Diversity and virulence of the fungus *Beauveria* (Cordycipitaceae) species associated with *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae) in South Africa

By

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Summary

Diversity and virulence of the fungus *Beauveria* (Cordycipitaceae) species associated with *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae) in South Africa

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In South African forestry, the Eucalyptus snout beetle, *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae), has become an economically important invasive pest. The adults and larvae feed on leaves and cause severe damage to *Eucalyptus* plantations. The egg parasitoid, *Anaphes nitens* Girault (Hymenoptera: Mymaridae) was introduced to control *Gonipterus* sp. n. 2 populations, but additional biological control is necessary. Entomopathogenic fungi (EPF) can potentially be used effectively together with *A. nitens*, as they can target different life stages. Entomopathogenic fungi are able to infect host insects by direct contact to the insect cuticle, killing the host and sporulating on the dead insect to infect unsuspecting healthy hosts. This study focussed on the *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) species associated with *Gonipterus* sp. n. 2, as well as their virulence towards adult beetles. The study found that *B. bassiana* cryptic species diversity was present in South Africa and associated with *Gonipterus* sp. n. 2. The newly described species, *B. namnaoensis*, as well as two novel species, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2 formed part of the *B. bassiana* cryptic species complex. *Beauveria* *pseudobassiana* species were also obtained from *Gonipterus* sp. n. 2 cadavers. The virulence of these species was analysed in virulence trials. Inter- and intraspecies variation in virulence were observed during the trials. The most virulent strains with the lowest survival of beetles belonged to *B. namnaoensis* and *Beauveria* sp. nov. 1. The least virulent strains with the highest beetle survivals recorded were that of two *Beauveria* sp. nov. 2 strains and one *B. pseudobassiana* strain. Moderate virulence and survival were attributed to one *B. bassiana*, *B. pseudobassiana* and two remaining *Beauveria* sp. nov. 2 strains. Thus, the most virulent strains of the newly described species, *B. namnaoensis*, as well as *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2 could be considered for *Gonipterus* sp. n. 2 biological control in South African forestry.

Declaration

I, **Marizanne Jones** declare that the dissertation/thesis, which I hereby submit for the degree Masters in Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Ethics statement

The author, whose name appears on the title page of this dissertation/thesis, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that s/he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

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Preface

Entomopathogenic fungi (EPF) are organisms that infect and kill insects. For over two centuries, these fungi have served as global tools to manage pest insect populations. Entomopathogenic fungi offer environmentally safer alternatives to chemical pesticides, including for humans and non-target insect species. Inter- and intraspecific diversity within entomopathogenic fungal genera provide unexplored options for biopesticide development. Some of the most well-known genera of EPF include *Beauveria*, *Isaria*, *Lecanicillium* and *Metarhizium.*

Several *Beauveria* (Cordycipitaceae) species have been used to produce biopesticides for the use in integrated pest management programmes in agriculture, but currently there are no biopesticides registered for the use against *Eucalyptus* insect pests in South Africa. Given the increasing threat of invasive insect pests and diseases, adding biopesticides to the forestry IPM toolbox will have substantial value for the industry. At present, classical biological control is one of the main pest management tactics used and biopesticides can be applied in a cooperative manner to improve pest management in cases where classical biological control is not sufficient to maintain the insect pest population below economic threshold levels, such as the case of the *Gonipterus scutellatus* cryptic species complex.

Gonipterus sp. n. 2, that forms part of the *G. scutellatus* cryptic species complex, is an economically important insect pest of *Eucalyptus* in South Africa. The classical biological control agent, *Anaphes nitens* was introduced into South Africa in 1926. The pest was considered to be under economic control by the 1950s, but pest resurgence has led to the need for developing additional pest management tactics that can be used together with *A. nitens* to suppress the population under economic threshold levels. Therefore, this study aims to uncover the diversity of *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) cryptic species associated with *Gonipterus* sp. n. 2 in South Africa and to evaluate the virulence potential of these fungi against this invasive pest.

Chapter 1 provides a literature review on factors that influence the diversity and virulence of entomopathogenic fungi and their use as biocontrol agents. The chapter provides a detailed overview of the diversity of EPF species, their unique modes of infection and their current use in biological control. In addition, abiotic and biotic factors affecting their efficacy were also discussed.

In Chapter 2 the diversity of *Beauveria bassiana* cryptic species associated with *Gonipterus* sp. n. 2 in South African *Eucalyptus* plantations is identified. Morphological characteristics are used to identify the fungal genera isolated from field collected adult beetles. Species level identifications of *Beauveria* isolates are completed using sequence data from the ITS, *rpb1* and *tef1* gene regions, from which phylogenetic trees are constructed, separately and in combination.

In Chapter 3, the pathogenicity and virulence of native *B. bassiana* cryptic species against *Gonipterus* sp. n. 2 adults were assessed. Nine *Beauveria* isolates were selected for virulence trials. The virulence of these strains is assessed under laboratory conditions at constant temperature and humidity. Percentage mortality and mean lethal time is used as parameters to evaluate virulence.

Chapter 1: Literature review

Factors influencing the diversity and virulence of entomopathogens and their use as biocontrol agents in plantation forestry

Introduction

Globally, more than 200 species of *Eucalyptus* (Myrtaceae) have been introduced from their native ranges in Australia for cultivation since the early 1800s, establishing it as one of the most widely planted genera worldwide (Rejmánek and Richardson, 2011). In South Africa, *Eucalyptus* plantations encompass a substantial 528 449 ha of the country's forested land (Forestry South Africa, 2021). Nevertheless, this valuable resource faces an increasing threat from numerous invasive insect pests, posing a significant challenge to the *Eucalyptus* industry's future success (Hurley et al., 2016). As a result, effective management strategies for these invasive insect pests have become paramount (Hurley et al., 2016; Lanfranco and Dungey, 2001; Loch and Floyd, 2001).

Managing invasive *Eucalyptus* pests involves employing various strategies, with biological control, chemical pesticides and the utilisation of resistant genotypes being the most prevalent. Yet, developing resistant genotypes is a time-consuming process and insect pests can adapt and develop resistance against them, creating ongoing challenges (Henery, 2011; Wingfield et al., 2008). Chemical control, while effective, is less favoured due to its high cost, incompatibility with biological control and adverse environmental impact (Carriger et al., 2006; Chen et al., 2021; Cothran et al., 2013; Gill and Garg, 2014; Meyers and Bull, 2002). Biological control, although promising, is not without its own set of challenges. It often demands considerable time for development and may, in some instances, lose its effectiveness over time. For example, despite the once-successful biological control programmes targeting invasive forestry pests in the genus *Gonipterus* (Coleoptera: Curculionidae), outbreaks continue to be observed (Lanfranco and Dungey, 2001; Schröder et al., 2020).

Given the escalating threat posed by forest insect pests, there is a pressing need for additional management strategies to ensure optimal control of invasive pests that lack natural enemies. In this context, entomopathogenic fungi (EPF) have emerged as promising biopesticides for integration into comprehensive integrated pest management (IPM) programmes aimed at enhancing *Eucalyptus* pest control.

Entomopathogenic fungi have found value within IPM systems due to their ability to infect insects at various life stages and persist in the environment for extended periods (Alfina and Haneda, 2022; Cole and Kendrick, 1981; Milner et al., 2003). They are effectively integrated with other pest management strategies, including chemical and classical biological control. For instance, the entomopathogen *Lecanicillium* *muscarium* (Hypocreales: Cordycipitaceae) is employed alongside the parasitoid *Aphidius colemani*, Viereck (Hymenoptera: Braconidae), to combat the green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) in laboratory settings, with promising prospects for environmental applications (Mohammed and Hatcher, 2017). In Brazil, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) is used successfully to control *Gonipterus platensis* Marelli (Coleoptera: Curculionidae) in laboratory conditions in combination with the parasitoid *Anaphes nitens* Girault (Hymenoptera: Mymaridae) (Jordan et al., 2021; Pérez-Otero et al., 2003). Numerous commercial EPF products have demonstrated success in environmental applications for controlling various pests. Extensive studies have focused on assessing their efficacy and practical implementation (Bamisile et al., 2021a; Khun et al., 2020; Mantzoukas et al., 2022; Mantzoukas et al., 2023; Mascarin et al., 2019).

Several factors constrain the success, persistence and diversity of EPF. One of the most critical limitations being the ability of selected EPF strains to adapt to their environment. Temperature plays a pivotal role in influencing the virulence of EPF and shifts in climatic conditions can alter the dynamics between EPF and their insect hosts (Acheampong et al., 2020; Fargues and Luz, 2000; Inglis et al., 1999; Jaronski, 2010; Uma Devi et al., 2005). Additionally, humidity, soil moisture and specifically UV radiation exerts a detrimental effect on fungal development by causing damage to conidia (Acheampong et al., 2020; Alves et al., 1998; Braga et al., 2001a, b; Coombes et al., 2016; Fernandes et al., 2015; Quesada-Moraga et al., 2023; Thompson et al., 2006). Lastly, for fungal strains to serve as effective biological control agents, they must exhibit adaptability to various climatic conditions (Quesada-Moraga et al., 2023). Given these considerations, researchers must hold the ability to characterise the diversity of EPF within agro-ecosystems, as this information is essential for addressing the above-mentioned challenges when utilising these fungi in pest management.

Entomopathogenic fungi are typically present in the environment infecting various insect species. The initial step in harnessing them for use as biological control agents against invasive insect pests involves characterising the native diversity of EPF within the target agro-ecosystem (Chen et al., 2021). Subsequently, researchers can assess the virulence of these species against economically significant insect species, identifying potential wild strains for targeting specific pests. Lastly, investigations into the factors influencing virulence can shed light on the optimal conditions for the application of biopesticides.

It is important to note that knowledge regarding the use of EPF in many forestry pest management systems remains limited. Alfina and Haneda (2022) conducted a systematic literature review on the implementation of EPF in forestry systems globally. Among the 51 articles on EPF implementation for controlling forestry pests published in the past two decades, the majority originate from the USA (Alfina and Haneda, 2022). China follows with the second-highest number of publications (eight), while Turkey has four. Additionally, Brazil, Ireland and New Zealand each have three publications, while Canada, Mexico and South Korea contribute two articles each. Finally, several countries, including Australia, Chile, Croatia, India, Indonesia, Kenya, Lithuania, Morocco, Poland and the United Kingdom, have only one publication each in this area (Alfina and Haneda, 2022). Notably, there were no published reports on the use of EPF as a management strategy against insect pests in forestry in South Africa at the time of our review.

This review will assess i) the diversity of EPF and their mode of action, ii) investigate how abiotic and biotic factors could influence the diversity and virulence of EPF and iii) investigate the compatibility of EPF with classical biological control of insect pests.

Diversity of entomopathogens

Entomopathogenic fungi constitute a diverse group of organisms, primarily comprising a wide array of fungal species that have evolved to infect various insect hosts. These fungi play pivotal roles in the natural regulation of insect populations (Araújo and Hughes, 2016; Niu et al., 2019), demonstrating their ability to infect insect hosts across all developmental stages, from eggs to adults, spanning at least 20 different insect orders. Through the course of evolution, EPF have adapted to their distinct ecological niches, giving rise to a remarkable diversity in both morphology and phylogeny. It is worth noting that this group of fungi encompasses an abundance of phylogenetic diversity, much of which remains largely unexplored (Araújo and Hughes, 2016).

Six major clades of EPF have emerged, each having evolved distinctive strategies to infect their insect hosts. These clades encompass the *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Entomophtoromycota*, *Microsporidia* and *Oomycetes* (**Figure 1**) (Araújo and Hughes, 2016; Hibbett et al., 2018; Litwin et al., 2020). Notably, *Oomycetes* exhibit unique biological characteristics, such as cellulose cell walls, zoospores equipped with two flagella, large nuclei and several other features that set them apart from fungi. It is worth mentioning that *Oomycetes* constitute a genetically and morphologically diverse clade that falls under the Kingdom *Straminipila* rather than Kingdom Fungi (Beakes and Thines, 2017; Volk, 2013) and thus, *Oomycetes* are not included in this review which focusses on EPF within the Kingdom Fungi.

Ascomycota, the largest group within the fungal Kingdom, boasts an impressive tally of approximately 64 000 described species. This diverse group include species thriving in a wide array of ecological niches, from decomposers to pathogens and even mutualistic organisms that engage in intricate relationships with their hosts (Alexopoulos et al., 1996; Kirk et al., 2008; Litwin et al., 2020). Within the *Ascomycota*, insect pathogens are predominantly situated in *Hypocreales*, although a few species can also be found in *Ascosphaerales*, *Myriangiales* and *Pleosporales* (Araújo and Hughes, 2016; Samson et al., 1988).

Ascomycota fungi are characterised by their filamentous nature, featuring septate hyphae and they have gained a reputation for their ability to attack a wide range of invertebrate hosts (Araújo and Hughes, 2016). Entomopathogenic fungi from *Ascomycota* grow within their insect hosts as hyphal bodies (Litwin et al., 2020; Prasertphon and Tanada, 1968). The majority of EPF belonging to *Ascomycota* exhibit a well-developed parasitic phase that involves infecting the body of their host. These fungi have been documented to target numerous species spanning across 13 different insect orders and play a prominent role in the realm of biopesticides. Among *Ascomycota*, several biopesticide products are globally available, hailing from genera like *Beauveria*, *Isaria*, *Lecanicillium*, *Metarhizium* and *Purpureocillium*. These biopesticides are distinguished for their ability to infect a wide variety of insect species, thanks to their impressively broad host ranges (Castro et al., 2016; Litwin et al., 2020; Mascarin and Jaronski, 2016; Ríos-Moreno et al., 2016).

Basidiomycota comprises of several essential decomposers specialising in colonising dead wood, breaking down cellulose and lignin, as well as processing leaf litter on forest floors (Braga-Neto et al., 2008). Interestingly, only three genera within *Basidiomycota* have been identified as insect pathogens. Among these, one *Fibularhizoctonia* species focusses on infecting the eggs of subterranean termites, while two *Uredinella* species and approximately 240 species within *Septobasidium* are known to target scale insects (Araújo and Hughes, 2016; Evans, 1989). *Fibularhizoctonia* adopts a parasitic lifestyle concerning subterranean termites, specifically *Reticulitermes*. Remarkably, its sclerotia (globose fungal structures) closely mimic the texture and diameter of termite eggs, deceiving the termite workers into tending to it as if it were their own (Matsuura et al., 2000). Some suggest that once the termites cease their care for the fungus, it may turn pathogenic and spread to infect the actual termite eggs (Araújo and Hughes, 2016; Matsuura, 2006). In the case of the two *Uredinella* species, this genus targets a single insect host of Diaspididae. Nevertheless, the demise of the insect host leads to the death of the fungus itself (Araújo and Hughes, 2016; Couch, 1937). In contrast, *Septobasidium* species go further, infecting an entire Diaspididae colony and providing life-long protection to these insects against predators (Couch, 1938). Nevertheless, not all colony members benefit from this protection; some get infected by *Septobasidium*, which results in castration and dwarfism of the affected individuals (Couch, 1938). *Septobasidium* species serve as a striking example of a fungus farming insects, as they encase all colony members in hyphal structures, rendering them unable to escape (Araújo and Hughes, 2016; Couch, 1931). Despite their intriguing behaviours, none of these fungal genera have been developed for use in biological control within forestry or agriculture, suggesting a potential avenue for further exploration.

Within *Chytridiomycota*, there are 64 EPF species spread across two genera: *Myiophagus* and *Myrmicinosporidium*. These fungal infections primarily target insect of the orders Blatodea, Diptera and Hymenoptera (Araújo and Hughes, 2016). A closely related phylum, *Blastocladiomycota*, shares several characteristics with *Chytridiomycota* and includes two genera of EPF: *Coelomycidium* and *Coelomomyces* (Kaczmarek and Boguś, 2021; Naranjo-Ortiz and Gabaldón, 2019).

Within *Blastocladiomycota*, several species have been identified that attack economically and medically significant insect pests. Notably, *Coelomomyces* species play a crucial role in this phylum, as they infect all life stages of important disease vector species, including *Aedes* Meigen (Diptera: Culicidae), *Anopheles* Meigen (Diptera: Culicidae), *Culex* Linnaeus (Diptera: Culicidae) and *Simulium* Latreille (Diptera: Simuliidae) (Araújo and Hughes, 2016). Beyond infection, *Coelomomyces* species also exert a notable influence on the behaviour and reproduction patterns of their hosts (Araújo and Hughes, 2016; Kaczmarek and Boguś, 2021). *Coelomomyces*, owing to its relative host specificity and its capacity to induce detrimental natural epizootics among mosquito larvae populations, has been suggested as a promising biological control agent for mosquitoes (Kaczmarek and Boguś, 2021). However, the unpredictability of *Coelomomyces* infection rates and the complexity of its lifecycle for mass production may potentially overshadow its advantages as a biological control agent (Scholte et al., 2004).

Among the most significant EPF phyla are *Entomophthoromycota*, known to infect a noteworthy 10 insect orders (Humber, 2012). Within this phylum, there are 19 genera comprising of EPF species, displaying the highest levels of insecticidal activities (Araújo and Hughes, 2016; Litwin et al., 2020). However, precisely determining the number of EPF species within these 19 genera remains a challenge, as they exhibit the ability to infect a wide array of insect hosts (Araújo and Hughes, 2016). *Entomophthoromycota* primarily target adult insects, except for two exceptional species, *Entomophthora aquatica* and *Entomophthora conglomerata*, which infect the larval stages of mosquitoes (Araújo and Hughes, 2016; Scholte et al., 2004). What sets *Entomophthoromycota* apart is their biotrophic lifestyle, wherein they consume their insect host while still alive (Roy et al., 2006). Intriguingly, despite the infection, insect hosts continue to exhibit normal behaviour (Araújo and Hughes, 2016). Unfortunately, the full potential of *Entomophthoromycota* as biological control agents remains largely untapped due to the extreme challenges associated with culturing and breeding them under laboratory conditions (Litwin et al., 2020).

Microsporidia possess a unique spore mechanism, functioning similar to a syringe, injecting protoplast material into their target hosts (Keeling and Fast, 2002). This group is renowned as insect pathogens, comprising 143 genera encompassing over 1200 species (Araújo and Hughes, 2016). Of these, around 93 genera are documented to infect insect species across 12 different insect orders. The economic impact of *Microsporidia* extends to both their role as pathogens of bees, as well as biological control agents. Notably, *Nosema bombycis* and *Nosema ceranae* have been responsible for severe losses in apiculture (Higes et al., 2006). Additionally, two species, *Tubulinosema* sp. and *Antonospora lucastae* (formerly *Nosema locustae*), are employed as biological control agents against beet webworm (*Loxostege sticticalis*) and grasshoppers, respectively. Furthermore, *Nosema ceranae*, initially isolated from honeybees, has been observed infecting beet webworm larvae under both natural and laboratory conditions (Kaczmarek and Boguś, 2021; Lange, 2005; Malysh et al., 2018; Solter et al., 2012; Zhou and Zhang, 2009). Regrettably, no fungal species from this group have been identified for the control of pests within forestry environments.

Mode of action

The infection process of EPF unfolds through several stages, requiring direct contact between the fungus and the cuticle surface of the target insect. Unlike bacteria and viruses, which require ingestion by an insect to initiate infection, EPF can invade their insect hosts through the cuticle or after ingestion (de Faria and Wraight, 2007; Mannino et al., 2019; Niu et al., 2019). Successful pathogenesis and infection by EPF hinges on various factors, including adhesins, lytic enzymes and secondary metabolites that are produced by fungi to overcome their host's natural defences (Litwin et al., 2020).

The initial stage of infection involves the adherence of fungal spores to the insect cuticle. During this phase, hydrophobins and adhesin proteins are synthesized to recognise and infect the insect host (Greenfield et al., 2014). In the subsequent steps, lytic enzymes are generated to hydrolyse the insect cuticle and facilitate the formation of the appressorium (Santi et al., 2010). During penetration, another lytic enzyme, phospholipase C, hydrolyses phosphodiester bonds, enabling fungal hyphae to invade the insect body cavity, infect the tissues and disrupt physiological processes (Donzelli and Krasnoff, 2016). At this stage, EPF produce secondary metabolites that impact the host immune responses and induce paralysis. Mechanical damage and nutrient depletion caused by EPF lead to the destruction of the insect body and organs. Once all available nutrients are consumed, fungal hyphae emerge from open segments of the cadaver and infectious spores are produced, enabling the fungi to spread and infect healthy individuals (Litwin et al., 2020; Skinner et al., 2014). The entire infection process can take up to 14 days, with initial symptoms appearing within 7 days, depending on the fungal species. Some studies have noted that while overall infection processes are similar, molecular mechanisms can vary among groups, for example in the case of *Beauveria bassiana* and *Metarhizium robertsii* (Ortiz-Urquiza and Keyhani, 2013). The subsequent sections will delve into the primary infection pathways of *Ascomycota*, given its status as the most diverse and abundant EPF genus.

a. Hydrophobins and adhesins

The initial phase of infection involves the adhesion of fungal spores to the target insect cuticle. During this stage, two types of proteins, hydrophobins and adhesins, play pivotal roles. Hydrophobins are responsible for breaking down the layers of the insect cuticle during sporulation. On the other hand, adhesins, including MAD1 and MAD2, facilitate both the attachment to the insect cuticle and the recognition of the fungal pathogen by its host (Greenfield et al., 2014; Litwin et al., 2020; Wang and St Leger, 2007).

b. Lytic enzymes

Lytic enzymes take the lead in the infection process, responsible for breaking down the components of the insect cuticle, thereby facilitating the penetration of appressoria into the outer layers of the target insects (Litwin et al., 2020). These enzymes come into play as soon as the spores attach to the insect, initiating the formation of the appressorium (Santi et al., 2010). Three primary types of lytic enzymes are involved: lipases, proteolytic enzymes and chitinase enzymes.

