

**ENTOMOPATHOGENIC NEMATODE INTERACTIONS WITH WHITE GRUBS FROM
FOREST AND SUGARCANE PLANTATIONS**

by

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SUMMARY

ENTOMOPATHOGENIC NEMATODE INTERACTIONS WITH WHITE GRUBS FROM FOREST AND SUGARCANE PLANTATIONS

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In South Africa, white grubs are important pests of sugarcane and wattle. There is need for a sustainable management alternative since their control has mainly been through the use of chemical insecticides. Such alternatives can include the use of entomopathogenic nematodes, EPNs. A review of the available literature revealed that EPNs can potentially provide a higher percentage control of white grubs, when compared to chemical insecticides. However, this control efficacy is characterized by several inconsistencies that can be attributed to differences in the EPN species, environmental conditions, and the resistance of white grubs to EPN infection. To increase efficacy of EPNs against white grubs, these inconsistencies need to be minimized, for example by using locally isolated EPN species that are better adapted to the local environment.

In this project, I investigated the potential use of locally isolated EPNs as an alternative biological control method for white grubs in South Africa. I examined EPN associations with white grubs collected from wattle and sugarcane plantations in the Mpumalanga and KwaZulu-Natal provinces of South Africa. Using bioassays, EPN pathogenicity towards white grubs as well as the white grub resistance mechanisms to EPN infection were evaluated using eleven locally isolated EPNs. The results indicated that the mortality percentage of the white grubs varied significantly but was low for most of the EPN species tested, except for the EPN *Heterorhabditis zealandica* MJ2C. When *H. zealandica* MJ2C was used in a probit test, its varying lethal dosages were revealed for the different white grubs. The experiments to investigate the physiological defence mechanisms of the grubs showed that the ability of the nematodes to penetrate the white grubs, the encapsulation rates and growth in the haemolymph varied between the EPNs and the white grub species. However, the different species of the EPN symbiotic bacteria were able to grow in the haemolymph of all the white grub species tested, implying that white grubs avoid initial attack of EPNs as a resistance mechanism.

During a survey for white grubs in the KwaZulu-Natal province in South Africa, sporadic incidence of larvae with symptoms of nematode infections were detected. Incubation of the infected cadavers resulted in isolation of three EPN species. This is the first report of locally occurring EPNs found naturally associated with white grub species in South Africa. This project also resulted in the description of a new EPN species from South Africa, described as *Steinernema bertusi* Katumanyane, Malan, Tiedt & Hurley (Rhabditida: Steinernematidae). The newly described EPN species was the fifth species to be included in the *Cameroonense*-clade, the 12th *Steinernema* species described from South Africa, of which only a 100 species are currently described in the world.

RESEARCH OUTPUTS

Journal articles

Katmanyane, A., Malan, A.P., Louwrens, R.T. & Hurley, B.P. 2020. *Steinernema bertusi* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Nematology* 22: 343–360. 10.1163/15685411-00003309

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DECLARATION

I declare that the dissertation/thesis, which I hereby submit for the degree Philosophiae Doctor (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 she has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Polict guidelines for responsible research.

Agil Katumanyane

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PREFACE

White grubs (Coleoptera: Scarabaeidae) are some of the target insect groups for biological control using entomopathogenic nematodes (EPNs). This is because of their soil dwelling nature, resistance to chemical insecticides and susceptibility to EPNs. EPNs are small worms that live in the soil and are capable of actively searching and infecting their hosts through natural openings such as the spiracles, mouth and anus. In addition, EPNs belonging to the genus *Heterorhabditis* are known to possess a dorsal tooth, which they use to pierce thin parts of the insect's cuticle by abrasion.

EPN's owe their pathogenicity mainly to the symbiotic bacteria which they carry in their intestines or specialized vesicles. The pathogenicity process is highly efficient, and the hosts normally die within 48 hours. EPNs are particularly suited for biological control of white grubs, as they have co-evolved with white grubs in the soil. For this reason, and due to advances in mass production technology, EPNs have been tested against various white grub species and some EPN based products are available on the market for biological control of white grubs.

Biological control of white grubs has been characterized by inconsistencies in efficacy, which is often attributed to different biotic and abiotic factors. Biotic factors include resistance mechanisms developed by white grubs to EPN infection. Chapter 1 reviews the literature on the EPN-white grub pathogenesis process. Different studies where EPNs were used for the biocontrol of white grubs are reviewed and the possible reasons for inconsistencies in the efficacy of EPNs against white grubs are examined. The behavioral, morphological, and physiological resistance mechanisms of white grubs to infection by EPNs as well as the classification of resistance mechanisms into primary, secondary and tertiary defense mechanisms are discussed. The chapter concludes by highlighting the potential approaches for improving EPN pathogenicity to white grubs in future field applications.

To date, approximately 100 *Steinernema* and 16 *Heterorhabditis* species have been described from different parts of the world. Thirteen of these have been described from South Africa,

suggesting a high EPN biodiversity. In Chapter 2, a new EPN species, *Steinernema bertusii* n. sp., is described based on both morphological and molecular characterizations. It was isolated from natural vegetation in Port Edward, KwaZulu-Natal, South Africa. This description is the 12th *Steinernema* species described from South Africa, which means South Africa currently has 12 % of the described species in this genus.

White grubs are the most important insect pests of sugarcane plantations in South Africa and the neighbouring Kingdom of Eswatini, formerly Swaziland. They are also important establishment pests of black wattle (*Acacia mearnsii*). The effort to control white grubs in these environments has been predominantly through the use of chemical insecticides, which has created a need for more environmentally friendly management alternatives. Chapter 3 examines the efficacy of native EPNs against species of white grubs that are pests in sugarcane and wattle plantations. Eleven locally isolated EPNs were tested for their potential to kill the white grub species *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Monochelus* sp. and *Maladera* sp. 4., under laboratory conditions. Concentration trials to determine lethal dosages for three of the white grub species were performed, using an EPN that had shown a high level of insecticidal capacity towards most of the white grub species.

Following from the results of Chapter 3, which showed that some of the white grubs had a low level of susceptibility to EPNs, some of the resistance mechanisms of white grubs towards nematodes were examined. These results are presented in Chapter 4. Laboratory bioassays were used to determine the ability of the EPNs to penetrate the white grubs, the ability of the EPN symbiotic bacteria to grow in their haemolymph, the encapsulation rates, as well as the nematode growth inside the insect haemolymph.

Sporadic incidence of larvae with symptoms of nematode infections were detected and isolated from wattle and sugarcane plantations during the survey and collection of white grubs in KwaZulu-Natal province in South Africa, presented in Chapter 3. Thus, larvae with symptoms of nematode infection were separated and examined for presence of nematodes. Nematodes

isolated/extracted from the white grubs were identified using molecular characterization. These results are presented in Chapter 5.

A summary at the end of this thesis presents the major findings of this study. All the chapters of this thesis have been formatted in the form of manuscripts. For this reason, it was inevitable to have repetitions, particularly in the introduction and discussion sections of some of the chapters. Appendices of all the chapters are included at the end of the thesis

Chapter 1

White grub defence mechanisms against entomopathogenic nematodes

Abstract

White grubs are important sporadic pests in commercial agriculture. Their control using conventional chemical control methods is difficult because they are soil dwelling. The use of entomopathogenic nematodes (EPNs) has offered promising results as an alternative to chemical control, as the beetles spend their entire larval stage in the soil, offering a long window of opportunity to apply EPNs. However, due to the natural co-existence of white grubs and EPNs in the soil, white grubs have developed a variety of behavioural, morphological and physiological defence mechanisms against the EPNs. Understanding how and when these defence mechanisms are used by the white grubs, and the extent to which they influence EPN efficacy, is important when considering biocontrol using EPNs. The defence mechanisms are separated into primary, secondary and tertiary defence mechanisms. The primary defences include association with other organisms, a cryptic nature, evasive behaviour and changes in respiratory patterns aimed at preventing the white grub from getting in contact with the EPNs. The secondary defences include the possession of spines and hairs to brush off invading nematodes, dense peritrophic membrane, hard exo-skeleton, active thrashing, and dropping or regurgitating once the white grub has been in contact with the EPNs. The tertiary defence mechanisms include the white grubs' cellular and humeral responses that act at a molecular level. Here we review what is known about these defence mechanisms and suggest how they can be manipulated to minimise their counteracting effect on EPN efficacy.

Introduction

White grubs (Coleoptera: Scarabaeidae) are the root feeding larvae of the Scarabidae beetles that are sporadic pests of various plants (Ritcher 1996; Jackson & Klein 2006). Members of the Scarabaeidae families Aphodiinae, Cetoniinae, Dynastinae, Melolonthinae and Rutelinae have been recorded as pests of various crops (Ritcher 1966; Harrison & Wingfield 2016). Morphologically, white grubs are easily differentiated by their C-shaped, white milky body with three prominent pairs of thoracic legs and a dark, strongly chitinized head capsule (Sutton & Stone Jr. 1974). The adults are known as chafer beetles or chafers (Harrison & Wingfield 2016). The grubs feed on plant roots, while adult beetles bore into underground stems and defoliate plants (Jackson & Klein 2006). White grubs are thus sporadic pests of various economically important crops globally, including lawns, turf, pastures, sugarcane, forestry, and many legumes and grains (Ritcher 1996; Jackson & Klein 2006).

Control of white grubs is difficult because of their soil dwelling nature and known resistance to chemical insecticides (Grewal *et al.* 2004). Moreover, the adult beetles are usually nocturnal, further complicating possible control strategies such as the use of contact chemical pesticides (Jackson & Klein 2006). Suggested management approaches include cultural control, such as avoiding planting if white grub populations are predicted to increase (Sutton & Stone Jr 1974), and biological control, using pathogenic, parasitic and predaceous organisms (Davis 1922; Fleming 1968). Chemical insecticides have generally been recommended as a preventative measure, as their efficacy is reduced when the larva develops into its third instar stage. Thus far, chemical insecticides have been the most used method to control white grubs with mixed control efficacies achieved (Grewal *et al.* 2004). Other cultural control methods include cultivation and tillage, but its success has been considered unjustifiable in no-tillage areas and dependant on the depth of larval localizations in tillage area (Oliveira *et al.* 2000).

Biological control using entomopathogenic nematodes (EPNs) has been shown to provide better control of specific white grub species than chemical insecticides (Grewal *et al.* 2005).

EPNs are naturally occurring small roundworms of the families Heterorhabditidae and Steinernematidae that can actively search for and infect their insect hosts in the soil environment (Kaya *et al.* 1993). However, the natural co-occurrence of white grubs and EPNs in the soil has resulted in the co-evolution of specific resistance mechanisms of white grubs against EPNs (Schmid-Hempel 2008). The resistance mechanisms include behavioural, morphological and physiological defences. The mechanisms used and their effectiveness can vary between different species and growth stages of white grub, as well as the EPN species and environmental conditions involved (Fig. 1). For example, caged and restrained larvae of *Popillia japonica* Newman (Coleoptera: Scarabaeidae) were observed to only remove (avoid) 13 % of invading nematodes when compared to 60 % in the unrestrained larvae (Gaugler 1994). Varying EPN encapsulation rates were observed between the second and the third instar grubs of the white grub, *Polyphylla adspersa* Motschulsky (Coleoptera: Scarabaeidae), when exposed to *Heterorhabditis bacteriophora* Poinar and *Steinernema glaseri* Steiner in laboratory bioassays (Alvandi *et al.* 2017).

The objective of this review is to synthesize knowledge on the different defence mechanisms employed by white grubs and provide suggestions on how this knowledge can be used to improve the control/management of white grubs using EPNs. EPNs and their potential as biocontrol agents of white grubs are discussed, followed by the exploration of the different defence mechanisms of white grubs. Possibilities for the manipulation of these defence mechanisms with the purpose of increasing the susceptibility of the white grubs to EPNs and improving the biocontrol potential EPNs are also discussed.

