present although it is believed to be a nutrient rich, moist and ideal environment for the growth of many saprophytic species of fungi. During this study I tested a hypothesis that the presence of antibiotic producing actinomycetes is responsible for the absence of other contaminating saprophytes in these environments dominated by the ophiostomatoid fungi. Following this hypothesis, the ophiostomatoid fungi would have some level of tolerance to the compounds produced, similar to fungus growing ants and the southern pine beetle where beneficial fungi have tolerance to antifungal compounds produced.

Following a culture-based approach, using selective media, actinomycetes were found to be present on the exoskeleton of *O. erosus* (Coleoptera: Scolytinae) and in the infructescences of *Protea repens* and *Protea neriifolia*. Most of the actinomycetes on the exoskeleton of *Orthotomicus erosus* had observable *in vitro* antifungal activity. These did, however also inhibit the growth of the main ophiostomatoid fungal symbiont of *O. erosus*. In the infructescences of *Protea* spp. several actinomycetes were isolated. On the basis of 16S rRNA gene phylogeny, these were clustered into five distinct groups. Members of some groups were present in both *Protea* spp. and had antifungal effects to which ophiostomatoid fungi have tolerance. Subsequently, the most common groups of isolates were further classified using a multi-locus sequence analysis (MLSA) approach. This analysis showed that our isolates include four potentially novel species.

Actinomycetes are present in the galleries of *O.* erosus but may not be responsible for creating a niche where only ophiostomatoid fungi can grow. This is primarily because of their low frequency of occurrence. However, *Streptomyces* spp. in *Protea* spp. infructescences may have some impact on the selectivity of the environment. This is because the compounds they produced were active against saprophyte fungi while the ophiostomatoid fungi had some tolerance.

Preface

Ophiostomatoid fungi, consisting of members of the fungal orders Microascales and Ophiostomatales often occur in niches from which many other common saprophytic fungi are absent (Marais and Wingfield, 1994; De Beer *et al.*, 2013). The galleries of bark beetles (Coleoptera: Scolytinae) is one such example (Kirisits, 2004). According to Zhou *et al.* (2001) the galleries of *Orthotomicus erosus* (Coleoptera: Scolytinae) in South Africa, were dominated by *Ophiostoma ips* in more than 60% of galleries sampled (Zhou *et al.*, 2001). In the infructescences of *Protea* spp. the same phenomenon is observed. Here, genera such as *Knoxdaviesa*, *Sporothrix* and *Ophiostoma* are the most common inhabitants (Roets *et al.*, 2013). The presence of antibiotic producing actinomycetes may provide a possible explanation for the restricted diversity of fungi observed in the niches dominated by the ophiostomatoid fungi. These bacteria are responsible for the production of most known antibiotics (Watve *et al.*, 2001; Clardy *et al.*, 2006) and are able to grow in niches where nutrients are scarce and the growth conditions not suitable for many other microbes (Kaltenpoth, 2009). These characteristics make them very valuable symbionts of plants and animals as they are beneficial through supplying strategic compounds required to control competition for nutrients within these ecosystems (Kaltenpoth, 2009). In this study, by focusing on bark beetle galleries and the infructescences of proteas, we investigated the hypothesis that actinomycetes may be producing antifungal compounds to which ophiostomatoid fungi have evolved tolerance.

Chapter 1 provides an overview of the associations between bacteria and insects. Insect-bacterial symbiosis may be in the form of nutritional interactions, reproductive manipulation or protective interactions (Dillon and Dillon, 2004; Kaltenpoth, 2009). The first section focuses on protective interactions where the bacteria benefit the insect. The second section of this chapter provides an overview of the characteristics of actinomycetes which make them suitable insect symbionts. The ability of actinomycetes, particularly *Streptomyces* spp., to form spores, their remarkable metabolic capabilities and their ability to produce antibiotics are discussed (Kaltenpoth, 2009; Seipke *et al.*, 2011).

Bark beetle galleries typically only contain ophiostomatoid fungi, many of which are tolerant to cycloheximide (Harrington, 1981; Malloch and Blackwell, 1993; De Beer *et al.*, 2013). The work investigating the presence of actinomycetes producing antifungal compounds in the galleries of a pine infesting bark beetle, *Orthotomicus erosus*, is presented in Chapter 2. We hypothesise that actinomycetes, producing antifungal compounds similar to cycloheximide, may be responsible for the observed fungal diversity and we isolated such bacteria using a culture-based approach. The possible antifungal activity of actinomycetes associated with *O. erosus* in South Africa was also determined.

Common saprophytic fungi, which are expected to grow in protected, nutrient rich environments, are seldom seen in the infructescences of *Protea* spp. (Marais and Wingfield, 1994). In Chapter 3 the presence of antifungal actinomycetes that may have an influence on this observed fungal distribution was investigated for two *Protea* spp. Here, culture-based methods to isolate actinomycetes were used, followed by subsequent dual-plate bioassays to

characterise antifungal activity. The identity of the isolated actinomycetes was also investigated using 16S rRNA gene sequence analysis.

Based on the 16S rRNA sequences of the actinomycetes isolated from *O. erosus* galleries and the infructescences of *Protea* spp. in South Africa, it was concluded that all the isolates belonged to the genus *Streptomyces*. In Chapter 4, these isolates were classified using a multi-locus sequence analysis approach. A phylogenetic analysis of the concatenated sequence data of the 16S rRNA, *gyrB*, *rpoB* and *trpB* genes was used. This approach is known to provide a high level of phylogenetic resolution and could also give an indication of whether some of these isolates could be members of previously undescribed species.

The work in this dissertation, therefore, aims to expand our knowledge of the ecology of environments dominated by ophiostomatoid fungi through attempting to provide a link between these fungi and the presence of specific actinomycetes. It also provides an indication of the diversity of actinomycetes in such environments in South Africa. The presence of actinomycetes and their role in unique South African ecosystems have not been studied extensively before.

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Chapter 1

Insect-microbe symbiosis and Actinomycetes: all the traits to be ideal partners

Introduction

Microbial symbiosis is a very important source of phenotypic variation in organisms belonging to all domains of life. Both animals and plants are influenced by the presence of bacteria and fungi, living on or in them or sharing a niche. In insects, microbial symbioses are particularly well characterized in terms of the nutritional benefits they provide to their hosts (Dillon and Dillon, 2004). This is because most insects utilize some form of plant material for nutrition, often without possessing the necessary metabolic capabilities to break these down to usable monomers (Dillon and Dillon, 2004).

The protective role that microbes can play for insect hosts has recently received much attention (Brownlie and Johnson, 2009; Kaltenpoth, 2009). Protective mutualisms have been described in a variety of insects (Currie *et al.*, 1999a; Cafaro and Currie, 2005; Kaltenpoth *et al.*, 2005; Scott *et al.*, 2008; Brownlie and Johnson, 2009; Kaltenpoth, 2009). Such protection might take the form of physical protection, or production of toxins or other secondary metabolites (Moran *et al.*, 2005; Brownlie and Johnson, 2009; Kaltenpoth, 2009). Bacteria are particularly prominent producers of a diversity of secondary metabolites in these symbioses.

Antibiotic producing bacteria are often responsible for facilitating protection of insect symbionts against parasites or pathogens (Currie *et al.*, 1999a; Cafaro and Currie, 2005; Kaltenpoth *et al.*, 2005; Scott *et al.*, 2008). Since actinobacteria are the most common producers of antibiotics, they are usually involved in such antibiotic-mediated protective interactions. For example, attine ants harbour different genera of actinomycetes which produce antibiotics specific against a co-evolved parasite of the ants, *Escovopsis* spp. (Currie *et al.*, 1999a; Cafaro and Currie, 2005). European beewolves have *Streptomyces* symbionts in their antennae that produce antibiotics that are applied to brood cells for protection against pathogens (Kaltenpoth *et al.*, 2005; Kaltenpoth *et al.*, 2006). Similarly, *Streptomyces* spp.

have been discovered in bark beetle galleries, with antifungal properties specific against a parasitic fungus, *Ophiostoma minus* (Scott *et al.*, 2008).

Actinomycetes are ideal partners for protective interactions (Kaltenpoth, 2009). As mentioned above, they are prolific producers of antibiotics and responsible for most antibiotic compounds known (Watve *et al.*, 2001; Clardy *et al*., 2006; Baltz, 2008). They further have traits that make them easy to acquire and maintain as symbionts. The ability to form spores not only facilitates transport or vectoring, but also allows synchronization with the insect life-cycle by remaining dormant during insect diapause (Kaltenpoth, 2009). Furthermore, actinomycetes are able to utilize nutrients inaccessible to their host, which eliminates the threat of competition by symbionts (Calderón-Cortés *et al.*, 2012). Finally, the ability to produce odours and volatiles may play an important part in their association with insects, although this remains largely unstudied (Gerber and Lechevalier, 1965).

This review provides an overview of insect-microbe symbiosis, with a focus on beneficial interactions. Emphasis is placed on examples of different types of protective symbioses and in particular actinomycete-mediated protective interactions. Finally, the review considers actinomycetes and the traits they possess to establish symbiotic interactions with insects.

Section A

Insect-microbe symbiosis

Symbiosis has long been recognized as important for individual organisms and ecosystems alike. Albert Frank first used this term in 1877 for “all the cases where different species live on or in one another” and DeBary in 1878 as “the living together of differently named organisms” (Sapp, 1994). These interactions can vary in terms of the level of host and symbiont cost and benefit, which determines whether the interaction is mutualistic, commensal or parasitic (Boucher *et al.*, 1982; Keeler, 1985; Sapp, 1994; Paracer and Ahmadjian, 2000; Klepzig *et al*., 2009). While much of the focus has often been on interactions amongst plants and animals, many animals engage in symbiosis with microorganisms (Douglas, 1998; Steinert *et al.*, 2000; Hooper *et al.*, 2001; Moran *et al.*, 2008).

Nutritional interactions

The best known symbiotic associations between microorganisms and insects involve the supplementation of nutrients to the insects, and usually the distribution and/or inoculation of the microorganisms to suitable environments or hosts. These interactions can be at a level where the benefit is either at the individual or the community level.

Nutritional symbiosis at an individual level

It is well known that species of the bacterial genus *Buchnera* exist as intracellular endosymbionts in specialized cells, referred to as mycetocytes or bacteriocytes, in certain aphids (Douglas, 1998). It has also been detected in psyllids, whiteflies and mealybugs (Homoptera: Sternorrhyncha) (Baumann, 2002). Sap-feeding insects have specialized mouthparts, enabling them to search the plant for phloem-sieves, where they feed on plant-sap, a substrate rich in carbohydrates, but deficient in important nitrogen derivatives such as essential amino acids (Sandstrom and Moran, 1999). As in the aphids, the other Sternorrhyncha obtain essential amino acids from these obligate endosymbionts (Douglas, 1998). These symbionts are vertically transmitted, enclosed in vesicles provided by the host and are essential for survival of the insect. Their elimination results in the death of the insect (Douglas, 1998; Baumann, 2002).

The association with primary or P-symbionts, such as *Buchnera* in aphids, *Wigglesworthia* in tsetse flies and *Blochmannia* in carpenter ants (Douglas, 1998; Sameshima *et al.*, 1999; Aksoy and Rio, 2005), is hypothesised to have originated between 100 to 200 million years ago (MYA) in aphids through infection of an insect by free living bacteria. It is thought that the insect most likely utilized the bacterium to expand its nutritional capabilities in order to overcome environmental pressures (Munson *et al.*, 1991; Moran *et al.*, 1993; Baumann, 2002). In carpenter ants, *Blochmannia* P-symbionts were shown to enhance the competitive ability of the insects through expansion of their metabolic capabilities, while in tsetse flies, the *Wigglesworthia* P-symbionts expand host metabolic abilities and their ability to reproduce (Sauer *et al.*, 2000; Feldhaar *et al.*, 2007; Pais *et al.*, 2008).

Nutritional symbiosis of whole communities

Fungus-growing ants

Various attine ants (Attini: Formicidae) are involved in mutualistic relationships with fungi. This interaction exists in at least 12 genera and more than 210 species and is thought to have originated about 50 MYA (Mueller et al., 1998; Poulsen and Currie, 2002). The lower attines, consisting of eight genera including *Cyphomyrmex* and *Apterostigma* have colonies of very few to a few thousand individuals (Schultz and Meier, 1995). Their gardens are kept small and they use insect faeces and plant detritus as substrate for fungal cultivation (Mueller and Wisclo, 1998). The remaining four genera are referred to as the higher attines, which include the leaf-cutter ants, *Atta* and *Acromyrmex*, which use fresh plant material as growth substrate for their fungal cultivars (North *et al.*, 1997; Currie, 2001; Poulsen and Currie, 2002).

Attine ants and their mutualist fungus, which belongs to the genus *Leugoagaricus*, have an obligate relationship as the fungus is the only source of food for ant larvae and the queen, while worker diets are supplemented with plant sap (Currie, 2001). In exchange, the ants provide growth substrate, a suitable environment and protection against parasites and pathogens. The ants protect their fungal cultivars through certain sanitary practices which include "weeding" and "grooming" the fungi and removing infected parts (Weber, 1958; Currie 2001; Currie and Stuart, 2001; Reynolds and Currie, 2004).

In the case of fungus-growing ants, the symbiont fungal cultivar is vertically transmitted by queens that transfer inoculum during their nuptial flight (Currie, 2001, Mueller *et al.*, 2001). New nests are established by virgin queens, which give rise to a new worker brood after mating and finding a suitable site for the new nest (Fernandez-Marin *et al*., 2004). The queen inoculates the fungus in a specialized chamber and tends to it through foraging or providing faecal fluid until workers are reared and assume care of the fungal garden (Currie, 2001). During this founding stage the colony is at its most vulnerable to predation, pathogen and parasite infection and failure to establish the symbiont culture (Fernandez-Marin et al., 2004).

Fungus-growing termites

A similar symbiosis to that of leaf-cutter ants exists in fungus-growing termites. Termites residing in the sub-family Macrotermitinae engage in an ectosymbiosis (symbiosis where the members are physically separated at one stage) with *Termitomyces* spp. (Aanen *et al.*, 2002;

Hyodo *et al.*, 2003). In contrast to the leaf-cutter ants, the plant material, usually dead cellulosic tissue collected from the surrounding environment, is first consumed by the termites. The faecal pellets of chewed, undigested plant-derived substrate are then packed into a structure known as a fungus comb (Martin and Martin, 1978; Darlington, 2004). Small nodules form on the surfaces of these combs that consist of synnemata and conidia of the fungus (Martin and Martin, 1978).

Several hypotheses have been proposed for the function of the symbiotic fungus in the termite *Termitomyces* mutualism (Hyodo *et al.*, 2003). These included supplementation of insect metabolic capabilities, provision of additional nitrogen from nitrogen poor substrates, and digestion of lignin in order to release cellulose that can then be used. Support for all three hypotheses can be found among the various termite genera, but not all genera rely on all these functions (Matsumoto, 1976; Martin and Martin, 1978; Hyodo *et al.*, 2000; Hyodo *et al.*, 2003).

Odontotermes spp. were shown to contain chitinolytic enzymes in their midgut, thus relying upon the fungus as a source of nutrition (Rouland *et al.*, 1991; Hyodo *et al.*, 2003). *Macrotermes* spp. generally depend on the fungus for digestion of lignin in the substrate, which makes cellulose accessible to be used as nutrients (Hyodo *et al.*, 2003). Similarly, *Macrotermes gilvus*, a fungus-growing termite from South-East Asia (Hyodo et al., 2000), selectively feeds on older parts of the fungus comb, where lower levels of lignin is present (Hyodo et al., 2000). This was evident as fresh fungus combs were shown to have much higher lignin to cellulose ratios than older combs (Hyodo et al., 2000). An African termite, *Macrotermes natalensis*, possesses all the enzymes, except cellulases, to digest wood that has not been decayed in any manner (Martin & Martin, 1978). By ingesting fungal nodules, they acquire the necessary cellulases to completely digest cellulose (Martin and Martin, 1978).

Termite phylogenies suggest a single evolutionary event leading to the mutualistic lifestyle with fungi (Aanen *et al.*, 2002). This hypothesis is based on the exclusive association with fungi that all members of this monophyletic termite lineage share. The exclusive association also suggests that there has been no reversion to a non-symbiotic lifestyle (Aanen et al., 2002). This relationship owes its success to the fact that most of the symbiont species are shared between termite species, suggesting that horizontal transmission of symbionts occurs (Aanen et al, 2002; Katoh *et al.*, 2002). Vertical transmission has, however, been detected in

Microtermes spp. and in *Macrotermes bellicosus* (Johnson *et al.*, 1981), both experimentally and in the field. Interestingly, the fungi of the latter species also lack sexual fruiting structures (Aanen *et al.*, 2002; Katoh *et al.*, 2002; Aanen, 2006). Despite this pattern of vertical transmission, the absence of monophyly for the fungus associated with *Microtermes* and *M. bellicosus* suggests that horizontal transfer still occurs, with vertical inheritance being a more recent development (Aanen *et al*., 2002).

A symbiotic community associated with bark beetles

Bark beetles (Coleoptera: Scolytinae) are important pests of many trees, but probably best known for attacking conifers (Malloch and Blackwell, 1993; Raffa *et al.*, 1993; Farrel *et al.*, 2001). These beetles construct egg galleries in the secondary phloem or inner bark of trees (Stark, 1982; Malloch and Blackwell, 1993; Paine *et al.*, 1997; Sauvard, 2004). They are also well known for their association with fungi, most commonly ascomycetes residing in the Microascales and Ophiostomatales (Malloch and Blackwell, 1993; Kirisits, 2004; Harrington, 2005; Six, 2012). These insects may benefit from this association in the form of nutrition and assisting in tree death, although some authors disagree (Raffa *et al.*, 1993; Harrington, 2005; Six and Wingfield, 2011). Bark beetle-fungi associations include many tree pathogens, such as the causative agents of Dutch elm disease, *Ophiostoma ulmi* and *O. novo-ulmi*, which are vectored by *Scolytus* spp. (Anderbrant and Schlyter, 1987; Webber, 1990; Brasier and Buck, 2001).