Lipases, the first to be produced, serve to hydrolyse lipids and lipoproteins found in the outer cuticle (epicuticle) of the target insect (Pedrini et al., 2007). These enzymes target ester bonds within triacylglycerols, breaking them down and releasing free fatty acids, diacylglycerols, monoacylglycerols, as well as glycerol (Silva et al., 2009). Lipases are widely present in most EPF across various classes, enhancing the adhesion of germinating spores to the insect cuticle by increasing hydrophobic interactions between the fungi and the cuticle surface (Mondal et al., 2016; Santi et al., 2010; Supakdamrongkul et al., 2010). For example, Phospholipase C, a type of lipase found in *Metarhizium brunneum* (formerly *Metarhizium anisopliae* var. *anisopliae*), works by hydrolysing phosphodiester bonds and degrading phospholipids in the insect membranes, allowing the fungus to enter the insect hemocoel and infect the tissues (Santi et al., 2010).

Proteolytic enzymes come into play by hydrolysing peptide bonds within insect cuticles, effectively exposing chitin fibrils. One of the first comprehensive proteases described, subtilisin, has the ability to degrade cuticle proteins and modify the surface cuticle, thereby facilitating spore adhesion (Litwin et al., 2020). This endoprotease is also found in *Metarhizium anisopliae* and *Beauveria bassiana* (Donatti et al., 2008; Shah et al., 2005). Additionally, several other proteases found in various EPF play essential roles in the pathogenesis process of infection (Zhao et al., 2016). These proteases have an additional role in the infection process—they actively participate in the inactivation of antifungal proteins present in the epidermis of the target insects (Litwin et al., 2020; Sotelo-Mundo et al., 2007).

Chitinase enzymes are categorized based on their specific actions on the chitin molecule. These classifications include endochitinases, which hydrolyse the Β-1,4-

glycosidic bond within the chitin molecule and exochitinases, which hydrolyse N-acetylglucosamine oligomers formed during the action of endochitinases. Numerous EPF have the presence of these chitin enzymes, with both endochitinases and exochitinases being essential for the complete degradation of the host insect cuticle (Duo-Chuan, 2006).

Entomopathogenic fungi in addition produce several other enzymes that do not directly participate in cuticle digestion and degradation. Still, these enzymes play vital roles in pathogenesis and contribute to the ability of certain EPF species to survive and thrive within their target host insects (Jin et al., 2015).

c. Secondary metabolites

Entomopathogenic fungi are prolific producers of low-molecular-weight organic compounds known as secondary metabolites (Litwin et al., 2020). These secondary metabolites play a pivotal role in the survival and infection processes of EPF when responding to environmental conditions. Their high production levels highlight their significance in maintaining crucial host functions and they contribute to effective pathogen infection by causing damage to the host nervous system, reducing its resistance (Donzelli and Krasnoff, 2016).

Among these secondary metabolites are toxins produced by EPF, which play a crucial role in killing insect hosts. They achieve this by breaking down organic structures, leading to cellular dehydration and the inability to regenerate tissue (Alfina and Haneda, 2022). Notable secondary metabolites in this category include beauvericins, beauverolides, bassianolides, and destruxins (Maina et al., 2018). Destruxins, primarily produced by *Metarhizium anisopliae*, serve to modulate the immune system responses of insects, rendering them more susceptible to infections (Mc Namara et al., 2017), In contrast, other EPF species such as *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae* and *Metarhizium flavoridae* (all belonging to the *Ascomycota*) produce secondary metabolites such as lytic enzymes that break down the insect host cuticle (Gao et al., 2015). A recent comprehensive review delves into the various secondary compounds and their roles in greater detail (Litwin et al., 2020).

The production of these compounds is influenced by factors such as water availability, temperature and a combination of environmental conditions (Medina et al., 2015; Rohlfs and Churchill, 2011). To cope with abiotic stresses and maintain activity, fungal species produce various secondary compounds like glycerol and erythritol. Each fungal species exhibits unique tolerance levels when it comes to abiotic stresses. A study from 2012 demonstrated significant variations in the production of secondary metabolites by lichenized fungi, which comprise half of all known *Ascomycetes*, across different geographical regions and altitudes. Nutrient availability in these regions also play a crucial role, influencing the active secondary metabolite pathways due to an abundance or lack of nutrients. It is important to note that these are intricate systems, making it increasingly complex to separate the effects of environmental factors, developmental stages of the fungi and other variables from one another (Deduke et al., 2012). Nevertheless, further studies are required to define the impacts of environmental factors on the production of secondary metabolites in EPF species (Medina et al., 2015).

Bioprospecting

Many EPF and potential novel compounds remain to be discovered and utilised. Bioprospecting is used to identify new compounds with potential as pharmaceuticals, bio-pesticides, food ingredients or agricultural inputs (Dara et al., 2019). Bioprospecting can also be aimed at local uses (bioprospecting with commercial prospect).

Advancements have been confounded by many contributing factors, in the hope of retaining an increased spectrum of application opportunities. Research obstacles need to be mitigated to maximize their utilisation, from basic research areas of taxonomy, ecology, fungal physiological aspects to research dealing with improving fungal culture, fermentation, formulation and sterilisation (Mascarin and Jaronski, 2016; Mascarin et al., 2019). Multiple development issues may occur at various points of the research and development pipeline, hence final research on the viability of a product, or their capability to replace chemical insecticides are necessary for certain IPM programmes (Bamisile et al., 2021b; Dara et al., 2019).

There are several South African studies that have looked at South African EPF species and strains as possible biological control agents (Acheampong et al., 2020; Coombes et al., 2016; FitzGerald et al., 2016; Goble et al. 2012; Hatting et al., 2019). However, several studies are still necessary for further development of these strains as potential commercial biopesticides for the use in both agricultural and forestry industries.

The use of EPF in biological control

Entomopathogenic fungi have been developed commercially in numerous countries as classical biocontrol agents for IPM against insect pests (Jaronski, 2010; Mascarin et al., 2019; Shah and Pell, 2003). These biopesticides were developed in part to replace chemical control products, which have severe negative effects on non-target insects, the environment and human health (Jaronski, 2010). Despite knowledge of their detrimental impact, chemical insecticides are often preferred to biopesticides due to their efficiency and ability to quickly eradicate target pests (Jaronski, 2010).

A few well-known EPF, such as *Beauveria bassiana*, *Isaria fumosorosea* and *Metarhizium anisopliae* (all *Ascomycota*), are widely used as biocontrol agents to manage agricultural, forestry and disease vector pests with great success rates (Edgington et al., 2007; Evans et al., 2018; Fang et al., 2015; Niu et al., 2019). The literature likewise highlights the research focus on *Beauveria* spp. and *Metarhizium* spp. as potential mycoinsecticides, while the presence of less common entomopathogens such as *Colletotrichum* spp., *Isaria* spp. and *Lecanicillium* spp. have been documented in recent studies (Mascarin et al., 2017; Mascarin et al., 2016). Further research on the less common species is required to fully understand their efficacy as potential mycoinsecticides (Mascarin et al., 2017; Mascarin et al., 2016).

One of the most well-known EPF biopesticides is *Beauveria bassiana*. Laboratory studies have demonstrated its efficacy against pests such as the coffee berry borer (CBB), *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidea) (Mascarin et al., 2013; Vicentini et al., 2001). Since natural infections of *B. bassiana* at low levels were observed in the field against CBB, mycoinsecticides derived from native strains of *B. bassiana* have become more readily available (Mascarin et al., 2019). Given the cryptic behaviour of CBB, the strategic application of *B. bassiana* is crucial for successful pest control (Cure et al., 1998). Applications involve treating trees during the females' transit period, when they colonise new coffee berries, or applying it to fallen berries to disrupt the insect reservoir (Bustillo et al., 1999; De La Rosa et al., 2000; Jaramillo et al., 2015). These approaches have reduced CBB populations by 34% and 50% annually compared to control plots (Mascarin et al., 2019).

In 2017, the use of *B. bassiana* saw a significant increase in managing *Bemisia tabaci* Genn (Hemiptera: Alcyrodidac) on soybean, despite limited scientific support for its success under field conditions (van Lenteren et al., 2017). Users turned to *B. bassiana* to combat chemical insecticide-resistant whiteflies due to the lack of success with chemical products (Mascarin et al., 2019). Recent studies have also indicated that *B. bassiana* is compatible with the release of the biological control agent for *Gonipterus platensis* Marelli (Coleoptera: Curculionidae), namely *Anaphes nitens* Girault (Hymenoptera: Mymaridae), due to the low infectivity of this parasitoid in Brazilian forestry systems (Jordan et al., 2021; Pérez-Otero et al., 2003).

Regarding forestry systems, a recent review highlighted that pathogenicity studies have tested 34 fungal species within 12 families against forest insect pest species (Alfina and Haneda, 2022). Notably, some families, like *Ophiocordycipitaceae*, *Nosematidae*, *Saccharomycetaceae* and *Myriangiaceae* have only one tested fungal species each, while others like *Davidiellaceae*, *Entomophthoraceae*, *Hypocreaceae* and *Trichomaceae* have two. *Nectriaceae*, *Clavicipitaceae* and *Cordycipitaceae* feature three, eight, and 10 tested fungal species, respectively. As the *Ascomycota* comprises the largest number of species (31), they span several genera including *Acremonium*, *Aschersonia*, *Aspergillus*, *Beauveria*, *Candida*, *Cladosporium*, *Colletotrichum*, *Cordyceps*, *Fusarium*, *Hirsutella*, *Hypocrea*, *Isaria*, *Lecanicillium*, *Metarhizium*, *Myriangium* and *Purpureocillium* (Alfina and Haneda, 2022). Despite the significant diversity recorded over the years, our understanding of which species dominate various agricultural and forestry soils remains limited.

Field applications of EPF come with several limitations, including their overall lack of persistence and low re-infection rates when environmental conditions are suboptimal (Bamisile et al., 2021b; Skinner et al., 2014). Solutions to overcome these challenges include selecting more tolerant and virulent strains through screening processes, as well as nutritional and physical manipulation of conidia during fungal growth to increase spore fitness and stress tolerance of EPF to be used as biological control agents (Jackson, 1997; Rangel et al., 2015). Additionally, the utilisation of wild EPF that have adapted to their environment, with mass production of optimal strains to control target insect pests, offers another avenue (Jordan et al., 2021).

Biological control products face a limitation in the diversity of fungal strains used in IPM (Mascarin et al., 2019). Previously tested products have been available commercially for several years and their efficacy has declined due to insect tolerance and a lack of diversity (Mascarin et al., 2019; Quesada-Moraga et al., 2023). Current research in Brazil aims to develop new fungal products utilising wild EPF in various cropping and forestry systems to enhance their efficacy against pest species (Jordan et al., 2021; Mascarin et al., 2019). The higher virulence recorded in wild EPF suggests the potential for developing new mycopesticides on a commercial scale (Mascarin et al., 2019).

Factors influencing the diversity and virulence of EPF

Entomopathogenic fungal species richness and virulence are subject to the influence of both abiotic and biotic factors (Fernandes et al., 2010; Karar et al., 2021; Qayyum et al., 2021; Quesada-Moraga et al., 2023). Abiotic factors, such as temperature, humidity and soil composition, play a prominent role in shaping the dynamics of EPF. Concurrently, biotic factors encompass elements like host behaviour, host temperature and host resistance (Fernandes et al., 2015; Jaronski, 2010; Mascarin and Jaronski, 2016; McCoy et al., 2002). It is crucial to recognise that abiotic and biotic factors do not operate in isolation but intricately intertwine, creating complex interactions that impact the persistence of EPF.

a. Abiotic factors

Temperature

Only a limited number of field studies have delved into the relationships between temperature and fungal growth, germination and infection for EPF, despite the numerous laboratory studies where relationships with temperature has been studied (Acheampong et al., 2020; Fargues and Luz, 2000; Inglis et al., 1999; Quesada-Moraga et al., 2023; Uma Devi et al., 2005). In general, the optimal temperature range for the germination and growth rates of EPF falls between 23°C and 28°C for both field and laboratory studies (Acheampong et al., 2020; Jaronski, 2010). On the other hand, when temperatures increase to 30°C and beyond, the growth of EPF experiences a rapid decline. Beyond 34°C to 37°C, EPF cease to grow entirely, primarily due to the negative impact on the germination of conidia when temperatures exceed 30°C (Jaronski, 2010).

Distinct species of EPF exhibit varying degrees of tolerance to temperature. For instance, certain strains of *B. bassiana sensu lato* flourish at higher temperatures, while others isolated from different hosts thrive at lower temperatures (Fargues et al., 1997). Furthermore, differences in optimal temperature ranges exist among different strains of fungi within the same species of EPF (Bugeme et al., 2009; Fargues et al., 1996). Consequently, the optimal temperatures for each fungal species significantly influence its persistence in the environment, potentially leading to spatio-temporal variations in species richness across climatic regions.

Temperature fluctuations can critically impact the ability of EPF to infect insect hosts (Jaronski, 2010). In conjunction with low temperatures, sporulation within insects is substantially reduced, allowing only a few hours each day during winter when temperatures are conducive for successful sporulation and infection (Jaronski, 2010). This extended time frame before insects succumb to fungal infections, provides ample opportunity for them to inflict damage on cultivated crops. Generally, *Beauveria* spp. exhibit an upper temperature growth limit of 34°C–36°C, with any temperature surpassing this range significantly diminishing their efficacy (Acheampong et al., 2020; Mascarin and Jaronski, 2016). Laboratory studies further underscore the impact of temperature, as *B. bassiana* infections are notably reduced at 35°C compared to 25°C (Noma and Strickler, 1999). In certain cases, fungal strains that can tolerate colder temperatures may prove more advantageous in cooler climates when compared to those requiring higher temperatures (De Croos and Bidochka, 1999; Li and Feng, 2009; Rath et al., 1995). For the majority of EPF, temperatures dipping below 16°C led to decreased growth rates, thereby jeopardizing their survival on target insect populations (Acheampong et al., 2020; Ihara et al., 2008; Inglis et al., 1999). Importantly, sporulation might not promptly resume even when temperatures become more favourable. For instance, isolates of *B. bassiana* and *M. anisopliae* exhibit a delayed response in normal sporulation after exposure to temperatures above their optimal threshold (Jaronski, 2010).

Until recently, the influence of temperature on fungal diversity in various forestry systems and climatic regions received limited attention (Romero et al., 2021). Recent studies have uncovered not only variations in fungal diversity among different climatic regions but also alterations in spore structures based on the temperature conditions to which they are exposed (Romero et al., 2021). These changes in spore structures serve as indicators of the temperature and humidity fluctuations within their environment (Romero et al., 2021).

Relative humidity (RH)

Changes in RH exert a significant influence on the growth, abundance and virulence of EPF populations (Zhang et al., 2021). Elevated RH generally favours fungal growth and fosters greater species richness within their respective habitats (Cao et al., 2022). It is important to note that the RH range necessary for insect infections varies considerably depending on species, strains and environmental conditions. In vitro, spore germination leading to infection typically requires approximately 75% RH, depending on the specific fungal species. For instance, the efficacy of *B. bassiana* against *Rhodnius prolixus* Stål demands a RH threshold exceeding 96% (Luz and Fargues, 1999), while reduced virulence has been observed against *Cyclas formicarius* Fabricius at RH levels below 43% (Yasuda et al., 1997). These disparities are believed to be influenced by the duration of exposure of insects to fungal spores during incubation (Luz and Fargues, 1999). In contrast, certain EPF, such as *H. thompsonii* and *Lecanicillium* spp, exemplify the extreme end of high RH requirements, with their efficacy hinging on exposure to high RH levels for at least 24 hours (McCoy, 1981). Consequently, RH stands as a pivotal environmental factor governing the effectiveness of *B. bassiana* both in laboratory settings and practical field applications (Acheampong et al., 2020; Mascarin and Jaronski, 2016). Further studies are needed to investigate the role of RH in the infection stage and sporulation of EPF and to understand RH within the broader context of the specific environment where EPF are employed as biological control agents for pest management.

Soil

Extensive research has been devoted to unravel the intricacies of fungal diversity within soil ecosystems. The factors steering fungal soil diversity are multifaceted and encompass variables such as soil moisture, pH, organic matter content and the intricate tapestry of soil microbe communities (Niu et al., 2019). Moreover, fungal growth and development are intricately linked to soil characteristics, including texture, chemical properties, fertilisation practices and nutrient content–factors that furnish the vital energy resources needed by these fungi (Johansson et al., 1999; Qayyum et al., 2021).

One pivotal determinant of fungal communities' hinges on soil texture, where soil particle sizes play a decisive role in shaping fungal hyphal growth and persistence within the soil matrix (Gupta and Germida, 2015). For instance, sandy soils exhibit a propensity for harbouring *I. fumosorosea* and *M. anisopliae*, whereas clay soils favour a higher prevalence of *B. bassiana* than *I. fumosorosea* and *M. anisopliae* (Tkaczuk et al., 2014). Notably, *B. bassiana* finds prominence in soils from natural habitats and orchards, diverging from the prevalence of *Metarhizium* species in agricultural soils (Meyling and Eilenberg, 2007; Qayyum et al., 2021). This variation in soil texture plays a pivotal role in the retention or facilitation of water, nutrients and chemicals, thus creating an optimal environment conducive to the growth of EPF.

Entomopathogenic fungi, exhibit a preference for soils characterised by a low pH, low electrical conductivity (EC), low temperature, and high moisture content (Qayyum et al., 2021). Notably, the diversity of EPF communities thrive in the presence of rich organic soils. This is attributable to the capacity of fungal communities to metabolise diverse carbon sources and organic matter, a process enabled by favourable fungal growth conditions (Jaronski, 2010; Johnson et al., 2017; Klingen et al., 2002; Kuzyakov, 2010; Uzman et al., 2019). Consequently, this robust microbial activity leads to an amplified population of EPF within the soil, enhancing the likelihood of insect infections.

While some studies have touched upon the influence of different soil types on EPF diversity and virulence, much of this complex relationship remains unexplored. This knowledge gap presents an abundant source for future researchers to delve into the ideal soil textures that facilitate EPF diversity and persistence.

b. Biotic factors

A multitude of biotic factors, ranging from host behaviour, host resistance and host susceptibility, exert a complex influence on the diversity and virulence of fungi within their environmental niches (Jaronski, 2010; Mascarin and Jaronski, 2016). Furthermore, other fungal species or insect pathogens can engage in competitive interactions with EPF, impacting their diversity within their native ecosystems (Jaronski, 2010).

Host behaviour

Insects have evolved behavioural defences to shield themselves from fungal infections. One of these defences is grooming, in which the insect host diligently removes any potential fungal spores from its cuticle before infection can take hold (Ortiz-Urquiza and Keyhani, 2013). Additionally, certain insect species have been observed removing the corpses of infected insects to safeguard healthy individuals from potential infection (Ortiz-Urquiza and Keyhani, 2013). These behavioural defence mechanisms represent just a subset of the strategies employed by insects to fend off fungal infections, with variations in mechanisms observed across different insect species.

Host tolerance

Certain insects exhibit the remarkable ability to actively regulate their body temperatures by harnessing heat directly from the sun and warm surfaces (Carruthers et al., 1992). This intriguing phenomenon is known as behavioural fever that becomes prevalent once these insects have been exposed to pathogenic infections (Clancy et al., 2018; Inglis et al., 1996; Kalsbeek et al., 2001; Quesada-Moraga et al., 2023; Watson et al., 1993). Behaviour like basking and the practice of behavioural fever thermoregulation, enable insects to elevate their body temperatures beyond the upper thermal limits tolerated by pathogens such as *B. bassiana*. On the other hand, there are insects whose body temperatures can plummet as low as 10°C for up to six hours each day (Jaronski, 2010). Consequently, the success rate of fungal infections is intricately linked to the specific insect species and its ecological interactions within its environment (Jaronski, 2010).