EPNs and their associated bacteria

EPNs are small, microscopic roundworms of the order Rhabditida, which are lethal to insects when they infect them. There are several commercial EPN species in the families Steinernematidae and Heterorhabditidae that are being used for the control of various insect pests (Kaya *et al.* 1993; Nobuyoshi 2002). Though phylogenetically distinct, these families have

evolved a similar insect parasitism mechanism through convergent evolution (Adams 2007). This mechanism involves the use of symbiotic bacteria to kill their insect host within a short period of time (less than 120 hrs), as stipulated by Dillman *et al.* (2012). The EPNs must retain their symbiotic bacteria for subsequent generations. Following this criterion, some members of the genus *Oscheius* have been redefined as true EPNs (Dillman *et al.* 2012).

The symbiotic bacteria associated with EPNs belong to the Enterobacteriaceae family. These bacterial species are carried inside the intestines of the infective juveniles (IJs) in Steinernematidae, while in Heterorhabditidae the IJs possess a specialised vesicle which serves to carry the bacteria (Hinchliffe *et al.* 2010). The free living IJs actively search for their potential hosts by staying close to the ground surface and ambushing any passing hosts or foraging through the soil profiles for potential hosts (Nobuyoshi 2002). The IJs enter their host via natural body openings such as the anus, spiracles, the mouth and sometimes by direct penetration through the insect's cuticle as in some heterorhabditids (Nobuyoshi 2002). Subsequently, the EPNs penetrate through the gut epithelium and release the symbiotic bacteria into the insect's haemocoel. In the haemocoel, the symbiotic bacteria secrete toxins which kill the host, usually within 48 h of infection. Host insect mortality happens due to septicaemia (Sandhu *et al.* 2012), which can occur even at very low concentrations of the bacteria (An & Grewal 2007; Goodrich-Blair & Clarke 2007). For example, An & Grewal (2007) compared the virulence of *Photorhabdus temperata* Fischer-Le Saux, Viallard, Brunel, Normand and Boemare and *Xenorhabdus koppenhoeferi* Tailliez, Pages, Ginibre and Boemare, the symbiotic bacteria of the nematodes *H. bacteriophora* and *Steinernema scarabaei* Stock and Koppenhöfer, respectively, to the three white grub species, *P. japonica*, *Rhizotrogus majalis* Razoumowsky and *Cyclocephala borealis* Arrow. Their results indicated that both bacteria were pathogenic to all three white grub species, even at two cells per white grub.

After the free living IJs have released their bacterial symbiont and toxic products are secreted inside the host, the nematodes undergo notable morphological changes in a process referred

to as activation/recovery. At this point, the nematodes become actively parasitic (Alonso *et al.* 2018). Recovered or activated nematodes feed on bacterial cells and decayed insect tissues to complete their lifecycle. This may continue for a number of generations until the resources are depleted. Upon depletion of resources, free living IJs are produced and they leave the cadaver to repeat the cycle (Kaya *et al.* 1993; Nobuyoshi 2002). In this process, the bacterium is primarily responsible for the mortality of the insect host and providing nutrition for the nematodes. However, in laboratory bioassays, some axenic *Steinernema* nematodes exhibit host immune suppression and host killing properties, although very inefficiently (Han & Ehlers 2000; Lu *et al.* 2017).

The pathogenic effect of the EPNs is attributed to the complex mixture of secondary products secreted by both the nematode and the bacteria upon successful infection and nematode activation. This complex mixture includes proteins, peptides, glycans, lipids and small organic molecules (Shepherd *et al.* 2015; Dreyer *et al.* 2018) and is known to be carried on the surface coat in some nematodes (Blaxter *et al.* 1992). For example, the body surface of *Steinernema feltiae* Filipjev has been shown to harbour chemical compounds which upon reaction with some host functional proteins, inhibits the host's defensive processes, while *Steinernema carpocapsae* Weiser secretes products from its body for its host immunosuppression (Brivio & Mastore 2018).

EPN-white grub pathogenesis

The EPN-white grub pathogenesis is a two-step synergistic process that require a successful penetration of the nematode into the insect and a subsequent successful infection of the insect's haemolymph by the IJs and their symbiotic bacteria (Lu *et al.* 2017). The survival and reproduction of EPNs in an insect host depends on the ability of its symbiotic bacteria to grow inside the host, and to make nutrients available to the nematodes (Han & Ehlers 2000; Ciche *et al.* 2006). The rate of bacterial growth is related to the rate of insect mortality (Clarke & Dowds 1991).

The nematode-bacteria duo, once inside the insect's haemocoel, is exposed to the insect's innate immune response, at which point it responds by avoiding detection and recognition by insect tissues as non-self or by suppressing the insect's immune response (Vallet-Gely *et al.* 2008). It is the synergistic effect of the nematodes and the bacteria, which suppresses the immune system of the host insects. A combination of complex protein compounds secreted from the surface of the nematodes and secondary toxins formed by the symbiotic bacteria inhibit the insect's phagocytic responses enabling a successful infection (Eleftherianos *et al.* 2010). The bacterial virulence factors that suppress host immune responses include toxin complexes, proteases, lipases and lipopolysaccharide (Wang *et al.* 1995; Forst *et al.* 1997; Jarosz 1998; Li *et al.* 2009, 2012; Dreyer *et al.* 2018).

Host recognition underlies some of the differences between EPN resistant and susceptible hosts (Li *et al.* 2007). Some EPN symbiont bacterial toxins have been shown to suppress eicosanoid biosynthesis, the downstream mediator of non-self-recognition. Eicosanoid suppression inhibits the insect's immunity and promotes pathogenicity (Kim *et al.* 2018).

The bacterial virulence factors are controlled at a molecular level. *Photorhabdus* and *Xenorhabdus* bacteria contain several genes encoding insecticidal toxins required for insect infection (Richards & Goodrich-Blair 2009; Rodou *et al.* 2010). Other studies such as Vizcaino *et al.* (2014). have also identified certain *Photorhabdus* and *Xenorhabdus* genes encoding molecules required for the normal growth and development of their nematode host. Notably, the *Photorhabdus luminescens* Poinar TT01 genome contains genes that play important roles in the regulation of both pathogenicity and mutualism, which suggests a link between these two contrasting lifestyles (Lango & Clarke 2010; Somvanshi *et al.* 2012). Moreover, distinct *Photorhabdus* genes encoding secreted proteins also perform dual functions as inhibitors of the growth of microbial competitors in the dead insect, and as potent suppressors of host insects' immune responses (Eleftherianos *et al.* 2017).

The use of EPNs for white grub control

EPNs are naturally soil dwelling and have emerged as excellent biocontrol agents of some below and above ground insect pests worldwide (Grewal *et al.* 2005; Lacey & Georgis 2012; Platt *et al.* 2020). White grubs are known to have long life cycles (1-3 years) of which a major part is in the soil, providing a long window of opportunity for the application of EPNs. Various laboratory and field studies have been performed to determine the potential for biocontrol of white grubs using EPNs. Some of these studies have resulted in high mortality of the target white grub species (Table 1), but the results can be difficult to predict, as the control efficacy recorded from the EPNs is inconsistent (Grewal *et al.* 2005). No single nematode species is known to provide the best control for a broad range of white grub species (Grewal *et al.* 2005). In fact, different strains of the same EPN species are known to provide varying degrees of control of the same grub species (Grewal *et al.* 2005; Georgis *et al.* 2006; Koppenhöfer *et al.* 2006).

The consensus is that inconsistencies in EPN efficacy to control white grubs can be explained on the basis of biotic factors such as host attractiveness, nematode dispersal rates and the nematode's poor ability to penetrate through the cuticle/gut wall (Georgis & Gaugler 1991; Koppenhöfer *et al.* 2007) and/or environmental factors such as moisture, temperature and soil physical properties (Kaya *et al.* 1993). Moisture, temperature, and soil physical properties are the most critical factors that influence EPN performance (Kaya *et al.* 1993).

Differences in the biology and behaviour of the nematode and the white grubs will influence the success of infection. One salient biological factor is the white grub's developmental stage (Karunakar *et al.* 2000; Grewal *et al.* 2002,2004; Lee *et al.* 2002; Koppenhöfer & Fuzy 2003a; Koppenhöfer *et al.* 2004, 2006; Li *et al.* 2007; An *et al.* 2012). The studies done in this regard show that irrespective of the white grub species, the first and second instars of white grubs are more susceptible to EPNs compared to the more resistant third instars. In their study, Ansari *et al.* (2008) showed that the third instars of *Hoplia philanthus* Füssly were generally resistant

to the tested nine EPNs when compared to the second instars. In another study, Alvandi *et al.* (2017) determined the immune response of *P. adspersa* larvae against the virulence of the EPNs, *H. bacteriophora* and *S. glaseri*, and showed that the third instar larvae were more resistant than the second instars. This poses challenges for using these EPNs for the management of white grubs, as the third instar grubs stay in the soil for a long period and are more damaging to the crops.

In terms of the biology of EPNs, the dispersal behaviour of the nematodes will affect their ability to locate and infect white grubs. EPNs can be classified either as cruisers or ambushers. Cruiser nematodes such as *H. bacteriophora* are highly mobile and adapted to infect less mobile hosts (Nobuyoshi 2002). Thus, in the field, cruiser nematodes are consequently adapted to control white grubs, which are known to be less mobile, with some exhibiting cocooning and overwintering patterns (Ritcher 1966). In comparison, ambusher nematodes such as *S. carpocapsae* are known to rather use a 'sit and wait' foraging strategy (Nobuyoshi 2002). This type of EPNs are suitable for the control of hosts that roam the surface of the soil. Ambusher EPNs are known to be triggered by movement and vibrations of the host (Kaya *et al.* 1993), a strategy which can be countered by white grubs when they remain quiescent in the soil.

Field application of EPNs for white grub control

The first EPN species that was used for control of scarabs on a widespread basis was *Steinernema glaseri* against the white grub *P. japonica* (Gaugler *et al.* 1992). Since then considerable success has been achieved in this regard. Many EPN species have shown a high potential for field use against white grubs (Peters 1996, Koppenhöfer & Fuzy 2003b; Koppenhöfer *et al.* 2003; Klein *et al.* 2007; An *et al.* 2012). Several EPN species have in fact been naturally isolated from white grubs, including *H. bacteriophora*, *Heterorhabditis megidis* Poinar, Jackson and Klein, *Steinernema arenarium* Artyukhovsky, *Steinernema kushidai* Mamiya, *S. scarabaei* and *S. glaseri*. However, it has been shown that EPNs can sometimes opportunistically infect weak white grubs in the field (Kaya *et al.* 1993), in which case this may

not necessarily translate into an exceptionally high control potential of such EPNs towards their natural hosts.

EPN based products are now available in many countries and labelled for the control of various pests including scarabaeid larvae (Shapiro-Ilan *et al.* 2010). Four species are commercially available for the control of white grubs, namely *H. bacteriophora*, *Heterorhabditis zealandica* Poinar, *Heterorhabditis marelatus* Liu and Berry, and *S. glaseri* (Grewal *et al.* 2005). Abate *et al.* (2017), however, warned against the use of non-native EPNs, due to possible negative effects on the ecosystems where they are introduced, such as non-target effects and negative interactions with native EPN species.

Compared to laboratory studies, the efficacy of field applied EPNs has been inconsistent and difficult to predict (Grewal *et al.* 2005). Good laboratory results do not always translate into good field results, as EPNs have to withstand the adverse effects of various biotic and abiotic factors in the field. Unfavourable or sub-optimal environmental conditions can adversely affect the dispersal, survival and persistence of field applied EPNs (Georgis *et al.* 2006; Shapiro-Ilan *et al.* 2010). Biotic factors may include survival, motility, behaviour and reproductive potential, whereas abiotic factors include soil characteristics such as soil type, texture, moisture, pH, temperature, light and oxygen concentration (Brown & Gaugler 1997; Grant & Villani 2003; Jabbour & Barbercheck 2008). Minimising the negative effects of these factors will be a step towards optimising EPN efficacy. One of the strategies to minimize abiotic and biotic effects on EPNs is to identify strains of EPNs that are suitable for the target host and environment. For example, Shapiro-Ilan *et al.* (2014b) showed that different nematodes have different levels of tolerance to desiccation and freezing.