A well-studied example of the symbiosis between bark beetles and fungi that infest conifers is that of the Southern Pine Beetle *(Dendroctonus frontalis*). It inoculates the phloem with fungi carried in its mycangia and on the surface of the exoskeleton (Paine et al. 1997; Six and Klepzig, 2004). The most common non-mycangial fungal associates of *D. frontalis* are *Ophiostoma minus*, a bluestain species, and *O. nigricarpum*, a non-staining fungus (Paine et al., 1997) found on its exoskeleton. It also has mycangial fungal symbionts *Ceratocystiopsis ranacalosa* and an undescribed *Entomocorticium* species (Barras and Perry, 1972; Bridges, 1983; Paine et al., 1997). The role of *O. minus* as an associate of *D. frontalis* is still heavily debated (Paine et al. 1997, Six and Klepzig, 2004, Harrington, 2005; Six and Wingfield, 2011), and is noteworthy as it causes an intense bluestain of sapwood (Seifert, 1993).

Ophiostoma minus has been suggested to be beneficial to *D. frontalis* through pathogenesis of the host tree (Paine et al., 1997), although many authors suggest otherwise. This is because

tree death also occurred when trees were attacked by beetles without *O. minus* (Bridges *et al.*, 1985, Six and Wingfield, 2011). The presence of *O. minus* is thus not a necessity for tree death to occur, but it may speed up tree death (Klepzig et al., 2001). Beetle colonisation of host trees is dependent on population numbers, as trees are able to defend themselves by exuding resin. High beetle populations cause resin levels to become depleted and lead to successful infestation (Christiansen *et al.*, 1987; Franceschi *et al.*, 2005). *Ophiostoma minus* in turn receive transport to new host tissue in this short-lived mutualism (Klepzig et al., 2001). After larvae hatch, *O. minus* is detrimental to beetle larvae and they may not complete development in galleries colonized by other bluestain fungi (Paine et al. 1997, Harrington, 2005; Six and Klepzig, 2006).

Other fungal associates of the Southern Pine Beetle, such as *Ceratocystiopsis ranacalosa* (Six and Klebzig, 2004) and *Entomocorticium dendroctoni* (Harrington, 2005, Paine et al., 1997), are carried in specialized mycangia that have evolved for carrying fungi. According to Six and Paine (1999), *Cop. ranucalosa* is a weak mutualist, but shows parallel cladogenesis with its bark beetle host, whilst *Entomcorticium* is a strong mutualist, but shows no parallel cladogenesis.

Female *D. frontalis* construct galleries where eggs are deposited and both mycangial fungi are inoculated into the walls of the galleries (Wagner, 1981; Sauvard, 2004). Once hatched, the larvae feed on these fungi (Ayres *et al.*, 2000; Klepzig *et al.*, 2001). Specialized feeding chambers colonized by a monoculture of one of the mycangial fungi are constructed at later stage (Six and Klepzig, 2004). Feeding on the mycangial fungi has shown a positive effect on larval brood development (Six and Klebzig, 2004, Harrington, 2005).

Klepzig et al. (2001) proposed that some of the fungi associated with the southern pine bark beetle ecosystem could be vectored by mites. Ninety-six species of mites have been associated with *D. frontalis*, with 14 of these being phoretic (Lombardero *et al.*, 2000; Klepzig *et al.*, 2001; Lombardero *et al.*, 2003; Hofstetter *et al.*, 2006). Three species of *Tarsonemus* (Acarina: Tarsonemidae) mites, namely *Tarsonemus ips, T. krantzi* and *T. fusarii* have been identified as close associates of *D. frontalis* (Lombardero et al., 2000, Lombardero et al. 2003). It is believed that the interactions between these mites and their fungal mutualists extends much deeper than phoresy (Lombardero *et al.*, 2000; Lombardero *et al.*, 2003). *Tarsonemus ips* and *T. kranzi* have sporotheceae, specialized flap-like structures of the

integument (Moser, 1985; Klepzig et al., 2001), for the transport of ascospores of *Cop. ranucalosa* and *O. minus* (Lombardero et al. 2000).

Entomocorticium has not been found on mites and is postulated that the mites do not vector this fungus as they do not derive any nutritional benefit from it (Lombardero *et al.*, 2000; Klepzig *et al.*, 2001). This has been confirmed by feeding studies, which have shown that the mites have positive population growth rates in the presence of *O. minus* and *Cop. ranuculosa*, but not when feeding on *Entomocorticium* monocultures (Lombardero et al., 2000). Thus, mites obtain benefit through transportation of these two species of fungus, while the fungi provide nutrition to the mites.

Bark beetle-fungus symbioses provide an ideal model to study the formation and stability of symbiotic associations. The role of fungi in bark beetle life strategies may differ from beneficial to detrimental (Six and Wingfield, 2011) and yet the symbionts remain part of the insect's associated microbiota. This would be a good example for the co-evolution of microorganisms to benefit from their insect associates (Farrell *et al.*, 2001; Mueller *et al.*, 2005). There is a great diversity of bark beetles and their associated fungi and the occasional exchange of symbionts and this also adds to the attractiveness of this system to study the ecology and evolution of symbiosis (Farrell *et al.*, 2001; Six and Klepzig, 2004)

Reproductive manipulation of insects by symbionts

Several bacteria can influence host reproduction. These include *Spiroplasma*, *Rickettsia* and *Wolbachia* (O'Neill *et al.*, 1992; Werren, 1997; Narita and Kageyama, 2006). Insect sex-determination is thought to be influenced by temperature, hormones and endosymbiotic bacteria (Narita *et al*, 2007).

Cytoplasmic incompatibility provides a mechanism through which endosymbiotic bacteria can influence insect reproduction. If adult insects that are infected with different strains of *Wolbachia* mate, cytoplasmic incompatibility can cause embryonic mortality (Werren, 1997; Bourtzis *et al.*, 2003). The mechanism by which this system functions is based on modification-rescue. In male insects, *Wolbachia* modifies insect sperm to kill the progeny during embryogenesis. If the female is infected with the same *Wolbachia* strain, the modification is removed to rescue the offspring (Werren, 1997; Poinsot *et al.*, 2003).

Cytoplasmic incompatibility due to bacterial infection occurs in flour beetles, *Tribolium confusum* (Stevens and Wicklow, 1992). It was found that when either *Streptomyces* or *Penicillium* was present, the insect was cured of cytoplasmic incompatibility (Stevens and Wicklow, 1992). In experiments conducted on these flour beetles, several species of *Streptomyces* and *Penicillium* were assayed for their ability to cure them of infection. *Streptomyces aureofaciens* was found to cure up to eight out of ten insects, which is linked to its ability to produce tetracycline (Stevens and Wicklow, 1992).

Parthenogenesis occurs widely in wasps (Hymenoptera) where unfertilised eggs can produce male offspring. Thelytokus parthogenesis is common in Hymenoptera and occurs when the offspring from unfertilised eggs are only females (Hagimori *et al*., 2006). One example is an endoparasitic wasp, *Neochrysocharis formosa*, in which thelytokus parthogenesis is induced by a *Rickettsia* species (Hagimori *et al.*, 2006). Parthenogenesis is also caused by *Wolbachia* in a different genus of parasitoid wasp, *Encarsia* spp. (Zchori-Fein *et al.*, 2001). Male-killing is a phenomenon where bacterial symbionts vertically transmitted by female insects kill male progeny (Hurst *et al.*, 1999). For example, the two spot ladybird, *Adalia bipunctata* are infected by a male killing *Rickettsia* sp. and a *Spiroplasma* sp. (Hurst *et al.*, 1999). Feminisation occurs when infection by bacterial symbionts cause male host insects to become female (Hiroki *et al.*, 2002). This has been reported *Encarsia* sp. wasps due to *Cardinum* spp. infection (Giorgini *et al.*, 2001).

Protective symbiosis of insects by microorganisms

Microbial metabolites are important in cooperation and antagonism with other organisms in ecosystems. Organisms from all domains of life can benefit or be negatively affected by chemicals produced by bacteria. These include algae, fungi, molluscs, nematodes, crustaceans, insects and vertebrates (Dillon and Dillon, 2004; Piel, 2004; Moya *et al.*, 2008) and other key references). Well known bacterial toxins are enteric toxins such as the Shiga toxin produced by *Shigella dysenteriae*, enterotoxins produced by *Salmonella* spp., *Escherichia coli*, *Vibrio cholerae* and several others that are responsible for many deaths worldwide (Gill, 1982; Middlebrook and Dorland, 1984; Sears and Kaper, 1996). Another mechanism by which bacteria manipulate their environment is through the production of

antibiotics. Although these compounds are well known for their clinical use, they are just as important for the survival of many organisms in different ecosystems.

Protection by various microbe-derived chemicals and toxins

Gut microbes can protect insects against the colonization of the gut by pathogens. This protection mechanism is referred to as "colonization resistance" and is commonly described in mammals (Araneo *et al.*, 1996), but has a much wider distribution in nature (Dillon & Dillon, 2004). For example, *Schistocerca gregaria*, a species of locust raised 'germ-free' by Charnley *et al*. (1985) was susceptible to fungal infection as opposed to infection resistant naturally raised locusts. This effect was attributed to phenolic production by the gut microbes (Charnley et al., 1985, Dillon and Dillon, 2004). Competition for nutrient sources is another mechanism of colonization resistance (Dillon and Dillon, 2004).

Paleamon macrodactylus, a marine shrimp is protected by bacterial symbionts (Gil-Turnes *et al.*, 1989) against a phycomycete fungus, *Lagenidium callinectes*, an important pathogen of marine crustaceans (Fisher, 1983b; Gil-Turnes, 1989). The fungus threatens the survival of *P. macrodactylus* embryos because infection always results in death. Embryos are, however, remarkably resistant to fungal infection and this could be attributed to the presence of bacteria on the egg and embryo surfaces that are responsible for producing antifungal compounds (Gil-Turnes *et al.*, 1989). Epizootic bacteria, belonging to the genus *Alteromonas* were isolated from healthy embryos (Fisher, 1983a). When tested, these bacteria had antifungal effects against several marine and freshwater fungal pathogens (Fisher, 1983a). More specifically it was later repeatedly isolated from healthy *P. macrodactylus* individuals and found to inhibit *L. callinectes in vitro* (Gil-Turnes *et al.*, 1989). The isolate of *Alteromonas* was found to produce 2, 3-indolinedione, proven to inhibit *L. callinectes* (Gil-Turnes *et al.*, 1989).

Rove beetles, belonging to the genus *Paederus* (Coleoptera: Staphylinidae) are protected from arthropod predators by the toxin pederin (Kellner, 2002a). This toxin is believed to be produced by an endosymbiotic bacterium belonging to the genus *Pseudomonas*, and closely related to the type species *Pseudomonas aeruginosa* (Kellner, 2002b). Pederin, contained in the insect haemolymph, inhibits protein synthesis in eukaryotes, but leaves prokaryotes unaffected and is passed from adults to juveniles through deposition into eggs (Kellner and Dettner, 1996). The offspring of these beetles are especially vulnerable to predation by wolf

spiders (Kellner and Dettner, 1996). Upon attack, *Paederus* larvae successfully employ this toxin as a chemical defence against wolf spiders (Kellner and Dettner, 1996; Kellner, 2002a).

Parasitoid wasps are very common parasites of different life stages of virtually all insects. Insects have evolved mechanisms to evade such parasitism. One form of protection is through the acquisition and maintenance of toxin producing symbionts. For example, *Acyrthosiphon pisum* (the pea aphid) is vulnerable to parasitism by a parasitoid wasp, *Arphidius ervi* (Oliver *et al.*, 2003). The level of parasitism by this wasp is highly variable, even in clonal populations (Oliver *et al.*, 2003). It was found that secondary symbionts, maintained in addition to its primary *Buchnera* symbionts, are responsible for this resistance. This resistance is based on secretion of compounds toxic to wasp larvae. The symbionts can also stunt larval development through alteration of host metabolism. These symbionts include members of the *Enterobacteriaceae, Rickettsia* and *Spiroplasma* (Ferrari *et al.*, 2004).

Protection by antibiotic producing microbes

Apart from the above-mentioned examples, there are numerous antibiotics that play important roles in protecting insects. Antibiotic mediated symbioses are frequently at a community level in social insects, where protection benefits whole insect communities rather than individuals. The antibiotic producing organisms often receive nutrients, protection and in most cases vectoring in return and in return produce valuable secondary metabolites (Kaltenpoth, 2009); Seipke *et al.*, 2012). Actinomycetes produce the greatest amounts of antibiotics and it is this feature that provides advantage to the macrosymbionts. The interactions of insects involving the actinomycetes are the best studied examples of the use of antibiotics and will be discussed in greater detail in the next sections.

Actinomycete-facilitated protection of fungus-growing ant cultivars

Escovopsis spp. is an ascomycete myco-parasite of the fungi used by fungus growing ants to produce nutrition for the colony (Currie *et al.*, 1999b). If an ant colony cannot eliminate an infection by this fungus, it may lose biomass, until the entire colony eventually dies (Currie, 2001). *Escovopsis* is attracted through chemotaxis towards the host fungi (Gerardo *et al.*, 2006; Caldera et al., 2009) and upon contact with the beneficial fungus, secrete compounds that degrade cells and cause nutrient loss (Reynold and Currie, 2004). *Escovopsis* has been described as an ancient member of this ant-microbe symbiosis (Reynold & Currie, 2004). In

spite of this co-evolved parasite, fungus growing ants have the ability to overcome this infection and maintain their colonies.

When investigating the ability of ant-colonies to overcome *Escovopsis* invasion, Currie *et al*. (1999) observed a powdery, whitish grey crust on the cuticle of the ants. They performed morphological and biochemical characterization of this crust and found that it was due to a filamentous bacterium initially identified as *Streptomyces* spp. but later found to be members of the genus *Pseudonocardia* (Currie *et al*., 1999a; Cafaro and Currie, 2005). Isolates of the *Pseudonocardia* showed no inhibitory activity towards several saprophytic fungi or entomopathogenic fungi (Currie et al., 1999a). This bacterium suppressed *Escovopsis*, spore germination completely in 25% of assays (Currie *et al*. 1999a). When symbionts were grown in association with the mutualist of fungus growing ants, an increase in biomass was recorded, indicating a growth-promoting effect (Currie *et al*., 1999a).

The symbionts of fungus growing ants are located on different body parts throughout the different genera (Currie *et al*., 2006). For example, in *Cyphomyrmex*, the bacteria were first noticed on its polypleural plates (Currie *et al*, 2006). Upon further investigation, a large cavity referred to as a fovea was discovered (Currie *et al*., 2006). Exocrine glands are also present on this fovea, which indicates the possibility of the ants secreting compounds into these specialized crypts for cultivation of symbionts (Currie *et al*., 2006). In the genera, *Trachymyrmex* and *Acromyrmex*, the bacteria are found directly on gland-associated parts of the exoskeleton located on the polypleural plates (Currie *et al*., 2006).

Currie *et al*. (1999a) postulated that actinomycete symbionts of leaf-cutting ants are co-evolved mutualists. Their presence on all fungus-growing ants, vertical transmission from parent to daughter nest, growth promoting effects for the basidiomycete mutualist, and the very specific inhibition towards *Escovopsis* is evidence to consider them as co-evolved. Phylogenetic analysis of 16S rRNA encoding genes grouped the bacteria isolated from each genus of ant separately, providing additional evidence of a specific co-evolved interaction and co-diversification (Cafaro & Currie, 2005).

Antibiotics from actinomycetes associated with attine ants have recently been identified. The first such compound was extracted from a *Pseudonocardia* isolate obtained from *Apterostigma dentigerum* (Oh *et al*., 2009). This isolate had strong antifungal activity against *Escovopsis* while no effects were observed against the ant mutualist cultivar (Oh *et al*., 2009).

A novel antibiotic was identified and named Dentigerumycin and was shown to be a strong inhibitor of *Escovopsis* (Oh *et al.*, 2009). Actinomycetes associated with ants from the genus *Acromyrmex* also produce a compound shown to inhibit growth of *Escovopsis* (Haeder *et al.*, 2009). Here, a strain belonging to *Streptomyces* was identified as producer of a compound subsequently named Cancidin (Haeder *et al.*, 2009).

European beewolves use symbiotic bacteria to protect their offspring

European beewolves, *Philantus triangulum* (Hymenoptera: Crabronidae) also rely on a mutualistic relationship with bacteria for survival. Commonly known as digger wasps, they hunt honey bees (*Apis mellifera*) to provide food for larvae (Strohm & Linsenmair, 1999, Kaltenpoth *et al*., 2005). A cocoon, which contains this prey, is spun by larvae in which they overwinter (Strohm & Linsenmair, 1999, Kaltenpoth *et al*., 2005). This environment contains nutrients and is moist and humid, presenting a favourable environment for bacteria and fungi and the natural microbial decomposers of bees (Strohm & Linsenmair, 2001). If microorganisms were to be able to infect these brood cells, they could have a detrimental effect on beewolf populations, killing the larvae and increasing reproductive stress (Strohm 2000, Strohm & Linsenmair, 2001).