Insects, like all organisms, rely on immune responses as another line of defence (Cooper and Eleftherianos, 2017). Their innate immune response serves as the initial barrier against invading pathogens. Interestingly, some insect species display a protective response after infection with a pathogen, which enhances their resistance to the same or similar pathogens (Cooper and Eleftherianos, 2017). These immune responses encompass both adaptive, involving immunological memory for heightened future responses, and humoral, characterised by antibody–mediated mechanisms (Cooper and Eleftherianos, 2017; Sheehan et al., 2020). In the case of the humoral immune response in insects, hemocytes produce an array of antimicrobial peptides known for their potent antifungal activity (Sheehan et al., 2020). It is clear that insect immune responses are intricately complex and tailored to the specific species and the pathogens they encounter.

It is evident that numerous studies have addressed biotic factors that influence fungal species diversity in a broader context. However, there is a notable shortage of research exclusively dedicated to understanding the impact of biotic factors on EPF. Substantial knowledge gaps persist on the biotic effects on EPF species diversity and how these effects correlate with virulence towards insect hosts.

Conclusion

Entomopathogenic fungi encompass a wide range of fungal and oomycete species capable of infecting insects at various developmental stages. Literature highlights the significant influence exerted by abiotic and biotic factors on the diversity of fungal species. Nevertheless, the shortage of studies exclusively dedicated to elucidating the impact of these factors on EPF diversity is apparent. Given the extensive diversity observed among EPF species, it is imperative for forthcoming investigations to shift their focus towards a comprehensive assessment of species found within cultivated and uncultivated ecosystems, as opposed to relying solely on established commercial products. This approach promises to provide researchers with a more comprehensive understanding of novel naturally occurring EPF, contributing to the effective management of invasive insect species through their exploitation as biopesticides in IPM programmes.

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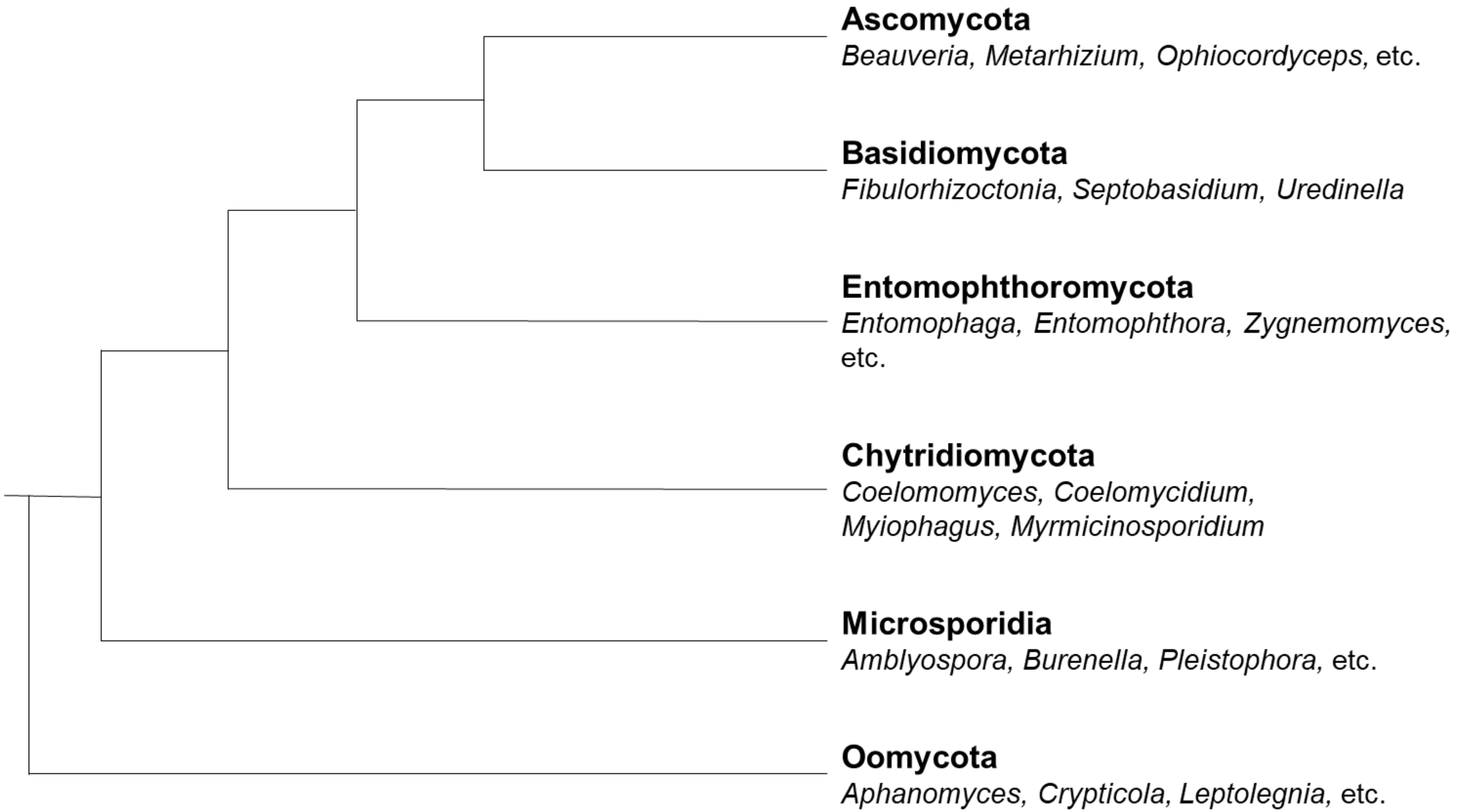

Figure 1. The relationship between the different fungal groups discussed in the review with some EPF species listed. Adapted from Araújo and Hughes (2016).

Chapter 2:

Beauveria (Hypocreales: Cordycipitaceae) cryptic species associated with *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae) from South African *Eucalyptus* plantations

Abstract

The Eucalyptus snout beetle, *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae), poses an economic threat to *Eucalyptus* (Myrtaceae) plantations in South Africa. While efforts to control this pest have relied on the egg parasitoid, *Anaphes nitens* Girault (Hymenoptera: Mymaridae), sporadic outbreaks of *Gonipterus* sp. n. 2 persist across the forestry landscape. Local entomopathogenic fungi (EPF) offer a potential complementary solution alongside classical biological control. This study aimed to explore the diversity of *Beauveria* (*Cordycipitaceae*) species associated with *Gonipterus* sp. n. 2 in South African forestry. Beetles collected from *Eucalyptus* plantations were incubated in moisture chambers until fungal growth appeared. The fungi were isolated and purified on Malt Extract Agar (MEA). Subsequently, DNA was extracted and sequences of the RNA polymerase II large subunit (*rpb1*), Translation Elongation Factor 1-alpha (*tef1*), Internal Transcribed Spacer region (ITS) and Beta-locus intergenic region (*Bloc*) were analysed to determine phylogenetic identifications of the isolates. Out of 128 isolates sequenced, 32% were identified as *Beauveria* spp., 48% as opportunistic EPF and 20% as other fungal genera such as *Alternaria*, *Aspergillus*, *Auxarthron*, *Cladosporium*, *Myriodontium*, *Penicillium*, *Purpureocillium* and *Trichothecium*. Within the *B. bassiana* species complex, three species namely, *B. namnaoensis*, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2 were identified, along with *B. pseudobassiana*. This study revealed the cryptic species diversity of *Beauveria* associated with *Gonipterus* sp. n. 2 in South African *Eucalyptus* plantations, laying a foundation for future work on the potential use these EPF to manage *Gonipterus* sp. n. 2.

Introduction

Entomopathogenic fungi (EPF) exhibit a diverse array of morphological and phylogenetic traits, specifically adapted to exploit insect hosts (Araújo and Hughes, 2016). These fungi belong to no less than five out of the eight fungal phyla and play a vital role in controlling insect populations (Araújo and Hughes, 2016; Goettel et al., 2005). Among the most renowned EPF species is *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), previously known as *Botrytis bassiana* (Balsamo-Crivelli, 1835a, b; Vuillemin, 1912). *Beauveria*, belonging to the phylum *Ascomycota* and the highly diverse *Hypocreales* (Araújo and Hughes, 2016), is recognised as a cosmopolitan genus primarily reproducing asexually. It thrives in soil and inhabits insect hosts, capable of infecting over 700 insect species worldwide

(Bissett and Widden, 1988; de Hoog and Rao, 1975; Imoulan et al., 2017; Khonsanit et al., 2020). The sexual phase of *Beauveria* is rarely observed in natural environments and proves to be exceptionally challenging to reproduce under laboratory conditions (Lee et al., 2010). *Beauveria* has gained substantial attention as a potential biological control agent in integrated pest management (IPM) programmes (Ali et al., 2018; Bara and Laing, 2020; Mascarin et al., 2019; McGuire and Northfield, 2020; Mohammed and Hatcher, 2017).

Beauveria is primarily recognised morphologically by its distinctive sympodial, short, globose to flask-shaped phialides, producing single-celled, holoblastic conidia (Khonsanit et al., 2020; Kobmoo et al., 2021; Rehner and Buckley, 2005; Rehner et al., 2011). Important morphological traits for identifying these species are the single-celled, hyaline and smooth-walled conidia, exhibiting varied shapes such as globose, cylindrical, ellipsoidal, vermiform or reniform (Khonsanit et al., 2020; Kobmoo et al., 2021).

Only *B. bassiana* and *B. pseudobassiana* species were previously recognised, based on morphology, but recent studies using molecular data have revealed numerous cryptic species within *Beauveria*. The taxonomy of the *B. bassiana* species complex has undergone revision using phylogenetic techniques, whole-genome sequencing and morphometric analyses (Clifton et al., 2023; Kobmoo et al., 2021; Rehner et al., 2011). The most used method for identifying cryptic species within *Beauveria* involves utilising the Beta-locus intergenic region (*Bloc*) (Castrillo et al., 2020; Clifton et al., 2023; Rehner et al., 2011). These analyses have revealed that *B. bassiana* comprises of multiple phylogenetically distinct lineages, incorporating approximately 12 cryptic species that cannot be distinguished through morphological techniques alone (Clifton et al., 2023; Rehner and Buckley, 2005; Rehner et al., 2011; Rehner et al., 2006). Significant divergence between three clades within *B. bassiana* has facilitated the identification of two distinct novel species: *B. namnaoensis* and *B. neobassiana* (Kobmoo et al., 2021). This highlights the crucial role of molecular identification in detecting cryptic species within the genus (Gautam et al., 2022; Kobmoo et al., 2021).

The distribution of EPF species can be influenced by several environmental factors (Cao et al., 2022; Jaronski, 2010; Quesada-Moraga et al., 2023). Among the most critical factors are temperature and relative humidity (RH). Entomopathogenic fungal species exhibit varying degrees of temperature tolerance. For example, certain EPF species thrive in higher temperatures, while others prevail in lower temperatures

(Fargues et al., 1997; Jaronski, 2010; Quesada-Moraga et al., 2023). Fluctuations in RH significantly impact the abundance and growth of EPF populations (Zhang et al., 2021). In general, higher RH favours greater fungal diversity and growth (Cao et al., 2022).

South Africa's plantation forests are threatened by several invasive insect pests. One particularly damaging pest is the Eucalyptus snout beetle, *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae), inflicting substantial yield losses (Schröder et al., 2020). Despite the successful establishment of a classical biological control agent, namely the egg parasitoid *Anaphes nitens* Girault (Hymenoptera: Mymaridae), frequent *Gonipterus* sp. n. 2 outbreaks persist (Cordero Rivera et al., 1999; Huber and Prinsloo, 1990; Loch, 2008; Schröder et al., 2020; Valente et al., 2004). Therefore, considering an additional biological control agent such as *Beauveria* for implementation in IPM might aid in curbing *Gonipterus* sp. n. 2 populations in forestry.

The current understanding of the diversity and distribution of *Beauveria* species in South Africa remains unknown (Goble et al., 2012; Morar-Bhana et al., 2011; Rong and Grobbelaar, 1998). Likewise, there is uncertainty regarding the presence of recently identified cryptic species within the *B. bassiana* complex in South Africa. This study aimed to explore the diversity of *Beauveria* species within the primary *Eucalyptus* (Myrtaceae) production regions in South Africa, specifically focusing on their association with the forestry pest, *Gonipterus* sp. n. 2. Additionally, this study aims to determine the presence of potential cryptic diversity within the *B. bassiana sensu lato* clade within the country.

Materials and methods

a. Sampling

Asymptomatic adult *Gonipterus* sp. n. 2 beetles were collected from *Eucalyptus* plantations within the main *Eucalyptus* production regions of South Africa, namely Mpumalanga and KwaZulu-Natal provinces (**Figure 1**). Ad hoc samples collected in Gauteng province from private properties were also included. A few insect cadavers were given to the project from the University of Pretoria’s biocontrol facility as these beetles showed infection and were removed from the rearing cultures (**Figure 1**).

b. Isolations

Collected adult beetles were placed in groups of 10, based on their collection sites, in moisture chambers at approximately 26.3 ± 0.05°C and 92.2 ± 2.0% RH until the beetles were dead and fungal growth was observed on the cadavers (**Figure 2**). Once fungal growth was observed from the insect cadavers, the mycelium penetrating the insect cuticle was isolated from each insect onto a generalist medium, Malt Extract Agar (MEA: 3% w/v malt extract, 2% w/v agar, streptomycin 3 ml/L). Plating was done in triplicates per insect aseptically. The isolates were incubated at room temperature set to 23°C for 7 days before each culture was purified by subculturing onto MEA and incubated for another 7 days at 23°C to obtain pure colonies.

The isolates for each geographic region were grouped together based on primary identifications using morphological characteristics, such as colour, texture, colony size and reproductive structures using a Zeiss Stemi 508 stereomicroscope (Samson et al., 2011). The cultures were then preserved in cryovials filled with 1 ml of 10% glycerol. For the preservations, approximately five agar blocks were cut out from the cultures to be preserved and placed inside the cryovials. The vials were vortexed to ensure all agar blocks were submerged in the glycerol before storage at -80°C.

In addition to the isolates obtained from insect cadavers, the commercial Eco-BB® strain R444 that forms part of Madumbi's BioManagement range, was selected based on its availability in small quantities and its classification as a *B. bassiana* strain.

c. DNA extractions, PCR amplifications and sequencing

Morphological representative strains from all *Beauveria* isolates from this study were selected for sequencing and molecular analyses. Subsequently, all isolates that could not be identified morphologically were selected for sequencing to confirm identifications to genus and species level. DNA was extracted from pure cultures. DNA extractions for pigmented fungi such as *Aspergillus*, *Beauveria*, *Penicillium* and other dematiaceous species (dark in colour) were carried out using the Zymo Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research Corporation, California, USA) and a similar protocol was followed as suggested by the manufacturers. DNA from *Fusarium* species, as well as unknown species with hyaline conidia was extracted using the PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) according to the manufacturer's instructions. These separate methods were selected because coloured spores or mycelia can interfere with DNA extraction, lowering the quality of the DNA.

PCR reactions were performed in a 25 µl total reaction mixture composed of 17.5 µl demineralised sterile water, 5 µl MyTaq™ PCR buffer (5 x MyTaq™ reaction buffer: 5

mM dNTPs, 15 mM $MgCl_2$, Bioline Reagents Ltd, United Kingdom), 0.5 µl of each primer (0.1 µM primer) and 0.15 µl MyTaq™ polymerase (2.5 U MyTaq™ DNA polymerase, Bioline Reagents Ltd, United Kingdom). The PCR amplification profiles, along with the primers used for PCR and sequencing are included in **Table 1**. PCR amplicons were separated using 1% agarose (SeaKem® LE Agarose, Lonza Bioscience) gel electrophoresis at 80 V for 40 min. PCR products were stained using GelRed® Nucleic Acid Gel Stain (Biotium, Hayward, California, USA) and electrophoresed alongside a 0.5 µg/µl GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific). The results were visualised under UV light using a Bio-Rad Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., USA).

The PCR products were cleaned for sequencing using ExoSAP. Using PCR strip tubes, 5 µl PCR product was combined with 2 µl ExoSAP-IT™ PCR clean-up reagent (1 U/µl FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific), 20 U/µl Exonuclease I (Thermo Fisher Scientific)) and incubated at 37°C for 15 min and then at 85°C for a further 15 min.

Bidirectional sequencing reactions were performed in 96 well plates. Reactions were set up to a total volume of 13 µl and were made up by 7.4 µl sterile water, 2.1 µl 5x BigDye™ Terminator v3.1 Sequencing buffer (Applied Biosystems, Foster City, CA, USA), 0.5 µl BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA), 1 µl primer, and 2 µl ExoSap product. Initial denaturation was performed at 94°C for 5 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 50 °C for 10 s and elongation at 60°C for 4 min. Reactions were kept at 4°C in foil until precipitation.

Reactions were precipitated with sodium acetate. A volume of 60 µl of a sodium acetate master mix [6 000 µl absolute ethanol, 240 µl 3 M sodium acetate (pH 4.6), and 960 µl of sterile water] was used for precipitation before sequencing, transferred to each reaction and the sealed plate centrifuged 3220 x g at 4°C for 30 min. The supernatant was discarded and the plate inverted on a paper towel and centrifuged for 15 s at 180 x g. The pellet was washed with 100 µl freshly prepared 70% (v/v) ethanol. The plate was incubated at room temperature for 5 min and then centrifuged for 10 min at 3220 x g. The ethanol was discarded and the plate inverted and centrifuged as before. The wash step was repeated and the plate inverted and centrifuged as before for 30 s. Excess ethanol was allowed to evaporate by air drying in a laminar flow for 10 min. A plastic film was used to seal the plate wells. The reactions were kept at 4°C in foil until sequencing.

Sequencing was conducted at the Sanger Sequencing Facility of the University of Pretoria (Bioinformatics and Computational Biology Unit, v 19.8.22) using an ABI PRISMTM 3500xl Auto-sequencer (Applied Biosystems, Foster City, CA, USA). The forward and reverse sequences of each strain were assembled into contigs with Geneious Prime 2019 (BioMatters Ltd., Auckland, New Zealand) and manually edited where necessary to remove any errors that might have been incorporated during sequencing.

d. Molecular identification and data analysis

Sequences obtained from this study were primarily identified using a BLASTn search against the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Fusarium MLST (http://www.westerdijkinstitute.nl/fusarium/) databases. From the results obtained, a sequence dataset of newly acquired sequences and closely related sequences were compiled and used for phylogenetic analyses. Reference sequences for *Beauveria* were from Kobmoo et al. (2021). Sequences that had low quality after DNA sequencing or inadequate sequence lengths were removed from the phylogenetic analysis.

All sequences were aligned using MAFFT v.7.305b (Katoh and Standley, 2013), with G-INS-I algorithm and were manually adjusted in Geneious Prime where needed. Prior to analysis, Neighbour Joining (NJ) analysis was performed on each individual dataset to determine any discrepancies. Individual and multi-gene maximum likelihood phylogenies were constructed using IQTree v.1.6 (Nguyen et al., 2014) and visualised in FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). The most suitable model for each partition was determined using ModelFinder (Kalyaanamoorthy et al., 2017) and bootstrapping was determined using UFBoot (Minh et al., 2013). Both ModelFinder and UFBoot are integrated into the IQtree software. After visualising the trees in FigTree, final editing was done in Affinity Designer v.1.6.1 (2019 Serif (Europe) Ltd, Nottingham, UK).

Results

1. Preliminary identifications

A total of 321 fungal isolates were obtained from insect cadavers of *Gonipterus* sp. n. 2 collected from plantations across Mpumalanga and KwaZulu-Natal, accompanied by ad hoc collections from Gauteng and cadavers from the University of Pretoria's biocontrol facility. Based on morphological traits, eight fungal genera were distinguished, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Beauveria*, *Bipolaris*, *Curvularia*, *Fusarium*, *Penicillium* and some genera that remained unidentified (**Figure 3**). Among these isolates, 66 could not be classified using morphological criteria.

From 128 fungal isolates either morphologically unidentifiable or initially labelled as *Beauveria* species, molecular analyses were conducted. The ITS gene region was used to distinguish between *Beauveria* and other fungal species for further molecular identification using more gene regions, since only *Beauveria* was of interest in the study. Sequencing uncovered a total of 17 genera within these 128 isolates (**Figure 4**). The identified genera consisted of *Alternaria*, *Aspergillus*, *Aureobasidium*, *Auxarthron*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Deflexula*, *Eppicoccum*, *Fusarium*, *Myriodontium*, *Penicillium*, *Pestalotiopsis*, *Phomatospora*, *Purpureocillium*, *Scopulariopsis* and *Trichothecium* with 40 species identified in total with the ITS gene region for non-target species and three additional gene regions (*Bloc*, *rpb1* and *tef1*) for *Beauveria* species (**Table 2; Figure 5**). Specifically, 40 fungal isolates out of the 128 sequenced were identified as *Beauveria* species, constituting 12.4% of the total collection frequency. Among these 40 isolates, five species were identified that consisted of *B. bassiana* (n=10), *B. namnaoensis* (n=4), *B. pseudobassiana* (n=5), *Beauveria* sp. nov. 1 (n=10) and *Beauveria* sp. nov. 2 (n=11) in the collection regions (**Figure 6**).