Defence mechanisms of white grubs against EPN attack

In general, insect larvae encounter numerous enemies, including humans, birds, mammals, amphibians, other arthropods and pathogens. Consequently, they have evolved various adaptations to avoid predation, parasitism and lethal infections. Such adaptations include

mimicry, crypsis, escape, startle, mutualism, parental care, feigning death (thantosis), aggregation, armour, fighting, aposematism, acoustic and chemical defences (Gaugler *et al.* 1994). In general, the defence mechanisms of insects can be separated into primary, secondary and tertiary levels (Figure 1). The three levels are sequential and spatially separated, with one being activated after the previous has failed (Smilanich *et al.* 2009), so as to minimise the energy costs of defence.

Primary defence mechanisms

Primary defence includes the morphological and behavioural mechanisms that the white grubs use to avoid encounters with their pathogens (Smilanich *et al.* 2009). In this case, the white grubs avoid the energy costs of defence and the risk of injury or death that may result from their interaction with pathogens. These mechanisms include the cryptic nature, evasive behaviour and changes in respiratory patterns of white grubs, and their association with other organisms.

Cryptic nature: Immature scarabs protect themselves simply by hiding in the densely compacted soil particles. The quiescent nature of white grubs also helps them to avoid contact with ambusher nematodes which are known to adopt a sit and wait mechanism and are triggered by movement and vibrations of the host (Kaya *et al.* 1993; Nobuyoshi 2002), thus the cocooning and overwintering behaviour of white grubs could act as an additional barrier to infection. The scarab larval cocoons and the pupal cells are also known to serve as an infection barrier (Gaugler 1988; Gross, 1993; Gaugler *et al.* 1994).

Evasive behaviour: Behavioural defences of scarab larvae involved in avoidance of pathogens include evasive behaviours that facilitate escape from the immediate environment (Gaugler *et al.* 1994). For example, in Petri dish bioassays, larvae of the Japanese beetle, *P. japonica*, responded to attack by IJs of *H. bacteriophora* with aggressive and evasive behaviours that involved brushing off the EPNs with the legs and rubbing the EPNs with the abrasive raster. The grooming and aggressive behaviours resulted in 60% death of the applied EPNs and a 13% reduction in white grub mortality (Gaugler *et al.* 1994, Alvandi *et al.* 2017). However, the

grooming and aggressive behaviours that dislodge, disable, or kill the attacking nematode could double as both primary and secondary defence as they are activated once the scarab larvae have been in contact with the pathogen.

Changes in respiratory patterns: White grubs avoid infection by EPNs by changing the pattern of their carbon dioxide release. Carbon dioxide concentration is an imperative cue in host location of some EPNs (Gaugler *et al.* 1988; Lewis *et al.* 1993). Releasing carbon dioxide in infrequent bursts rather than continuously, assists white grubs to avoid detection by EPNs (Gaugler 1988, Kaya *et al.* 1993; Gaugler *et al.* 1994, Grewal *et al.* 2005).

Association with other organisms: Natural enemies with antagonistic effects on EPNs include bacteria, protozoans, turbellarians, other nematodes, collembolans, mites, tardigrades, oligochaetes, fungi and scavenger insects (Kaya *et al.* 1998; Kaya 2002). A study by Karagoz *et al.* (2007) found that *Sancassania* sp. (Acari: Acaridae) mites isolated from field-collected scarab larvae were predaceous towards infective juveniles (IJs) of *S. feltiae* under laboratory conditions. Adult female mites consumed more than 80% of the IJs of *S. feltiae* within 24 h and reduced their infectivity towards the larvae of *Galleria mellonella* Linnaeus. In another recent study, Skowronek *et al.* (2020) found that bacteria from the midgut of the common white grub *Melolontha melolontha* L. larvae exhibited antagonistic activity against EPN bacteria. There is need for further research on organisms associated with white grubs and their role in the resistance of white grubs to EPNs.

Secondary defence mechanisms

The secondary defence mechanisms are those activated once the larva has been detected or attacked by the pathogen. These include the use of spines, hairs, regurgitating, thrashing or dropping (Smilanich *et al.* 2009). EPNs, being endoparasitoids must overcome the first line of morphological defences consisting of the white grub's hard exoskeleton, spiracles covered by a dense layer of impenetrable sieve plates and mucosal tissues of the external openings (Brivio & Mastore 2018). Scarab spiracles, a key portal of nematode entry in most insects (Georgis &

Hague 1991; Nguyen & Smart, 1991), are protected by impenetrable sieve plates (Gaugler *et al.* 1994; Forschler & Gardner 1991) that protect white grubs from being penetrated by EPNs. In addition, the white grub gut's dense peritrophic membrane may delay penetration through the gut, allowing the food passage to remove nematodes from the vulnerable alimentary tract (Forschler & Gardner 1991; Gaugler *et al.* 1994, Grewal *et al.* 2005). However, some EPNs manage to avoid these defence mechanisms. For example, the IJs of *H. bacteriophora* possess an anterior tooth which they use to directly pierce and penetrate the cuticle of insects along the thin walls (Grewal *et al.* 2005) and access the larval haemocoel.

Tertiary defence

The tertiary defence includes the physiological defence mechanisms or the innate immune system and is aimed at providing resistance to the insect host (Smilanich *et al.* 2009). This defence level is divided into cellular and humoral immunity. The cellular system includes phagocytosis, nodulation and encapsulation mediated by haemocytes (Strand 2008). The humoral immune response includes the production of soluble effector molecules; antimicrobial peptides, lysozyme, lectins, activation of phenoloxidase cascades that relates with coagulation and melanisation of haemolymph and production of reactive oxygen species (Hoffmann 2003; Kanost *et al.* 2004; Tsakas & Marmaras 2010; Strand 2008; Alvandi *et al.* 2017). For white grubs, the innate immunity is aimed at the EPNs, or the bacteria that the EPNs release before they are killed (Stokwe *et al.* 2017).

Humoral and cellular defences can be activated simultaneously to recognise pathogens and their encapsulation (Götz & Vey 1987; Götz *et al.* 1981; Rizki & Rizki 1987; Alvandi *et al.* 2017). Humoral defences that kill or incapacitate invading EPNs are well known for many insects including scarabs (Dunphy & Thurston 1990; Wang *et al.* 1994). The principal defence response of insects against small pathogens such as bacteria and fungi is phagocytosis, whereas encapsulation is used for large parasites such as EPNs.

Encapsulation involves the use of multiple haemocytes (granulocytes and plasmatocytes) to attach to the invading EPN (Schmidt *et al.* 2001; Strand 2008) and aggregate on the surface coat of the nematode's cuticle. Finally, the encapsulated nematode will be killed by the by-products of phenoloxidase cascade (e.g., quinone and melanin) within a cellular capsule entrapped within a necrotic internal layer of haemocytes (Strand 2008; Kanost & Gorman 2008; Alvandi *et al.* 2017). Granulocytes and plasmatocytes have been reported to be the most abundant haemocytes in the white grub, *P. adspersa* (Alvandi *et al.* 2017) and are involved in the cellular encapsulation that leads to capsule formation (Lavine & Strand 2002).

Suppression of bacterial growth

The haemocoel of the longhorned beetle *Cacosceles newmannii* Thompson (Coleoptera: Cerambycidae) and the woolly apple aphid *Eriosoma lanigerum* Hausmann (Hemiptera: Aphididae) have been shown to suppress the growth of symbiotic bacteria of some EPNs (Stokwe *et al.* 2017; Javal *et al.* 2019). Inhibition of EPN bacterial growth in turn inhibits EPN growth and reproduction. For example, when Javal *et al.* (2019) tested three locally isolated *Steinernema* species against the larvae of *C. newmannii*, a very low level of pathogenicity was observed. This was attributed to the suppression of symbiotic bacteria of EPNs in the insect's haemocoel and thus inability of the infective juveniles to access their main nutrient source.

Potential for maximisation of EPN pathogenicity to white grubs

The management of white grubs with EPNs still remains challenging because of the various primary, secondary and tertiary defences that different white grub species have evolved, as well as the other biotic and abiotic influences on EPNs in the environment where they are applied. An understanding the biological functioning of white grubs in relation to EPNs and the use of that knowledge when using EPNs to manage white grubs can enhance the effectiveness of this management tool. In the section below, we discuss different factors that can be explored to improve the virulence of EPN against white grubs.

Isolating and screening various EPN species/strains

Most of the inconsistencies in the efficacy of EPNs to control white grubs can be explained by the use of an unsuitable nematode strain or adverse environmental conditions (Grewal *et al.* 2005; see Section 4 and 5). It is thus necessary to scout for and screen available EPN species and strains for their potential to control specific white grub species for application under specific conditions (Shapiro-Ilan *et al.* 2014a). Using local EPNs that are better adapted to the local environmental conditions should be a preferred option as this reduces the effect of environmental stress on EPNs and prevent the introduction of alien EPN species into new environments (Abate *et al.* 2017). For example, in their laboratory study, Abate *et al.* (2019) showed that a local strain of *H. bacteriophora* provided better control for *Heteronychus licas* Klug when compared to an imported strain of the same nematode species and that of *S. feltiae*.

The process to isolate, screen and select the most suitable EPNs for particular environment conditions requires accurate identification of both the white grub species and the EPN species. Even though there is a lack of taxonomists, the advancement in molecular identification using DNA sequence data has allowed faster and more accurate identifications of EPNs and their symbiotic bacteria.

Timing of application of EPNs

The timing of EPN application can be an important factor in determining their efficacy to control white grubs. This is because the first and second instar larvae are the most susceptible to EPNs, while the third instars are most resistant (Grewal *et al.* 2004; Alvandi *et al.* 2017). Knowledge on the life cycle of the white grub species and its abundance in the environment can thus be used to inform the potential application time of EPNs. The best application time of EPNs against white grubs would likely be in spring and summer when temperatures are warm for EPN movement and when the second and third instars migrate to the surface to feed (Sutton & Stone Jr 1974; Choo *et al.* 2002). However, the occurrence of overlapping generations of white grubs, or a mixture of white grub species with differing phenology, within the same

plantation can complicate the timing of application. Timely monitoring and accurate identification of the white grubs is thus needed. Preventative application of EPNs is also a viable option as the nematodes can stay dormant for a very long time in the soil or maintained in the soil by being recycled in the host. In addition, more than one round of application of EPNs might be necessary to bring the white grub populations to a manageable level.

Use of synergistic entomopathogens and insecticides

Different entomopathogenic fungi (EPFs), such as the commonly used *Beauveria bassiana* Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, as well as the soil dwelling bacteria *Bacillus thuringiensis* Berliner, have been shown to have an additive and synergistic interaction with EPNs for scarab grub control (Koppenhofer & Kaya 1997; Choo *et al.* 2002; Ansari *et al.* 2004). In this case, the EPF or the bacteria reduces the immunity of the host which is then attacked by the EPNs. However, in their study Wu *et al.* (2014) showed both synergistic and antagonistic associations between EPNs and EPFs and recommended that more virulent fungal strains or species may be required to achieve a stronger synergistic/positive interaction with nematodes in the management of the white grub, *Cyclocephala lurida* Bland.

The addition of an insecticide within the integrated pest management program for control of the grubs can further increase mortality of the target white grubs. For example, Koppenhöfer *et al.* (2003b) used neonicotinoid insecticides and entomopathogenic fungi along with EPNs to reduce the density and damage of several species of white grubs. Insecticides lower the immune response of the white grub, thus making the grubs more susceptible to EPN infection. Infective juveniles of EPNs have been found to be tolerant to short exposures (2–6 h) of most acaricides, fungicides, herbicides and insecticides (Rovesti *et al.* 2013; Laznik&Trdan 2014). They can, therefore, be applied simultaneously with many pesticides.