Kaltenpoth *et al.* (2005) found that a whitish substance was secreted onto the brood cell by female wasps prior to oviposition. This substance was found squeezed from the antennal glands and spread out on the brood cell (Kaltenpoth et al., 2005) Scanning electron microscopy revealed that this white substance contained rod-like and branched structures roughly 0.5 µm in size (Kaltenpoth et al., 2005). DNA from beewolf antennae was isolated and PCR amplified using actinomycete-specific primers, and then sequenced, which lead to the discovery of *Streptomyces* bacteria (Kaltenpoth *et al.*, 2005; Kaltenpoth *et al.*, 2006). To establish the extent of this relationship of *Streptomyces* bacteria to beewolves, 11 individuals from four different populations were examined and were found to have identical sequences (Kaltenpoth et al., 2005), which strongly suggests a mutualistic relationship between microbe and animal.

Multi-partner protective symbiosis in bark beetles

Scott *et al.* (2008) investigated the possibility of bacterial symbionts associated with *Dendroctonus. frontalis*. They hypothesized that *D. frontalis* engages mutualistic actinomycete bacteria, that would inhibit *Ophiostoma minus* but not *Entomocorticium* (Scott *et al.*, 2008). Isolation experiments were performed on mycangia from collected beetles. Isolated bacterial strains were challenged with *Entomocorticium* and *O. minus* (Scott *et al.* 2008) using dual-plate bioassays (Scott *et al.*, 2008). The 16S rRNA analysis showed that they all grouped in a clade closely related to *Streptomyces thermosacchari* (Scott *et al.*, 2008). *In vitro* bioassays indicated strong inhibition of *O. minus* while *Entomocorticium* was only slightly affected (Scott *et al.* 2008). Upon isolation and purification, the inhibiting substance was identified as a polyene peroxide, which the investigators named mycangimycin (Scott et al., 2008). Inhibition of *O. minus* and not the mycangial fungi adds to the argument that most ophiostomatoid fungi carried by conifer–infesting insects do not benefit the insects (Six and Wingfield, 2011).

Section B

Actinomycetes as ideal symbionts

Actinomycete diversity

The actinomycetes are filamentous, gram-positive bacteria that all exhibit some sort of branching morphology (Lechevalier and Lechevalier, 1967; Stackebrandt *et al.*, 1997; Kampfer, 2006). This grouping includes well-known genera such as *Micrococcus*, *Frankia*, *Propionibacterium*, *Nocardia* and *Streptomyces*. The formation of hyphae and spores are characters shared with fungi. For this reason, their taxonomic position was uncertain until the late 1950's, as mycologists considered them as fungi and diseases caused by them were frequently included in books on medical mycology (Conant *et al.* 1954; Lechevalier and Lechevalier, 1967). In contrast, bacteriologists insisted that they are bacteria because of their small size and sensitivity to antibacterial compounds (Lechevalier and Lechevalier, 1967).

The taxonomy of the actinomycetes is important because they are responsible for the production of numerous economically important secondary metabolites, including antibiotics. Due to the extensive screening for economically important metabolites and taxonomic problems during the early 1900's, the genus *Streptomyces* became a dumping ground for species, with over 3000 species described by 1970 (Trejo, 1970; Guo *et al.*, 2008). These were originally dealt with by the International *Streptomyces* Project (ISP), which involved the identification of a standard set of criteria for the description of *Streptomyces* species and the identification of type strains to be stored in culture collections around the world (Shirling and Gottlieb, 1966). This was followed by a numerical classification system, based on phenotypic data and found to be very successful at the time for separating them into species groups (Williams *et al.*, 1983; Anderson and Wellington, 2001). These phenotypic characters included whole cell protein profiles, usually done through poly-acrylamide gel electrophoresis (PAGE), fatty acid methyl ester analysis (FAME), biochemical characterization of enzymes, phage typing, serology and whole cell analysis (Anderson and Wellington, 2001; Kampfer, 2006)

Recently, genotyping methods have proved most successful for the taxonomy of *Steptomyces* spp. These studies have predominantly been based on members belonging to the family Streptomycetaceae (Anderson and Wellington, 2001). These methods include DNA-DNA hybridization (Labeda, 1992), restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA PCR (RAPD-PCR) assays (Roberts and Crawford,

2000; Anderson and Wellington, 2001). The introduction of DNA sequencing technology has changed the landscape of bacterial taxonomy in general and has also contributed significantly to *Streptomyces* taxonomy. Sequence comparisons based on the 16S rRNA gene have been very successful and the use of three regions within this gene can give resolution up to species level (Takeuchi *et al.*, 1996; Kataoka *et al.*, 1997; Anderson and Wellington, 2001; Maidak *et al.*, 2001). Recently, the development of multi-locus sequence analysis schemes has improved the discriminatory power even further to refine species belonging to *Streptomyces* (Guo *et al.*, 2008; Rong *et al.*, 2009; Rong and Huang, 2010; Labeda, 2011; Rong and Huang, 2012).

Spore formation

Actinomycetes are non-motile bacteria and rely on spores for dispersal. This is an important mechanism for survival in a soil environment and also makes them very suitable as insect symbionts. These spores are generally more resistant to heat, dessication and acid, than vegetative *Streptomyces* cells (Lee and Rho, 1993). Spore formation in *Streptomyces* is believed to be an adaptation to survive being frequently consumed by soil invertebrates (Chater and Chandra, 2008).

The ability to produce spores provides bacteria and fungi with an ideal mechanism to be acquired by insects that subsequently introduce them into new environments (Kaltenpoth, 2009). Also, due to the electrochemical nature of some species of actinomycetes' spores, it may be difficult for an insect to lose such a symbiont. For instance, if spores attach to an insect and are introduced into its nesting niche (soil, foliage or wood), it would provide a possible growth substrate to the actinomycete and spores would initiate germination (Cafaro and Currie, 2005; Poulsen *et al.*, 2005). Once the bacteria have sporulated, spores may once again attach to larvae or nymphs in due time (Kaltenpoth *et al.*, 2010). Another benefit of spores is that it is a resting structure and can therefore be synchronized with the life cycle of an insect (Kaltenpoth *et al.*, 2010). Once an insect enters diapause and conditions are not suitable for bacteria, they can sporulate and re-engage the insect once it starts an active lifestyle again.

Several arthropods that frequently occur in soil ecosystems have shown the ability to vector actinomycete spores on their exoskeletons (Ruddick and Williams, 1972; Cafaro and Currie, 2005; Poulsen *et al.*, 2005; Kaltenpoth *et al.*, 2010). For example, mites have been shown to vector *Streptomyces* spores at a high incidence (Ruddick and Williams, 1972). In a study by

Ruddick and Williams (1972), they found that *S. griseus* grew in the tracks the arthropods had moved in, whilst no significant growth was recorded with faecal pellets from these animals (Ruddick and Williams, 1972). This would be indicative of actinomycete dispersal on arthropod exoskeletons. Several structures on these arthropods are ideally suited for such a purpose. *Streptomyces griseus* spores proved to be easily acquired by mites. Scanning electron microscopy (SEM) analyses revealed that spores could become attached to appendages and the body surface and occasionally spore chains attach themselves around cuticular hair of mites (Ruddick and Williams, 1972).

Metabolism

The ability for an insect to tolerate and maintain a bacterium as symbiont is likely to be highly dependent on its metabolism. In establishing symbiotic associations, the level of competition between the host and microsymbiont needs to be minimal. Actinomycetes are able to live off complex carbohydrates, often regarded as inaccessible to other organisms and may also be able to survive of animal waste products and excreta (Cochrane and Conn, 1950; Crawford, 1978; Kaltenpoth, 2009; Calderón-Cortés *et al.*, 2012). Furthermore, actinomycetes would be able to metabolize other substrates inaccessible to the insect and even provide some simpler metabolites from complex sources (Crawford, 1978; Antai and Crawford, 1981; Adams *et al.*, 2011; Calderón-Cortés *et al.*, 2012)

Streptomyces spp. in particular are known for their ability to degrade several polymers such as hemicelluloses, pectin, keratin and chitin amongst others (Goodfellow and Williams, 1983; McCarthy and Williams, 1992; Hesketh *et al.*, 2002; Borodina *et al.*, 2005). This was confirmed through the analysis of the genome of *S. coelicolor*. Bentley *et al.* (2002) identified 819 potential secreted proteins of which hydrolases are the most abundant. These include peptidases, chitinases, cellulases and several others for utilization of a diverse variety of nutrient sources. This ability also serves an important purpose in recycling precious nutrients because these compounds are made available for the utilization by other organisms. The diversity of excreted hydrolytic enzymes by *Streptomyces* is an important contribution to several insects, such as the occurrence of actinomycetes in termite guts (Kaltenpoth, 2009).

Chitin, an insoluble, nitrogen-containing polysaccharide is the second most abundant polysaccharide in nature, occurring in fungal cell walls and arthropod exoskeletons (Chater *et al.*, 2010). Chitin functions as a very useful source of nutrition for actinomycetes including members of the genus *Streptomyces*. It contains both nitrogen and carbon, which is an ideal

nutrient source if hydrolysed entirely (Chater *et al.*, 2010). Streptomycetes are some of very few organisms able to produce several different families of chitinases, the enzyme responsible for digestion of chitin (Chater *et al*., 2010). Chitinases cleave glycosidic bonds to yield oligosaccharides (Chater *et al*., 2010). The *S. coelicolor* genome revealed that it produces 13 chitinases in two families. This would indicate an ability to utilize several different sources of chitin with different chemistry (Chater *et al*., 2010). Many different homologues of chitin binding proteins (CHB) exist in different *Streptomyces* sp., an important first step in its digestion (Chater *et al*., 2010). CHBs do not have any enzymatic activity, and are found only on the substrate (Schrempf, 2001). CHBs bind and stick to chitin, thereby attaching the bacterium to its substrate (Schrempf 2001). When *S. olivaceus* and *Aspergillus proliferans* are grown in the presence of one another, the streptomycete will take advantage of the fungus and grow at its expense. CHB will bind to the chitin on the fungal cell walls and stick the bacterium to the fungus (Chater *et al*., 2010). Streptomycetes can also survive well on the chitin in the guts of insects that consume fungi (Chater *et al.*, 2010). Several actinomycetes are detrimental to other organisms through digestion of chitin in fungal hyphae, production of chitinase inhibitors, both prokaryote and eukaryote (Chater *et al.* 2010).

Production of volatile compounds

Volatile compounds, produced by actinomycetes could provide a chemical cue for insects to locate the bacteria for exploitation, as well as having some defensive properties in ecosystems. It has been shown that some of these compounds are produced during insects' aggregation behaviour and this may well be an adaptation learnt from associations with microorganisms, such as locust gut bacteria being responsible for their aggregation pheromone (Dillon *et al.*, 2002; Leroy *et al.*, 2011). These compounds have also been shown to have antimicrobial activities and this can serve as deter predators, parasites and pathogens. Odorous compounds are abundant in nature and could provide a means by which actinomycetes are able to manipulate the ecosystems they exist in, and thereby become symbionts of insects. The influence of volatiles from bacteria is believed to be a method of inter- and intraspecies communication, carbon release and possibly selective growth promotion or inhibition of organisms sharing its environment (Kai *et al.*, 2009).

Volatile compounds such as geosmin are important in niche specialization in bacteria, because they allow the bacteria to manipulate other organisms at a distance through water and air (Kai *et al.*, 2009). Actinomycetes have been linked to this smell through the production of the volatile compound geosmin (Gerber and Lechevalier, 1965; Gust *et al.*, 2003). Geosmin consists mainly of hydrogen and carbon, while nitrogen is completely absent (Gerber and Lechevalier, 1965). *Streptomyces griseus* is an example of an actinomycete producing large amounts of this compound (Gerber and Lechevalier, 1965).

Volatiles from *S. griseus* were shown to negatively affect sporulation of the fungus *Gleosporium* while having a positive effect on sclerotia formation in the fungi *Rhizoctonia solani* and *Sclerotium cepivorum*. Li *et al.*, (2010) showed that *S. globisporus* produces volatile compounds that could have an effect on sporulation, conidial germination and vegetative growth of another fungus, *Penicillium italicum*. The presence of these volatiles prevented the sporulation of *P. italicum*, thereby preventing blue mould of citrus. Fumigation with these compounds has subsequently been investigated as a possible preventative treatment of postharvest disease (Li *et al.*, 2010).

Other odorous volatiles produced by Streptomycetes are methylisoborneol and 2-methyl-3-hydroxypyrazine (Gerber, 1977). Řezanka and Sigler, (2008) evaluated several strains of *Streptomyces* for production of volatile compounds and found that pyrazines are produced by several species of *Streptomyces*. Methylisoborneol is also produced by various cyanobacteria. Izaguirre *et al.* (1982) found that this compound is produced by *Oscillatoria* spp. and *Anabaena* spp. and is responsible for musty odours and tastes in drinking water supplies.

A study on the seven-spot ladybird beetle (*Coccinella septempunctata*) investigated the role of alkyl-methoxypyrazines in these insects (Abassi *et al.*, 1998). That study showed that these compounds are responsible for the smell of these beetles, and is an important warning odour of would-be predators, as well as serving as an aggregation pheromone (Abassi *et al.*, 1998). Harlequin bugs (*Murgantia histrionica*) secrete a frothy liquid from their prothorax (Zahn *et al.*, 2008). This secretion contains 2-isopropyl-3-methoxy-pyrazine and is believed to serve as a warning odour (Zahn *et al.*, 2008). These volatiles are also present in several actinomycetes as mentioned above. It is believed that this volatile compound in insects is produced from precursors they receive from plant hosts (Zahn *et al.*, 2008).

Pyrazines are ecologically important odourous compounds that have been detected in the genus *Streptomyces*. For example, *Lymantria dispar*, the gypsy moth, has been hypothesized to produce pyrazines involved in defence. Furthermore, it has been suggested that certain co-evolved parasites may use this odour as host-finding kairomone (Aldrich *et al.*, 1997). The compound 2-isopropyl-3-methoxy-pyrazine was also detected in the gum of peachtrees (Derksen *et al.*, 2007). In laboratory studies, the peach tree borer *Synanthedon exitiosa* (Lepidoptera: Sesiidae) responded to this compound (Derksen *et al.*, 2007). In combination with other compounds, it also induced or increased oviposition rates of these moths (Derksen *et al.*, 2007). This demonstrates further ability of actinomycetes to influence other organisms with which they share a niche.

Antibiotic Production

Actinomycetes produce great numbers of different antibiotics which can be a great asset to insect symbionts. These range from bactericidal, fungicidal and nematicidal (Clardy *et al*., 2006; Baltz, 2007; Baltz, 2008). Of the antibiotics produced by actinomycetes, 70-80% are produced by *Streptomyces* spp. with other producers being species of *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora* and *Actinoplanes* (Challis and Hopwood, 2003). Watve *et al*. (2001) used mathematical modelling to estimate that the number of antibiotics in the genus *Streptomyces* might be as high as 150,000.

Streptomyces and many other actinomycete genera are thought to have evolved in soil. This provides an environment with a number of abiotic stressors. In this environment there are also many potential competitors for already scarce nutrients. Given that actinomycetes are non-motile, they need a competitive strategy that allows them to defend their resources locally (Challis & Hopwood, 2003). It is thus thought that *Streptomyces* antibiotics are produced upon nutrient exhaustion to enable them to defend their scarce resources (Challis & Hopwood, 2003).

Objectives and aims

Insects benefit from associations with microorganisms in many different ways. Among these, protective symbiosis is important for the survival of many different insect species. Protective interactions can have many forms that include physical and chemical defence. In associations involving actinomycete bacteria, antibiotics are valuable to symbionts. This is clear in several insect-microbe associations, including attine ants (Currie *et al.*, 1999a; Cafaro and Currie,

2005), European beewolves (Kaltenpoth *et al.*, 2005; Kaltenpoth *et al.*, 2006) and bark beetles (Scott *et al.*, 2008). These insects all benefit from antibiotics produced by various actinomycetes, through protection of colonies or nests, food source or of individuals.

Actinomycetes are responsible for the production of most antibiotics known. Of these, most are produced by members of the genus *Streptomyces*. This feature makes actinomycetes ideal symbionts to insects for a variety of uses. Actinomycetes have several other traits that make them easily acquirable symbionts. This includes spore formation and the ability to utilize a variety of nutrients, including compounds inaccessible to metabolism of many other organisms (Kaltenpoth, 2009; Seipke *et al.*, 2012).

Actinomycetes as symbionts of other organisms seem to be widespread (Seipke *et al.*, 2012). Except for insects, interactions between actinomycetes and plants have also been recorded (Seipke *et al.*, 2012). The fact that these interactions appear to be so common is encouraging studies in more environments to look for the presence of actinomycetes and to determine whether there are detectable interactions with animals and plants. Further encouragement for such studies is the fact that this may be a source of valuable and novel antibiotics (Crawford and Clardy, 2011).

Given the common occurrence of actinomycete bacteria in insect systems, and the common role as protective symbionts against other invading microbes, we explore two systems in this thesis to look for such associations. In both cases we hypothesise that the presence of antibiotic producing actinomycetes may provide a beneficial environment to ophiostomatoid fungi associated with insects, while negatively affecting other common saprophytes in these environments.