2. Multigene identification of *Beauveria* cryptic species

The phylogenetic analysis included 40 *Beauveria* strains obtained in this study, as well as one commercial strain R444 and several reference strains from Kobmoo et al. (2021) (**Figure 6**). The analysis was based on multiple gene regions: the Internal Transcribed Spacer region (ITS), RNA polymerase II large subunit (*rpb1*) and Translation Elongation Factor 1-alpha (*tef1*). Among these 40 isolates, the distribution comprised of *B. bassiana* (25%), *B. pseudobassiana* (12.5%), the recently described *B. namnaoensis* (10%) and two novel *Beauveria* species, *Beauveria* sp. nov. 1 (25%) and *Beauveria* sp. nov. 2 (27.5%) isolated during this study. The topology of the phylogenies was evaluated by 1000 bootstrap replications.

A combined maximum likelihood phylogeny was constructed using *rpb1*, *tef1* and ITS (**Figure 7**). This analysis revealed that 26 strains, including the commercial strain R444, grouped within the *B. bassiana sensu lato* clade. Markedly, within this clade, none of the strains from this study were identified as *B. bassiana* or *B. neobassiana*.

However, five strains were identified as *B. pseudobassiana*, four as *B. namnaoensis*, 10 grouped in the *Beauveria* sp. nov. 1 and 11 grouped in the *Beauveria* sp. nov. 2 clades (**Figure 7**). In addition, the strains from this study were named based on a concordance approach.

In the ITS maximum likelihood phylogeny, the average sequence length for the ITS region in this study was 570 bp. Notably from the results, no *B. bassiana* or *B. neobassiana* species were identified from the *Gonipterus* sp. n. 2 cadavers during this study. Twenty-five strains grouped with the *B. bassiana sensu lato* clade, alongside the commercial strain R444. In addition, five strains were classified as *B. pseudobassiana*, four as the newly described *B. namnaoensis*, 10 grouped in the novel clade named *Beauveria* sp. nov. 1 and 11 grouped in another novel clade named *Beauveria* sp. nov. 2 (**Figure 8**).

The maximum likelihood *rpb1* phylogeny showcased average sequence lengths of 660 bp. Within the phylogeny, no *B. bassiana* or *B. neobassiana* species were identified. Notably, 25 strains were grouped within the *B. bassiana sensu lato* clade, along with the commercial strain R444. Furthermore, five strains grouped with *B. pseudobassiana*, while four were identified as the recently described species *B. namnaoensis*. Additionally, 10 and 11 strains were grouped within the novel clades *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2, respectively (**Figure 9**).

Regarding the *tef1* phylogeny, the average sequence length for the *tef1* region was 925 bp in this study. Intriguingly, no *B. bassiana* or *B. neobassiana* species were identified as with all previous phylogenies. Due to compromised sequence quality and distorted topology, four strains (EPF001A7, EPF001B9, EPF003D8 and EPF003E2) were excluded from analysis. Among the remaining 22 strains, including the commercial strain R444, all strains grouped with the *B. bassiana sensu lato* clade. Moreover, five strains grouped with *B. pseudobassiana*, four grouped with the recently described species *B. namnaoensis*, 10 grouped with *Beauveria* sp. nov. 1 and seven grouped with *Beauveria* sp. nov. 2 (**Figure 10**).

Discussion

The present study recovered 321 fungal isolates from *Gonipterus* sp. n. 2 cadavers, representing 17 fungal genera. Sequencing revealed that *Beauveria* accounted for 40 isolates, consisting of 12.4% isolation frequency in the surveyed areas. Alongside *B. pseudobassiana* and *B. namnaoensis*, two newly discovered species, *Beauveria* sp.

nov. 1 and *Beauveria* sp. nov. 2, were identified during the study. This is the first study to confirm the presence of *B. bassiana* cryptic species in KwaZulu-Natal, Mpumalanga and Gauteng, South Africa.

Previous studies have highlighted the pivotal role of environmental conditions in the distribution of EPF (Quesada-Moraga et al., 2023). Mpumalanga exhibits a temperate to subtropical climate (Kruger and Shongwe, 2004). KwaZulu-Natal, on the other hand, displays a varied climate, with coastal regions experiencing a subtropical climate featuring warm to hot temperatures, while the midlands have a temperate and relatively mild climate (Kruger and Shongwe, 2004). Gauteng experiences a temperate climate with warm to hot summers and mild winters (Kruger and Shongwe, 2004). The study suggests a potential impact on distribution due to environmental and climatic conditions, urging future research to delve deeper into the intricate interactions between EPF and their environments (Jaronski, 2010; Quesada-Moraga et al., 2023).

It was evident that the *B. bassiana sensu lato* clade harboured multiple cryptic species, as indicated by several previous studies (Clifton et al., 2023; Jordan et al., 2021; Khonsanit et al., 2020; Kobmoo et al., 2021; Wang et al., 2022). Newly described species within the *B. bassiana sensu lato* clade were morphologically indistinguishable from one another in this study, as has been observed in prior research (Kobmoo et al., 2021). For instance, it was not possible to distinguish *B. pseudobassiana*, *B. bassiana* and *B. namnaoensis* isolates collected in this study through morphological traits alone. However, employing multigene phylogenetic analyses allowed successful differentiation among these species (Kobmoo et al., 2021). The difficulty in morphologically distinguishing *Beauveria* species highlights the significance of molecular analysis in identifying cryptic species within the *B. bassiana* clade (Gautam et al., 2022; Khonsanit et al., 2020; Kobmoo et al., 2021).

Despite the *Bloc* region typically being an effective marker for identifying *Beauveria* cryptic species (Castrillo et al., 2020; Clifton et al., 2023; Rehner et al., 2011), this study encountered challenges in obtaining sequences of optimal length and quality for robust phylogenetic analysis. The average sequence length obtained was 600 bp, notably shorter than the preferred >1500 bp observed in previous studies. Similarly, additional sequences removed from the *tef1* region exhibited similar issues to those in the *Bloc* region, resulting in distorted topologies in the *tef1* phylogeny. A thorough re-sequencing and analysis of all sequences will be conducted to assess the persistence of these trends. Should these trends persist, exploring the possibility of gene variations by sequencing the genome of these strains will be essential (Castrillo et al., 2020).

Distinct separation among *Beauveria* species was most evident in the *rpb1* and *tef1* regions, while the ITS region exhibited less effective separation. The observed topology in the ITS phylogeny was as anticipated; despite being a universal barcode for fungal species, ITS does not offer optimal resolution for identifying cryptic fungal species (Badotti et al., 2017). In contrast to the findings reported by Kobmoo et al. (2021), the *rpb1* phylogeny alone did not provide exclusive separation among the species and strains, as *tef1* also displayed satisfactory differentiation. The combined phylogeny of *rpb1*, *tef1* and ITS exhibited the most distinct separation among the species and strains studied, guiding the nomenclature of strains through a concordance approach. Thus, further investigation into the taxonomy of these *Beauveria* species is necessary before new *Beauveria* species from South Africa can be accepted.

The comprehensive outcomes of the phylogenetic analyses confirm the absence of both *B. bassiana* and *B. neobassiana* species within this study. Among the isolates, five strains were identified as *B. pseudobassiana*, while an additional four strains grouped with the recently described species, *B. namnaoensis*. Notably, our findings indicate the presence of cryptic species associated with *Gonipterus* sp. n. 2 in South Africa. Moreover, the phylogenetic analyses revealed the existence of two novel species, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov 2. Although these species were not extensively investigated in this study, a revision of the *Beauveria* phylogeny is essential to validate the identification of these novel *Beauveria* species originating from South Africa.

The commercial strain R444 emerged as a potential novel cryptic *Beauveria* species. In the ITS analysis, strain R444 formed a distinct cryptic clade, closely associated with *Beauveria* sp. nov. 1. Its average sequence length in ITS was 513 bp. Similarly, in the *rpb1* phylogeny, strain R444 aligned closely with the *B. namnaoensis* clade, yet retained its own distinct cryptic clade, with an average sequence length of 658 bp. In the *tef1* region, strain R444's sequence length averaged 929 bp. Corresponding to the ITS and *rpb1* findings, strain R444 manifested as a cryptic clade in *tef1*, but closely linked with *B. neobassiana*. In the combined phylogeny, strain R444 grouped closely with *B. neobassiana*, but remained its own clade. This outcome warrants further investigation, given the recent evaluation of diverse cryptic species within the *B.*

bassiana sensu lato clade (Clifton et al., 2023; Jordan et al., 2021; Khonsanit et al., 2020; Kobmoo et al., 2021; Wang et al., 2022). Despite these observations, strain R444 consistently grouped within the *B. bassiana sensu lato* clade across all assessed phylogenies. Moreover, it is important to note that strain R444 has been in circulation for several years, registered prior to the description of new species within the *B. bassiana sensu lato* clade (Kobmoo et al., 2021; Mascarin et al., 2019).

The findings from this study bear significant implications for IPM strategies targeting *Gonipterus* sp. n. 2. The finding of novel species within the *B. bassiana* cryptic clade could potentially enhance the biological control of this invasive insect pest. Likewise, it holds promise for cultivating a diverse range of novel, locally occurring strains suited for biological control, effectively adapted to their specific environments for optimal insect infection (Jaronski, 2010; Jordan et al., 2021; Mascarin and Jaronski, 2016). Future research avenues should concentrate on refining the taxonomy of *Beauveria* and describing the cryptic species within the *B. bassiana sensu lato* clade discovered in South Africa before officially recognising new species. Furthermore, additional studies should explore the potential of these strains for biological control and the possible development of selected strains as commercial biopesticides.

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Table 1. Summary of the PCR protocols used during the study for each gene region.

Locus region	Primer	Sequence (5'–3')	Initial denaturation	Cycle	Denaturation	Annealing	Extension	Final extension	Reference
ITS	ITS1F	CTTGGTCATTTA GAGGAAGTAA	5 min at 94°C	30	40 s at 94°C	30 s at 57°C	60 s at 72°C	10 min at 72°C	Castlebury et al., 2004; White et al., 1990
	ITS4	TCCTCCGCTTAT TGATATGC							
rpb1	CRPB1	CCWGGYTTY ATCAAGAARGT	3 min at 94°C	34	60 s at 94°C	90 s at 50°C	180 s at 72°C	10 min at 72°C	Castlebury et al., 2004; Khonsanit et al., 2020
	RPB1Cr	CCNGCDATNTC R TTRTCCATRTA							
tef1	EF1-983F	GCYCCYGGHCA Y CGTGAYTTYAT	3 min at 94°C	35	45 s at 94°C	45 s at 56°C	90 s at 72°C	8 min at 72°C	Khonsanit et al., 2020
	EF1-2218R	ATGACACCRAC R GCRACRGTYTG							
Bloc	B5.1F	CGACCCGGCCA A CTACTTTGA	2 min at 94°C	40	30 s at 94°C	30 s at 56.3°C	120 s at 72°C	15 min at 72°C	Rehner et al., 2011; Rehner et al., 2006
	B3.1R	GTCTTCCAGTAC CACTACGCC							

Table 2. Summary of fungal species isolated in Mpumalanga, KwaZulu-Natal and Gauteng. Molecular identifications are based on 128 isolates that were morphologically unidentified or identified as *Beauveria* species. Only *Beauveria* species were considered target species during the study.

Species	Localities			Total number of isolates
	Mpumalanga	KwaZulu-Natal	Gauteng	
Alternaria solani	_	1	_	1
Aspergillus flavus	2	_	_	2
Aspergillus niger	_	_	1	1
Aspergillus pseudoustus	_	_	1	1
Aureobasidium pullulans	_	1	1	2
Auxarthron umbrinum	_	_	3	3
Beauveria bassiana	_	10	_	10
Beauveria namnaoensis	4	_	_	4
Beauveria pseudobassiana	_	5	_	5
Beauveria sp. nov. 1	_	5	5	10
Beauveria sp. nov 2	3	4	4	11
Cladosporium cladosporioides	2	2	4	8
Cladosporium delicatulum	_	1	_	1
Cladosporium perangustum	_	_	1	1

Species	Localities			Total number of isolates
	Mpumalanga	KwaZulu-Natal	Gauteng	
Cladosporium pseudocladosporioides	3	_	_	3
Clonostachys rosea	1	_	_	1
Deflexula secundiramea	_	1	_	1
Eppicoccum nigrum	1	_	_	1
Fusarium annulatum	2	_	6	8
Fusarium equiseti	2	3	2	7
Fusarium fujikuroi	2	_	_	2
Fusarium glycines	5	_	2	7
Fusarium oxysporum	1	_	_	1
Fusarium tardicrescens	_	1	_	1
Fusarium tricinctum	1	_	_	1
Fusarium triseptatum	2	2	_	4
Fusarium verticillioides	2	_	2	4
Myriodontium keratinophilum	_	_	3	3
Penicillium brevicompactum	_	_	1	1
Penicillium cairsense	_	1	_	1
Penicillium corvianum	_	_	1	1

Species	Localities			Total number of isolates
	Mpumalanga	KwaZulu-Natal	Gauteng	
Penicillium fagi	3	2	2	7
Penicillium glabrum	2	_	_	2
Penicillium repensicola	_	1	_	1
Penicillium thomii	_	_	2	2
Pestalotiopsis uvicola	_	_	1	1
Phomatospora biseriata	_	_	1	1
Purpureocillium lilacinum	_	_	2	2
Scopulariopsis brevicaulis	_	1	1	2
Trichothecium roseum	1	_	2	3
Total number of isolates	**39**	**41**	**48**	**128**

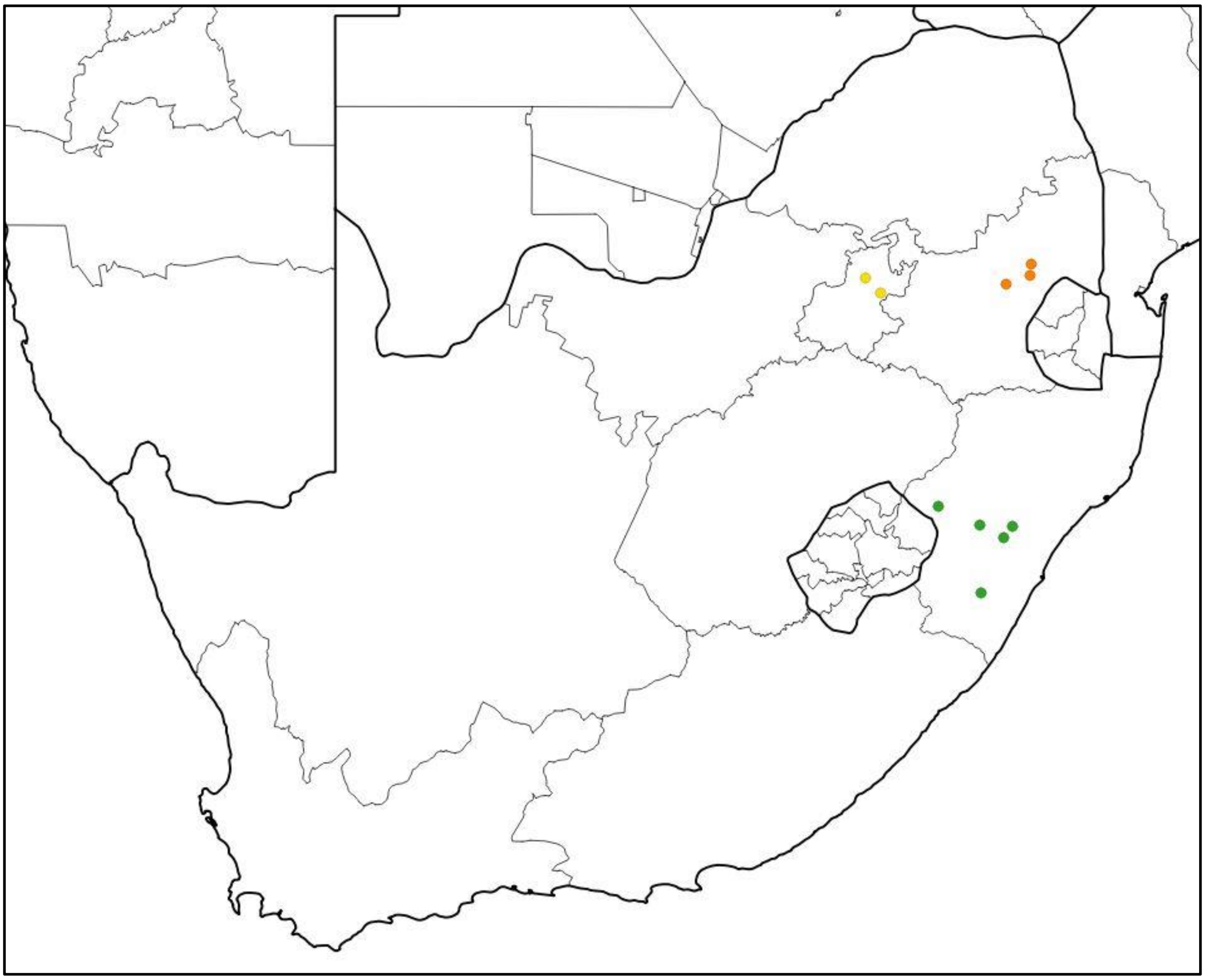

Figure 1. A map indicating the locations where *Gonipterus* sp. n. 2 were collected from *Eucalyptus* plantations in the main *Eucalyptus* production regions of South Africa. Mpumalanga sites are indicated in orange, KwaZulu-Natal sites are indicated in green and Gauteng sites are indicated in yellow.


Figure 2. *Gonipterus* sp. n. 2 cadaver displaying *Beauveria* sporulation.

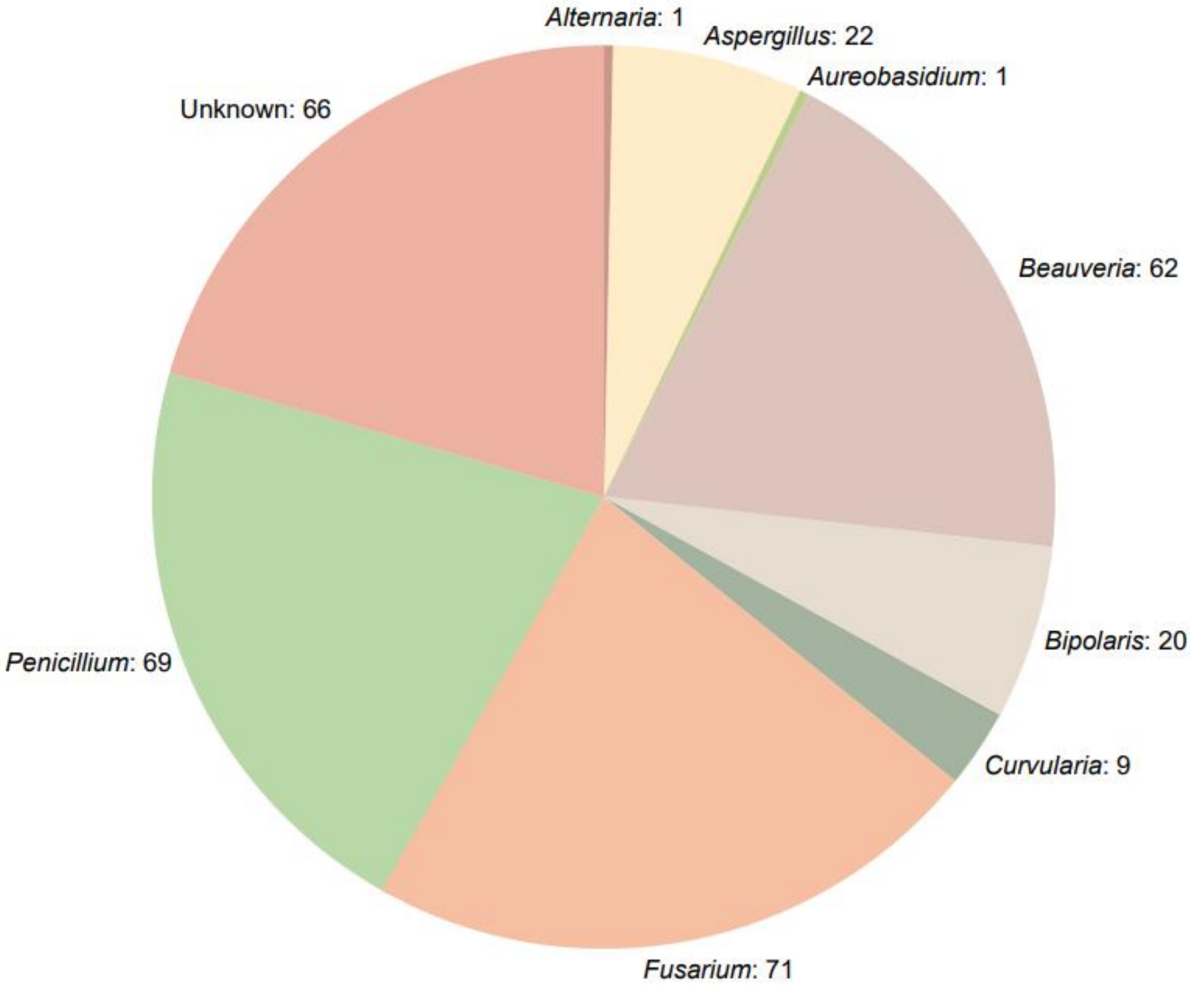

Figure 3. Morphological identifications of 321 isolates obtained from insect cadavers during the study.