Reducing the effect of environmental stress on EPNs

It has been determined that most of the inconsistencies in the efficacy of EPNs to control white grubs can partly be explained by adverse environmental conditions (Grewal *et al.* 2005). The most common environmental factors that affect the survival ability of EPNs include moisture, temperature and soil physical properties (Kaya *et al.* 1993). Adjustments such as applying irrigation after EPN application or applying the EPNs during a season where the environmental factors are suitable, can increase EPN performance. Use of EPN strains that are locally isolated will provide more adaptation benefits, because the EPNs are naturally adjusted to the environment (Alpert 2006). Reducing environmental stress should be coupled with improvement of formulation and application technologies (Cruz-Martínez *et al.* 2017). Different methods of nematode formulation have been evaluated. These include formulation for storage and transport; aqueous suspension, synthetic sponges, gels, clay and powder and formulation for direct application in the field which include the use of gels or infected cadaver (Cruz-Martínez *et al.* 2017). For example, Abate *et al.* (2019) showed that formulating the EPNs in a polyacrylamide gel medium, rather than direct inundation in water, increased the EPN survival as the gel has greater water holding capacity.

EPN genomics and manipulation

Biocontrol efficacy can be improved through strain discovery, selection, hybridization and transgenic manipulation (Gaugler *et al.* 1997). Genetic manipulation of the EPNs for the control of white grubs can focus on genetic traits such as host finding and penetration ability, dispersal and desiccation tolerance (Bai *et al.* 2005; Salame *et al.* 2010). For example, in their study, Shapiro-Ilan *et al.* (2014b) compared inter- and intraspecific variation in freeze and desiccation tolerance among different different EPNs. The results showed different levels of tolerance to desiccation and freezing among EPN species. Intraspecific variation was found for freeze tolerance and not for desiccation tolerance. Genome sequencing of EPNs and their symbionts can help in the overall understanding of genes responsible for such inter- and intraspecific

characters which can help in genetic improvement for EPN efficacy. Such can be done by facilitating the selection of candidate genes for hypothesis-driven studies of gene-trait relationships or by genomics-assisted breeding for desirable traits (Lu *et al.* 2016).

Switching bacterial symbionts between EPN species

The EPN bacterial symbionts affect the pathogenesis process of insects as they contribute to the EPNs virulence and reproduction (Kaya *et al.* 1993). The relationship between the EPN and its symbiotic bacteria is very specific. Each EPN naturally isolated from the environment has been found in association with its own specific bacteria (Goodrich-Blair & Clarke 2007). Each *Steinernema/Heterorhabditis* species associates with a single *Xenorhabdus/Photorhabdus* species, but one bacterial species can form associations with more than one EPN host of the same genus (Boemare 2002; Goodrich-Blair & Clarke 2007).

The possibility of producing monoxenic cultures of both nematodes and bacteria (Goodrich-Blair *et al.* 2009) has made it possible to study the relationship between EPNs and their (non) associate bacteria. The results show that some EPNs are able to feed on non-cognate bacteria, but typically do not retain it in the second generation (Akhurst 1983; McMullen II *et al.* 2018). A recent study by McMullen II *et al.* (2018) showed that non-cognate nematode-bacteria pairings resulted in reductions for multiple measures of success, including total progeny production and virulence. The IJs also carried fewer bacterial cells when colonized by a non-cognate symbiont. This suggests that level of specificity is dictated by events occurring at the molecular and cellular interface between the host and the bacteria during the colonization process (Goodrich-Blair and Clarke 2007). This suggests in turn that understanding the molecular functioning of the two organisms can help to identify how to pair the most virulent bacteria, with the most virulent EPNs for biocontrol of insects, including white grubs.

Conclusion

This review discusses many examples that show that biological control of Scarabidae pests is possible using EPNs and can provide an even higher percentage control than chemical control. The greatest challenge, however, has been to eliminate the inconsistencies in the efficacy of EPNs to control white grubs. These inconsistencies have been attributed to resistance of the white grubs to EPNs, the use of the wrong EPN species or application of the EPN during unsuitable environmental conditions. All these are issues that can be addressed through thorough research.

To increase control efficacy, a greater understanding of the patterns and mechanisms of resistance of white grubs to EPNs is needed. Such knowledge will assist in selecting the most suitable EPN species for target white grubs. Related to this, studies on genome-assisted breeding of EPNs and investigating potential synergistic microbes and materials to increase EPN efficacy is needed. Furthermore, timely monitoring and proper identification of the white grubs in the field is crucial.

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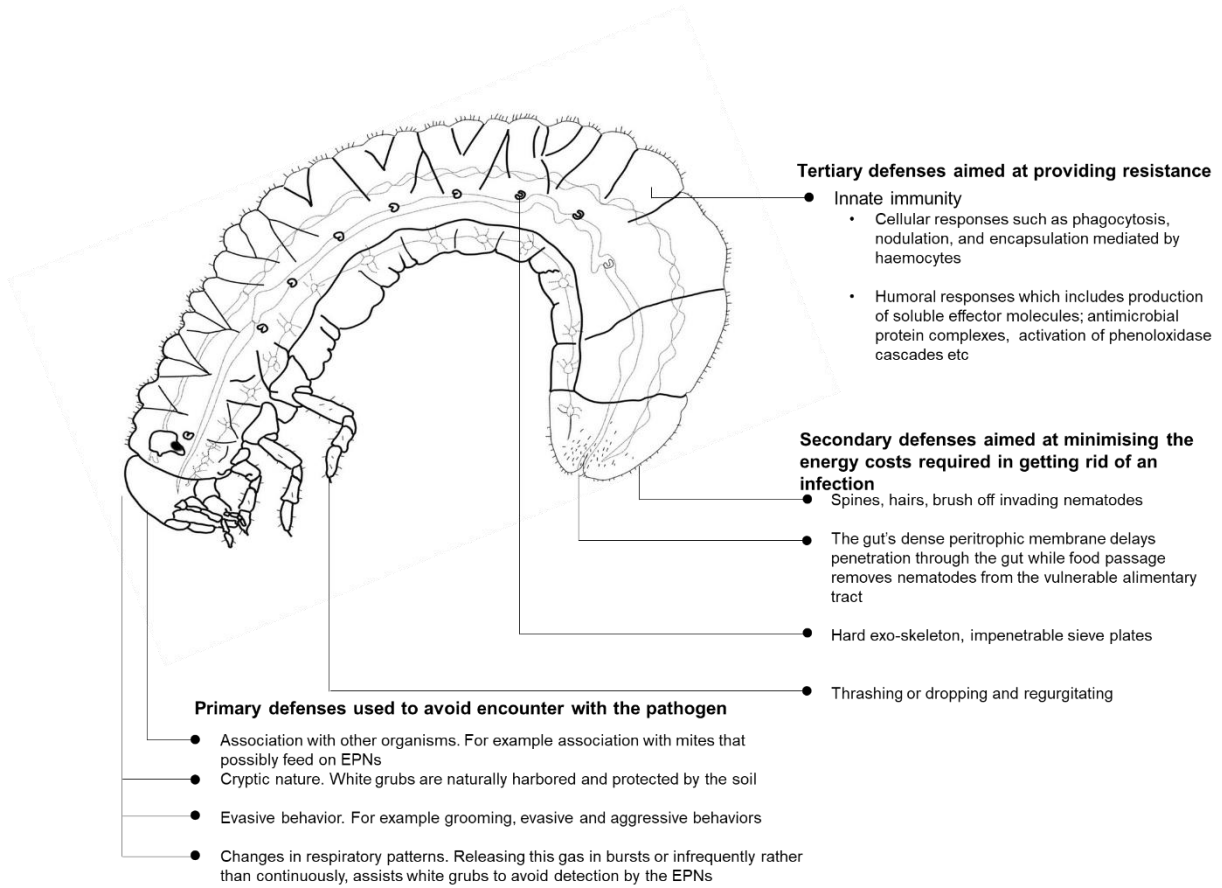
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Table 1. 1 List of some of the most susceptible white grubs to entomopathogenic nematodes (EPNs), and the percent mortality obtained from each study.

White grub species	EPN species	Field (F)/ Laboratory (L)/Pot (P)	IJs/larva - (lab) IJs/ha - (Field/Pot)	Efficacy (% control)	Time interval for efficacy (days)	References
<i>Popillia japonica</i>	<i>S. scarabaei</i> AMK001	L	400	80-100	14	Koppenhöfer & Fuzy 2003a
	<i>H. bacteriophora</i> GPS11	F	2.5 x 10 ⁹	97	35	Grewal <i>et al.</i> 2004
	<i>H. bacteriophora</i> TF <i>H. bacteriophora</i> , <i>S. glaseri</i> , <i>S. scarabaei</i>	L	400	65-92	14	Koppenhöfer & Fuzy 2003a Koppenhöfer <i>et al.</i> 2004 Koppenhöfer <i>et al.</i> 2004 Koppenhöfer <i>et al.</i> 2004
		L	400	86 – 100	14	
		L	400	86 – 100	14	
L	400	86 – 100	14			
	<i>H. zealandica</i> X1	F	2.5 x 10 ⁹	98	35	Grewal <i>et al.</i> 2004
<i>Anomala orientalis</i>	<i>S. scarabaei</i>	L	400	98	14	Koppenhöfer & Fuzy 2003a Koppenhöfer & Fuzy 2009 Koppenhöfer <i>et al.</i> 2004
	<i>S. scarabaei</i>	F		77-100		
	<i>S. scarabaei</i>	L	400	>90	14	
<i>Maladera castanea</i>	<i>S. scarabaei</i>	L		100	14	Koppenhöfer & Fuzy 2003b Koppenhöfer <i>et al.</i> 2004
	<i>S. scarabaei</i>	L	400	> 90%		
<i>Rhizotrogus majalis</i>	<i>S. scarabaei</i>	L	400	100	14	Koppenhöfer & Fuzy 2003a Cappaert & Koppenhöfer <i>et al.</i> 2003 Koppenhöfer <i>et al.</i> 2004
	<i>S. scarabaei</i>	F	2.5 x 10 ⁹	88	21	
	<i>S. scarabaei</i>	L	400	>98	14	
<i>Cyclocephala borealis</i>	<i>H. zealandica</i> X1	F	2.5 x 10 ⁹	96	35	Grewal <i>et al.</i> 2004; Koppenhöfer & Fuzy, 2003a Grewal <i>et al.</i> 2004
	<i>S. scarabaei</i>	F	2.5 x 10 ⁹	84	21	
	<i>H. bacteriophora</i> GPS11	F	2.5 x 10 ⁹	83	35	
<i>Hoplia philanthus</i>	<i>S. glaseri</i>	P	7.5 x 10 ⁹	>80	42	Ansari <i>et al.</i> 2006 Ansari <i>et al.</i> 2008
	<i>H. megidis</i>	P	7.5 x 10 ⁹	>70	42	
	<i>H. megidis</i> <i>S. glaseri</i>	L	10,000	100	7	
L		10,000	100	7		
<i>Melolontha melolontha</i>	<i>S. glaseri</i>	L	5000	70-80	27	Peters 2000
<i>Phyllophaga crinite</i>	<i>S. scarabaei</i>	L	400	Highly susceptible	14	Koppenhöfer <i>et al.</i> 2004 Koppenhöfer <i>et al.</i> 2004
<i>Phyllophaga congrua</i>	<i>S. scarabaei</i>	L	400	Highly susceptible	14	
<i>Ataenius spretulus</i>	<i>H. bacteriophora</i>	L	400	Very susceptible	14	

<i>Phyllophaga</i> (Sub. <i>Phytalus</i>) <i>georgiana</i>	<i>S. scarabaei</i>	L P	400 2.5×10^9	Highly susceptible 76-100	14 21	Koppenhöfer <i>et al.</i> 2008
<i>Polyphylla</i> <i>adpersa</i>	<i>S. glaseri</i>	L	400	Very susceptible	14	Koppenhöfer <i>et al.</i> 2004

Figure 1. 1 Defence mechanisms used by white grubs to evade attack by nematodes. Based on Gaugler 1988; Forschler & Gardner 1991; Gross, 1993; Kaya *et al.* 1993; Gaugler *et al.* 1994; Hoffmann 2003; Kanost *et al.* 2004; Grewal *et al.* 2005; Karagoz *et al.* 2007; Strand 2008; Smilanich *et al.* 2009; Tsakas & Marmaras 2010; Alvandi *et al.* 2017; Brivio & Mastore 2018.