A diverse selection of actinomycetes has been described from South Africa. *Gordonia lacunae*, *Nonomuraea candida*, *Actinomadura rudentiformis*, *Actinomadura*, *napierensis* and *Nocardia gamkensis* have all been described from South Africa (Meyers *et al.*, 2004; Cook *et al.*, 2005; Le Roes and Meyers, 2006; Le Roes and Meyers, 2007; Le Roes and Meyers, 2008). Kaltenpoth *et al.* (2006) reported *Candidatus* Streptomyces philanthi from various *Philanthus* spp. wasps in South Africa. Furthermore, Visser and coworkers (2012) found several actinomycetes from fungus growing termites in South Africa. In experiments on colonies of *Macrotermes natalensis*, *Microtermes* sp. and an *Odontotermes* sp. they found several actinomycetes, belonging to the genera *Streptomyces*, *Micromonospora* and

Actinomadura. (Visser *et al.*, 2012). Many of the isolated actinomycetes had inhibitory activity against, *Pseudoxylaria* sp., a parasite of termite colonies, as well as the termite mutualist fungus *Termitomyces* (Visser *et al.*, 2012)..

In this dissertation we firstly considered invasive bark beetles in South African pine plantations. Bark beetles have intricate relationships with fungi belonging to the orders Ophiostomatales and Microascales, collectively referred to as ophiostomatoid fungi. The most dominant pine-infesting bark beetle in South Africa, *Orthotomicus erosus* (Scolytinae, Coleoptera), has fungal associates belonging to the Ophiostomatales. *Ophiostoma ips* was detected in more than 60% of beetles sampled, along with a few *Leptographium* spp. (Zhou *et al.*, 2001). Non-ophiostomatoid fungi were generally not present (Zhou *et al.*, 2001).

In addition, I also considered the occurrence of ophiostomatoid fungi in infructescences of native *Protea* spp. in South Africa. Proteas are serotonous planst that are native throughout sub-saharan Africa, with most species concentrated in the Cape Floristic Region in the Western Cape province of South Africa (Rourke, 1980). *Protea* infructescences are commonly, and almost exclusively in some cases, colonized by ophiostomatoid fungi (Marais and Wingfield, 1994; Roets *et al.*, 2006).

The aims of this thesis were:

- **To isolate and identify actinomycetes associated with the pine bark beetle *Orthotomicus erosus* in South Africa.**
- **To isolate and identify actinomycetes associated with infructescences from *Protea repens* and *Protea neriifolia* in South Africa.**
- **To test the antifungal activity of the actinomycetes obtained in this study against common saprophytic and ophiostomatoid fungi.**
- **To determine the taxonomic position of the most commonly isolated actinomycetes obtained from this study.**

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Chapter 2

Antibiotic producing actinomycetes associated with the pine bark beetle, *Orthotomicus erosus* Wollaston (Coleoptera: Scolytinae) in South Africa

Abstract

Bacteria are commonly associated with insects and sometimes they provide a protective benefit to their hosts. An example of this phenomenon has recently been described for the southern pine beetle, *Dendroctonus frontalis* and its mutualistic fungal symbiont, which is protected against a competitor, *Ophiostoma minus*. The aim of this study was to investigate the presence of potentially protective actinomycetes associated with *Orthotomicus erosus*, an alien invasive pine bark beetle in South Africa. This bark beetle and its relatives have an association with the ophiostomatoid fungi, which are commonly the only fungi found in the bark beetle galleries. We hypothesised that antibiotic producing actinomycetes could be responsible for the paucity of other fungi in the galleries by producing compounds to which ophiostomatoid fungi are tolerant. Several actinomycetes in the genus *Streptomyces* and one *Gordonia* sp. were identified. Remarkably, several of the isolates were closely related to actinomycetes associated with other pine-infesting insects, including bark beetles and the woodwasp *Sirex noctilio*, in other parts of the world. Most actinomycetes isolates had antifungal properties against the test fungi, including *Ophiostoma ips*, which is the most common fungal symbiont of *O. erosus*. Overall, the results suggest that *O. erosus* is closely associated with actinomycetes and that they could influence the fungal communities with which it is associated.

Introduction

The European bark beetle *Orthotomicus erosus* (Curculionidae: Scolytinae) is an introduced pine-infesting pest in South Africa (Tribe, 1990). It typically infests s stressed or dying trees it introduces blue stain fungi that invade the sap-wood and depreciate the timber value (Tribe, 1990; Hurley *et al*. 2012). The blue-stain fungus, *Ophiostoma ips* is the dominant associate of *O. erosus* in South Africa, but several other ophiostomatoid fungi co-occur with this species in the galleries of the beetles (Zhou *et al.* 2001). Although the fresh bark beetle galleries represent an environment rich in nutrients and other growth substrates, it is remarkable that this niche is seldom overgrown with common mould fungi.

The presence of primarily Ophiostomatoid fungi and the lack of contaminating moulds in the galleries of the beetles has raised the question as to whether there are factors that increase the fitness of fungi commonly associated with the insect over other fungi. A possible factor that could influence this outcome is a symbiotic relationship between the insects, fungi and antibiotic producing bacteria. The ophiostomatoid fungi presumably have resistance to this substance, but that negatively affect the fitness of other fungi in this nutrient-rich environment. This situation exists in many other symbiotic communities where actinomycetes have been found to produce metabolites that are exploited to provide protection to various insects and other microorganisms (Currie *et al.*, 1999; Cafaro and Currie, 2005; Scott *et al.*, 2008; Kaltenpoth, 2009). It would thus be a case of the fungus exploiting conditions in this niche to its own benefit.

Examples of actinomycetes involved in multi-species interactions include Attine ants (Attini: Formicidae) that have co-evolved with actinomycetes from the genus *Pseudonocardia* to protect their food source against a parasite (Currie *et al.* 1999). The ants cultivate a basidiomycete fungus which is used for nutrition (Currie *et al.* 1999), but the fungal garden can be parasitized by another fungus (*Escovopsis* sp.), threatening the survival of the entire colony. Metabolites produced by the actinomycetes residing on the ants' integuments protect the crop by inhibiting the growth of *Escovopsis* (Currie *et al.* 1999; Cafaro & Currie, 2005). Actinomycete-insect symbioses also occur with the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytinae), in its native environment in the USA (Scott *et al.* 2008). Survival of larvae in the galleries of these beetles is negatively impacted by *Ophiostoma minus*, a fungal symbiont of mites that competes with the fungal mutualist, an *Entomocorticium* species, of the beetle. *Streptomyces* symbionts in the mycangium of *D.*

frontalis produce antibiotics that inhibit the growth of *O. minus* whilst the mutualistic fungus is tolerant to the antibiotics (Scott *et al.* 2008).

Symbiotic interactions involving actinomycete bacteria are common. Actinomycetes belong to the bacterial order Actinomycetales, members of which all share some form of branching cell structure, referred to as mycelia. They are generally considered to be saprophytes, most commonly occurring in soil (Kampfer, 2006). Actinomycetes are the most important producers of antibiotics known (Lechevalier & Lechevalier, 1967) and it is estimated that more than 100 000 antibiotic compounds may be produced by members of the genus *Streptomyces* (Watve *et al.* 2001). The formation of heat and desiccation resistant spores is a common feature (Lechevalier & Lechevalier, 1967) and the hydrophobicity of these spores can facilitate their transport (Ruddick & Williams, 1972). All these features could be important for their association with arthropods such as insects and mites (Kaltenpoth, 2009)

The aim of this study was to isolate and identify possible actinomycete symbionts from the invasive *O. erosus* in South Africa, and to determine whether they produce antibiotics that might be of importance in this niche. We hypothesized that actinomycete symbionts of *O. erosus* produce antifungal compounds, similar to cycloheximide, which is known to have broad antifungal effects except for members of the Ophiostomatales (Whiffen, 1950; Harrington, 1981) and which negatively affect the fitness of potentially competing saprophytic fungi from the galleries.

Materials and Methods

Bacterial isolation

Orthotomicus erosus galleries were collected from dead *Pinus patula* trees in the Lothair plantation, Mpumalanga Province, South Africa. Forty beetles were removed from these galleries and crushed in sterilized 10% phosphate buffered saline solution (PBS). Three tenfold serial dilutions were prepared for each sample using 10% PBS.

An aliquot of 100 µl of each dilution was inoculated onto chitin agar in duplicate (Hsu & Lockwood, 1975), supplemented with antibiotics (Cycloheximide 5 mg/L and Nystatin 10 000 units/L) (Cafaro & Currie, 2005). These plates were incubated for approximately 30 days at 28°C while being monitored daily. Isolates presumed to be actinomycetes were selected and inoculated onto yeast malt extract glucose agar (YMEA) (1% Biolab malt

extract (Biolab Diagnostics, Midrand, South Africa), 0.4% Oxoid yeast extract (Oxoid, Hampshire, England), 0.4% D-glucose (Merck Chemicals, Wadeville, South Africa), 0.12% Biolab Bacteriological Agar (Biolab Diagnostics, Midrand, South Africa) (Cafaro and Currie, 2005) and incubated at 28°C until sufficient growth had occurred.

DNA sequencing

Fifteen isolates were collected and DNA was extracted using a Quick-gDNA™ MiniPrep kit (Zymo Research). The 16S rRNA gene was amplified using an Applied Biosystems Veriti™ Thermal Cycler. The reaction mixture consisted of 2.5 mM dNTPs, 0.1 µM of each primer pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pH (5'-AAG GAG GTG ATC CAG CCG CA-3'), 0.1 u/µl Super-Therm Taq polymerase (Southern Cross Biotechnology, Cape Town, South Africa), 10X PCR buffer, 2.5 mM $MgCl_2$ and nuclease-free water to a total volume of 25 µl. The PCR protocol included 94°C for 10 minutes, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute, ending with a single cycle of 72°C for 5 minutes. PCR products were verified using a 1% agarose gel electrophoresis. The PCR products were cleaned by adding 1 µl of *E. coli* Exonuclease 1, and 4 µl of Alkaline Phosphatase, to 20 µl of the PCR product. The reaction was incubated at 37°C for 15 minutes, followed by incubation at 85°C for 15 minutes.

Sequencing of PCR productss were performed using 0.5 µl ABI BigDye Terminator v3.1 (Applied Biosystems), 2.5 µl sequencing buffer, 4 µl template DNA, and 2.5 µM of any one of the three primers pA, pH and *pD. Nuclease-free water (4.7 µl) was added to obtain a final reaction volume of 12 µl. The following cycles were run: 5 seconds at 96°C, 25 cycles of 10 seconds at 96°C , 5 seconds at 58°C, and 4 minutes and 15 seconds at 60°C. Precipitation was done by adding 2 µl 3 M sodium acetate and 16 µl 100% ethanol to the sequencing reaction, followed by centrifugation at 14 000 rpm for 30 minutes. The supernatant was removed and the pellet washed by adding 150 µl of 70% ethanol. The sequencing product was again centrifuged at 14 000 rpm for 5 minutes. The sequencing products were analysed on an ABI 3130 sequence analyser (Applied Biosystems).

Contigs were constructed from forward and reverse sequences obtained with different primers for each isolate using the CLC Main Workbench version 6.0 software package (CLC Bio, Aarhus, Denmark). A BLASTN (Altschul *et al.* 1990) search was performed to identify the closest matching sequences in GenBank (Benson *et al.* 2005). A search was also

performed against the Ribosomal Database Project (Maidak *et al.* 2001) using the Seqmatch platform. This search compared all sequences obtained in this study to those of all bacterial type strains. Similar sequences were downloaded for phylogenetic analyses.

Phylogenetic analyses

To determine the relationship between sequences obtained in the present study and the published reference sequences, phylogenetic trees were constructed employing neighbour joining analyses. Nucleotide sequences were aligned using the online version of MAFFT version 6 (Katoh *et al.* 2005). Phylogenetic tree construction was performed using the Neighbour-Joining approach in PAUP with 1000 bootstrap replicates (Swofford, 2002). Trees were visualized using Mega 5.05. (Tamura *et al.* 2011).

Dual-plate bioassay challenges

A preliminary assay was performed for all 15 isolates to serve as a selection step for further antifungal assays. Four different isolates were inoculated onto the four quadrants of an YMEA plate and these were incubated for two weeks. These test plates were then inoculated with a *Trichoderma* sp. by placing a plug, 15 mm in diameter, at the centre of the pre-inoculated plate. Any cultures showing antifungal activity were subjected to further *in vitro* assays.

Bioassay challenges were done for the selected isolates following the approach of Cafaro and Currie (2005). *Streptomyces* isolates were inoculated (10 mm in diameter) onto a 90 mm petri dish containing YMEA (Cafaro and Currie, 2005). These plates were incubated for 21 days. Three fungal species were chosen for use in bioassays, namely a beetle associate fungus (*O. ips*), a common saprophyte (*Trichoderma* sp.) and a pine endophyte (*Diplodia pinea*). *Diplodia pinea* and *Trichoderma* spp. are regularly isolated from pine wood and are potential competitors of *O. ips.* Isolates were obtained from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. A single 15 mm fungal plug was inoculated at the edge of the 21 day old plates and incubated at 25 °C until sufficient growth on control plates was observed. Two repeats were performed for each pairing. Plates were examined and the average zone of inhibition measured for all the bioassay challenges.

Following the above mentioned challenges, the beetle symbiont fungus *O. ips*, and one of the bacteria (isolate BB9) were simultaneously inoculated on YMEA plates. Isolate BB9, used as a representative of a recurring phylogenetic grouping, was inoculated at the centre of a 90mm Petri dishes and a single 15mm *O. ips* plug was inoculated at the edge of the same plate. This plate was incubated at 25°C until sufficient growth had been observed and the result was recorded. This trial was repeated twice.

Results

Isolates and DNA sequence based identifications

Fifteen actinomycete isolates were obtained from forty *O. erosus* individuals collected in this study (Table 1).

Partial 16S rRNA sequences (700 – 1400 bp) were obtained for all 15 isolates.. Isolates were all identified based on the best matches for the 16S rRNA gene sequences in GenBank and the RDP database. Based on these data, all but one isolate belonged to the genus *Streptomyces* and the remaining isolate represented a species of *Gordonia* (Table 1).

Phylogenetic analysis

Eight of the 15 isolates were closely related to the type strain of *S. ambofaciens*. These isolates grouped in a single clade together with isolates from other pine-infesting insects and had 66 % bootstrap support (Fig. 1). This clade formed part of a larger clade including other pine-infesting insect-associated isolates with 99% support.

Six isolates grouped with type strains of *Streptomyces* spp. other than *S. ambofaciens*. One of these isolates grouped with the type strains of *S. griseochromogenes* and *S. resistomycificus* (95% bootstrap support), although it was clearly separated from these species (Fig 1). Two isolates grouped with *S. atratus* with 70% support, while two more clustered close to *S. sanglieri* and *S. atratus*, although the entire group received limited support. There was one isolate that grouped with *S. alni* with 70% support, while another occurred on the same branch as *Gordonia sinesedis* with 100% support.

Dual-Plate Bioassay Challenges

In preliminary antifungal assays, 11 of the 15 cultures were found to have moderate to strong inhibitory effects to the *Trichoderma* sp. These 11 isolates were used in the subsequent *in*

vitro antifungal assays (Table 2). All actinomycete strains inhibited the three fungal species *Diplodia pinea*, *Trichoderma* sp. and *O. ips*, but to varying degrees. Of the three fungi, *O. ips* was most strongly inhibited (Fig. 2a).

Isolates, phylogenetically related to each other, had similar levels of activity against the test fungi (Table 2). The isolates most similar to *S. ambofaciens* (Bex, BB9, BB10.5, BB12, BB44, BB47 BB155.3, BB155.4) all displayed moderate to strong (6 - 10 mm) levels of inhibition against both the *Trichoderma* sp. and *D. pinea*. These isolates had even higher inhibition when tested against *O. ips*. Most other isolates with antifungal activity had moderate to strong inhibitory activity against *Trichoderma* sp. and *D. pinea*, with a higher or very strong activity against *O. ips*. Isolate B1, most similar to *S. phaeoluteichromatogenes* had very strong inhibitory activity against all test fungi.

When isolate BB9, representing the group of isolates that were phylogenetically related and most similar to *S. ambofaciens* and *O. ips* were simultaneously inoculated on fresh growth medium, fungal growth still occurred (Fig. 2a). Furthermore, living fungal material could still be isolated from the edges of the *O. ips* culture despite inhibition, showing that it had not been killed by the actinomycete.

Discussion

In this study, 15 actinomycete isolates were collected from adult *O. erosus* beetles that infest *Pinus* spp. in South Africa. These bacteria were identified based on colony morphology and comparisons of the 16S rRNA sequence data. The majority of these isolates represented *Streptomyces* spp. Although relatively few isolates of actinomycetes were recovered in this preliminary study, these bacteria appear to be associated with *O. erosus*. This is the first time that members of the actinomycetes have been reported from this, or any other tree-infesting bark beetle in South Africa.

Based on the 16S rRNA phylogeny, three groups of bacteria were consistently isolated. Comparison of the strains isolated from *O. erosus* revealed that eight isolates grouped within one of the three clades of species that were identified by Hulcr *et al.* (2011). This clade also included a strain isolated from *D. frontalis* by Scott *et al.* (2008) and cellulose degrading *Streptomyces* spp. associated with a pine infesting siricid wasp, *Sirex noctilio* (Adams *et al.* 2011; Hulcr *et al.* 2011). The data thus suggest a consistent association between insects associated with pine trees and the *Streptomyces* isolates forming part of this clade.