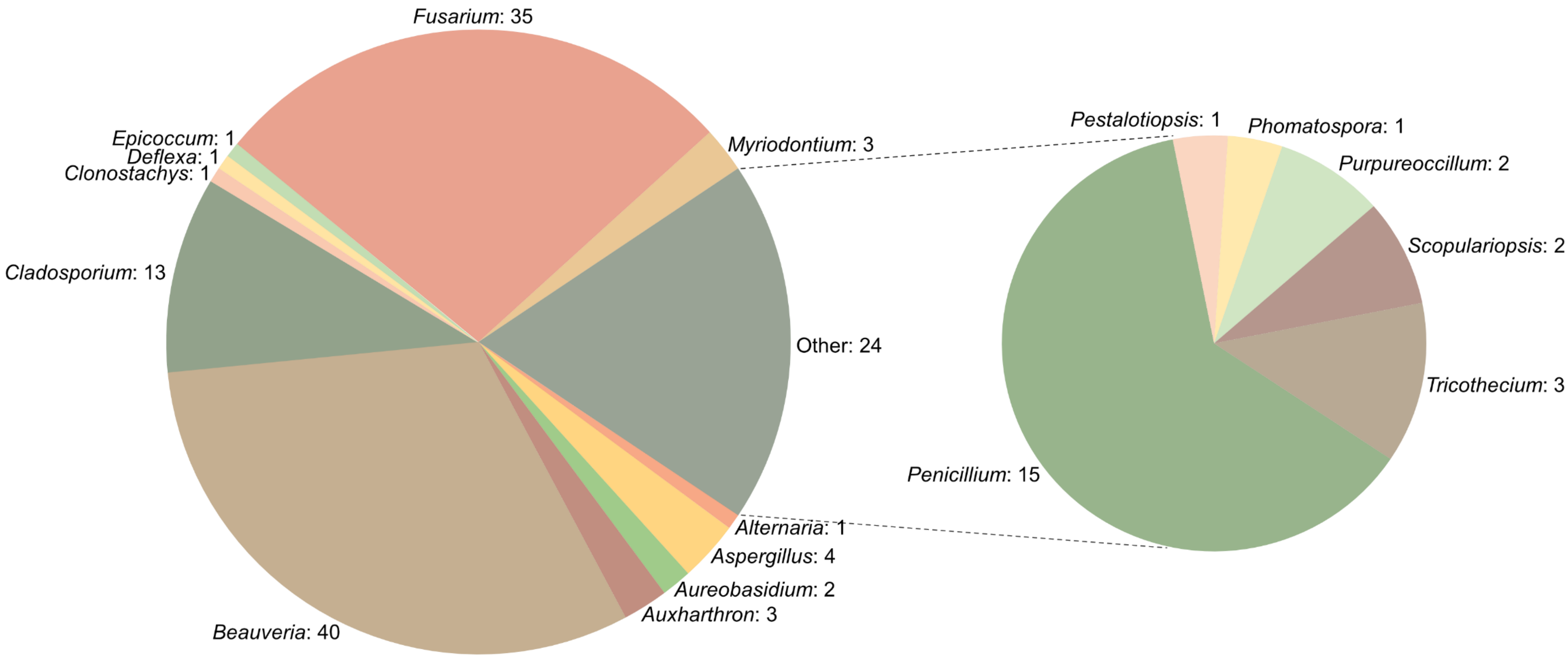

Figure 4. Molecular identifications of 128 selected isolates that were morphologically unidentified or identified as *Beauveria* species during the study.

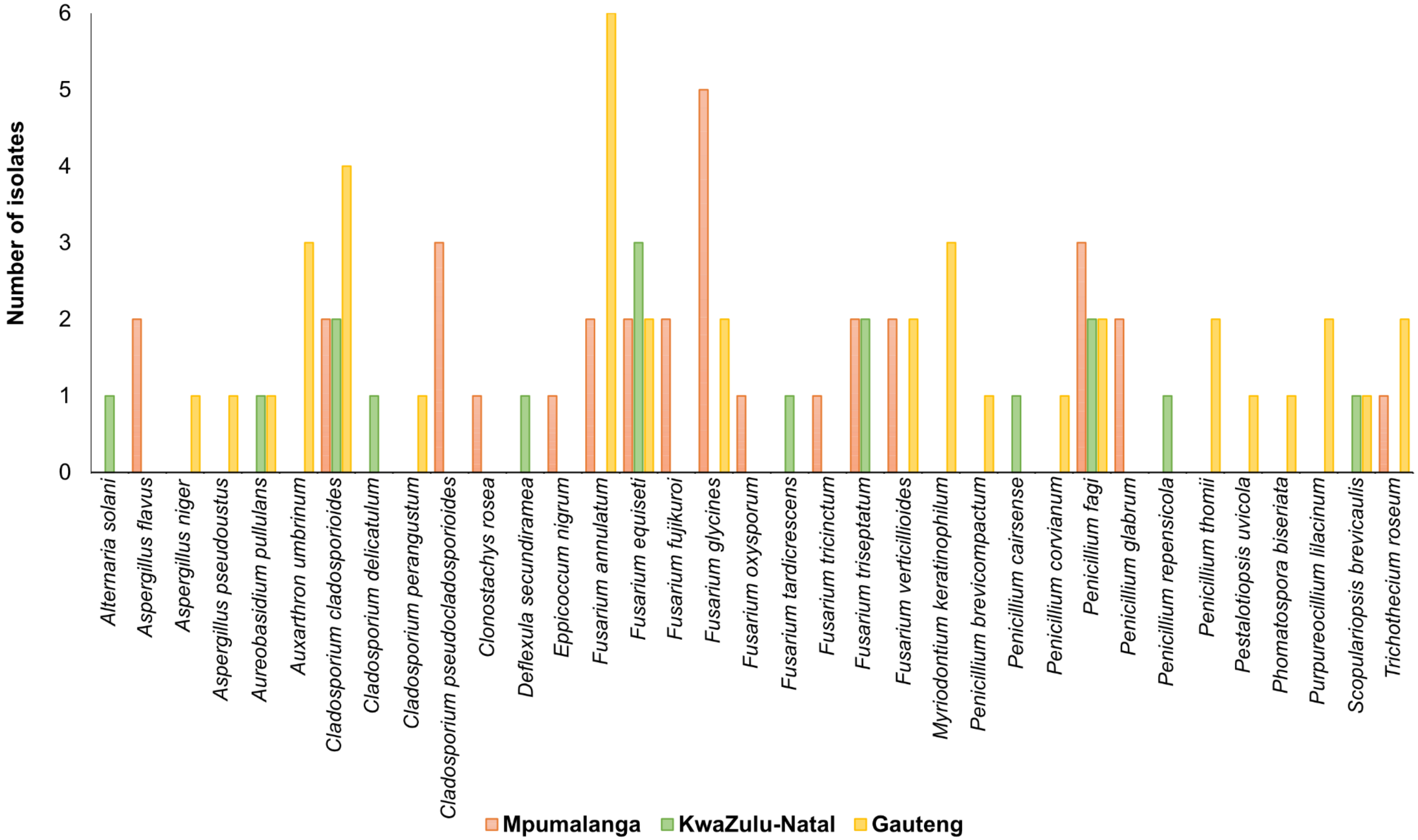

Figure 5. Summary of 128 non-target species isolated in Mpumalanga, KwaZulu-Natal and Gauteng.

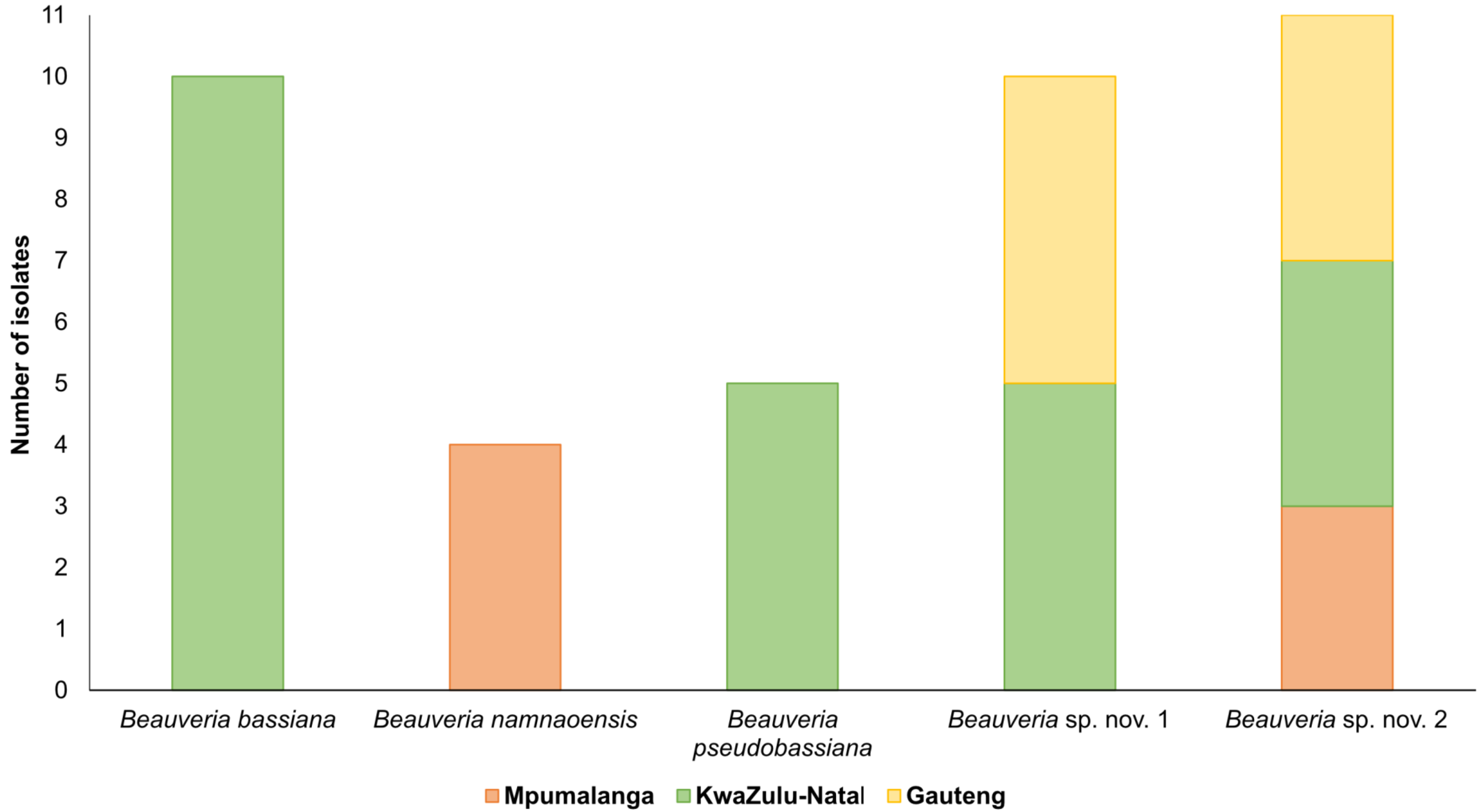

Figure 6. Summary of total number of *Beauveria* isolates collected in Mpumalanga, KwaZulu-Natal and Gauteng from insect cadavers.

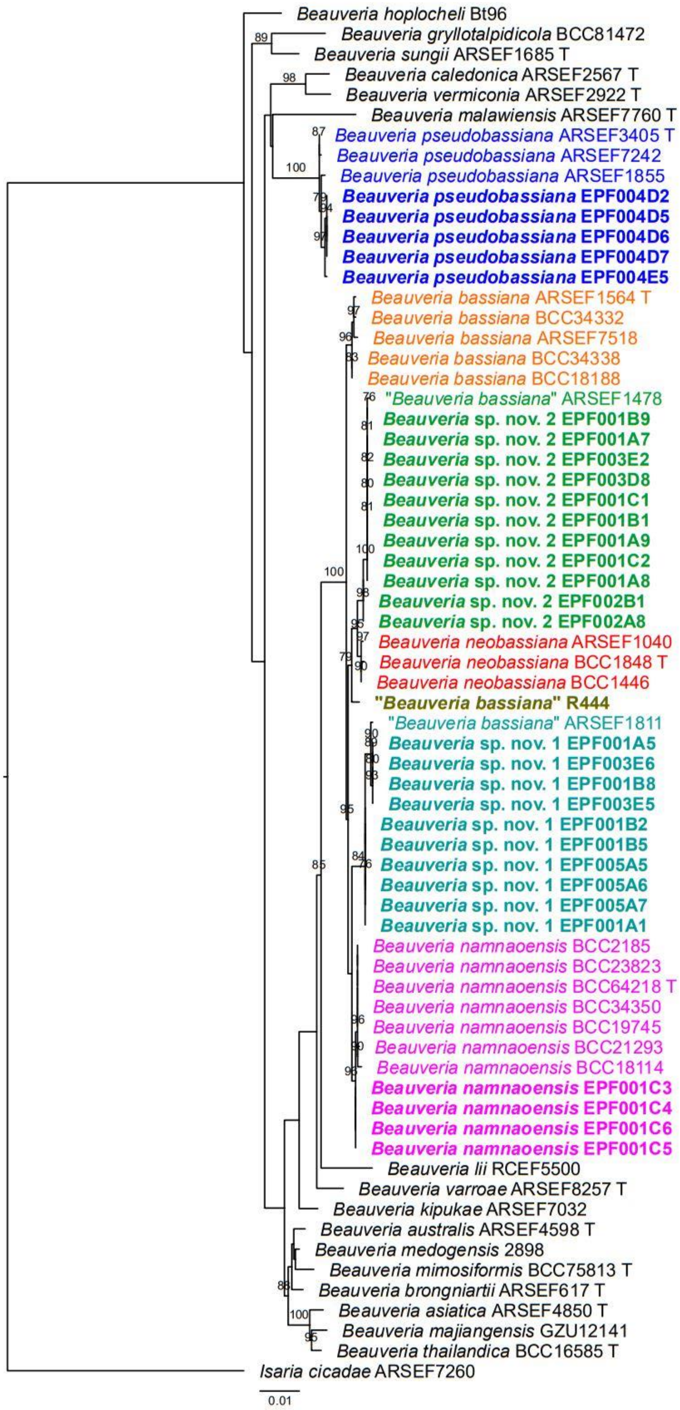

Figure 7. Maximum likelihood combined phylogeny based on *rpb1*, *tef1* and ITS showing the identities of *Beauveria* isolates associated with *Gonipterus* sp. n. 2 collected in various South African *Eucalyptus* plantations. Sequences obtained from ex-type cultures are indicated by T. CBS numbers are included at the end of the strain name. Bootstrap values ≥75% are indicated on the branches. Sequences isolated during this study are indicated in **bold**. *Isaria cicadae* ARSEF7260, was selected as the out group. Clades are represented by different colours. Dark blue represents *B. pseudobassiana* clade, orange represents *B. bassiana*, red represents *B. neobassiana*, pink represents *B. namnaoensis*, teal represents *Beauveria* sp. nov. 1, green represents *Beauveria* sp. nov. 2 and army green represents strain R444.

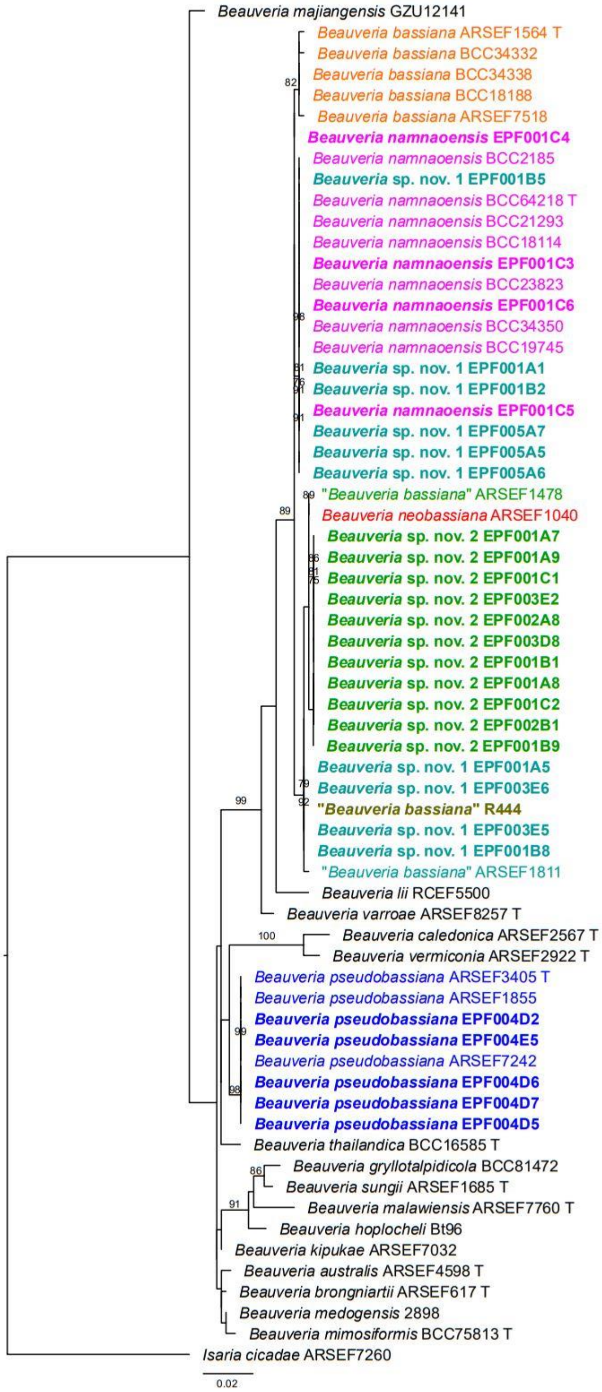

Figure 8. Maximum likelihood phylogeny based on ITS showing the identities of *Beauveria* isolates associated with *Gonipterus* sp. n. 2 collected in various South African *Eucalyptus* plantations. Sequences obtained from ex-type cultures are indicated by T. CBS numbers are included at the end of the strain name. Bootstrap values ≥75% are indicated on the branches. Sequences isolated during this study are indicated in **bold**. *Isaria cicadae* ARSEF7260, was selected as the out group. Clades are represented by different colours. Dark blue represents *B. pseudobassiana* clade, orange represents *B. bassiana*, red represents *B. neobassiana*, pink represents *B. namnaoensis*, teal represents *Beauveria* sp. nov. 1, green represents *Beauveria* sp. nov. 2 and army green represents strain R444.