Chapter 2
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***Steinernema bertusi* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic
nematode from South Africa**

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Summary - Two isolates of *Steinernema bertusi* n. sp. were separately recovered from Tito, Mpumalanga and Port Edward, Kwa Zulu Natal, South Africa. In this paper, we describe the isolates as a new entomopathogenic nematode (EPN) species using molecular and morphological methodologies. The new species belongs to the *cameroonense*-clade, which consists of nematodes only isolated from the African continent. *Steinernema bertusi* n. sp. is characterised by having the longest infective juvenile (IJ) for this clade at 716 (628-814) μm . The IJ is further characterised by a body diam. of 32 (28-36) μm and the pattern for the arrangement of the lateral ridges from head to tail is 2, 4, 5, 4, 2. The first-generation male spicule and gubernaculum length is 82 (72-88) μm and 63 (54-72) μm , respectively. Only 25% of the second generation males possess a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double flapped epiptygmata and a mucron at the posterior end. The new EPN species is most closely related to *S. sacchari* and is the fifth species to be included in the *cameroonense*-clade.

Keywords - *cameroonense*-clade, molecular, morphology, morphometrics, new species, taxonomy.

Introduction

Entomopathogenic nematodes (EPN) belonging to the order Rhabditida are known to parasitise various below- and above-ground insects (Grewal *et al.* 2005; Lacey & Georgis, 2012). For this reason, they are used worldwide in biological control and integrated pest programs of various agricultural insect pests. EPN kill their insect hosts with the help of symbiotic bacteria, which they carry in their digestive system and sometimes in specialised bacterial chambers (Kaya *et al.* 1993; Nobuyoshi, 2002). Death of the insect normally occurs within 48 h after infection. The advancement in *in vitro* mass production and formulation technology has allowed for a prolonged shelf life of EPN and ease of transportation over long distances. Thus, EPN are available on the market for export to different countries, in some of which, they are exempted from registration. The two families of EPN: Steinernematidae and Heterorhabditidae are easily commercialised, partly because of their ease of mass production (Shapiro-Ilan *et al.* 2010).

Approximately 100 *Steinernema* and 16 *Heterorhabditis* valid species have been described from different parts of the world (Nguyen, 2016). In South Africa, the first EPN species description was in 2006 for *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006. To date, 13 EPN species have been described from South Africa, including recent descriptions of *Steinernema beitlechemi* Çimen, Půža, Nermut', Hatting, Ramakuwela, Faktorová & Hazir, 2016, *Steinernema biddulphi* Harum, Çimen, Vladimír & Půža, 2015, *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley, 2016 and *Steinernema nguyeni* Malan, Knoetze & Tiedt, 2016, as well as another six locally isolated species, already reported from other countries (Malan & Ferreira, 2017; Steyn *et al.* 2017; Abate *et al.* 2018). The discovery and description of new EPN species is important, because it allows to study the diversity among species, and the discovery of different genetic traits that can subsequently be used for genetic studies. As regards EPN as biocontrol agents of insect pests, it has also been determined that some EPN are highly host specific, thus discovering new EPN species will reassure more precise control EPN of specific insect pests in localised areas (Adams & Nguyen, 2002).

Spiridonov & Subbotin (2015) deduced from biogeographical analysis using *Steinernema* species descriptions up till 2015 that colonisation of the African continent occurred five times, with the resultant moderate diversity, for example in comparison to Asia, with the latter having repeated colonisation from other regions. They regarded the five ancestral colonisation events for Africa as: i) *Steinernema citrae* Stokwe, Malan, Nguyen, Knoetze & Tiedt, 2011 (*feltiae*-clade); ii) *Steinernema yirgalemense* Tallosi, Peters & Ehlers, 1995 (*bicornutum*-clade); iii) *Steinernema tophus* Çimen, Lee, Hatting, Hazir & Stock, 2014, *Steinernema innovationi* Çimen, Lee, Hatting, Hazir & Stock, 2014, *S. khoisanae* and *Steinernema jeffreyense* Malan, Knoetze & Tiedt, 2015 (*khoisanae*-clade); iv) *Steinernema karii* Waturu, Hunt & Reid, 1997 and *Steinernema ethiopiense* Tamiru, Waeyenberge, Hailu, Ehlers, Půža & Mráček, 2012 (*glaseri*-clade) to originate from Asia; and v) *Steinernema cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 (*cameroonense*-clade) to have originated from the Americas. Since the publication of the book of Hunt & Nguyen (2016), new South African species have been added to four of the five clades, including *S. nguyeni* to the *feltiae*-clade, *S. biddulphi* to the *bicornutum*-clade, *S. fabii* to the *khoisanae*-clade, and *S. beitlechemi* to the *cameroonense*-clade.

In the current study, a new *Steinernema* species from South Africa, referred to as *S. bertusi* n. sp., is described using morphology and molecular characterisation. This will also be the twelfth *Steinernema* species described from South Africa, some 12% of the world species descriptions for this genus.

Materials and methods

EPN source

The first isolate, called Tito 13 (Mpumalanga: 27°33.49S 30°28.85E), of the new species of EPN was obtained through baiting from soil samples from a wattle forest plantation, using the last instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). This isolation of the EPN in 2016, was part of a larger survey for EPNs from commercial forestry

areas in South Africa (Abate *et al.* 2018). After being molecularly identified using the ITS and D2-D3 region, the isolate culture was lost as it proved impossible to be successfully maintained on *Galleria* or *Tenebrio molitor* Linnaeus 1758 larvae. During a different survey by Steyn *et al.* (2017), the isolate WS22 (Natural forest reserve, Port Edward, KwaZulu-Natal: 30°59.699' 30°15.108'E) was identified using molecular techniques, as being identical to Tito 13 and was used for the molecular and morphological description of the new *Steinernema* as *S. bertusi* n. sp.

Light microscopy

Individuals belonging to different growth stages of the nematode, which included the infective juvenile (IJ), first and second-generation males and females, were obtained for morphological observations using a light microscope. A sample of IJ was stored in vented tissue culture flasks (12°C) for a period of 2 months to obtain ex-sheathed IJ. Both temporary and permanent slides were prepared for the different growth stages of the nematode. To obtain these stages, ten *G. mellonella* larvae were placed in Petri dishes lined with moist Whatmann filter paper and inoculated with 200 IJ per larva of *G. mellonella*. The plates were placed in a plastic container lined with wet paper towels (100% moisture), and kept in a growth chamber (MRC 358, Labotec), at 25°C for a period of 48 h. Dead larvae were removed after 48 h, rinsed with distilled water, placed in different Petri dishes and returned to the growth chamber. A few individuals were removed daily and dissected in Ringers solution to observe the stage of growth of the nematodes. First generation male and female adults were obtained after 4-5 days post infection, while second generation adults were obtained after 6-7 days.

The IJ were harvested by using a modified White trap (Woodring & Kaya, 1988). The different growth stages of the nematodes were permanently fixed in hot TAF (2% triethanolamine, 8% formalin in distilled water) at 85°C (Courtney *et al.* 1955). Specimens were then processed to pure glycerin, using the modified Seinhorst technique (Seinhorst, 1959), after which they were mounted in pure glycerin to obtain permanent slides. The individual nematodes on the

permanent slides were used for the morphometric studies. Measurements and drawings were made by means of a Zeiss compound microscope (Zeiss Axio Scope A1), fitted with a digital camera, and computer with ZEN lite digital imaging software (ZEN black 2.3 SP1 / blue 2.6).

Scanning electron microscopy (SEM)

For the SEM, the samples (first and second generation adults, fresh IJ and unsheathed IJ) were fixed in TAF. They were then washed three times in 0.05 M cacodylate buffer for 15 min each, and then washed three times in distilled water for 15 min each, after which they were dehydrated in a graded ethanol series (70, 80, 90 and 2 × 100%). The samples were critical point dried with liquid CO₂, mounted on SEM stubs and sputter coated with 20 nm gold/palladium (66/33%). The samples were viewed with a FEI Quanta 200 ESEM, operating at 10 kV under high vacuum mode.

DNA extraction

The protocols outlined in Nguyen (2007) were used to extract DNA from a single young female nematode. The lysis buffer consisted of 50 mM MgCl₂, 10 mM DTT, 4.5% Tween-20, 0.1% gelatine and 1 µl of proteinase K at 60 µg m⁻¹. The first generation female was placed in 30 µl drop of the lysis buffer pipetted on the upper side of a 0.5 ml microcentrifuge tube. The nematode was cut into a few pieces, using a sterile insulin needle and the contents were immediately placed on ice and transferred to –80 °C for 20 min. For total lysis of the cells and digestion of the proteins, the tubes were incubated at 65°C for 1 h and at 95°C for 10 min in a thermocycler (GeneAmp 2720). The tube was cooled on ice and centrifuged at 11,600 g at 10°C for 2 min and 5 µl were pipetted from the supernatant and used in the PCR amplification.

PCR amplification

The protocols described in Nguyen (2007) for PCR amplification of both the ITS and the D2-D3 regions were followed. The ITS regions and D2-D3 fragment of the 28S region of the ribosomal DNA were amplified in a 25 µl reaction. The PCR primers used to amplify the ITS region were

the 18S primer (5-TTGATTACGTCCCTGCCCTTT-3) and the 28S primer (5-TTTCACCTCGCCGTTACTAAGG-3) as described by Vrain *et al.* (1992). The primers used to amplify the D2-D3 regions of 28S rDNA were the D2F (5-CCTTAGTAACGGCGAGTGAAA-3) in Nguyen *et al.* (2007), and 536 (5-CAGCTATCCTGAGGAAAC-3) in Stock *et al.* (2001). PCR amplifications were carried out in tubes containing 5 µl nematode lysate, together with 0.5 µm of each primer and 12.5 µl KAPA2G™ Robust Hotstart ReadyMix (KAPA Biosystems). The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 s, annealing at 50-55°C for 30 s, and extension at 72°C for 45 s, with all conditions being repeated for 35 cycles. A 2-min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

The PCR product was then run on 1% agarose gel in a 1 × TBE buffer and visualised by means of ethidium bromide staining. Post-PCR purification was done using the NucleoFast Purification System (Macherey Nagel). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by electrophoresis on the 3730 × 1 DNA Analyser (Applied Biosystems) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University). The ITS primers 18S and 28S, and additional two internal primers, KN58 (5-GTATGTTTGGTTGAAGGTC-3) and KNRV (5-CACGCTCATACAACTGCTC-3), suggested by Nguyen *et al.* (2007), were used for the sequencing of the complete ITS region. Likewise, primers 502 (5-CAAGTACCGTGAGGGAAAGTTGC-3) and 503 (5-CCTTGGTCCGTGTTTCAAGACG-3), reported by Stock *et al.* (2001) were used for sequencing of the D2-D3 regions.