The low frequency at which the actinomycetes were isolated corresponded with the findings of Hulcr *et al.* (2011) who found *Streptomyces* associates of North American bark beetles at low frequency. Such low frequencies preclude definite conclusions regarding specific interactions between beetles and *Actinobacteria*. Such low frequency of isolation would suggest that the association is not essential for the beetles or fungi involved. However, it was sufficiently consistent to suggest that it is also most likely not completely random. Wider sampling, throughout the life-cycle of the beetle, and using more sensitive techniques (e.g. next-generation sequencing) will be needed to conclude on the true frequency of interaction between these organisms. The association of actinomycetes with *O. erosus* may indicate that they could have some effect on the microbial consortium present in the beetle galleries. The alternative would be that *O. erosus* may frequent environments where these antibiotic producing actinomycetes grow, but that they have little impact on the biology of the insect. The latter seems more likely in this instance, as the infrequent occurrence of these associates indicate that they are unlikely to play an important part in beetle fitness.

The bioassays to test the potential effect of the *Streptomyces* spp. on fungi in *O. erosus* galleries showed that several of these bacteria have antifungal properties. The selection of test fungi used for the assay included a common saprophyte (*Trichoderma* sp.), an endophyte and opportunistic pathogen of *Pinus* spp. (*D. pinea*), and the fungal symbiont of *O. erosus*. The levels of inhibition varied amongst test strains ranging from weak to very strong. The most frequently isolated strains were able to inhibit all test fungi, including *O. ips*, the most common fungal symbiont to *O. erosus*. This result suggests that *Streptomyces* isolates collected in this study are unlikely to have co-evolved as symbionts of *O. erosus.*

Although it did not appear that the isolated *Streptomyces* spp. directly benefited *O. ips*, it is still possible that they play some role in the ecology of these fungi and the associated beetles. For example, the symbionts of the beetles such as *O. ips* are inoculated into the newly formed galleries at the time of infestation, either directly from the beetle's bodies or with the help of mites (Moser *et al.* 1989; Klepzig *et al.* 2001). These fungi become established, and dominate the niche and it is likely that contaminating saprophytes enter the niche only at a later stage. If the antibiotic producing *Actinobacteria* are introduced at the same time as the fungal associates, there would be sufficient opportunity for the fungus to establish itself and penetrate the wood before widespread colonization of the bacteria. However, once the bacteria are established and producing antibiotics in the galleries, these would then be

protected against possible harmful saprophytes that are expected to enter later. Simultaneous inoculation of *Streptomyces* sp. and the fungal symbiont on medium showed that *O. ips* can colonize large amounts of the resource and grow up to the edge of the bacterial colony before inhibition is exhibited. The results might suggest that *O. ips* may survive, while other saprophytes subsequently introduced may be inhibited completely.

This study represents the first investigation of actinomycetes associated with insects in South Africa. *Streptomyces* spp. are occasional symbionts of *O. erosus* in South Africa, with several phylogenetically similar symbionts encountered. These are very similar to symbionts of other bark beetles and a pine infesting woodwasp in North America (Scott *et al.*, 2008; Hulcr *et al.*, 2011). The source of these symbionts is unknown, but given that they are apparently common to those associated with other pine-infesting bark beetles, it is likely that they entered South Africa with *O. erosus*, which is native to Mediterranean Europe. The most common isolates were found to contain widespread antifungal activity. These findings are preliminary and based on limited sampling, but we were successful in isolating antifungal actinomycetes from *O. erosus*. Future work should investigate the presence of similar *Streptomyces* spp. on other insects associated with *Pinus* sp. across different geographical ranges. *O. erosus* and its galleries should be surveyed using culture-independent methods.

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Tables and figures

Table 1: Actinomycetes isolates obtained from *Orthotomicus erosus* from *Pinus patula* in South Africa, with the results of BLAST searches in RDP Seqmatch and NCBI

Isolate	RDP Match		NCBI Match		Similarity
	Taxon label	**Similarity**	**Taxon label**	**Source of isolate**	**Similarity**
2BB2	*Streptomyces drozdowiczii*	98%	*S. drozdowiczii*	*Lolium perenne* rhizosphere	99%
B1	*S. phaeoluteichromatogenes*	100%	*Streptomyces* sp. SXY37	Soil	100%
B1.2	*S. flavovariabilis*	95%	Uncultured soil bacterium clone D1B5	Soil	99%
B155.4	*S. coelescens*	94%	*Streptomyces* sp. SA3_actG	*Sirex noctilio*	99%
B1Ex	*S. ambofaciens*	97%	*Streptomyces* sp. SA3_actG	*Sirex noctilio*	99%
BB1.1	*S. alni*	96%	*Streptomyces* sp. S5.TSA.009	Brazilian atlantic forest soil	100%
BB12	*S. ambofaciens*	97%	*Streptomyces* sp. SA3_actG	*Sirex noctilio*	99%
BB155.3	*S. ambofaciens*	94%	*Streptomyces* sp. SA3_actG	*Sirex noctilio*	99%
BB17	*S. sanglieri*	100%	Actinobacterium ZXY009	Bamboo forest soil	99%
BB2.1.1.	*Gordonia sinesedis*	95%	*Gordonia* sp. PDA4	Soil	98%
BB20.5	*S.luridiscabiei*	100%	*Streptomyces* sp. LYG-1	Soil	100%
BB21	*S. drozdowiczii*	98%	*Streptomyces* sp. G4_9	Soil	100%
BB44	*S. ambofaciens*	98%	*Streptomyces* sp. 23bC	*Sirex noctilio*	99%
BB70	*S. sanglieri*	*100%*	*Streptomyces* sp. S9(2010)	*Acromyrmex octospinosus*	99%
BB9	*S. fulvissimus*	97%	*Streptomyces* sp. 3bA	*Sirex noctilio*	99%

Table 2: Results of bioassays where Actinomycetes isolates were tested for their ability to inhibit growth of Trichoderma sp. (saprophyte), D. pinea (endophyte) and O. ips (fungal symbiont)

	Fungal isolates		
Actinomycete Isolates	***Trichoderma* sp.**	***Diplodia pinea***	***Ophiostoma ips***
B1	+++	+++	+++
BB12	++	++	+++
BB155.3	++	++	+++
BB155.4	++	++	+++
BB44	++	++	+++
BB47	++	++	+++
BB9	++	++	+++
BEx	++	++	+++
BB70	+	++	+++
B1.2	++	+	++
BB21	+	+	++

(Inhibition zones of 15 mm are indicated by +++, 10 mm by ++, and 5 mm by +)

Figure 1: NJ tree of isolated all isolates from this study (black) with closest matching type strains (green), isolates from other pine infesting insects from literature (blue).

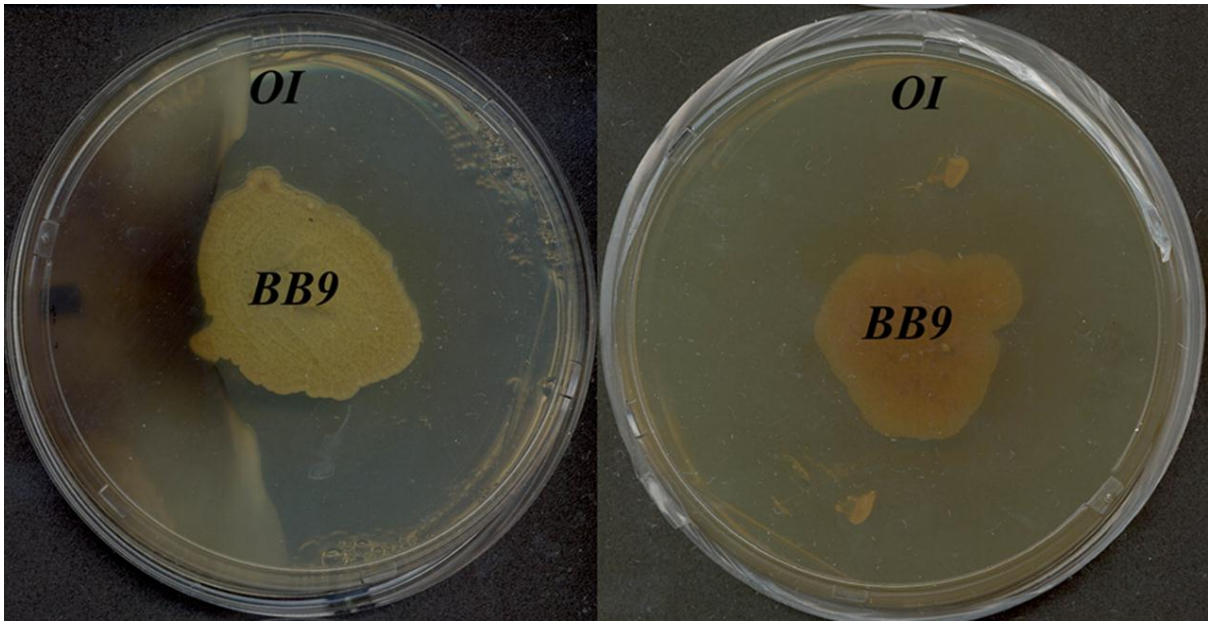

Figure 2: Bioassay challenge with isolate BB9 and *O. ips* simultaneously inoculated and bacteria inoculated two weeks before fungi on yeast malt extract agar. This figure illustrates how a fungal isolate can grow uninhibited with a bacterial culture when inoculated at the same time.

Chapter 3

Actinomycetes inhabiting the infructescensces of *Protea* spp. in South Africa and their antifungal activity

Abstract

The known fungal community in *Protea* infructescences is mainly restricted to specialized saprophytic species that reside in the genera *Ophiostoma*, *Sporothrix* (Ophiostomatales) and *Knoxdaviesa* (Microascales). Other than these ophiostomatoid fungi, common saprophytic fungi are seldom present, which is strange given the abundance of mainly dead plant tissue in this moist protected environment. We hypothesised that the absence of common saprophytic fungi in *Protea* infructescences could be due to the presence of microbes producing anti-fungal compounds. Using a culture based survey, employing selective media and *in vitro* antifungal assays, we isolated antibiotic producing actinomycetes from infructescences of *Protea repens* and *P. neriifolia* from two geographically separated areas. Isolates were grouped into five different morphological groups. These groups were supported in a 16S rRNA gene tree and were identified as *Streptomyces* spp. Two of the groups had strong antifungal activity *in vitro.* The ophiostomatoid fungi used in the antifungal assays had higher levels of tolerance to compounds produced by *Streptomyces* sp. group 3 and *Streptomyces* sp. group 4, compared to the inhibitory effect of these bacteria on other saprophytes. *Streptomyces* sp. group 1 had inhibitory activity against all the test fungi. The identified actinomycetes appear to be common in the *Protea* spp. used in this study. The observed antifungal activity of the isolated actinomycetes could contribute to protection of the plant material against common saprophytic fungi and at the same time benefit the ophiostomatoid fungi by reducing competition. The results of this study provide some explanation for the exclusive presence of ophiostomatoid fungi in *Protea* infructescences.

Introduction

Species of the genus *Protea* (Proteaceae) are shrubs and small trees distributed throughout sub-Saharan Africa (Rourke, 1980). A total of 114 species are known of which the greatest diversity occurs in the Cape Floristic Region (Rourke, 1980). The flowers of *Protea* spp. are arranged into inflorescences (Rourke, 1980) and the mature seeds are stored for long periods of time in these structures before they are released when environmental conditions are suitable for seed germination (Bond, 1985). The infructescences of many species remain closed until the time of dispersal and consist of abundant dead plant tissue in a moist, warm and often insect-colonised environment.

A remarkable feature of the closed infructescences of *Protea* spp. is that very few fungi are found colonizing them at any given time, despite the fact that they provide the perfect conditions in which saprotrophic fungi would typically thrive (Marais and Wingfield, 1994; Roets *et al*., 2013). One would expect to find common saprophytes such as species of *Trichoderma, Penicillium* and the many other fungi frequently encountered under similar conditions in nature, but this is not the case. A number of studies have shown that this enclosure is mainly occupied by a specialised group of fungi residing in the Ophiostomatales and Microascales (Marais and Wingfield, 1994; Marais *et al.*, 1998; Wingfield *et al.*, 1999; Roets *et al.*, 2008, 2010, 2013). Collectively, fungi from these two ascomycete orders are referred to as the ophiostomatoid fungi (De Beer *et al*., 2013a) because they produce similar spore-bearing structures specifically adapted for insect dispersal (Malloch and Blackwell, 1993). The ophiostomatoid associates of *Protea* infructescences from Southern Africa mainly reside in the genera *Ophiostoma* and *Knoxdaviesia* (= *Gondwanamyces*) (Marais and Wingfield, 1994; Marais *et al.*, 1998; Wingfield *et al.*, 1999; Roets *et al.*, 2008, 2010, 2013; De Beer et al. 2013b).

The majority of ophiostomatoid fungi are associated with bark beetles (Scolytinae, Coleoptera; Six and Klepzig, 2004; Kirisits 2004) or nitidulid beetles (Coleoptera: Nitidulidae; Heath *et al*. 2009; Kamgan Nkuekam *et al*. 2011). Some species, however, associate with mites present in bark beetle ecosystems, rather than with the beetles themselves (Roets *et al*., 2007). Indeed, recent studies suggest that mites are probably the primary vectors of the ophiostomatoid fungi in *Protea* infructescences (Roets et al. 2007,

2013). Furthermore, the preference of mites for specific ophiostomatoid species plays an important role in the distribution pattern of these fungi inside the infructescenses (Roets *et al*., 2011, 2013). However, this does not explain the exclusion of common saprophytes from these environments.

One hypothesis to explain the absence of saprophytes in the infructescences is that antibiotic producing bacteria may suppress their growth in these environments. This phenomenon is well known from other insect-fungus associations, where a selective antagonism is observed against fungal mutualists, parasites or pathogens (Currie *et al.*, 1999; Cafaro and Currie, 2005; Scott *et al.*, 2008). These interactions might have a direct benefit to the fungus involved, and could for that reason be essential, albeit indirectly, for the survival of the third partner (the insect). The aim of this study was to consider whether antibiotic producing bacteria occur in the infructescences of *Protea* spp. Furthermore, we explored the antifungal activity of these actinomycetes as a possible contributing factor to the preferential occurrence of ophiostomatoid fungi in these structures.

Materials and Methods

Isolation

Protea repens infructescences were collected at Pringle Bay and at the J.S. Marais Botanical gardens near Stellenbosch, and *Protea neriifolia* infructescences from Pringle Bay and Franschhoek, in the Western Cape Province of South Africa. The infructescences were aseptically opened and parts from the individual flowers, outer involucral bracts, inner involucral bracts and involucral receptacle were removed. Tissue samples from the different floral parts were suspended in a solution of 6 % yeast extract and 0.05 % SDS (Sodium Dodecyl Sulphate) and incubated at 40 °C for 20 min (Hayakawa and Nonomura, 1989). A 10^{-6} dilution was performed to remove the antimicrobial detergent effect of SDS (Hayakawa and Nonomura, 1989). These suspensions were then plated onto three types of artificial media: YMA (0.4 % g yeast extract (Oxoid, Basingstoke, Hampshire, England), 1% g malt extract (Biolab Diagnostics, Wadeville, South Africa), 0.4 % D-glucose (Biolab Diagnostics, Wadeville, South Africa) 1.5 % bacteriological agar (Biolab Diagnostics, Wadeville, South Africa) as described by Cafaro and Currie (2005), glycerol-asparagine agar (1 % glycerol (Merck, Wadeville, South Africa), 0.01 % g L-aspargine (BDH, Dubai, UAE), 0.12 % Biolab agar (Biolab Diagnostics, Wadeville, Gauteng)) (Pridham and Lyons, 1961) and starch casein

agar (0.15 % starch (UNILAB, Mandaluyong, The Philippines), 0.4 % casein (Sigma-Aldrich, St. Louis, MO, USA) (Kuster and Williams, 1964).

Plates were incubated at 28 °C for approximately 14 days, whilst being continuously monitored. Colonies provisionally identified as actinomycetes based on morphology were obtained in pure culture by repeated isolation on YMA (Cafaro and Currie, 2005). Isolates were then grouped based on similar colony morphology (colour, size and colour of pigments). Representatives were selected from each group for further study. Care was taken to include isolates from each host species and location sampled.

DNA extraction and sequencing

Genomic DNA was isolated by adding mycelium from each single pure colony into 50 µl Prepman Ultra™ solution (Applied Biosystems). This mixture was vortexed and incubated at 96 °C for 5 min. The mycelial samples were crushed using a micropestle followed by a second incubation at 96 °C for 5 min. Prepman Ultra™ reactions were centrifuged at 9,000 g's for 5 min at and the supernatant transferred to sterile 1.5 ml Eppendorf tubes. These supernatants were used as template DNA.

The 16S ribosomal RNA gene region was selected for PCR and sequencing. A PCR reaction was done using 0.1 µM each of primers pA (5' - AGA GTT TGA TCC TGG CTC AG – 3') and pH (5' - AAG GAG GTG ATC CAG CCG CA – 3') (Edwards *et al.*, 1989), 10 mM dNTPs, 2.5 mM $MgCl_2$, 10X PCR buffer, 0.1 u/µl SuperTherm *taq* polymerase (Southern Cross Biotechnology, Cape Town, South Africa), 1 µl DNA, and made up to a total volume of 25 µl with nuclease-free water. The initial PCR cycles were 10 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 1 min at 58 °C, and 90 s at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were purified by adding *E. coli* Exonuclease I (Fermentas) and Alkaline Phosphatase (Fermentas), and incubation the mixture for 15 min at 37 °C. Enzymes were inactivated by heating the reactions to 85 °C for 15 min.