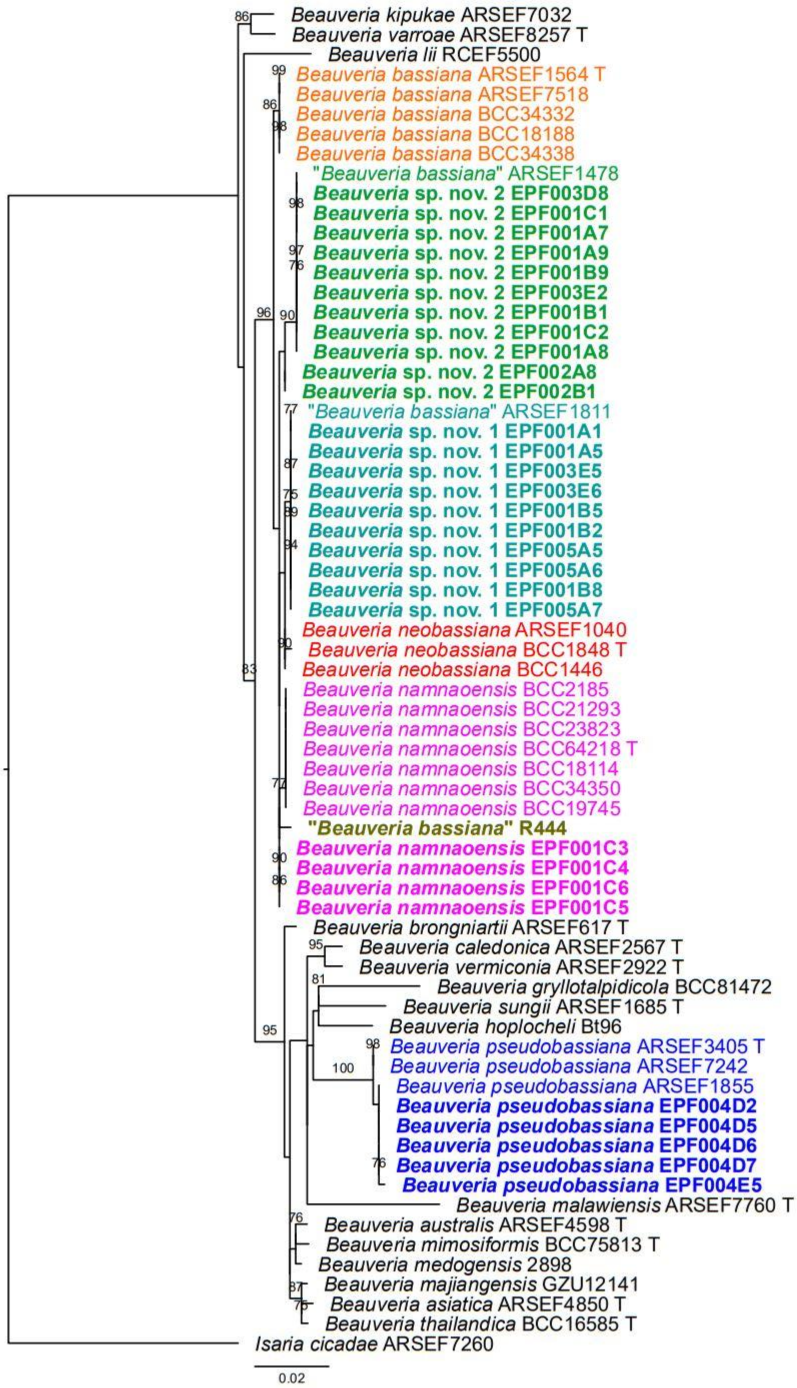

Figure 9. Maximum likelihood phylogeny based on *rpb1* showing the identities of *Beauveria* isolates associated with *Gonipterus* sp. n. 2 collected in various South African *Eucalyptus* plantations. Sequences obtained from ex-type cultures are indicated by T. CBS numbers are included at the end of the strain name. Bootstrap values ≥75% are indicated on the branches. Sequences isolated during this study are indicated in **bold**. *Isaria cicadae* ARSEF7260, was selected as the out group. Clades are represented by different colours. Dark blue represents *B. pseudobassiana* clade, orange represents *B. bassiana*, red represents *B. neobassiana*, pink represents *B. namnaoensis*, teal represents *Beauveria* sp. nov. 1, green represents *Beauveria* sp. nov. 2 and army green represents strain R444.

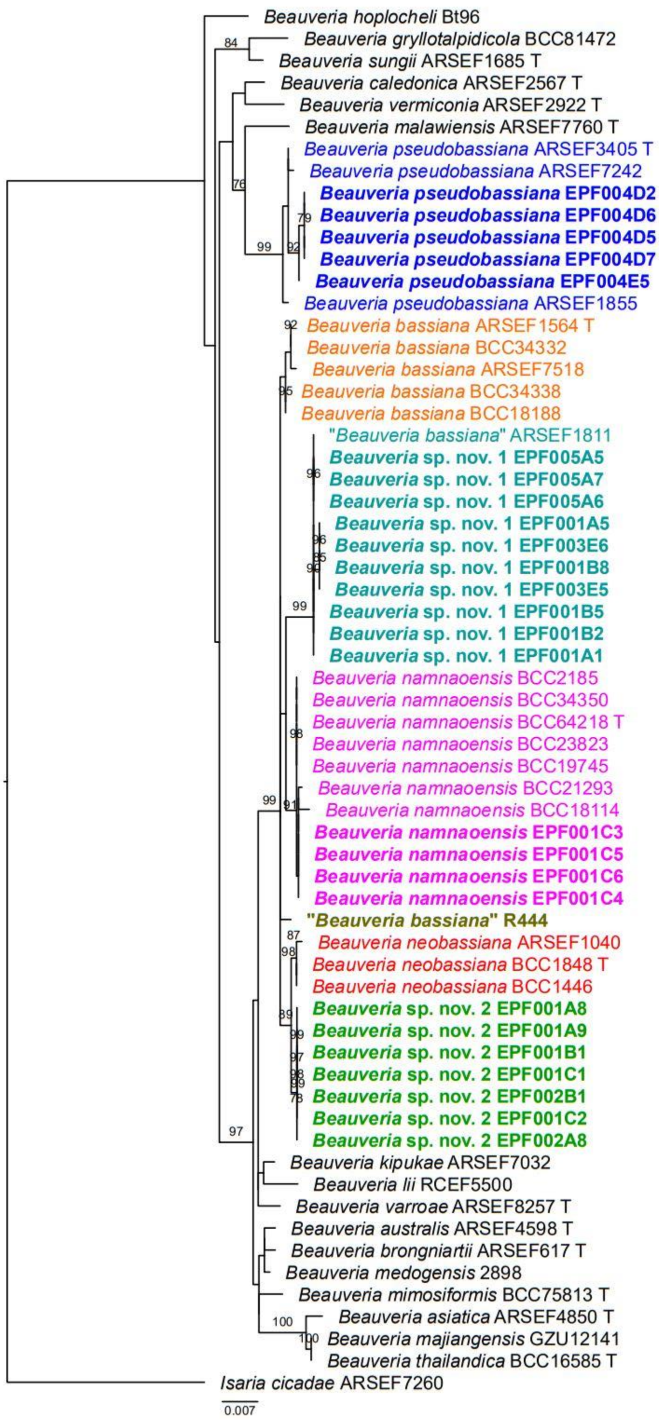

Figure 10. Maximum likelihood phylogeny based on *tef1* showing the identities of *Beauveria* isolates associated with *Gonipterus* sp. n. 2 collected in various South African *Eucalyptus* plantations. Sequences obtained from ex-type cultures are indicated by T. CBS numbers are included at the end of the strain name. Bootstrap values ≥75% are indicated on the branches. Sequences isolated during this study are indicated in **bold**. *Isaria cicadae* ARSEF7260, was selected as the out group. Clades are represented by different colours. Dark blue represents *B. pseudobassiana* clade, orange represents *B. bassiana*, red represents *B. neobassiana*, pink represents *B. namnaoensis*, teal represents *Beauveria* sp. nov. 1, green represents *Beauveria* sp. nov. 2 and army green represents strain R444.

Chapter 3:

Virulence of *Beauveria* (Hypocreales: Cordycipitaceae) cryptic species against *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae)

Abstract

Several biopesticide products are commercially available for implementation in integrated pest management (IPM) programmes, but none are registered for use against forestry pests in South Africa. *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) species is one of the most well-known entomopathogens used in biopesticides in IPM. In recent years, several cryptic species within the *Beauveria bassiana* clade have been identified. One of the recently described species, *B. namnaoensis*, and two novel species, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2, have been isolated from *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae) cadavers in South Africa. This study aimed to compare the virulence of these *B. bassiana* cryptic species on *Gonipterus* sp. n. 2. The virulence of nine *Beauveria* strains, namely one *B. bassiana* strain, one *B. namnaoensis* strain, one *Beauveria* sp. nov. 1 strain, four *Beauveria* sp. nov. 2 strains and two *B. pseudobassiana* strains were compared in the laboratory. A negative control and *Beauveria bassiana* strain R444 as the commercial control were also included. Ten field collected beetles were used per strain. Mortality and fungal growth were recorded daily for 14 days at 26.3 ± 0.05°C and 74.2 ± 0.89% RH. Intra- and interspecific variation in virulence was found. Three strains namely, *B. namnaoensis*, *Beauveria* sp. nov. 1 and *B. pseudobassiana* had the highest virulence with LT_{50} values of five, four and nine days, respectively. The commercial strain showed no pathogenicity on *Gonipterus* sp. n. 2. The virulence potential of these species suggest that it would be worth further evaluating these strains as biological control agents for *Gonipterus* sp. n. 2.

Introduction

Entomopathogenic fungi (EPF) have served as biological control agents against insect pests for over two centuries (Bamisile et al., 2021). This diverse group encompasses unique microbial, soil-dwelling, plant endophyte and saprotrophic species capable of infecting and killing insects by penetration of the host cuticle (Bilgo et al., 2018; Kazartsev and Lednev, 2021; Vega et al., 2008). Their heterogeneity extends across various characteristics and plays pivotal roles in ecological processes, notably in controlling insect populations (Litwin et al., 2020; Roy and Cottrell, 2008). Moreover, EPF hold significant importance in integrated pest management (IPM) strategies, contributing to both classical and augmentative biological control. They induce frequent epizootics within insect populations, showcasing their vital role in sustainable pest management (Lacey et al., 2015; Lord, 2005b; Mascarin and Jaronski, 2016; Mohammed and Hatcher, 2017).

Several commercially available and significant species of EPF include *Beauveria*, *Isaria*, *Lecanicillium* and *Metarhizium* (Jaronski and Mascarin, 2017; Mascarin and Jaronski, 2016; Mascarin et al., 2019). The *Beauveria bassiana* clade is widely utilised in managing various agricultural, forestry and veterinary pests worldwide, representing 40% of global commercial biopesticides (de Faria and Wraight, 2007; Ebani and Mancianti, 2021; Jaronski and Mascarin, 2017; Mascarin and Jaronski, 2016). The earliest recorded attempt to use *Beauveria* was to control chinch bugs, *Blissus leucopterus* Say (Hemiptera: Blissidae), in the mid-1800s, shortly after the description of *B. bassiana*, within the US Midwest (Lord, 2005a; Mascarin and Jaronski, 2016). By 2016, the global market featured 61 commercial products based on *B. bassiana*, including four products originating from South Africa (Mascarin and Jaronski, 2016).

Inter- and intraspecies variation in virulence of *B. bassiana* species have been documented across various studies. Interspecies variation in virulence of *B. bassiana* and *B. pseudobassiana* was reported in previous studies when tested against target insects such as phytophagous pests in South Africa (Goble et al., 2012; Hatting et al., 2019; Maistrou et al., 2020; Romón et al., 2017). Studies that tested various strains of *B. bassiana* against a number of target insects, reported intraspecies variation in virulence (Hussain et al., 2016; Maistrou et al., 2020; Valero-Jiménez et al., 2014). Jordan et al. (2021) recorded intraspecies variation in virulence of several *B. bassiana* strains that have been isolated from *Gonipterus platensis* Marelli (Coleoptera: Curculionidae).

Cryptic species within the *B. bassiana* clade have been discovered, bearing significant implications for the utilisation of EPF in IPM (Clifton et al., 2023; Kobmoo et al., 2021; Wang et al., 2022). Currently, the variation in virulence among cryptic species on different insect hosts remain poorly understood. Advances in identification techniques, such as chemical profiling, Next Generation Sequencing (NGS), genomic data and multigene phylogenetic analyses, have led to the description of more novel *Beauveria* species (Clifton et al., 2023; Khonsanit et al., 2020; Kobmoo et al., 2021; Wang et al., 2022).

Recent studies have confirmed that *B. bassiana* species, both known and cryptic, directly sourced from the environment, exhibit higher virulence compared to commercial *Beauveria* products (Clifton et al., 2023; Jordan et al., 2021; Quesada-Moraga et al., 2023; Wang et al., 2022). In a study conducted in Brazil, *B. bassiana* species collected from natural environments demonstrated 95% mortality against target pests, contrasting with the 5% mortality recorded with *B. bassiana* commercial products (Jordan et al., 2021). Clifton et al. (2023) and Wang et al. (2022) highlighted the presence of cryptic species within *Beauveria bassiana* during efficacy tests of certain strains against insect pests, revealing variations in virulence among these cryptic species. The comparison of the virulence and efficacy of recently described cryptic species, *B. namnaoensis* and *B. neobassiana* (Kobmoo et al., 2021), with known *Beauveria* species remains unclear. Furthermore, the *B. bassiana* cryptic species identified by Kobmoo et al. (2021) were also found in South Africa in association with *Gonipterus* sp. n. 2 (Coleoptera: Curculionidae) (**Chapter 2**).

Gonipterus sp. n. 2, known as the Eucalyptus snout beetle, is a significant economic pest of *Eucalyptus* forestry in South Africa. Originating from south east Australia, its presence was first noted in South Africa in 1916 (Tooke, 1955). It forms part of the *Gonipterus scutellatus* species complex, together with *G. platensis* Marelli and *G. pulverulentus* Lea (Schröder et al., 2020). Adults and larvae feed on young leaves, causing a distinct scalloped appearance, with larvae accounting for the majority of the damage inflicted (Mally, 1924). To manage the populations of *Gonipterus* sp. n. 2, the egg parasitoid *Anaphes nitens* Girault (Hymenoptera: Mymaridae), native to Australia, was introduced into South Africa as a classical biological control agent in 1926 (Tooke, 1955). *Anaphes nitens* females lay a single egg within the capsule of a *Gonipterus* egg. Upon hatching, *A. nitens* larvae consume the yolk of their host egg (Tooke, 1955). The complete life cycle of *A. nitens* spans between 17–32 days (Santolamazza-Carbone et al., 2006; Tooke, 1955). Despite the introduction of *A. nitens*, outbreaks of *Gonipterus* sp. n. 2 still occur frequently (Cordero Rivera et al., 1999; Huber and Prinsloo, 1990; Loch, 2008; Schröder et al., 2020; Valente et al., 2004). Consequently, further investigation into additional biological control strategies is necessary to effectively manage these insect populations and ease the considerable economic impact caused by *Gonipterus* sp. n. 2.

Entomopathogens such as *Beauveria* spp. have the potential to be used as biological control agents against *Gonipterus* spp., as demonstrated with commercial products (Dara et al., 2019; Echeverri-Molina and Santolamazza-Carbone, 2010; Mejía et al., 2024). A recent study in Brazil, showed that wild strains of *Beauveria* species isolated from *G. platensis* cadavers not only cause high mortality, but also displayed variation in virulence and efficacy between isolates (Jordan et al., 2021; Kobmoo et al., 2021; Talaei-Hassanloui et al., 2006). Their results indicated that an optimal strategy to implement EPF as biopesticides in *Gonipterus* IPM in South Africa would be to identify and understand the diversity of wild *Beauveria* strains in the South African plantations associated with *Gonipterus* sp. n. 2.

The objective of the study was to test the pathogenicity and virulence of two *B. bassiana* cryptic species naturally associated with *Gonipterus* sp. n. 2 in South African *Eucalyptus* plantations against adults of *Gonipterus* sp. n. 2. The first aim of the study was to determine if these wild strains of *B. bassiana* cryptic species were pathogenic against *Gonipterus* sp. n. 2 under laboratory conditions. The second aim was to evaluate inter- and intraspecies variation in virulence of *B. bassiana* cryptic species, *B. namnaoensis* and *B. neobassiana*, against *Gonipterus* sp. n. 2. Results from this study are valuable to determine the potential use of these EPF as biopesticides against *Gonipterus* sp. n. 2 in South Africa.

Materials and methods

a. Collection of *Gonipterus* beetles

Gonipterus sp. n. 2 beetles were collected from plantations in KwaZulu-Natal Midlands. The adults were fed fresh shoots of *Eucalyptus dunni* (Myrtales: Myrtaceae) obtained from the biocontrol facility nursery. Adult beetles were kept in clean containers with fresh leaves and sterile paper towel to prevent moisture buildup in the containers. Leaves were substituted three times weekly with fresh leaves before the containers were placed back in the incubators until the insects were needed for the virulence trials. The incubators (Memmert IPP260 Plus: 640 mm W x 800 mm H x 468 mm D, with 32 mm Peltier fan) were set at 22°C with 14:10 h light and dark photoperiods.

b. Fungal strain selection

The fungal strains selected for the virulence trials were isolated from *Gonipterus* sp. n. 2 cadavers collected in Mpumalanga, KwaZulu-Natal and Gauteng (**Table 1**; **Chapter 2**). The identities of the selected strains were confirmed through molecular analyses of three gene regions (ITS, *rpb1* and *tef1*). In total, nine representative strains (pure isolates of a fungus) were randomly selected for virulence trials. The representatives were only subsets of the species and strains isolated from **Chapter 2** to assess their inter- and intraspecies variation in virulence. These representatives were made up of two *B. pseudobassiana* strains, one *B. namnaoensis* strain, one *Beauveria* sp. nov. 1 strain and four *Beauveria* sp. nov. 2 strains with an additional *B. bassiana* strain (**Table 1**). The commercial *B. bassiana* strain (R444) was used as reference. Sterile SABAX water (Adcock Ingram Critical Care (PTY) LTD, Johannesburg) was used as the negative control.

c. Strain viability

Prior to the virulence trials, spores of the strains were harvested from 14-day old cultures on MEA media by superficial scraping with a sterile scalpel. Spore suspensions for each strain were prepared by adding the scraped spores to sterile SABAX water and sterile Tween-60 solution (0.1% v/v) to prevent hydrophobic interactions with the insect cuticle. Viability of the spores was assessed by spreading 100 µl spore suspension (10^6 spore ml^{-1}), prepared with sterile SABAX water, on MEA media in 60 X 15 mm petri dishes and incubated at approximately 25°C for 16 h prior to evaluation. Spores were scored as viable if the germ tube was the length of the spore diameter (Mustafa and Kaur, 2010). A total of 100 spores were scored per sample under 40 X magnification using a phase-contrast microscope. Only strains with a spore viability above 90% were considered viable. Similar viability tests were conducted on the commercial *B. bassiana* strain (R444).

d. Spore concentration

The concentration of the fungal spore suspension was determined using a haemocytometer at 40 X magnification using a light microscope. The concentration of the commercial *B. bassiana* strain (R444) was prepared similar to other treatments to keep the consistency. Twenty microlitres of the spore suspension was pipetted to each side (side 1 and side 2) of the haemocytometer under a cover slip. Each side had nine quadratic squares. The number of spores in five quadratic squares (i, ii, iii, iv and v) were counted using a light microscope. The area of a quadratic square is equivalent to 1 mm^2 (1 mm x 1 mm). The depth of the haemocytometer view side is 0.1 mm. Thus, the volume of a quadratic square is equivalent to 0.1 mm^3 (0.1 mm x 1mm^2). Furthermore, 0.1 mm^3 is equivalent to 0.1 µl (or 100 nl). Virulence trials with 10^6 spores ml^{-1} were done. Spore concentrations were similar to those recommended for the commercial strain formulation as a base-reference for all treatments during the trials for similarity as the recommended dose is not stipulated elsewhere (Mascarin and Jaronski, 2016). All spore concentrations were recorded (**Table 2**).

Average number of spores per square:

$$\mathbf{A} = \frac{\text{side 1(i+ii+iii+iv+v)+side 2(i+ii+iii+iv+v)}}{10}$$

Total number of cells in 1 ml:

$$\mathbf{B} = \frac{1000 \text{ x } \mathbf{A}}{0.1 \text{ (Volume of one quadratic square)}}$$

e. Virulence trials

Virulence trials were conducted with spore suspensions prepared as described above. Trials were conducted twice with new batches of spore suspensions to ensure spore viability. Experimental units consisted of 10 adult beetles per strain, kept in separate petri-dishes for the duration of both trials. The petri-dishes containing the adult beetles were placed in 9.5 L plastic containers. NaCl suspensions (75 g NaCl/100 ml H2O) were placed into the 9.5 L containers alongside the petri-dishes to sustain the constant RH, expected to be 75% and measured using an i-Button (iButton® hygrochron, DS1923-F5#). Due to NaCl's hygroscopicity (the ability to absorb moisture from the environment), it was expected to stabilise the RH within the containers (Zhang et al., 2020a). The NaCl solutions were replaced weekly in separate petri dishes. The 9.5 L containers were sealed to ensure stable temperature and humidity during the duration of the trials. Temperature and humidity within the 9.5 L containers were monitored with the aid of an i-Button that recorded environmental data within the 9.5 L containers during both trials at two-hour intervals. Temperature and RH were recorded at 26.3 ± 0.05°C and 74.2 ± 0.89%, respectively. Virulence trials took place over a period of two weeks while data were recorded.

f. Inoculation and observation

During inoculation, spore suspensions of 10 µl containing 10^6 spores ml^{-1} and sterile Tween-60 solution (0.1% v/v) of each fungal strain was pipetted directly on the insect cuticle. Once inoculated, the insects remained in petri dishes for 24 h without a food source where conditions were kept to approximately 26.3 ± 0.05°C and 74.2 ± 0.89% RH to ensure residual contact of the spores with the target insects. The method ensured direct contamination by topical spore deposition on the insect cuticle. After the 24 h incubation period, the insects were fed fresh *Eucalyptus* leaves that were replaced daily.