Sequence alignment and phylogenetic analysis

Sequences were assembled, analysed and edited using the CLC DNA main Workbench ver. 8.1. The sequences of both the ITS region of the rDNA gene and that of the D2-D3 region of the 28S gene were aligned and compared with other *Steinernema* species sequences available on GenBank (NCBI). Sequence alignment was done using ClustalX 2.1 (Thompson *et al.* 1997),

while phylogenetic analyses of sequence data were done using the Maximum Parsimony (MP) method in MEGA5 (Tamura *et al.* 2011). Support for tree branches was evaluated statistically by means of a bootstrap analysis based on 1000 re-samplings of the dataset. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by means of the random addition of sequences (ten replicates). *Caenorhabditis elegans* (FJ589008) was used as outgroup during the calculation of the trees based on the ITS sequences, while *Cervidellus alutus* (AF331911) was used as outgroup for the calculation of the tree based on the D2-D3 sequences.

Results

*Steinernema bertusi** n. sp.

= isolate WS22 of Steyn *et al.* (2017) and isolate Tito 13 of Abate *et al.* (2018)

(Figs. 2. 1-4)

Measurements

See Tables 1-4.

* Patronym in honour of Albertus J. Meyer, or ‘Bertus’ as he was affectionately known, a pioneer of Nematology in South Africa.

Description

First generation male

Body curved ventrally posteriorly. Body J-shaped when heat relaxed. Cuticle smooth under light microscope, but striations visible with SEM. Head rounded, two amphidial apertures observed. Posterior part of stoma funnel shaped. Testis monorchic, reflexed posteriorly. Head rounded with four cephalic papillae and six labial papillae. Pharynx with cylindrical procorpus, metacarpus slightly swollen, excretory pore located anterior to nerve ring and close to metacarpus. Testis reflex variable. Each spicule with two internal ribs, narrow velum running from rostrum to spicule tip. Gubernaculum boat-shaped in lateral view, anterior end curved. Copulatory papillae totalling 21 and comprising a single precloacal, midventral papillae and ten pairs arranged as: six pairs precloacal subventral, one pair adcloacal, one pair lateral and two pairs subterminal. Anal body diam. larger than tail length, no mucron observed in first generation males.

Second generation male

Six labial papillae more prominent than cephalic papillae, which occur further back on head. Labial region less rounded and continuous with body shape. Body more slender than first generation, with smaller spicule and gubernaculum. Mucron observed in 25% of individuals.

First generation female

Body stout, C-shaped when heat-relaxed and when fixed with TAF. Body cuticle smooth under light microscope, but faint annules observed under SEM. Labial region rounded and continuous with body, six labial papillae and four cephalic papillae. Cheilorhabdions large, well sclerotised. Stoma prominent, posterior part funnel shaped. Amphidial apertures not observed. Pharynx with cylindrical procorpus, metacarpus slightly swollen, isthmus distinct, basal bulb enlarged. Nerve ring surrounding isthmus and just anterior to basal bulb. Excretory pore anterior to nerve ring in mid-pharynx region. Cardia prominent. Gonads amphidelphic, reflexed. Vulva a median

transverse slit, slightly protruding from body surface, situated in mid-body region, with long double flapped epiptygmata. Tail length shorter than body anal diam. Tail terminus short and tapering with mucron.

Second generation female

Morphology much like first generation but body much shorter and smaller. Tail tapering, no mucron observed.

Infective juvenile

Cuticle (second-stage cuticle) present but lost in storage. Cuticle marked with prominent transverse striations. Body elongate and tapering posteriorly at both ends. Body slightly curved from middle towards both ends when heat relaxed. Visible striations on body under light microscope. Ensheathed juvenile with six labial and four cephalic papillae. Cephalic region smooth and continuous with body. Amphidial apertures prominent. Pharynx with a thin corpus and a slightly swollen metacarpus. Excretory pore located at mid-pharynx level. Excretory pore anterior to nerve ring. Bacterial chamber prominent and located in anterior intestine. Lateral field beginning anteriorly with two ridges and forming the following formula: 2, 4, 5, 4, 2. Portion with five ridges forming greatest part of lateral field. Ridges more prominent towards posterior end up to end of tail.

Type host and locality

Natural host unknown. The isolate was collected by baiting with larvae of *Galleria mellonella*, although this was not a preferred host as the population declined after each re-cycling. Isolated from the Natural Forest Reserve in Port Edward, KwaZulu-Natal: 30°59.699'S 30°15.108'E (Steyn *et al.* 2017).

Type material

Holotype first generation male, paratype males and paratype females and IJ, will be deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa. In addition to these, paratypes of males, females will be deposited in the United States Department of Agriculture Nematode Collection (USDANC), Beltsville, MD, USA.

Diagnosis and relationships

Steinernema bertusi n. sp. is characterised by differences in the morphology and the morphometrics of the IJ and adults, as shown in Tables 2. 1-4. The IJ of the new species can be recognised by a body length of 716 (628-814) μm , a body diam. of 32 (28-36) μm and a tail length of 66 (58-74) μm . The pattern of the lateral field showed 2, 4, 5, 4, 2 ridges. The first-generation male has a spicule and gubernaculum of 82 (72-88) μm and 63 (54-72) μm , respectively, and lacks a mucron. The second-generation males have similar morphological characters to the first generation, except only 25% of the tails have a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double-flapped epiptygmata and a mucron at the posterior end. The first generation female tail is shorter than the anal body diam. and possesses a mucron. The new species has the longest IJ body length of the six EPN species in the *cameroonense*-clade. Of the *cameroonense*-clade, only *S. bertusi* n. sp. and *S. sacchari* have no mucron in the first generation males.

Steinernema bertusi n. sp. differs from *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 by having a longer body length of the IJ of 716 (628-814) vs 648 (565-708) μm , and a longer mean pharynx length of 123 (112-137) vs 114 (104-128) μm , although the ranges overlap. The first-generation male of *S. bertusi* n. sp. has gubernaculum length with a longer mean length of 63 (54-72) vs 53 (40-62) μm in the case of *S. nyetense* (Fig. 2. 2; Tables 2. 3-4), with overlapping ranges. The tail of the first generation female of *S. bertusi* n. sp. is dome shaped, while that of *S. nyetense* is conical and pointed (Table 2. 4).

The IJ of *S. bertusi* n. sp. differs from *S. cameroonense* by their longer body length of 716 (628-814) vs 622 (490-694) μm (Table 2. 2). Even though having a longer IJ, the relative position of the excretory pore and the nerve ring of *S. bertusi* is similar to that of *S. cameroonense* at 59 (53-65) vs 54 (45-64) μm and 91 (76-110) vs 85 (69-100) μm respectively (Table 2. 2). The spicule and gubernaculum of the first-generation male of *S. bertusi* n. sp. is longer at 82 (72-88) vs 69 (51-85) μm and 63 (54-72) vs 45 (37-57) μm (Table 2. 3). The first-generation male of *S. bertusi* also differs from that of *S. cameroonense* by the lack of a mucron (Tables 3, 4).

Steinernema bertusi n. sp. differs from *Steinernema sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan, 2014 by its longer IJ length of 716 (628-814) vs 680 (630-722) μm (Table 2. 2). The first generation males of *S. bertusi* n. sp. spicules do not have prominent rostrum such as in the case of *S. sacchari* with a prominent rostrum (Table 2. 4). The males of *S. bertusi* n. sp. are also narrower in body diam. at 116 (84-152) vs 145 (86-205) μm (Table 2. 3). The genital papillae of *S. bertusi* n. sp. are in 20 + 1 (Fig. 2. 2C, D) arrangement, while those of *S. sacchari* are in a 24 + 1 arrangement. The vulva of *S. bertusi* n. sp. is slightly protruding, while that of *S. sacchari* does not protrude (Table 2. 4).

Steinernema bertusi n. sp. differs from *S. fabii* by the longer length of the IJ at 716 (628-814) vs 641 (590-697) μm (Table 2. 2). However the pharynx length of *S. bertusi* n. sp. is shorter than that *S. fabii* at 123 (112-137) vs 132 (120-146) μm (Table 2. 2). The first generation males of *S. bertusi* n. sp. however have shorter spicule lengths at 82 (72-88) vs 90 (79-106) and are also narrower in body diam. at 116 (84-152) vs 138 (102-196) μm (Table 2. 3). *Steinernema bertusi* n. sp. males are longer than those of *S. fabii* at 716 (628-814) vs 641 (590-697) μm (Table 2. 2).

Steinernema bertusi n. sp. differs from *S. beitlechemi* by the first generation males having shorter spicules at 82 (72-88) vs 93 (88-110) and also a mean narrower body diam. at 116 (84-152) vs 138 (102-196) μm (Table 2. 3). The first generation females of *S. bertusi* n. sp. have

slightly protruding double flapped epiptygmata, while those of *S. beitlechemi* have indistinct, small epiptygmata (Table 2. 4).

Molecular characterisation

Steinernema bertusi n. sp. clustered with *S. nyetense*, *S. sacchari*, *S. cameroonense*, *S. sacchari*, *S. fabii*, and *S. beitlechemi* in both the ITS and D2-D3 phylogenetic analyses. All these species belong to the *cameroonense*-clade, a clade only reported from the African continent. According to Spiridonov & Subbotin (2016), the ancestors of the *cameroonense*-clade have their origins in the Americas.

Steinernema bertusi n. sp. is characterised genetically by the sequences of the ITS (ITS1 + 5.8S + ITS2) (KY082902) and the D2-D3 (KY08290) rDNA regions (Figs. 2. 5-6). Pairwise distances using the ITS region show that the new species differs from its closest relatives *S. nyetense* by 16 bp and *S. cameroonense* by 26 bp and from *S. sacchari* by 28 bp, while differing from all other closely related species by larger differences in bp (Table 2. 5). Pairwise comparison using the D2-D3 regions is presented in Table 2. 6. The bp difference between *S. bertusi* n. sp. and its closest relative *S. sacchari* was 4 bp and 6 bp between *S. beitlechemi*, *S. cameroonense*, *S. fabii*, and *S. nyetense*. This small bp difference can be attributed to the conserved nature of the 28S (D2-D3) region of EPN.

Conclusion

According to molecular and morphological observations, evidence is presented to confirm *S. bertusi* n. sp. as a new EPN from South Africa. The species belongs to the *cameroonense*-clade, with its origin proposed to be from the Americas (Spiridonov & Subbotin, 2016). The IJ of *S. bertusi* n. sp. has the longest body length of all the described species in this clade. The first generation males of *S. bertusi* n. sp. have a long spicule and gubernaculum, with no mucron, while 25% of the second generation males have a mucron. The first-generation females of *S. bertusi* n. sp. have a slightly protruding vulva, with double flapped epiptygmata

and with a mucron at the posterior end. Molecularly it differs in the ITS region with 16 bp and 26 bp from its closest relatives, *S. nyetense* and *S. cameroonense* respectively. This new description adds a fifth species to the *cameroonense*-clade and becomes the fourteenth EPN described from South Africa.

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Table 2. 1 Morphometrics of different stages of *Steinernema bertusi* n. sp. All measurements in μm and in the form: mean \pm s.d. (range).