Amplicons were sequenced using the pA (Edwards *et al.*, 1989) primer to obtain the species specific α-region of the 16S rRNA (Kataoka *et al.*, 1997). Purified PCR products were sequenced using the ABI BigDye system (Applied Biosystems) with the following reaction: 2.5 µM primer pA , 0.5 µl BigDye 3.1 (Applied Biosystems), 2.5 µl sequencing buffer, and 4

µl template DNA, made up to a final volume of 12 µl with nuclease-free water. The following cycles were run: 96 °C for 5 s, followed by 25 cycles of 10 s at 96 °C, 5 s at 58 °C, and for 4 min 15 s at 60 °C. The sequencing reactions were precipitated by adding 2 µl 3 M sodium acetate and 16 µl 100 % ethanol. These reactions were centrifuged at 14000 rpm for 30 min. The supernatant was removed and pellets were washed by adding 150 µl of 70 % ethanol. Reactions were spun at 14000 rpm for 5 min. The wash step was repeated once. Excess ethanol was removed by heating reactions to 90 °C for 3 min. Sequences were analysed on an ABI 3130 sequence analyser (Applied Biosystems)

Phylogenetic analysis

Isolates obtained were initially identified by comparing sequences to all type strains available in the RDP database using the Seqmatch program hosted by the Ribosomal Database Project II (RDP-II) (Maidak *et al.*, 2001). The closest matching sequences of validly described species were downloaded and included in a single dataset, with a strain of *Mycobacterium tuberculosis* as outgroup. Sequence ends were trimmed using Bioedit (Hall, 1999) and the sequences aligned using MAFFT (Katoh *et al*., 2005). The best-fitting nucleotide substitution model was determined using JModeltest version 0.1 (Posada, 2008) and selected using the Akaike Information Criteria (Posada and Buckley, 2004). A maximum-likelihood phylogeny was produced with PhyML version 3.0 (Guindon *et al*., 2009) using the TIM3 nucleotide substitution model. Tree confidence was measured using a bootstrap search with 1000 replicates (Felsenstein, 1985). The tree was visualised using Mega 4 (Tamura *et al.*, 2007).

***In vitro* antifungal assays**

Bacterial isolates used in bioassays were randomly selected, with at least two isolates chosen for common groups and only one isolate for less frequently occurring groups. Antifungal bioassays were performed using the agar diffusion method described by Visser *et al*., (2011). Uniform 10 mm wide inoculations were made of bacterial cultures across YMA plates. Plates were incubated for 14 days at 28° C to allow secondary metabolites to diffuse into sections of clear agar. Agar plugs were removed with a cork borer (16 mm diam.) from clear zones neighbouring the zones where test organisms were growing. The purpose of this technique was to collect diffusible compounds without transferring actively growing culture.

Five fungal cultures isolated from *Protea* infructescences in previous studies were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. These included three ophiostomatoid fungi commonly found in *Protea* infructescences (*Knoxdaviesa capense*, *Ophiostoma splendens* and *Sporothrix variecibatus*), and one isolate each of a *Fusarium* sp. and a *Geosmithia* sp. collected from the outer surface of *Protea* infructescences. Fungal cultures were prepared for the assays by inoculating 10 ml of 2 % malt extract broth (2 % Biolab Malt extract, Biolab diagnostics, Wadeville, South Africa) with mycelial discs (10 mm diam.) of the test fungi and incubating them for 7 days at 25°C. These liquid cultures were then homogenized and evenly spread onto YMEA using a sterile spreader. Three replicates were prepared for every bacterial isolate to be tested.

The nine bacterial cultures were tested in all possible combinations against the five fungal isolates. Each combination was repeated three times and negative controls for each consisted of agar plugs from uninoculated plates. A 16 mm agar plug containing diffused antibiotics without any bacterial growth was retrieved from the actinomycete cultures as described above and placed at the centres of the plates inoculated with test fungi. These were incubated at 25° C until negative control plates were overgrown by the test fungi. The clear zones between fungal growth and antibiotic containing agar plugs were measured. This is referred to as the zone of inhibition and is used as an indication of the potency of antibiotic and the level of susceptibility of the test organism (Cafaro and Currie, 2005; Visser *et al.*, 2011).

Results

Isolations

A total of 86 isolates of putative actinomycetes were obtained. All of these could be classified in one of five morphological groups. Isolates residing in *Streptomyces sp.* group 1 had soft black colonies, produced grey spores and a yellow substrate pigment was observed in surrounding growth medium. Members belonging to *Streptomyces* sp. group 2 produced yellow translucent colonies with grey spores. A red substrate pigment was produced by all isolates in this group. A single isolate resided in *Streptomyces* sp. group 3, which produced a translucent colony, covered with white spores and no substrate pigments could be observed. *Streptomyces* sp. group 4 had white colonies and produced light grey spores while a red

substrate pigment was observed. *Streptomyces* sp. Group 5 had translucent colonies and white spore rings on the outer edge of the colony and produced no observable pigment. From the total number of isolates, 22 representing the different morphological groups were selected (Table 1) for further detailed identification.

Isolates of *Streptomyces* sp. Group 1 were from *P. repens* in Pringle Bay and Stellenbosch, and *P. neriifolia* in Franschhoek. *Streptomyces* sp. Group 2 isolates were from both hosts at all locations sampled. The single isolate representing *Streptomyces* sp. Group 3 was from *P. neriifolia* in Pringle Bay. *Streptomyces* sp. Group 4 isolates were from *P. neriifolia* in Pringle Bay and *P. repens* in Pringle Bay and SB, while members of *Streptomyces* sp. Group 5 were from *P. neriifolia* at both locations.

DNA sequencing and phylogenetic analysis

PCR resulted in amplicons of approximately 1400 bp. The aligned data set consisting of sequences obtained in the present study and those of type strains from RDP database consisted of approximately 650 characters from the species-specific α-region of the 16S rRNA gene sequence (Katoh *et al.*, 1997). Maximum likelihood analysis confirmed that isolates having a similar morphology also grouped together based on 16S rRNA sequences (Fig. 1). Furthermore, all the isolates collected in this tudy grouped with species of *Streptomyces*.

Isolates belonging to *Streptomyces* sp. group 1 formed a well-supported, distinct clade with the type strains of *S. griseofuscus*, *S. murinus*, *S. phaegriseochromogenes*, *S. costaricanus* and an undescribed *Streptomyces* sp. NRRL 30562 from snakevine in Australia (Fig. 1). Our isolates had identical sequences to all type strains and *Streptomyces* sp. NRRL 30562, while there was a 1 bp difference to *S. phaegriseochromogenes.* Isolates in *Streptomyces* sp. group 2 also clustered together, close to, but in a clade distinct from type strains of *S. misionensis*, *S. levis* and *S. phaeoluteichromatogenes* (Fig. 1). All of abovementioned type strains had 2 bp differences to members of *Streptomyces* sp. group 2. The single isolate representing *Streptomyces* sp. group 3 formed a cluster with 100 % bootstrap support together with the type strains of *S. hydrogenans* and *S. albidoflavus* (Fig. 1) and had identical sequences. Members of *Streptomyces* sp. group 4 formed a distinct cluster with 93 % bootstrap support together with an unidentified strain (MG3) from soil in Germany. The partial 16S rRNA

sequence of *Streptomyces* sp. MG3 differed by 6 bp to our isolates while *S. atratus* and *S. sanglieri* differed by 4 and 5 bp, respectively. The two isolates belonging to *Streptomyces* sp. group 5 formed a distinct clade basal to the other *Streptomyces* spp., without significant support. Members of *Streptomyces sp.* group 5 had 7 bp that were different to the type strain of *S. yanglinensis* and 13 different to *S. paucisporeus.*

***In vitro* antifungal assays**

Nine bacterial isolates were included in the assays and these represented the five different groups identified from *Protea* infructescences (Table 2). Isolates residing in *Streptomyces* Group 1 generally had strong inhibitory activities (Fig. 2, Table 2), especially against the *K. capense, Fusarium* and *Geosmithia* isolates. Levels of inhibition against *O. splendens* were similar to those against the saprophytic test fungi, while inhibition against *S. variecibatus*, was lower than against other test organisms.

Isolates belonging to *Streptomyces* Group 2 had low levels of antagonism against ophiostomatoid fungi, with isolate PrNe1I9 showing some inhibition against *O. splendens*, but none against other ophiostomatoid fungi. Isolate PrRe1I17 had no activity against any of the ophiostomatoid test fungi. Both these isolates had inhibitory activity against the non-ophiostomatoid test fungi and stronger activitiy against the non-ophiostomatoid than ophiostomatoid test fungi.

The single isolate residing in *Streptomyces* Group 3 had no observable activity against any of the test fungi. *Streptomyces* sp. Group 4 isolates had low inhibitory activities against the ophiostomatoid fungi. PrRe3I5 exhibited very low levels of inhibition against *O. splendens* and *K. capense,* while PrRe4I4 had no observable antagonistic effects against any of the ophiostomatoid test fungi. Both these isolates inhibited the non-ophiostomatoid test fungi to some degree and these effects were always stronger than those recorded against the ophiostomatoid fungi. The isolate residing in *Streptomyces* sp. Group 5 had no observable effects on the ophiostomatoid fungi and a only slight effect on *Geosmithia* sp.

Discussion

This study represents the first discovery of antibiotic producing actinomycete bacteria in the infructescences of *Protea* spp. Actinomycetes were also found to be common inhabitants in this niche. In all, five different groups of actinomycetes were identified and they were all species of *Streptomyces*. Bioassays showed that some of these bacteria were antagonistic to

saprotrophic fungi found on the outside of *Protea* infructescences, while ophiostomatoid fungi that typically occur inside the infructescences, were more tolerant to actinomycete activity. Results of this study suggest that the presence of actinomycetes that produce antifungal compounds, to which the ophiostomatoid fungi have some levels of tolerance, might contribute to the absence of saprotrophic fungi in the nutrient rich, moist and warm environment found in Protea infructescenses. .

Members of *Streptomyces* group 1 were most closely related with *S. murinus*, *S. costaricanus*, and *S. griseofuscus*. *Streptomyces* sp. NRRL 30562, which had identical partial 16S rRNA sequence to the isolates in this study, was identified as *S. padanus*, a strain that does not meet the criteria for validly described species. Strains identified as *S. padanus*, which was originally isolated from soil, have been detected in soil and mountain laurel (Ericaceae) (Nishimura *et al*., 2002; Xiong *et al.*, 2012). *Streptomyces* sp. AOK-30 was isolated from mountain laurel and seedlings of this plant successfully reinfected to make them more resistant to fungal pathogens (Nishimura *et al.*, 2002). Strains identified as *S. padanus* generally produce the antibiotics actinomycin (Kurosawa *et al.*, 2006) and fungichromin (Shih *et al.*, 2003). *Streptomyces costaricanus* has been isolated from nematode-suppressive soils and found to be effective in biocontrol of nematodes (Esnard *et al.*, 1995). No information is available for the *S. murinus* and *S. phaeogreichromogenes* type strains, and according to the USDA Agricultural Research Service Culture Collection (http://nrrl.ncaur.usda.gov), *S. griseofuscus* had been isolated from soil. We recorded antifungal activity against all test fungi for isolates from this group.

Streptomyces sp. group 2 isolates from this study were most closely related to *S. phaeoluteichromatogenes*, *S. misionensis* and *S. levis*. The origin of *S. phaeoluteichromatogenes* and *S. levis* is not known, but *S. misionensis*, was originally isolated from soil in Argentina. Strains identified as *S. misionensis* have been used as biocontrol agents against *Fusarium* pathogens of *Lilium* spp. (Chung *et al.*, 2011). *S. misionensis* has also been detected on ants (*Allomerus* spp.) and was found to antagonise fungal pathogens of the ant colony (Seipke *et al.*, 2012). Our isolates in this group had antifungal activity against *Geosmithia* and *Fusarium sp.* while the ophiostomatoid fungi appeared to tolerate its presence. Isolates residing in *Streptomyces* sp. Group 3 were most similar to the type strain of *S. albidoflavus.* This species has been isolated from soil, and one

isolate produced dibutyl phthalate, a compound active against many bacteria and fungi (Roy *et al.*, 2006). It has also been reported as an endophyte on mangrove plants, where it produces the antibiotic Antimycin (Yan *et al.*, 2010). There is s no information available on the source of the type strains of *S. hydrogenans*. The single isolate in this group emerging from the present study had no antifungal activity against any of the test fungi.

Phylogenetic analyses of our sequencing results suggest that *Streptomyces* sp. Group 4 isolates group closest to *S. atratus* and *S. sanglieri*, both species of which have been reported from soil (Manfio *et al.*, 2003). *Streptomyces sanglieri* produces the antibiotic Lactonamycin Z that has antibacterial and antitumor activity (Zhang *et al.*, 2008), while *S. atratus* has recently reported in arsenic containing soil (Delavat *et al.*, 2012). *Streptomyces* sp. MG3 was isolated from soil in Germany and had strong antifungal activity due to the production of a chitinase (Hoster *et al.*, 2005). Our isolates belonging to this group had strong antifungal effects against *Fusarium* and *Geosmithia* sp., compared to a relatively lower level of inhibition against the ophiostomatoid test fungi.

Three groups of isolates were found in both *P. repens* and *P. neriifolia* infructescences, while the single member of *Streptomyces* sp. Group 3 and members of *Streptomyces* sp. Group 5 were isolated only from *P. neriifolia.* There was no clear distribution pattern from these results, but it seems clear that actinomycetes are commonly present in the infructescences of *Protea* plants. Closely related type strains and isolates similar to these type strains have also been isolated from plants, with one from a member the family Proteaceae. Isolates residing in *Streptomyces* sp. Group 1 are very similar to those found in *Kennedia nigriscans* in Australia, which produce munumbicin A, B, C and D, active against many pathogenic bacteria and fungi (Castillo *et al.*, 2002). *Grevillea pteridifolia*, a member of the Proteaceae from Australia, produces the range of antibiotics known as the kakadumycins (Castillo *et al.*, 2003). *Monstera* sp. harbours *Streptomyces* endophytes producing the coronamycin antibiotics (Ezra *et al.*, 2004). All these are thought to provide some protection to the host plant (Strobel, 2003).

Most of the species related to the isolates obtained in this study originate predominately from soil, where *Streptomyces* spp. most commonly occur (Kampfer, 2006). Members of the mite family Edbakerellidae commonly occur in soil, and several new species of this family have recently been discovered in *Protea* infructescences (Theron *et al.*, 2012). *Streptomyces* spp.

have spores that are well-adapted to arthropod dispersal (Ruddick and Williams, 1972; Chater, 2006). Furthermore, it has been shown that *S. griseus* spores adhere to the exoskeletons of mites, effectively facilitating its spread (Goodfellow and Williams, 1983) and it is reasonable to hypothesise that these mites play a role in maintaining *Streptomyces* spp. in *Protea* infructescences.

Streptomycetes are able to digest several complex carbohydrates and nitrogenous wastes including a multitude of nutrients inaccessible to many other organisms (Kaltenpoth, 2009). They also utilize chitin from fungal debris or insect exoskeletons, lignocellulose, and several other plant associated polymers (Crawford, 1978; Kampfer, 2006). Due to the potential of *Streptomyces* to grow in very diverse environments and the ease with which they are dispersed by arthropods (Ruddick and Williams, 1972; Goodfellow and Williams, 1983), they could potentially play an important role in the interaction between insects, mites, fungi and *Protea* spp.

There are two potential benefits provided to the plant by *Streptomyces* spp. symbionts. *Protea* infructescences provide an enclosed environment with the ideal requirements for fungal growth. They are, therefore, at some risk from invasion by degrading saprophytes and pathogens. Furthermore, once seeds have spread and come into contact with soil, they will come into contact with more potentially harmful microbes. *Streptomyces* spp. have the ability to provide continued protection as has been shown in maize where the levels of seed pathogenic fungi have been reduced through treatment with a mixture of these strains (Bressan, 2003). Thus, *Streptomyces* spp. most likely provide various advantages to *Protea* infructescences and these would have provided a positive force to maintain the relationship between them.

Future studies should determine the mechanism of inoculation of *Streptomyces* spp. in *Protea* spp., including their ability to be spread and grazed upon by arthropods inhabiting *Protea* infructescences. The possibility of a symbiosis needs to be established, which can be achieved by wider sampling across geographic regions and over time, and should include the effect of more variables such as temperature, rainfall, soil type and associated animal diversity. Functional metagenomics is an emerging field of study that may also be applied to this niche. Knowledge of the fungal and bacterial metabolism, including carbohydrate active

enzymes and secondary metabolites may provide very important answers towards understanding the complex interactions between *Protea* spp. and the diversity of microorganisms and animals and associated with them.

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Tables and figures

Table 1: Isolates of *Streptomyces* obtained during the present study, their respective sources, and groupings based on culture morphology and 16S rRNA gene sequence

Group	Isolate nr.	Host	Location
1	**PrRe2I4**	*P. repens*	Pringle Bay
1	**PrRe3I9**	*P. repens*	J.S. Marais Gardens, Stellenbosch
1	**PrRe2I22**	*P. repens*	Pringle Bay
1	**PrRe3I7**	*P. repens*	J.S. Marais Gardens, Stellenbosch
1	**PrNe0I20**	*P. neriifolia*	Franschhoek
1	**PrRe3I4**	*P. repens*	J.S. Marais Gardens, Stellenbosch
2	**PrNe0I9**	*P. neriifolia*	Franschhoek
2	**PrNe0I10**	*P. neriifolia*	Franschhoek
2	**PrRe2I24**	*P. repens*	Pringle Bay
2	**PrNe1I9**	*P. neriifolia*	Pringle Bay
2	**PrRe1I17**	*P. repens*	J.S. Marais Gardens, Stellenbosch
3	**PrNe2I8**	*P. neriifolia*	Pringle Bay
4	**PrRe3I6**	*P. repens*	J.S. Marais Gardens, Stellenbosch
4	**PrNe2I2**	*P. neriifolia*	Pringle Bay
4	**PrRe3I13**	*P. repens*	J.S. Marais Gardens, Stellenbosch
4	**PrRe4I4**	*P. repens*	Pringle Bay
4	**PrRe1I9**	*P. repens*	J.S. Marais Gardens, Stellenbosch
4	**PrRe3I1**	*P. repens*	J.S. Marais Gardens, Stellenbosch
4	**PrRe4I7**	*P. repens*	Pringle Bay
4	**PrRe3I5**	*P. repens*	J.S. Marais Gardens, Stellenbosch
5	**PrNe2I3**	*P. neriifolia*	Pringle Bay
5	**PrNe0I4**	*P. neriifolia*	Franschhoek

Table 2: Zone of inhibition recorded for bioassay pairings. Values are averages of three replicates in mm.