Adult beetles were monitored daily for two weeks in order to assess mortality and fungal growth. For the observation of fungal growth, once deceased, cadavers were removed from their original 9.5 L containers and placed in new 9.5 L containers where they were incubated at approximately 26.3 ± 0.05°C and 92.2 ± 2.0% RH for two additional weeks and conditions measured with an i-Button.

g. Confirmation of strain identifications

Once fungal growth was observed, fungal hyphae were isolated from insect cadavers, plated onto MEA media and isolates were identified based on DNA sequences. The DNA of the fungi was extracted using the Zymo Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research Corporation, California, USA) as suggested by the manufacturers. This step ensured to exclude any potential fungal spores that were introduced with the field collected beetles from the fungal strains used to infect the beetles through phylogenetic analysis and molecular identification.

PCR reactions were performed in a 25 µl total reaction mixture composed of 17.5 µl demineralised sterile water, 5 µl MyTaq™ PCR buffer (5 x MyTaq™ reaction buffer: 5 mM dNTPs, 15 mM $MgCl_2$, Bioline Reagents Ltd, United Kingdom), 0.5 µl of each primer (0.1 µM primer) and 0.15 µl MyTaq™ polymerase (2.5 U MyTaq™ DNA polymerase, Bioline Reagents Ltd, United Kingdom). The PCR conditions and primers used for PCR and sequencing of ITS, *rpb1* and *tef1* are given in **Table 3**. PCR amplicons were separated using 1% agarose (SeaKem® LE Agarose, Lonza Bioscience) gel electrophoresis at 80 V for 40 min. PCR products were stained using GelRed® Nucleic Acid Gel Stain (Biotium, Hayward, California, USA) and electrophoresed alongside a 0.5 µg/µl GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific). The results were digitally photographed for visualisation under UV light using a Bio-Rad Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., USA).

The PCR products were cleaned using ExoSAP-IT™ PCR clean-up reagent (1 U/µl FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific), 20 U/µl Exonuclease I (Thermo Fisher Scientific)) according to the manufacturer's instructions. Sequencing reactions were set up using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the same sets of primers used during PCR amplification (**Table 3**). Sequencing products were cleaned using sodium acetate precipitation. Sequencing was conducted at the Sanger Sequencing Facility of the University of Pretoria (Bioinformatics and Computational

Biology Unit, v 19.8.22) using an ABI PRISMTM 3500xl Auto-sequencer (Applied Biosystems, Foster City, CA, USA). The forward and reverse sequences of each strain were assembled into contigs with Geneious Prime 2019 (BioMatters Ltd., Auckland, New Zealand) and manually edited where necessary to remove any errors that might have been incorporated during sequencing.

All sequences were aligned using MAFFT v.7.305b (Katoh and Standley, 2013), using G-INS-I algorithm and were manually adjusted in Geneious Prime where needed. Prior to analysis, Neighbour Joining (NJ) analysis was performed on each individual dataset to determine any discrepancies. Individual and multi-gene maximum likelihood phylogenies were constructed using IQTree v.1.6 (Nguyen et al., 2014) and visualised in FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). The most suitable model for each partition was determined using ModelFinder (Kalyaanamoorthy et al., 2017) and bootstrapping was determined using UFBoot (Minh et al., 2013). Both ModelFinder and UFBoot are integrated into the IQtree software. After visualising the trees in FigTree, final editing was done in Affinity Designer v.1.6.1 (2019 Serif (Europe) Ltd, Nottingham, UK).

h. Data analysis

The virulence of the treatments was evaluated according to the percentage overall mortality, estimated survival curves and the number of days it took for 50% of the beetles to die (LT_{50}). For mortality assessment, logistic regression within a Generalised Linear Mixed Model (GLMM) was applied to the beetle mortality data. The 'lme4' package in R was used for this analysis, treating the two trials as random effects in the mixed logit model (Jaeger, 2008; Mascarin et al., 2013). F-statistics, derived from a Wald-type III test for strains (fixed effect) in the mixed logit model, explained significant differences between means. Significantly different means were identified by Tukey’s honestly significant difference (Tukey’s HSD) test at $p \leq 0.05$.

Kaplan-Meier survival curves were used to determine the survival probability and LT_{50} values across the 14-day observation period. Utilising the 'survival' package in R, specifically the 'survfit' function, we compared the survival curves among the various fungal strains. The Cox proportional hazards model aided in this comparison in order to assess the relationships between the survival time of the beetles and the strains treated with, while accommodating censored data (Bender et al., 2005; Clifton et al., 2023). Estimated LT_{50} values were derived from the Kaplan-Meier survival curves, and comparisons were based on the overlap of their 95% confidence intervals (95% CI). Data from both trials were combined for this survival analysis.

All analyses were conducted in RStudio version 4.2.1 (R Core Team, 2022). This comprehensive approach allowed for a robust evaluation of fungal virulence and survivorship dynamics among the beetle populations under different fungal strains.

Results

All selected strains had a spore viability of >90%. All EPF originally isolated from *Gonipterus* sp. n. 2 cadavers (**Table 1**) were pathogenic against *Gonipterus* sp. n. 2 adults, with varying levels of virulence (Type III Wald chi-square test=45.96; df=10; $p<0.001$) (**Figure 1**). The average mortality observed for insects in the control treatment was 5%. No mortality was recorded for the insects treated with the commercial strain (Eco-BB®: strain R444). For the remaining treatments, virulence ranged from 10% to 95%. Groups treated with *B. namnaoensis* (EPF001C5), *Beauveria* sp. nov. 1 (EPF005A6) and *B. pseudobassiana* (EPF004D7) strains had the highest mortality ranging between 65% and 95%, which was significantly higher than the mortality observed in the beetle groups treated with the control (5%), as well as with *B. pseudobassiana* (EPF004E5) and two *Beauveria* sp. nov. 2 (EPF001C1; EPF002B1) strains, which ranged between 10% and 30%. Strains of *B. bassiana* (EPF004E9) and *Beauveria* sp. nov. 2 (EPF001A7; EPF3D8) did not differ significantly to the other treatments (**Figure 1**).

The Kaplan-Meier survival curves clearly indicated that beetles showed high survival in the negative control and commercial strains (**Figure 2**). Furthermore, high survival was recorded for two *Beauveria* sp. nov. 2 strains (EPF001C1; EPF002B1) and one *B. pseudobassiana* strain (EPF004E5), with more than half of the population surviving by the end of the 14-day trials. Moderate survival was recorded for strains of *B. bassiana* (EPF004E9), *Beauveria* sp. nov. 2 strains (EPF001A7; EPF003D8) and *B. pseudobassiana* (EPF004D7) with at least half of the population dead by the end of the 14-day trials (**Figure 2**). The lowest survival of beetle populations was recorded for *B. namnaoensis* (EPF001C5) and *Beauveria* sp. nov. 1 (EPF005A6) with the majority of the beetle populations dead by the end of the trials (**Figure 2**).

All isolates decreased survival rates of *Gonipterus* sp. n. 2 adults with the exception of Eco-BB® strain R444 and the control treatment (Cox Proportional Hazards model: Concordance= 0.672 ± 0.032; Likelihood ratio test= 45.96 on 10 df, $p< 0.05$; Wald test=

9.37 on 10 df, p=0.5; Logrank test= 29.33 on 10 df, p< 0.001). The LT_{50} values calculated from the Kaplan-Meier survival curves indicated that *B. namnaoensis* (EPF001C5) and *Beauveria* sp. nov. 1 (EPF005A6) killed 50% of the adult beetles at five- and four-days post-inoculation, indicating the highest virulence that corresponds to the Kaplan-Meier survival curves (**Figure 2**, **Table 4**). *Beauveria* sp. nov. 2 strains (EPF001C1; EPF002B1), as well as *B. pseudobassiana* (EPF004E5) failed to kill half of the adult beetle populations within 14 days and thus no LT_{50} values could be calculated (**Figure 2**, **Table 4**). The rest of the LT_{50} values were measured at six, seven, nine and 11 days for strains of *Beauveria* sp. nov. 2 (EPF001A7: EPF003D8), *B. pseudobassiana* (EPF004D7) and *B. bassiana* (EPF004E9), respectively (**Table 4**).

All beetle mortalities were confirmed to have occurred due to *Beauveria* infection. Sporulation was observed on all *Gonipterus* cadavers (**Figure 3**). The identifications of the spores were confirmed to belong to the strains chosen for the virulence trials through DNA sequencing. The concatenated phylogeny displays the identifications of these strains in relation to the original selected isolates (**Figure 3**).

Discussion

This study was the initial evaluation of *B. namnaoensis* along with two novel species, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2, regarding their virulence against *Gonipterus* sp. n. 2. It confirmed the presence of both inter- and intraspecies variation in the virulence of *B. bassiana* cryptic species against *Gonipterus* sp. n. 2. Among the strains tested, a *B. namnaoensis* strain (EPF001C5) and a *Beauveria* sp. nov. 1 strain (EPF005A6) exhibited the highest virulence against *Gonipterus* sp. n. 2. In contrast, two strains of *Beauveria* sp. nov. 2 (EPF001C1 and EPF002B1) displayed the lowest virulence among the tested *B. bassiana* cryptic species. Notably, all inoculated beetles exhibited fungal sporulation within 14 days following mortality due to infection.

Inter- and intraspecies variation in virulence between *B. bassiana* species have been observed in previous studies (Clifton et al., 2023; Jordan et al., 2021; Kryukov et al., 2010; Talaei-Hassanloui et al., 2006; Valero-Jiménez et al., 2014; Wang et al., 2022; Wraight et al., 2010). Additionally, variation in virulence between *Beauveria* cryptic species have also been documented (Clifton et al., 2023; Wang et al., 2022). Accurate identification of cryptic species will improve the knowledge of their virulence potential against different host insects when compared to known species on the same host. This study successfully assigned species names to the *Beauveria* strains used in the virulence trials, thereby contributing to a clearer comprehension of their identities.

Environmental factors, such as temperature and relative humidity, significantly influence both inter- and intraspecies variation in EPF virulence. Jaronski (2010) highlighted intraspecies variation in the virulence of *B. bassiana* strains under different temperature conditions, noting strains adapted to temperatures as low as 5°C and others displaying higher efficacy at temperatures exceeding 30°C (Acheampong et al., 2020; Jaronski, 2010; Kryukov et al., 2012). The impact of relative humidity spans a wide range, approximately 40%–98%, significantly affecting EPF virulence towards host insects (Acheampong et al., 2020; Fernández-Bravo et al., 2016; Jaronski, 2010; Quesada-Moraga et al., 2023; Quesada-Moraga et al., 2006; Valero-Jiménez et al., 2014). These environmental nuances play a pivotal role in shaping the effectiveness of EPF in combatting insect pests across varied conditions.

The present study evaluated various *Beauveria* cryptic species under constant temperature of 26.3 ± 0.05°C and 74.2 ± 0.89% RH, revealing notable interspecies variation in virulence. Comparatively, *B. bassiana* exhibited higher virulence than *B. pseudobassiana* and other *Beauveria* species towards a similar insect host under stable conditions (Jaronski, 2010; Maistrou et al., 2020; Romón et al., 2017). Specifically, *B. namnaoensis* and *Beauveria* sp. nov. 1 demonstrated considerably greater virulence when contrasted with *Beauveria* sp. nov. 2 and *B. pseudobassiana* within the same temperature and RH parameters. Furthermore, the study delved into the intraspecies variation in virulence among *Beauveria* species under this temperature and RH conditions (Jordan et al., 2021; Quesada-Moraga et al., 2023; Quesada-Moraga et al., 2006; Valero-Jiménez et al., 2014). Notably, four strains of *Beauveria* sp. nov. 2 (EPF001A7; EPF001C1; EPF002B1; EPF003D8) and two strains of *B. pseudobassiana* (EPF004D7; EPF004E5) exhibited intraspecies variations in virulence against *Gonipterus* sp. n. 2 at constant conditions. Temperature and RH is known to play a role in the virulence of EPF (Acheampong et al., 2020; Jaronski, 2010; Quesada-Moraga et al., 2023), therefore, further investigation under a range of different representative field conditions will need to be obtained for a better understanding of variation in virulence between strains. Changes in these environmental factors may yield varying outcomes based on the strain employed due to their distinct temperature and RH thresholds (Clifton et al., 2023; Coombes et al., 2016; Jaronski, 2010; Quesada-Moraga et al., 2023).

The origin of an EPF strain significantly influences its capacity to withstand varying temperatures and fluctuations in RH (Cao et al., 2022; Coombes et al., 2016; Jaronski, 2010; Quesada-Moraga et al., 2023). Entomopathogenic fungi demonstrate a remarkable ability to adapt to their natural habitats, enhancing their overall environmental competence (Robinson, 2001). Notably, native EPF species exhibited superior effectiveness as potential biological control agents when compared to non-native strains due to their adeptness in their specific environments (Clifton et al., 2023; Quesada-Moraga et al., 2023; Valero-Jiménez et al., 2014). A study documented the robust adaptation of *B. bassiana* strains to arid environments, despite their preference for higher optimal RH (Fernández-Bravo et al., 2016). While this study didn't specifically investigate the origins of individual strains, it prompts a hypothesis that the origin of each strain could have an effect on its virulence when temperature and RH were kept constant that was not representative of their original environment.

The efficacy of EPF can be influenced by various characteristics of their insect hosts (Jaronski, 2010; Quesada-Moraga et al., 2023). Host behaviours, such as behavioural fever or chilling, wherein insects deliberately raise or lower their body temperatures above or below EPF thresholds, significantly impact the EPF virulence towards host species (Carruthers et al., 1992; Jaronski, 2010; Liu et al., 2019). It remains unknown whether *Gonipterus* sp. n. 2 exhibits any behavioural mechanisms to counter EPF infection. In certain beetle species, like the red flour beetle, *Tribolium casteneum* Herbst (Coleoptera: Tenebrionidae), the secretion of defensive compounds called benzoquinones onto their cuticles renders them resistant to *B. bassiana* infection (Pedrini et al., 2015). Similarly, the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) demonstrates an antioxidant immune response when confronted with EPF species. During infection, it increases the expression of catalase and peroxidase genes as a defensive measure (Hussain et al., 2017; Puertollano et al., 2011). Further research is imperative to gain a deeper understanding of the influence of host-mediated effects on both inter- and intraspecies variations in the virulence of EPF species (Quesada-Moraga et al., 2023).

The lack of pathogenicity of the commercial strain observed in the present study could be due to some degree of specificity. Notably, the commercial product used during the study *Beauveria* strain (R444) primarily targets red spider mites, lepidopteran pests and other agricultural pests rather than Coleopteran species (Mascarin and Jaronski, 2016). Consequently, this explanation could shed light on why the commercial product exhibited no pathogenic effects towards adult *Gonipterus* sp. n. 2 (Deka et al., 2021; Wang et al., 2022; Wang et al., 2020). The virulence of an EPF strain can also pivot on the phenotypic adaptability of the target host and genetic variations among EPF strains within a single species (Quesada-Moraga et al., 2023). Nevertheless, comprehensive investigations into the impact of genetic variation on both inter- and intraspecies variations in virulence are key for a thorough comprehension of the specificity and virulence of EPF species towards their respective insect hosts (Clifton et al., 2023; Zhang et al., 2020b).

A limit is placed on the production of novel biopesticides for commercial use, due to limited research on the number of known virulent strains (Clifton et al., 2023; Jordan et al., 2021; Mascarin et al., 2013). The global utilisation of a small number of fungal strains poses challenges as it diminishes the likelihood of success across regions, primarily due to a lack of environmental adaptability (Quesada-Moraga et al., 2023). Hence, it is imperative not only to comprehend virulence but also to establish production methods capable of sustaining the virulence of these species during large-scale manufacturing (FitzGerald et al., 2016; Mascarin and Jaronski, 2016; Mascarin et al., 2019).

Beauveria bassiana species are suitable for implementation alongside biological control strategies (Dara et al., 2019; Echeverri-Molina and Santolamazza-Carbone, 2010; Pérez-Otero et al., 2003). The current study highlights the potential of *B. bassiana* species as effective biological control agents, consistent with prior research (Dara et al., 2019; Echeverri-Molina and Santolamazza-Carbone, 2010). Additionally, two separate studies highlighted the compatibility of *B. bassiana* species with parasitoids, affirming their integration potential within classical biological control methods (Mohammed and Hatcher, 2017; Pérez-Otero et al., 2003).

The study revealed the pathogenicity of *B. bassiana* cryptic species against *Gonipterus* sp. n. 2, highlighting both inter- and intraspecies variations in virulence. However, future studies should consider testing all strains isolated from *Gonipterus* sp. n. 2 for their potential in inter- and intraspecies variation of virulence. *Beauveria namnaoensis* (EPF001C5) and *Beauveria* sp. nov. 1 (EPF005A6) emerge as promising candidates for potential biological control agents in *Eucalyptus* IPM. Notably, the absence of commercial biopesticides tailored for South African forestry opens avenues for developing these wild strains for this specific purpose. Future investigations aimed at enhancing our understanding of *B. bassiana* cryptic species' interactions could involve assessing their virulence against the larval stage of *Gonipterus* sp. n. 2. Moreover, delving into the influence of environmental conditions on the variations in the virulence of *B. bassiana* cryptic species warrants further exploration.

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Table 1. Representative strains from various localities in South Africa that were selected for virulence trials to test their efficacy against *Gonipterus* sp. n. 2.

Treatment	Species	Strain name	Locality
1	SABAX (negative control)	N/A	N/A
2	*B. bassiana*	R444	Unknown
3	*B. bassiana*	EPF004E9	KwaZulu-Natal
4	*B. namnaoensis*	EPF001C5	Mpumalanga
5	*Beauveria* sp. nov. 1	EPF005A6	JHB-West
6	*Beauveria* sp. nov. 2	EPF001A7	KwaZulu-Natal
7	*Beauveria* sp. nov. 2	EPF001C1	Mpumalanga
8	*Beauveria* sp. nov. 2	EPF002B1	JHB-West
9	*Beauveria* sp. nov. 2	EPF003D8	Biocontrol facility
10	*B. pseudobassiana*	EPF004D7	KwaZulu-Natal
11	*B. pseudobassiana*	EPF004E5	KwaZulu-Natal

Table 2. Spore concentrations (10^6 spore/ml) of selected *Beauveria* strains obtained and adjusted prior to both virulence trials.

Treatment	Species	Strain name	Spore count (spores/ml)	
			Trial 1	**Trial 2**
1	SABAX	N/A	N/A	N/A
2	*B. bassiana*	R444	4×10^6	4×10^6
3	*B. bassiana*	EPF004E9	6.1×10^6	1.5×10^6
4	*B. namnaoensis*	EPF001C5	3.8×10^6	1.3×10^6
5	*Beauveria* sp. nov. 1	EPF005A6	8.3×10^6	1.3×10^6
6	*Beauveria* sp. nov. 2	EPF001A7	6.5×10^6	1.3×10^6
7	*Beauveria* sp. nov. 2	EPF001C1	1×10^6	6.3×10^6
8	*Beauveria* sp. nov. 2	EPF002B1	2.1×10^6	2×10^6
9	*Beauveria* sp. nov. 2	EPF003D8	2.6×10^6	2.2×10^6
10	*B. pseudobassiana*	EPF004D7	3×10^6	1.1×10^6
11	*B. pseudobassiana*	EPF004E5	1×10^6	1×10^6

Note: All spores exceeded 90% viability. Formulas used to calculate the spore concentration with the use of a hemacytometer.

Table 3. PCR protocols and primers used for each gene region during the study.

Locus region	Primers used	Initial denaturation	Cycles	Denaturation	Annealing	Extension	Final extension
Internal Transcribed Spacer (ITS)	ITS1F ITS4	5 min at 94°C	30	40 s at 94°C	30 s at 57°C	60 s at 72°C	10 min at 72°C
RNA polymerase II large subunit (*rpb1*)	CRPB1 RPB1Cr	3 min at 94°C	34	60 s at 94°C	90 s at 50°C	180 s at 72°C	10 min at 72°C
Translation Elongation Factor 1-alpha (*tef1*)	EF1-983F EPF1-2218R	3 min at 94°C	35	45 s at 94°C	45 s at 56°C	90 s at 72°C	8 min at 72°C

Table 4. The virulence (LT_{50}) observed for different treatments of different fungal strains against *Gonipterus* sp. n. 2 adults over a period of 14 days.