Character	First generation			Second generation		Infective juvenile
	Male		Female	Male	Female	
	Holotype	Paratype	Paratypes	Paratypes	Paratypes	
<i>N</i>	-	20	20	20	20	25
<i>L</i>	1798	1789 \pm 195 (1474-2211)	4567 \pm 719 3075-6002	1187 \pm 172 (994-1741)	1399 \pm 197 (1121-1810)	716 \pm 46 (628-814)
<i>A</i>	15	16 \pm 1.7 (13-19)	24 \pm 4.3 (15-33)	16 \pm 2.4 (6.9-19)	17 \pm 1.1 (16-19)	23 \pm 1.5 (20-25)
<i>b</i>	12	11 \pm 0.8 (9.8-13)	21 \pm 5.5 2.4-29	8.6 \pm 1.0 (7.5-12)	9.1 \pm 1.1 (7.8-11)	5.8 \pm 0.2 (5.3-6.3)
<i>c</i>	60	61 \pm 12 (43-83)	197 \pm 56 (43-291)	43 \pm 7.9 (32-65)	35 \pm 8.1 (21-49)	11 \pm 0.6 (9.7-12)
<i>c'</i>	0.7	0.6 \pm 0.1 (0.5-0.8)	0.4 \pm 0.1 (0.3-0.7)	0.7 \pm 0.1 (0.6-0.8)	0.5 \pm 0.1 (0.4-0.8)	1.8 \pm 0.1 (1.5-2.0)
<i>V</i>	-	-	54 \pm 3.5 (43-61)	-	0.6 \pm 0.01 (0.5-0.6)	-
Body diam. (BD)	122	116 \pm 18 (84-152)	191 \pm 22 (167-247)	72 \pm 13 (55-108)	81 \pm 7.9 (69-95)	32 \pm 2.5 (28-36)
Stoma length	4.44	5.9 \pm 0.9 4.4-7.7	9.2 \pm 2.0 6.5-15.5	5.8 \pm 0.7 4.4-6.9	6.8 \pm 0.9 5.3-9.5	-
Stoma diam.	6.99	7.8 \pm 0.8 6.2-9.5	11.1 \pm 1.5 8.8-14.0	7.6 \pm 0.8 6.3-8.9	8.7 \pm 0.9 6.7-10.6	-
Excretory pore (EP)	93	91 \pm 12 (59-108)	94 \pm 22 (66-160)	74 \pm 9.1 (43-87)	153 \pm 7.6 (138-166)	59 \pm 3.1 (53-65)
Nerve ring (NR)	113	116 \pm 6.5 (99-127)	140 \pm 11 (114-156)	98 \pm 13 (80-109)	111 \pm 6.5 (99-124)	91 \pm 7.0 (76-110)
Pharynx length (ES)	156	160 \pm 9.4 (142-174)	203 \pm 94 (185-217)	136 \pm 9.3 (116-150)	71 \pm 6.7 (61-86)	123 \pm 6.7 (112-137)
Testis reflex	520	463 \pm 70 (317-628)	-	268 \pm 66 (146-385)	-	-
Tail length (T)	30	30 \pm 6.2 (22-47)	-	28 \pm 4.3 (19-34)	41 \pm 5.6 (34-55)	66 \pm 4.0 (58-74)
Anal body diam. (ABD)	44	48 \pm 7.9 (37-66)	67 \pm 21 (45-140)	41 \pm 4.3 (35-48)	34 \pm 4.3 (28-42)	18 \pm 1.4 (16-22)
Mucron		-	8.5 \pm 2.5 (3.3-12.7)	5.6 \pm 1.3 (4.6-7.8)	-	-
Hyaline (H)		-	-	-	-	37 \pm 3.2 (31-44)
Spicule length (SL)*	80	82 \pm 4.6 (72-88)	-	71 \pm 3.2 (67-81)	-	-
Spicule width (SW)	19	18 \pm 1.3 (16-20)	-	14 \pm 1.1 (13-17)	-	-
Sp. Head length	24	22 \pm 2.8 (18-28)	-	19 \pm 3.3 (14-25)	-	-
Sp. Head width	21	20 \pm 1.7 (17-23)	-	16 \pm 1.6 (14-21)	-	-
Gubernaculum length (GL)	59	63 \pm 5.7 (54-72)	-	49 \pm 4.2 (41-59)	-	-
Gubernaculum width	10	11 \pm 1.4 (8.2-13)	-	10 \pm 1.4 (8.1-13)	-	-
D% = EP/ES x 100	59	57 \pm 6.2 (40-66)	46 \pm 11 (31-80)	54 \pm 6.5 (31-62)	216 \pm 17 (191-251)	48 \pm 2.5 (43-53)
E% = EP/T x 100	310	314 \pm 68 (185-432)	397 \pm 137 (105-817)	275 \pm 54 (138-383)	385 \pm 60 (262-491)	89 \pm 6.1 (78-104)
SW% = SL/ABD x 100	183	173 \pm 30 (130-236)	-	176 \pm 19 (147-202)	-	-
GS% = GL/SL x 100	73	77 \pm 6.57 (60-89)	-	69 \pm 4.6 (61-78)	-	-
H% = Hyaline/T x 100		-	-	-	-	56 \pm 4.0 (49-68)
T/ABW	0.68	0.62 \pm 0.09 (0.50-0.80)	-	0.67 \pm 0.06 (0.55-0.78)	-	-

V% = distance from anterior end to vulva/body length; EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of oesophagus,; * = measured according to the arc.

Table 2. 2 Comparative morphometrics of the third-stage infective juveniles of *Steinernema bertusi* n. sp. and related *Steinernema* spp. (in descending order of body length). Measurements are in μm and in the form: mean (range).

Species	Morphometric character ^a									n	References
	(Range)										
	L	BW	EP	NR	ES	T	A	b	c		
<i>S. schliemanii</i>	934 (842-	35 (30-	72 (61-80)	- -	148 (127-162)	88 (76-95)	26 (23-30)	6 (6-7)	11 (10-11)	20	Spiridonov <i>et al.</i> 2010
<i>S. ashuense</i>	768 (720-800)	30 (28-	55 (51-59)	86 (77-91)	119 (113-128)	71 (66-76)	25 (24-27)	6 (6-7)	11 (10-12)	20	Phan <i>et al.</i> 2006
<i>S.</i>	757 (730-790)	35 (32-	61 (58-68)	93 (88-100)	117 (109-125)	77 (71-85)	22 (21-23)	6.5 (6.0-7.1)	9.8 (8.7-11)	20	Ma <i>et al.</i> 2012
<i>S. bertusii</i> n.	716 (628-814)	32 (28-	59 (53-65)	91 (76-110)	123 (112-137)	66 (58-74)	23 (20-25)	6 (5.3-6.3)	11 (9.7-12)	25	-
<i>S.</i>	712 (642-778)	28 (26-	56 (50-68)	84 (80-100)	120 (115-152)	75 (68-92)	25 (18-29)	6 (4-6)	10 -	25	Phan <i>et al.</i> 2005
<i>S. monticolum</i>	706 (612-821)	37 (32-	58 (54-62)	88 (81-93)	124 (120-131)	77 (71-95)	19 (14-22)	6 (5-6)	9 (7.6-	-	Stock <i>et al.</i> 1997
<i>S. beitlechemi</i>	696 (606-768)	27 (24-	58 (51-62)	96 (86-105)	126 (111-143)	65 (57-70)	26 (23-28)	6 (5-6)	11 (10-13)	20	Çimen <i>et al.</i> 2016a
<i>S. sacchari</i>	680 (630-722)	37 (30-	53 (49-58)	84 (78-97)	113 (104-127)	64 (51-74)	19 (14-23)	6 (6-7)	11 (10-12)	25	Nthenga <i>et al.</i> 2014
<i>S. nyetense</i>	648 (565-708)	32 (25-	52 (46-57)	85 (72-102)	114 (104-128)	82 (54-	21 (19-26)	6 (5-6)	8 (6-11)	20	Kanga <i>et al.</i> 2012
<i>S. fabii</i>	641 (590-697)	28 (26-	53 (49-57)	65 (55-84)	132 (120-146)	58 (52-64)	24 (21-41)	4.8 (4.4-5.3)	11 (10-12)	25	Abate <i>et al.</i> 2016
<i>S.</i>	622 (490-694)	30 (24-	54 (45-64)	85 (69-100)	113 (105-125)	76 (52-	21 (17-25)	6 (5-6)	9 (6-12)	20	Kanga <i>et al.</i> 2012

Abbreviations are as in Table 2.1.

Table 2. 3 Comparative morphometrics of first-generation males of *Steinernema bertusi* n. sp. and related *Steinernema* spp. (in descending order of spicule length). Measurements are in μm and in the form: mean (range).

Species ^b	Morphometric characters ^a							n
	Spicule	Gubern	W	D%	SW%	GS%	MU C	
<i>S. beitlechemi</i>	93 (88-100)	62 (56-72)	138 (102-196)	71 (64-89)	152 (109-179)	67 (57-74)	P	2 0
<i>S. fabii</i>	90 (79-106)	66 (56-77)	126 (97-153)	64 (52-75)	177 (126-224)	73 (63-86)	P	2 0
<i>S. sacchari</i>	83 (73-89)	61 (50-68)	145 (86-205)	67 (54-88)	171 (146-210)	73 (66-81)	A	2 9
<i>S. bertusii</i> n. sp.	82 (72-88)	63 (54-72)	116 (84-152)	57 (40-66)	173 (130-236)	77 (60-89)	A	2 0
<i>S. nyetense</i>	80 (67-98)	53 (40-62)	106 (62-159)	55 (40-70)	199 (125-283)	66 (51-77)	P	2 0
<i>S. schliemanni</i>	72 (61-81)	53 (43-64)	87 (76-120)	54 (50-58)	- -	- -	P	1 5
<i>S. monticolum</i>	70 (61-80)	45 (35-54)	160 (117-206)	55 (49-61)	140 (120-150)	60 (50-70)	P	2 0
<i>S. cameroonense</i>	69 (51-85)	45 (37-57)	90 (65-124)	64 (48-76)	170 (131-201)	64 (47-76)	P	2 0
<i>S. changbaiense</i>	63 (56-70)	40 (33-46)	126 (96-139)	58 (52-66)	149 (114-156)	65 (54-72)	P	2 0
<i>S. ashiuense</i>	59 (50-65)	37 (25-43)	106 (80-125)	50 (44-56)	149 (128-167)	63 (43-73)	P	2 0
<i>S. robustispiculum</i>	58	41	127	56	129	70	P	2 0

(51-65)	(36-44)	(105- 150)	(50- 63)	(111- 150)	(64- 79)
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^a Abbreviations as in Table 2.1 and references as in Table 2.2
- Measurements not available.

Table 2. 4 Comparative morphology of *Steinernema bertusi* n. sp. and closely related species.

Species	IJ	Male 1st generation				Male 2nd generation	Female 1st generation		
	Lateral line	Spicule	Gubernaculum	Genital papillae	Mucron	Mucron	Vulva	Tail	Post-anal swelling
<i>S. bertusii</i> n. sp.	1, 4, 5, 4, 2	Spicule brown, blunt, no velum present	Boat-shaped in lateral view, anterior end curved, cuneus	20+1	P	A	Slightly protruding, double flapped epiptygmata	Dome-shaped, with terminal mucron	P
<i>S. ashiuense</i>	5 equal ridges in midbody	Slightly yellowish, velum large, not covering spicule tip	Boat shaped, cuneus long, needle-shaped, wing of corpus expanding laterally	20 + 1	P	P	Protruding, no epiptygmata	Dome-shaped, with terminal peg	-
<i>S. cameroonense</i>	2, 4, 5, 4, 3, 2	Yellow, brown, velum present	Boat-shaped in lateral view, cuneus needle shaped	22 + 1	P	P	Protruding with epiptygmata	Conical pointed, with mucron	P
<i>S. monticolum</i>	8 unequal ridges in midbody	Brown-orange, velum present, spicule tip pointed	Arcuate, large, posterior end forked	21/23 ± 1	P	P	Not protruding, no epiptygmata	Short, blunt, with mucron	P
<i>S. nyetense</i>	2, 4, 5, 4, 3, 2	Yellow brown, velum large	Boat-shaped in lateral view, cuneus needle-shaped	22 + 1	P	P	Protruding with epiptygmata	Conoid and pointed, mucron on the tip	P
<i>S. robustispiculum</i>	8 unequal ridges in midbody	Yellow-brown, prominent rostrum, velum large	Boat-shaped, cuneus long	22 + 1	P	-	Protruding, with epiptygmata	Dome shaped, with terminal peg	P
<i>S. beitlechemi</i>	2, 5, 6, 5, 2	Golden brown-slightly curved, manubrium elongate	Boat-shaped, manubrium curved ventrally	22+1	P	P	Indistinct, small epiptygmata	Obese and rounded, mucron on tip	-
<i>S. changbaiense</i>	2, 5, 6, 4, 2	Golden/yellowish, moderately curved, slender, manubrium usually elongate	Boat-shaped in lateral view, cuneus V-shaped	20/22+1	P	P	Symmetrical, slightly protruding	Conoid and short, mucron present	P
<i>S. fabii</i>	5 equal ridges in midbody	Bright yellow, velum prominent, posterior end doesn't reach spicule tip	Boat -shaped, cuneus needle shaped	22+1	P	P	Protruding with epiptygmata	Dome shaped, with terminal peg	A

<i>S. sacchari</i>	5 equal ridges in midbody	Yellow-brown, prominent rostrum, velum not reaching the spicule tip, spicule tip blunt	Boat -shaped, cuneus long	24 + 1	A	P	Not protruding with epiptygmata	Dome shaped, with terminal peg	A
<i>S. schliemanni</i>	8 equal ridges at midbody	Anterior ward projection on ventral edge of spicule proximal end	Cuneus absent	22 + 1	P	P	Slightly protruding vulva no epiptygmata	Conical, with rounded terminus	A

A, absent; P, present; -, information not available

Table 2. 5 Estimates of evolutionary divergence between ITS sequences. The number of base differences per sequence from between sequences are shown. Standard error estimate(s) are shown above the diagonal. gaps and missing data were eliminated.