		BACTERIAL STRAINS								
	Group →	**1**	**1**	**1**	**2**	**3**	**3**	**4**	**4**	**5**
	Species ↓	**Isolates ↓ →**	**PrRe3I4**	**PrRe3I7**	**PrRe2I22**	**PrNe2I8**	**PrRe1I17**	**PrNe1I9**	**PrRe3I5**	**PrRe4I4**	**PrNe2I3**
FUNGAL ISOLATES	***K. capense***	**CMW 1147**	19	10	24	0	2	0	6	1	0
	S. variecibatus	**CMW 23051**	1	6	9	0	0	0	1	1	0
	O. splendens	**CMW 20679**	6	10	13	2	0	8	5	2	0
	***Fusarium* sp.**	**PENDING**	10	12	17	2	7	11	11	9	2
	***Geosmithia* sp.**	**PENDING**	9	12	18	1	7	18	7	24	7

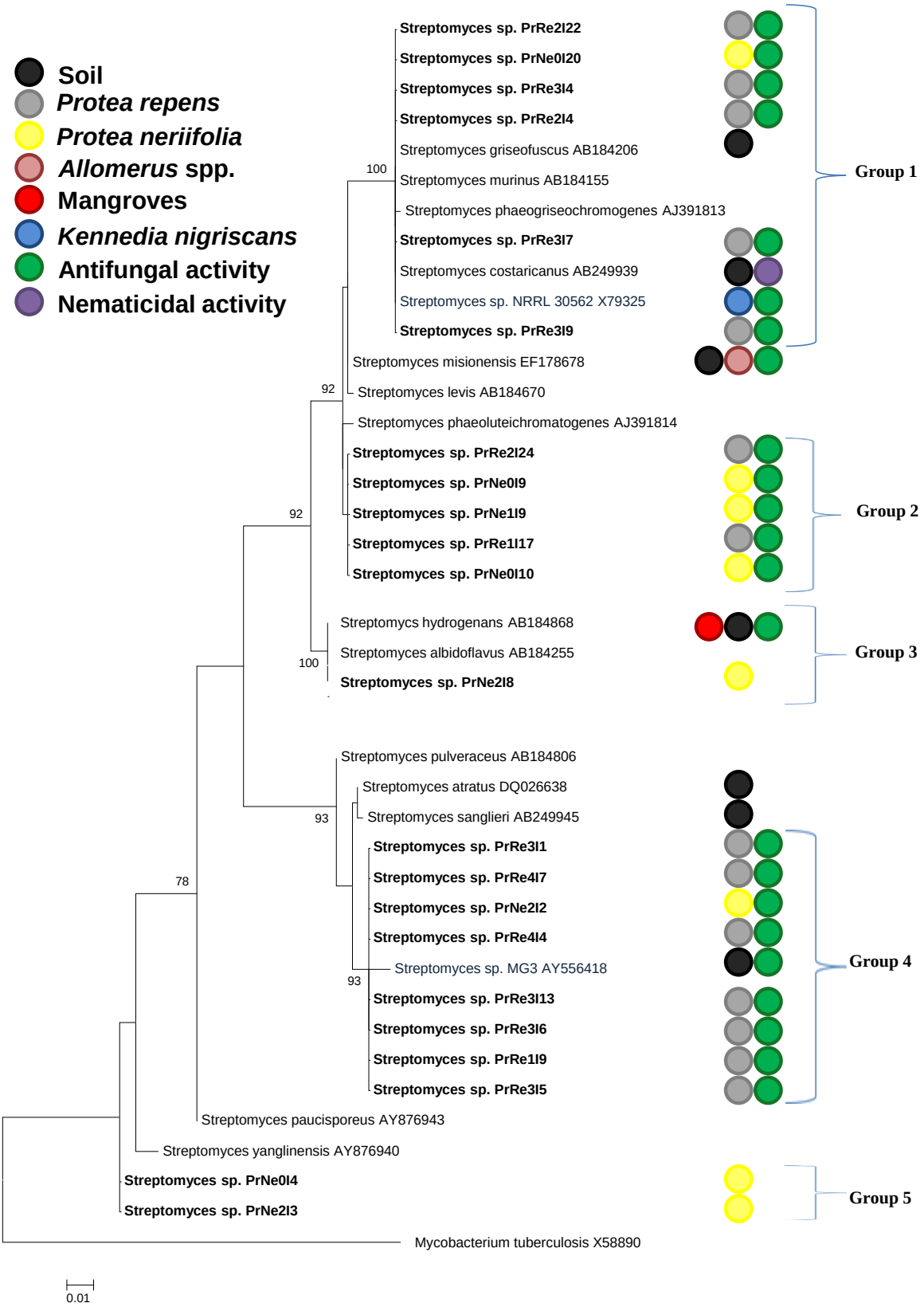

FIG. 1: A ML tree exhibiting the phylogenetic relationships based on 16S rRNA gene sequence of *Streptomyces* species obtained during the present study. Isolate numbers from this study are printed in bold type. Colour codes represent sources of isolates and possible antibiotic activities for strains from this study and related species from literature.

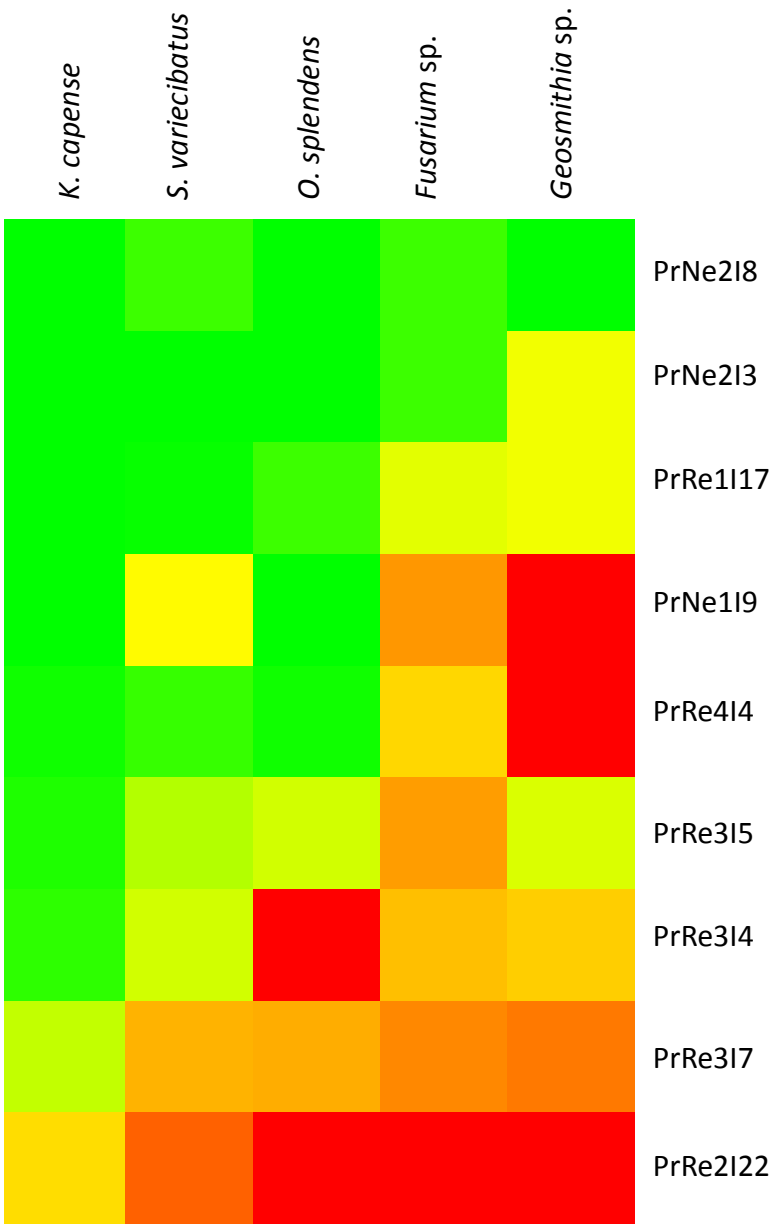

FIG. 2: A graphical illustration of bioassay results. Complete inhibition is illustrated by red and tolerance in green. Vertical entries represent fungi used in bioassays. Horisontal entries refer to bacterial strains.

Chapter 4

Identification of *Streptomyces* spp. associated with Ophiostomatoid fungi in bark beetle galleries and *Protea* infructescenses using multi-locus sequence analysis

Abstract

Members of the actinomycete genus *Streptomyces* are associated with environments where the diversity of fungi is restricted. A number of putative *Streptomyces* isolates were recently isolated from the galleries of the pine bark beetle *Orthotomicus erosus* and the infructescences of *Protea* spp. that are well-known niches for Ophiostomatoid fungi. The aim of this study was to identify *Streptomyces* spp. using multi-locus sequence analysis (MLSA). For this purpose, the DNA sequence for four gene regions were used. These included the 16S rRNA, *rpoB*, *gyrB*, *trpB*. The MLSA revealed that the isolates represented four novel species of *Streptomyces*, three from *Protea* and one from *O. erosus*. A fifth species grouped very close to *S. albidoflavus*.

Introduction

The European bark beetle, *Orthotomicus erosus* (Coleoptera: Scolytinae), is a secondary pest of *Pinus* spp. in South African plantations (Tribe, 1990). As with most Scolytine bark beetles, it has a unique association with fungi belonging to the Ophiostomatales (Ascomycota) (Zhou *et al.*, 2001). A similar pattern exists in the infructescences of *Protea* spp. These infructescences have been found to primarily harbour *Ophiostoma* (Ophiostomatales) and *Knoxdaviesia* (Microascales) species (Marais and Wingfield, 1994; Marais *et al.*, 1998; Wingfield *et al.*, 1999; Roets *et al.*, 2008, 2010, 2013), which are collectively treated as the Ophiostomatoid fungi (De Beer *et al.*, 2013).

The almost exclusive presence of ophiostomatoid fungi, and relative absence of common saprophytic fungi that would normally inhabit such nutrient rich niches, is intriguing. One possible explanation for the lack of common mould fungi in these environments is that actinomycetes that co-exist with arthropods and ophiostomatoid fungi in these niches produce compounds that suppress the growth of competing fungal saprophytes. This would be consistent with recent finding showing the presence of several actinomycetes belonging to the genus *Streptomyces* in *O. erosus* galleries and *Protea* infructescences in Southern Africa (Chapters 2 and 3).

Members of the genus *Streptomyces* are well known and often scrutinized for their ability to produce economically and medically important secondary metabolites. They are responsible for the production of at least 5000 known antibiotics (Anderson and Wellington, 2001) and have been estimated to produce as many as a 100 000 antibiotics (Watve *et al.*, 2001). The taxonomy of *Streptomyces* spp. has been complex and an attempt to resolve this situation was made through the establishment of the International Streptomyces Project (ISP) that has recommended criteria to be used for identification of *Streptomyces* species. (Shirling and Gottlieb, 1966). These criteria were mainly based on phenotypic characteristics and included the use of culture and spore morphology, pigment production, melanin production and utilization of different carbon sources (Shirling and Gottlieb, 1966). Another important contribution by the ISP was the description of 450 type strains, deposited in international culture collections (Anderson and Wellington, 2001).

Genotypic methods such as DNA hybridization are considered essential to delimit bacterial species (Stackebrandt and Goebel, 1994; Tindall *et al.*, 2010). With a relative DNA binding ratio of 70 % or higher to a type strain, the strain under investigation is considered to belong to the same species (Wayne *et al.*, 1987; Tindall *et al.*, 2010). The use of the small ribosomal subunit (16S rRNA) has also become important in the classification of bacterial species where sequence similarity higher than 97% is considered to correspond to a DNA-DNA hybridization value of more than 70% (Stackebrand and Goebel, 1994). Other gene regions have also been investigated and the RNA polymerase β-subunit (*rpoB*) has been useful in identifying and differentiating between *Streptomyces* spp. (Kim *et al.*, 2004; Mun *et al.*, 2007).

Multi-locus sequence analysis (MLSA) uses sequences from several housekeeping genes to delineate species and show genomic coherence (Gevers *et al.*, 2005). This approach was adopted for *Streptomyces* by Guo *et al.*, (2008) who used *atpD* (ATP synthase β-subunit), *gyrB* (DNA gyrase β-subunit), *trpB* (tryptophan synthase ß-subunit), *recA* (DNA recombinase α-subunit) and *rpoB* (RNA polymerase β-subunit) to construct a concatenated tree congruent with the 16S rRNA tree, but with much higher discriminatory power. The

findings emerging from this study was also in agreement with DNA:DNA hybridization values. Labeda (2011) used a similar approach, but focused only *rpoB*, *recA*, *atpD* and *trpB* when determining the taxonomic relationships of type strains representing phytopathogenic and related *Streptomyces* species. They found these four genes were also useful for the identification of unknown strains without the need for DNA:DNA hybridization experiments and suggested that even fewer genes may provide equally useful results (Labeda, 2011).

The objective of this study was to use MLSA to determine the taxonomic position of *Streptomyces* isolates obtained from pine bark beetles and *Protea* infructescences in South Africa. These strains were compared with the type strains of all species considered to be closely related to them.

Materials and methods

Isolates

Several strains isolated from the galleries of *O. erosus* (Chapter 2) and the infructescences of *Protea repens* and *P. neriifolia* (Chapter 3) were identified as belonging to the genus *Streptomyces*. These identifications were based on the comparison of the 16S rRNA sequence data against the Ribosomal Database Project Type Strain database (Maidak *et al.*, 2001). Five isolates from bark beetles and 16 isolates from *Protea* infructescences (Table 1) were included in the present study, together with 25 type strains of known *Streptomyces* spp. (Table 2). Four type strains were acquired from the CBS Actinomycete collection (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), and an additional twenty one strains were obtained from the ARS Culture Collection (Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois, USA).

DNA extraction, amplification and sequencing

Cultures were grown on Yeast-Malt extract agar (YMEA) (0.15 % Bacteriological Agar (Biolab Diagnostics, Midrand, South Africa), 0.1 % Malt extract powder (Biolab Diagnostics, Midrand, South Africa), 0.4 % yeast extract (Oxoid, Basingstoke, Hampshire, England), 0.4 % glucose (Biolab Diagnostics, Midrand, South Africa (Shirling and Gottlieb, 1966) at 28°C for five days. For DNA extraction, single colonies were selected, suspended in 50 µl Prepman Ultra™ solution (Applied Biosystems) and incubated for 10 min at 96°C. These reactions were pelleted and the supernatant containing the extracted DNA was used for amplification.

All 25 µl PCR reactions contained 50 - 200 ng template DNA, 2.5 µl 10X PCR buffer, 20 µM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 2.5 units/µl SuperTherm *taq* polymerase (Southern Cross Biotechnology, Cape Town, South Africa) and 2.5 µl DMSO (Sigma-Aldrich, St Louis, MO, USA). The 16S rRNA gene was amplified using primers pH and pA (Edwards *et al.*, 1989) and an annealing temperature of 58°C. The *trpB* gene was amplified with primers *trp*BPF and *trp*BPR at an annealing temperature at 66°C (Guo *et al.*, 2008). For *rpo*B amplification, primers *rpo*BPF and *rpo*BPR with an annealing temperature of 65°C was used (Guo *et al.*, 2008). Amplification of *gyr*B was achieved with the primers *gyr*BPFA and *gyrB*PRA (Rong and Huang, 2009). Reaction cycles used included an initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 sec, 60 sec at the above mentioned annealing temperatures for the respective primer pairs (Guo *et al.*, 2008; Rong and Huang, 2009), 72°C for 90 sec, and a final extension step at 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel.

A sequencing PCR reaction was performed using the amplification products as template. The 12 µl reactions contained 4 µl template, 2.5 µl sequencing buffer, 0.5 µl BigDye v3.1 (Applied Biosystems) and 200µM of the appropriate primer. The primer *rpo*BF1 was used for *rpo*B, *trp*BF for *trp*B (Guo *et al.*, 2008), *gyrBPFA* for *gyrB* (Rong *et al*., 2009) and pA for the 400 bp α-region of the 16S rRNA gene (Edwards *et al*., 1989). The reaction cycles were 96°C for 5 sec, then 25 cycles of 96°C for 10 sec, 58°C for 5 sec and 60°C for 4 min 15 sec. The amplified products were analysed on an ABI 3500 sequencer (Sequencing Unit, University of Pretoria).

Data analysis
All sequences were aligned using MAFFT version 6 (Katoh *et al.*, 2005) and trimmed using BioEdit (Hall, 1999). Sequence alignments (514 bp of *rpoB*, 486 bp of *gyrB* and 491 bp of *trpB* and the α –region of the 16S rRNA gene) were concatenated with SequenceMatrix (Vaidya *et al.*, 2010). A nucleotide substitution model was selected using JModeltest 2.1.1. (Guindon and Gascuel, 2003; Darriba *et al.*, 2012). Maximum likelihood analyses were performed using PhyML 3.0 (Guindon *et al.*, 2010), where the 16S rRNA data used the Timura-Nei (TrN) nucleotide substitution model and the concatenated alignments used the Transversion (TVM) nucleotide substitution model.