Species	Treatment	LT_{50}[a] (±SE)	95% CI[b]	
			Lower	Upper
N/A	Control	N/A[c]	_	_
B. bassiana	R444	N/A	_	_
	EPF004E9	11 ± 0.05	10.9	11.1
B. namnaoensis	EPF001C5	5 ± 0.1	4.8	5.2
Beauveria sp. nov. 1	EPF005A6	4 ± 0.1	3.8	4.2
Beauveria sp. nov. 2	EPF001A7	6 ± 0.05	5.9	6.1
	EPF001C1	N/A	_	_
	EPF002B1	N/A	_	_
	EPF003D8	7 ± 0.07	6.9	7.1
B. pseudobassiana	EPF004D7	9 ± 0.08	8.8	9.2
	EPF004E5	N/A	_	_

[a] LT_{50} values for mortality were estimated by Kaplan-Meier survivorship analysis with censored data for insects surviving longer than the 14-day period of the trial.

[b] 95% confidence intervals (CI) that did not overlap indicate differences between LT_{50} values

[c] N/A: adult survival exceeded 50% after 14 days and therefore, LT_{50} values could not be calculated as data was removed from analysis

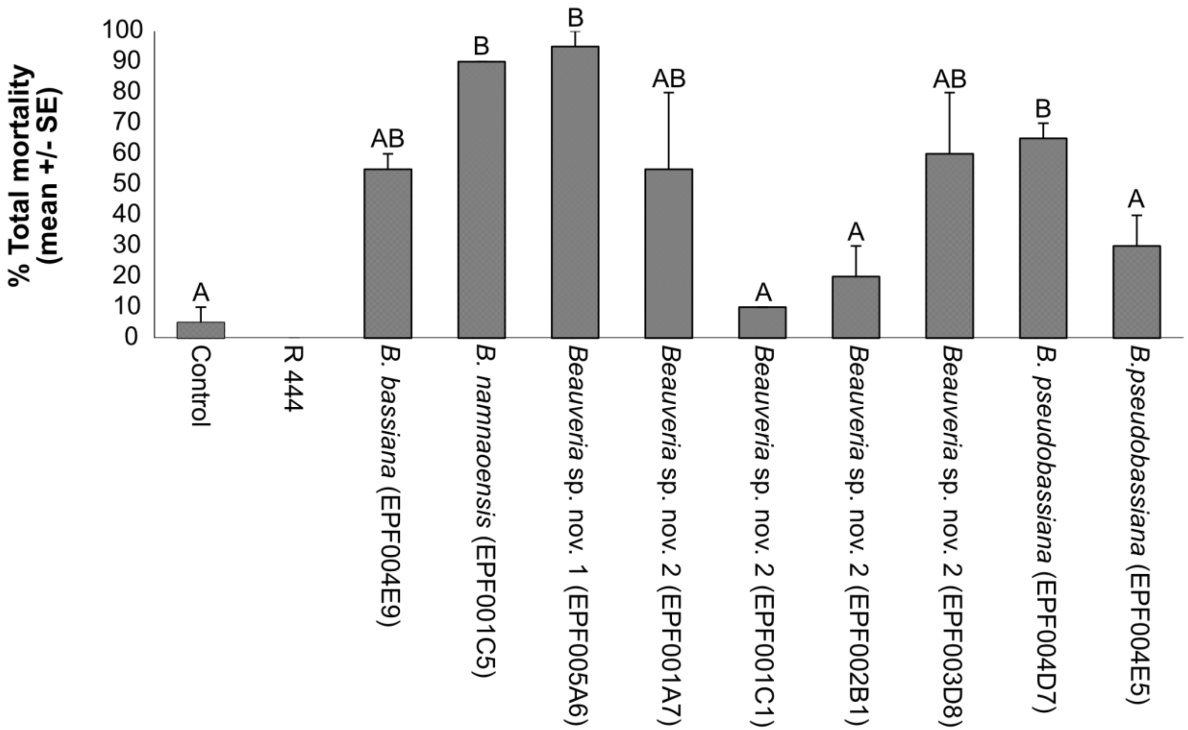

Figure 1. Percentage mortality (mean ± SE) of *Gonipterus* sp. n. 2 adults observed after exposure to different strains of *B. bassiana* (R444 and EPF004E9), *B. namnaoensis* (EPF001C5), *Beauveria* sp. nov. 1 (EPF005A6), *Beauveria* sp. nov. 2 (EPF001A7, EPF001C1, EPF002B1, EPF003D8) and *B. pseudobassiana* (EPF004D7, EPF004E5) at 14 days post-inoculation. All insects were exposed to 10 µl spore suspensions that contained 10^6 spores ml^{-1}. No mortality occurred for the commercial treatment (R444) and data were excluded from all statistical analyses. *Beauveria* mortality of a field introduced strain occurred in the negative control. Error bars represent the standard error (SE) of the average mortality in both trials. Bars that display similar letters are not significantly different (Tukey's HSD, $p \leq 0.05$).

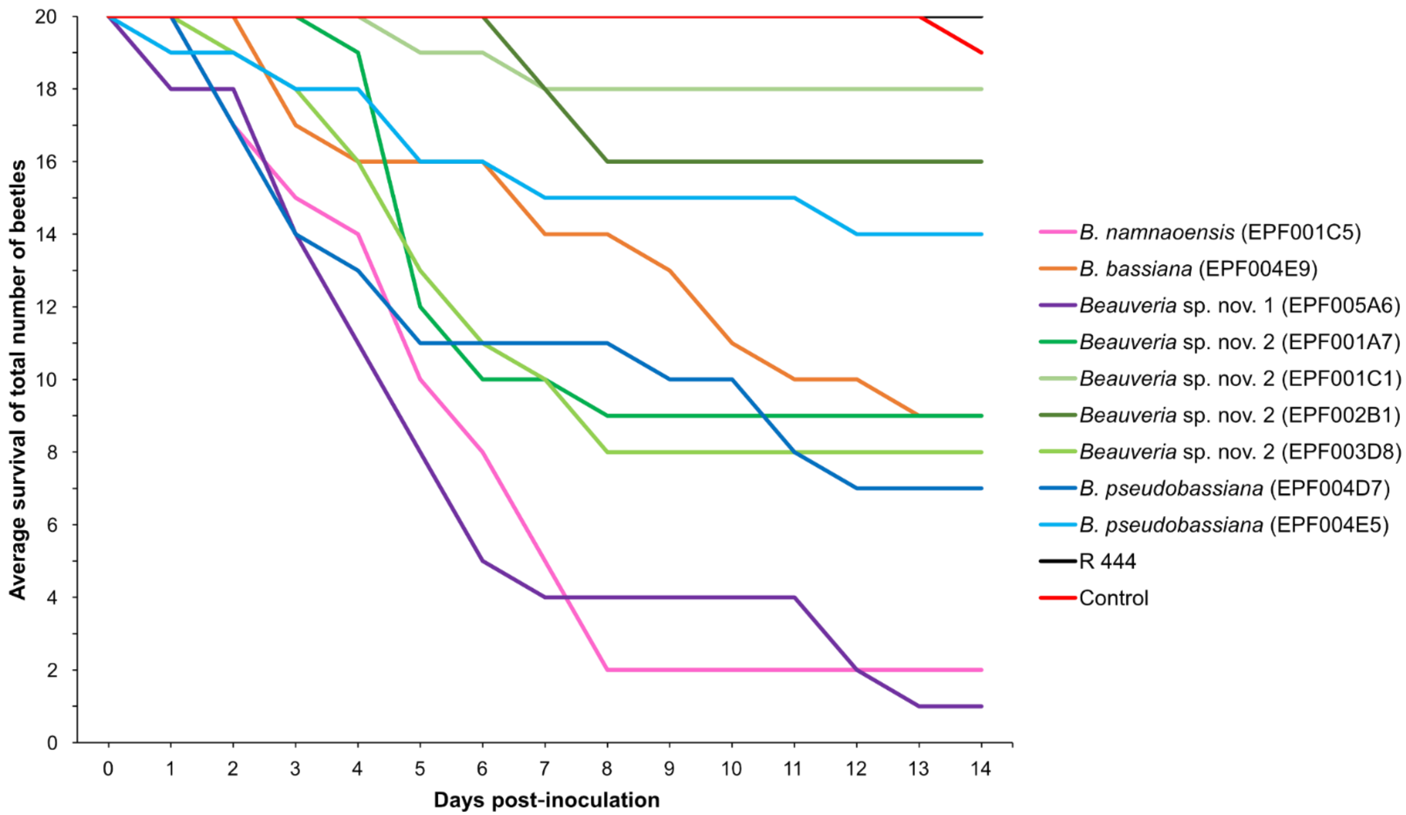

Figure 2. Kaplan-Meier survival curves of *Gonipterus* sp. n. 2 adults 14 days post-inoculation with 10^6 spores ml^{-1} suspensions. The various spore suspensions were made with spores from *B. bassiana* (R444 and EPF004E9), *B. namnaoensis* (EPF001C5), *Beauveria* sp. nov. 1 (EPF005A6), *Beauveria* sp. nov. 2 (EPF001A7, EPF001C1, EPF002B1, EPF003D8) and *B. pseudobassiana* (EPF004D7, EPF004E5) that made up the treatments for the virulence trials. Cox Proportional Hazards model: Concordance= 0.672 (SE=0.032); Likelihood ratio test= 45.96 on 10 df, $p<0.05$; Wald test= 9.37 on 10 df, $p=0.5$; Logrank test= 29.33 on 10 df, $p<0.001$.

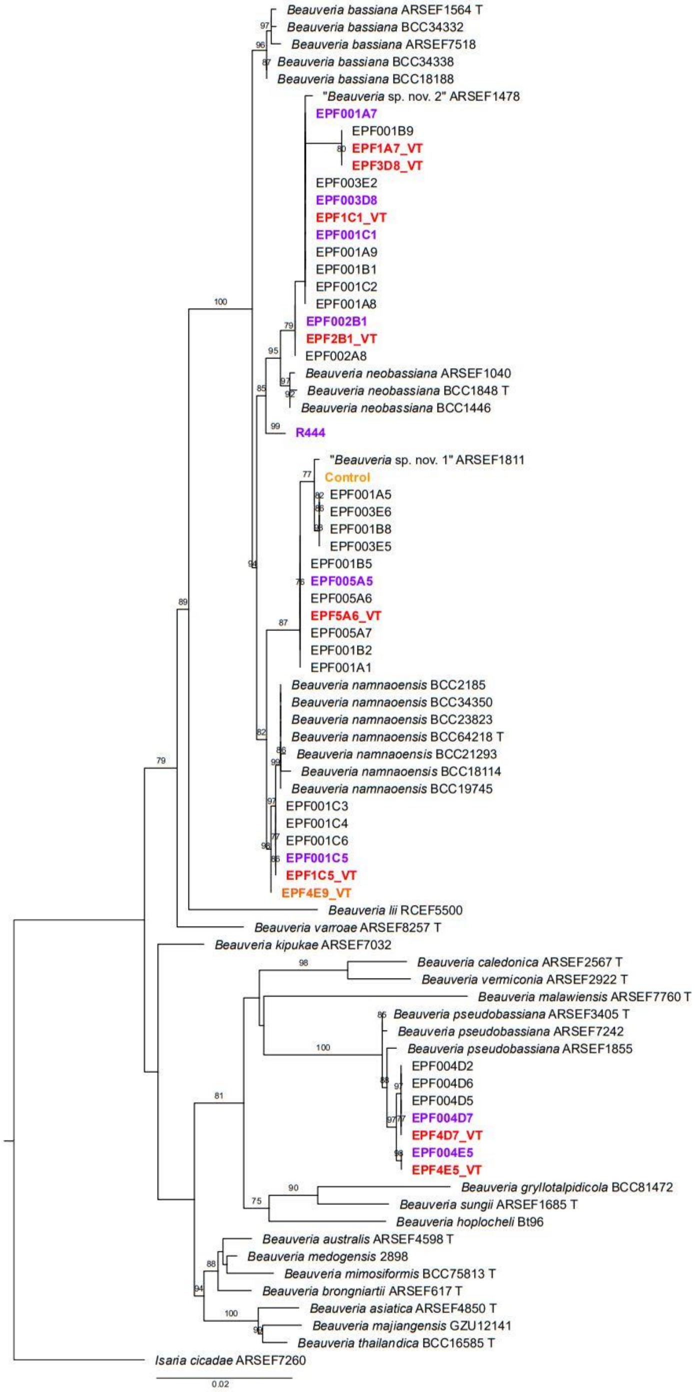

Figure 3. Maximum likelihood combined phylogeny based on the *rpb1*, *tef1* and ITS gene regions showing the identifications of *Beauveria* isolates that have been used in virulence trials against *Gonipterus* sp. n. 2. Sequences that have been obtained from ex-type cultures are indicated by a T at the end of the accession number. VT at the end of each strain number indicates isolates obtained from cadavers during the virulence trials. Sequences in purple represent the original sequences chosen and red represent the sequences obtained from the virulence trials and are indicated in **bold**. Orange indicates sequences chosen before final phylogenetic analysis as well as molecular identification of the control isolate obtained during trials. Bootstrap values ≥75% are shown. The tree was rooted to *Isaria cicadae*.

Chapter 4:

General discussion and conclusion

General discussion and conclusion

Entomopathogenic fungi include species with a diverse ecology in five fungal phyla that play a pivotal role in insect population control (Araújo and Hughes, 2016; Goettel et al., 2005). *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) is one of the best known EPF species and has been recorded to infect more than 700 insect species globally (Imoulan et al., 2017; Khonsanit et al., 2020). Literature indicates that the *B. bassiana sensu lato* clade consists of more than 12 phylogenetically distinct cryptic species (Clifton et al., 2023; Rehner and Buckley, 2005; Rehner et al., 2006). Studies that evaluated cryptic *Beauveria* species, confirmed wild *B. bassiana* cryptic species to be more virulent towards target insects when compared to commercial *Beauveria* biopesticides (Clifton et al., 2023; Jordan et al., 2021; Wang et al., 2022). On the other hand, the limited knowledge on the virulence of strains of *Beauveria* limits the number of novel biopesticides that can be produced for commercial use (Clifton et al., 2023; Jordan et al., 2021; Mascarin et al., 2013).

The invasive forestry pest, *Gonipterus* sp. n. 2 (Coleoptera: Curculionodae), causes severe damage and defoliation to *Eucalyptus* (Myrtaceae) plantations in South Africa (Schröder et al., 2020). Initially, the egg parasitoid, *Anaphes nitens* Girault (Hymenoptera: Mymaridae), managed to suppress *Gonipterus* sp. n. 2 populations below economic injury level, but outbreaks were still observed. Pest resurgences have been reported since the 1990s and outbreaks at low elevation regions have been observed where *Gonipterus* was previously reported to be under economic control. Additional pest management tools, compatible with the classical biological control agent, are thus needed to suppress the pest population below the economic threshold (Hurley et al., 2016; Schröder et al., 2020).

The literature review presented in this thesis (**Chapter 1**) highlighted the importance of EPF as biopesticides and the effects of abiotic and biotic factors on their efficacy and virulence. Globally, several commercial biopesticides have been developed using EPF for the implementation in integrated pest management (IPM) programmes (Mascarin et al., 2017; Mascarin and Jaronski, 2016; Mascarin et al., 2019). *Beauveria bassiana* species are one of the most well-known EPF used to develop biopesticides with 61 products available globally (Mascarin and Jaronski, 2016). The literature review further highlighted the impact of abiotic and biotic factors on the diversity and efficacy of fungal species. Abiotic factors that affect their efficacy and virulence include, but is not limited to, temperature, relative humidity (RH), soil type and UV irradiation. Biotic factors with similar effects are host behaviour, immune response and host tolerance (Acheampong et al., 2020; Cao et al., 2022; Jaronski, 2010; Quesada-Moraga et al., 2023). Future studies should focus on the impact of these factors on EPF, as studies in this area are currently lacking. A broader knowledge on the impact of these factors, could improve the understanding of these diverse species when it comes to integration into IPM programmes and this defined the focus of this thesis.

In the study on the diversity of fungi associated with *Gonipterus* sp. n. 2, we identified 17 genera, which included the species *B. pseudobassiana* and three species within the *B. bassiana* cryptic species clade, namely *B. namnaoensis*, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2. *Beauveria namnaoensis* has not previously been reported from South Africa and two novel species, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2, were also discovered. These results indicate that the diversity of cryptic *Beauveria* species hold significant implications for IPM strategies that target *Gonipterus* sp. n. 2. Similarly, the discovery of novel species within the *B. bassiana* cryptic clade, indicates a diverse range of possible novel species that remain to be discovered, that could be expected to be adapted to their local environment (Jaronski, 2010; Jordan et al., 2021; Mascarin and Jaronski, 2016). The potential use of these species as novel biopesticides opens alternative research questions to be explored.

Beauveria bassiana cryptic species were isolated from *Gonipterus* sp. n. 2 cadavers collected in Mpumalanga, KwaZulu-Natal and Gauteng and identified through phylogenetic analysis. The results showed that *B. pseudobassiana* species, as well as the recently described species *B. namnaoensis* were isolated from insect cadavers. In addition, two novel *Beauveria* species were obtained from insect cadavers, *Beauveria* sp. nov. 1 and *Beauveria* sp. nov. 2. The commercial product, Eco-BB® strain R444 did not resolve in a clade with any known species and might be a new species. Eco-BB® strain R444 is registered for the use against pests such as red spider mites and lepidopteran pests. The identification of the *B. bassiana* cryptic species strains proved to be challenging. The recommended identification region for *Beauveria* cryptic species, the Beta-locus intergenic region (*Bloc*) (Rehner et al., 2011), did not deliver optimal DNA sequences for use in phylogenetic analysis. The gene regions that were utilised for the identifications delivered satisfactory results, but future studies should focus on further improving these sequences and subsequent identifications using the *Bloc* region.

The third chapter considered the virulence of nine cryptic *Beauveria* species obtained from cadavers against adult *Gonipterus* sp. n. 2 beetles. Inter- and intraspecific variation in virulence was observed between the strains of the *Beauveria* species tested. This was the first study that tested the efficacy of *B. namnaoensis* and additional cryptic *Beauveria* species against *Gonipterus* sp. n. 2, with the results indicating the potential role of these EPF as biological control agents against *Gonipterus* sp. n. 2 in South Africa. This is an important finding, as of yet there are no registered biopesticides in South Africa for use against *Gonipterus* sp. n. 2. To determine the most effective strain for biological control, future research should include all cryptic *B. bassiana* strains obtained during the study when conducting virulence trials against *Gonipterus* sp. n. 2. Furthermore, laboratory-reared beetles should be used to prevent the possible introduction of field-related strains or diseases.

The discovery of *B. bassiana* cryptic species, indicates that these species are present in South African plantation forestry systems. These diverse species can be expected to be adapted to their natural environments, making them more efficient against target pests in their natural environments (Acheampong et al., 2020; Quesada-Moraga et al., 2023). The large diversity of EPF on a single pest species could be beneficial for commercial production to improve the target range of insect pests (Jordan et al., 2021). The development and application of these *B. bassiana* cryptic species obtained from *Gonipterus* sp. n. 2 cadavers would greatly improve IPM in South African forestry, as no such products are currently available. In addition, previous studies did indicate that *Beauveria* species are safe to be used in combination with the egg parasitoid, *A. nitens*, for *Gonipterus* population control (Jordan et al., 2021; Pérez-Otero et al., 2003). However, further investigation to determine the impact of these cryptic species on non-target organisms and their ability to persist in the environment is needed.

The description of new species in the *B. bassiana* clade could have an impact on already available products. For example, the commercial product used in the study, Eco-BB® strain R444, is classified as *B. bassiana*, but was identified as a unique, cryptic *Beauveria* species using phylogenetic analysis in this study. However, more research on the taxonomy of the *B. bassiana sensu lato* clade needs to be done before revision of the strain's identification. Although this strain was not effective against *Gonipterus* sp. n. 2, as no pathogenicity was recorded, it was produced for agricultural pests in the order Lepidoptera and not forestry pests, such as *Gonipterus* sp. n. 2 in the order Coleoptera.

The inter- and intraspecies variation in virulence obtained during the study, indicate that a large diversity of *Beauveria* species remain untapped. This corresponds to the study conducted in Brazil, where similar variation in virulence was observed between *B. bassiana* species (Jordan et al., 2021), as well as other studies that noted variation in virulence between *Beauveria* cryptic species (Clifton et al., 2023; Wang et al., 2022). If these species can be further researched or produced for commercial implementation, it could have a significant impact on the success of IPM programmes. Therefore, research is needed for the commercial development of these strains that might span several years before being available for implementation on a large, commercial scale. The research should not be limited to forestry systems, but could be explored in agriculture to improve IPM programmes on a larger scale.

This was the first study to identify the diversity and virulence of *B. bassiana* cryptic species in South Africa, with two novel species discovered. *Beauveria bassiana* cryptic species obtained from *Gonipterus* sp. n. 2 cadavers in South African *Eucalyptus* plantations, could be further investigated for their use and development as biopesticides. Future studies needed include identifying EPF from more insect genera and species present in *Eucalyptus* plantations. In addition, all commercial products available at present, as well as novel species and strains of *B. bassiana*, should be evaluated at a range of temperatures and relative humidities.

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