Results

Maximum likelihood analysis of the 400 bp α-region of 16S rRNA gene sequences separated the 22 isolates into five clades (Fig. 1). The isolates from *Protea* spp. formed clades 1, 2, 4 and 5, while the isolates from *O. erosus* formed clade 3. The MLSA tree (Fig. 2) provided better resolution of the different clades compared to the 16S rRNA tree. Type strains were well separated using MLSA and the isolates from *Protea* and *O. erosus* clustered in the same clades as those observed in the 16S rRNA tree.

Strains from Protea infructescences (Clades 1, 2, 4 and 5)
Results of the 16S rRNA analyses revealed isolates in Clade 1 grouped most closely with *S. murinus*, *S. lanatus*, *S. phaeogriseochromogenes* and *S. costaricanus* (Fig. 1). This clade had bootstrap support of 96%. Seven *Protea* isolates formed a distinct cluster (Clade 2) that had an 82% bootstrap support. Although comparisons done using the RDP seqmatch database indicated that Clade 2 isolates were most similar to *S. misionensis* and *S. phaeoluteichromatogenes*, the isolates and type strains did not group together in the phylogenetic analysis (Fig 1). A single isolate (PrNe2I8) did not group with any of the other isolates from that niche (Clade 4), but formed an unsupported lineage on a major branch with *S. hydrogenans*, *S. resistomycificus*, *S. albidoflavus* and *S. griseochromogenes*. Clade 5 had isolates that were most similar to, but clearly distinct from *S. atratus* and *S. sanglieri*.

In the MSLA tree (Fig. 2), isolates resided in four clades consistent with those emerging from the 16S rRNA (Fig. 2). In Clade 1, all isolates formed a well-supported clade (94%) with the type strain of *S. murinus* (Fig. 2), although the *S. murinus* type strain and isolate PrNe0I20 were clearly distinct from the other three isolates. Strains residing in Clade 2 of the 16S rRNA tree, all clustered together with 100% bootstrap support in the MLSA tree (Fig. 2). These isolates grouped distant from all the type strains. Isolates in this clade could be further separated into two sub- groups, although with low support. In the MLSA tree (Fig. 2), the single isolate from Clade 4 was clearly separated from the type strains of *S. hydrogenans*, *S. resistomycificus*, and *S. griseochromogenes*, but formed part of a well-supported linage

(100%) closely related, but distinct from *S. albidoflavus*. The isolates that made up Clade 5 five in the 16S rRNA analysis, grouped separately based on the multi-gene concatenated alignments (Fig. 2). These isolates formed a clade with 100 % bootstrap support and were well separated from *S. atratus*, which was the most closely related type strain.

Strains from bark beetles (Clade 3)
The five isolates from were *O. erosus* grouped together (Clade 3) in the 16S rRNA and MLSA trees (Fig. 1, 2), well separated from the cladess accommodating isolates from *Protea* infructescences. These isolates grouped together with high bootstrap support (100%) distinct from any of the known types strains. When compared to the Ribosomal Database Project Type Strain database (Maidak *et al.*, 2001) these isolates were most similar to the type strain of *S. ambofaciens* but with a similarity of only 93%.

Discussion

Based on preliminary identifications using 16S rRNA and subsequent MLSA analyses using *gyrB*, *rpoB* and *trpB* gene sequences, it was possible to identify a collection of *Streptomyces* spp. from the galleries of *O. erosus* and the infructescences of two *Protea* spp. Four of these species appear to represent novel taxa.

MLSA made it possible to clearly distinguish isolates from related type strains. This was even in cases where there was a high level of similarity between the isolates and the type strains based on their 16S rRNA sequences. Guo *et al*. (2008) first demonstrated the potential of MLSA to discriminate between *Streptomyces* spp. The loci suggested by these authors were located far apart on *Streptomyces* chromosomes, which suggest no genetic linkage. They also found the housekeeping genes *gyr*B, *rpo*B, *rec*A, *trp*B and *atp*D to be highly informative given its high polymorphism and the congruence between the trees from the different genes (Guo *et al.*, 2008). This fulfilled some of the requirements set out by Stackebrandt *et al.* (2002) for the use of multi gene phylogenies. Rong and Huang (2010) reported that a MLSA evolutionary distance of at least 0.007 corresponds to DNA hybridization values of 70% or higher, and suggests that it is a s a robust alternative to DNA: DNA hybridization (also see Rong and Huang, 2012).

The four isolates residing in Clade 1 of the MLSA phylogenetic tree were closely related to *S. murinus*, but they could represent a novel species. Those residing in Clade 2 clearly represent a new species distant from the type strains of described taxa in *Streptomyces*. The five isolates from *O. erosus* residing in Clade 3 were most closely related to *S. albidoflavus*, but also appear to represent a novel taxon. The single isolate residing in Clades 4 was identified as *S. albidoflavus*, but also may belong to novel taxon. Isolates residing in Clade 5 represent a novel taxon, well separated from *S. atratus*.

The fact that so many novel *Streptomyces* taxa emerged from a relatively limited study, clearly demonstrate that these organisms have been poorly sampled in South Africa. It is also evident that unique environments in Southern Africa, such as those in the Fynbos Biome (Linder, 2007), represent untapped potential for the discovery of novel species of antibiotic producing actinomycetes.

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Tables and figures

Table 1: *Streptomyces* isolates from South Africa included in the present study.

Clade	Isolate	Source
1	**PrNe0I20**	*P. neriifolia*
1	**PrRe2I22**	*P. repens*
1	**PrRe2I4**	*P. rotea repens*
1	**PrRe3I4**	*P. repens*
2	**PrNe0I10**	*P. neriifolia*
2	**PrNe0I9**	*P. neriifolia*
2	**PrNe1I9**	*P. neriifolia*
2	**PrRe1I13**	*P. repens*
2	**PrRe2I1**	*P. repens*
2	**PrRe2I24**	*P. repens*
2	**PrRe2I3**	*P. neriifolia*
3	**BBMP10.5**	*O. erosus*
3	**BBMP12**	*O. erosus*
3	**BBMP12**	*O. erosus*
3	**BBMP47**	*O. erosus*
3	**BBMP9**	*O. erosus*
4	**PrNe2I8**	*P. neriifolia*
5	**PrNe2I2**	*P. neriifolia*
5	**PrNe2I2**	*P. neriifolia*
5	**PrRe3I6**	*P. repens*
5	**PrRe4I4**	*P. repens*

Table 2: Type strains of known *Streptomyces* spp. included for reference purposes. Accession numbers of sequences generated in the present study are printed in bold type.

Type Strain	ARS NRRL[1]	CBS[2]	16S rRNA	*atpD*	*rpoB*	*gyrB*
S. alboflavus	B-1273		EF178699	**PENDING**	**PENDING**	**PENDING**
S. ambofaciens	ISP-5053		AB184182	**PENDING**	**PENDING**	**PENDING**
S. collinus	B-5412		AB184123	**PENDING**	**PENDING**	**PENDING**
S. costaricanus	B-16897		AB249939	**PENDING**	**PENDING**	**PENDING**
S. flaveolus		128.20	AB184764	**PENDING**	**PENDING**	**PENDING**
S. flavofungini	B-7866		AB184359	**PENDING**	**PENDING**	**PENDING**
S. fulvissimus	B-12307		AB184787	**PENDING**	**PENDING**	**PENDING**
S. griseochromogenes		714.72	AJ399491	**PENDING**	**PENDING**	**PENDING**
S. griseoflavus	B-5312		AJ781322	**PENDING**	**PENDING**	**PENDING**
S. hydrogenans	B-12091		AB184868	**PENDING**	**PENDING**	**PENDING**
S. intermedius	B-2670		AB184277	**PENDING**	**PENDING**	**PENDING**
S. janthinus		909.68	AB184851	**PENDING**	**PENDING**	**PENDING**
S. lanatus	B-2291		AB184845	**PENDING**	**PENDING**	**PENDING**
S. levis	B-16370		AB184670	**PENDING**	**PENDING**	**PENDING**
S. lienomycini	B-16371		AJ781353	**PENDING**	**PENDING**	**PENDING**
S. massasporeus	ISP-5035		AB184152	**PENDING**	**PENDING**	**PENDING**
S. misionensis		885.69	ED178678	**PENDING**	**PENDING**	**PENDING**
S. murinus	B-2286		AB184155	**PENDING**	**PENDING**	**PENDING**
S. pactum	ISP-5530		AB184398	**PENDING**	**PENDING**	**PENDING**
S. paradoxus	B-3483		AB184628	**PENDING**	**PENDING**	**PENDING**
S. phaegriseochromogenes	B-2834		AJ391813	**PENDING**	**PENDING**	**PENDING**
S. phaeoluteichromatogenes	B-5799		AJ391814	**PENDING**	**PENDING**	**PENDING**
S. resistomycificus		556.68	AJ399472	**PENDING**	**PENDING**	**PENDING**
S. rubrogriseus	B-16375		AB184681	**PENDING**	**PENDING**	**PENDING**
S. variegatus	B-16380		AJ781371	**PENDING**	**PENDING**	**PENDING**

[1] ARS NRRL = United States Department of Agriculture Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA

[2] CBS = Centraalbereau voor Schimmelcultures, Utrecht, The Netherlands

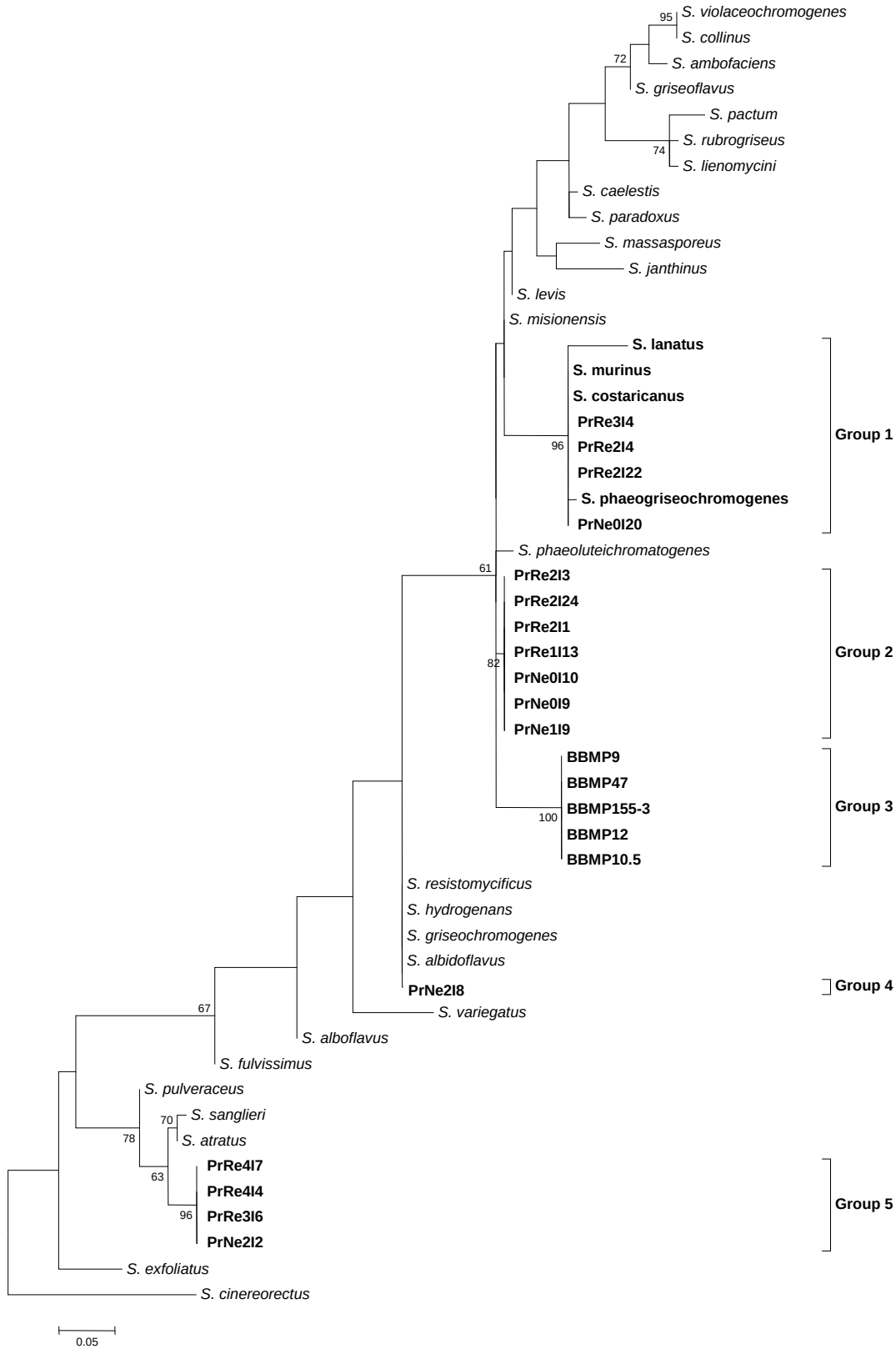

Fig. 1: Maximum likelihood tree of all strains based on 16S rRNA sequences. *Streptomyces cinereorectus* was used as outgroup. Isolate numbers of *Streptomyces* isolates sequenced in the present study are printed in bold type. Values at nodes represent percentages of bootstrap support.

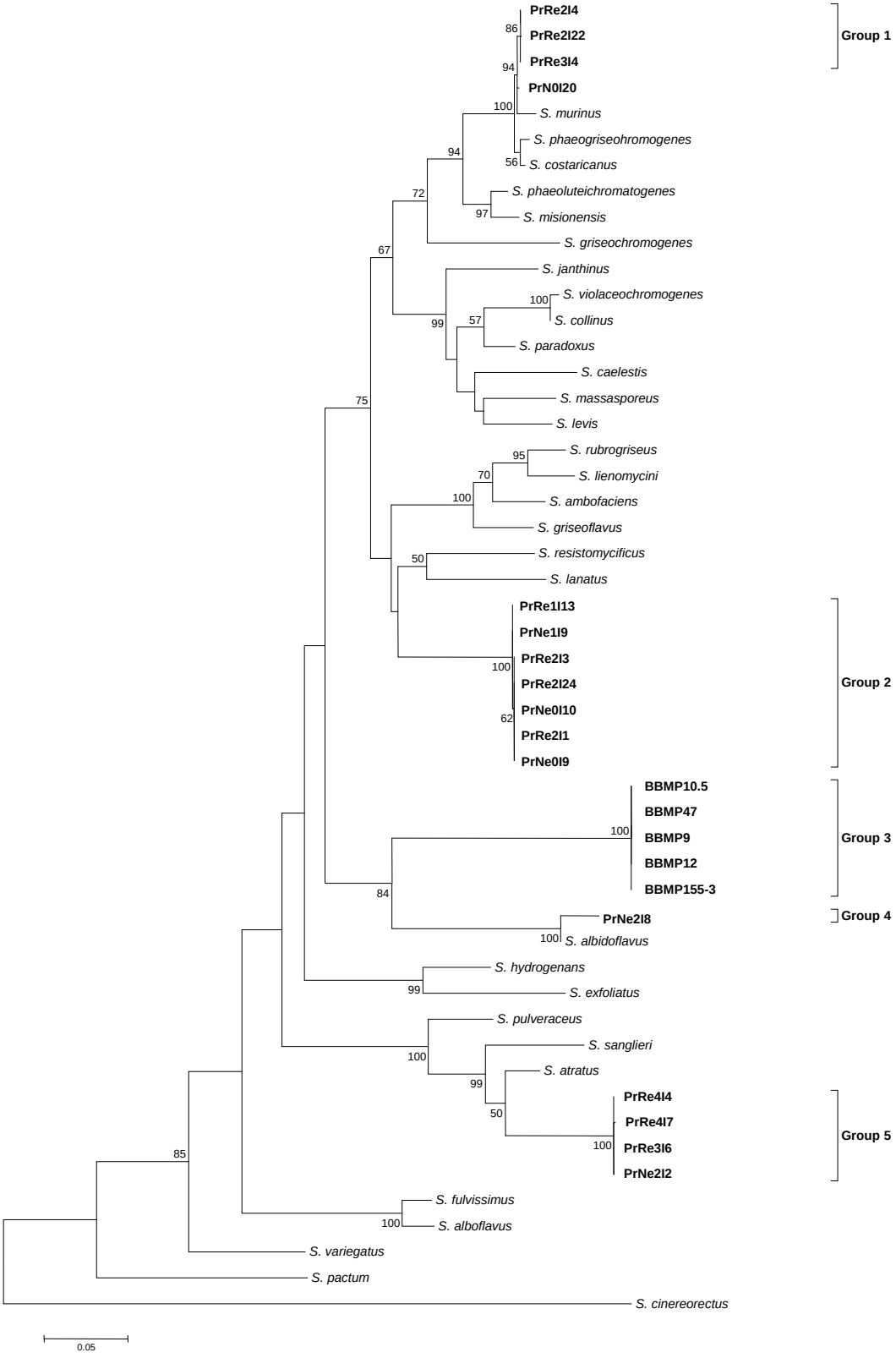

Fig. 2: Maximum likelihood phylogenetic analysis of four-gene alignments. *Streptomyces cinereorectus* was used as outgroup. Isolate numbers of *Streptomyces* isolates sequenced in the present study are printed in bold type. Values at nodes represent percentages of bootstrap support.