Species/GenBank number	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>S. bertusii</i> n. sp. KY082902		0.97	3.70	4.69	4.97	5.30	5.36	9.36	9.49	9.42	9.55	9.54	11.27
2 <i>S. bertusii</i> Tito13 KR527218	1		3.86	4.74	5.06	5.37	5.46	9.41	9.53	9.45	9.59	9.60	11.21
3 <i>S. nyetense</i> X985266	16	17		4.33	4.47	5.40	5.59	9.38	9.63	9.59	9.69	9.67	11.15
4 <i>S. cameroonense</i> JX985267	26	27	21		4.88	5.50	5.15	9.25	9.42	9.28	9.35	9.27	11.17
5 <i>S. sacchari</i> KC633095	28	29	23	29		5.25	4.94	9.03	9.24	9.22	9.31	9.30	11.09
6 <i>S. beitlechemi</i> KT373856	34	35	35	36	35		4.47	8.86	9.09	9.01	9.06	9.05	10.99
7 <i>S. fabii</i> KR527216	34	35	34	32	30	22		9.16	9.29	9.28	9.30	9.38	11.17
8 <i>S. monticolum</i> AF122017	115	116	114	111	113	107	107		4.85	4.44	4.93	8.41	11.04
9 <i>S. changbaiense</i> JN865168	118	119	118	115	119	115	114	28		3.03	3.70	8.68	10.79
10 <i>S. robustispiculum</i> AY355442	119	120	119	115	119	113	113	23	11		3.25	8.66	10.78
11 <i>S. ashuense</i> DQ354694	120	121	120	115	119	113	113	28	16	12		8.63	10.75
12 <i>S. schliemanni</i> HM778112	124	125	125	125	124	120	125	91	100	99	101		10.78
13 <i>C. elegans</i> FJ589008	250	251	257	258	257	261	261	275	281	278	278	271	

Table 2. 6 Estimates of evolutionary divergence between D2-D3 sequences. The number of base differences per sequence from between sequences are shown. Standard error estimate(s) are shown above the diagonal.

Species/GenBank number	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>S. bertusii</i> n. sp. KY082902		0.97	3.70	4.69	4.97	5.30	5.36	9.36	9.49	9.42	9.55	9.54	11.27
2 <i>S. bertusii</i> Tito13 KR527218	1		3.86	4.74	5.06	5.37	5.46	9.41	9.53	9.45	9.59	9.60	11.21
3 <i>S. nyetense</i> X985266	16	17		4.33	4.47	5.40	5.59	9.38	9.63	9.59	9.69	9.67	11.15
4 <i>S. cameroonense</i> JX985267	26	27	21		4.88	5.50	5.15	9.25	9.42	9.28	9.35	9.27	11.17
5 <i>S. sacchari</i> KC633095	28	29	23	29		5.25	4.94	9.03	9.24	9.22	9.31	9.30	11.09
6 <i>S. beitlechemi</i> KT373856	34	35	35	36	35		4.47	8.86	9.09	9.01	9.06	9.05	10.99
7 <i>S. fabii</i> KR527216	34	35	34	32	30	22		9.16	9.29	9.28	9.30	9.38	11.17
8 <i>S. monticolum</i> AF122017	115	116	114	111	113	107	107		4.85	4.44	4.93	8.41	11.04
9 <i>S. changbaiense</i> JN865168	118	119	118	115	119	115	114	28		3.03	3.70	8.68	10.79
10 <i>S. robustispiculum</i> AY355442	119	120	119	115	119	113	113	23	11		3.25	8.66	10.78
11 <i>S. ashuense</i> DQ354694	120	121	120	115	119	113	113	28	16	12		8.63	10.75
12 <i>S. schliemanni</i> HM778112	124	125	125	125	124	120	125	91	100	99	101		10.78
13 <i>C. elegans</i> FJ589008	250	251	257	258	257	261	261	275	281	278	278	271	

List of figures

Figure 2. 1 *Steinernema bertusi* n. sp. A: Tail region of second generation male. Female: B, H, I. B: Tail region; H: Vulva and epiptygmata; I: Anterior region. Infective juvenile. D: Tail region. First generation male C, E, F, G. C: Gubernaculum; E: Tail region; F: Spicule; G: Body shape. (Scale bars: A = 10 μm ; B, D, E, I: 20 μm ; C, F, H: 1000 μm ; G: 200 μm ,,)

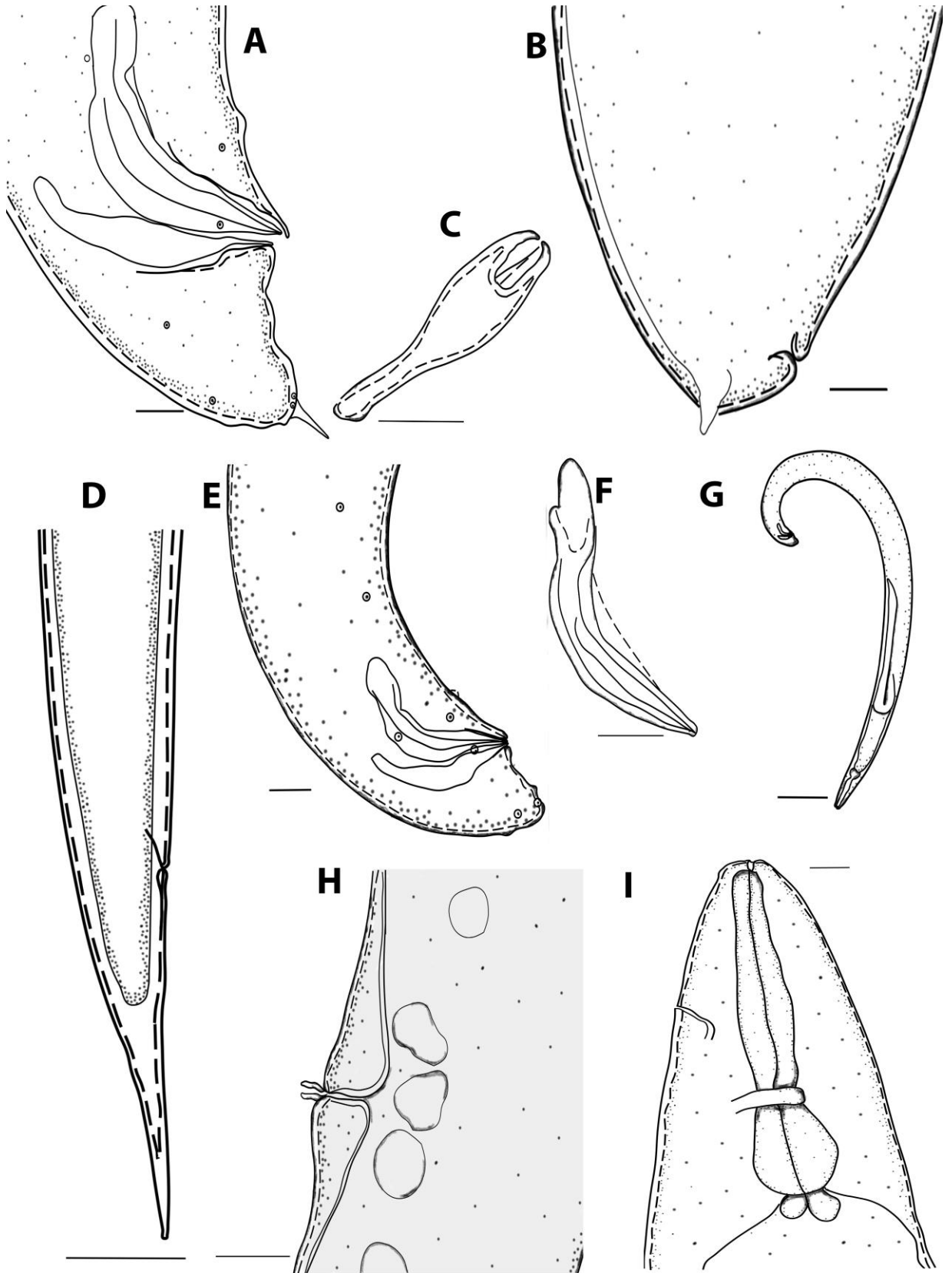


Figure 2. 2 Adult male of *Steinernema bertusi* n. sp. A: *En face* view showing four cephalic papillae and six labial papillae; B: Excretory pore (arrow); C: First generation male tail region showing papillae (numbered); D: Second generation tail region showing shape of tail, papillae and midventral papilla (numbered); E: Tail region of second generation male; F: Spicule. (Scale bars: A = 10 μm ; B, D-F= 20 μm ; C = 100 μm .)

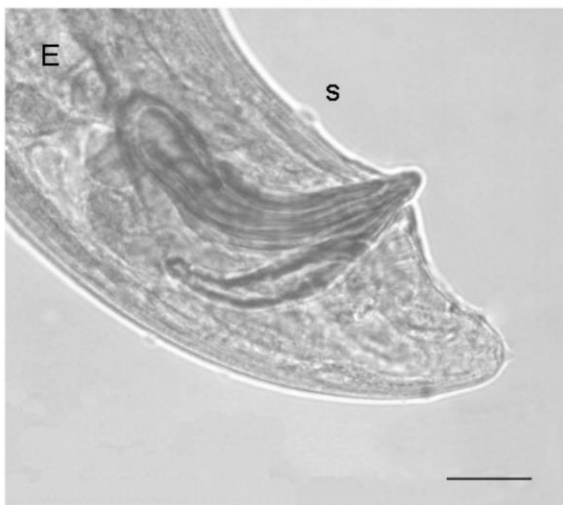
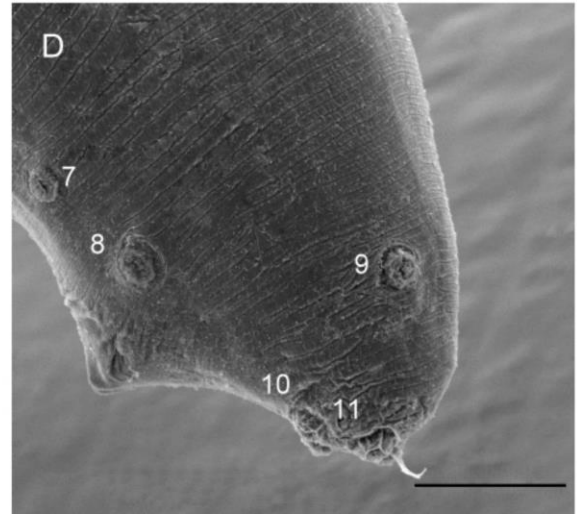
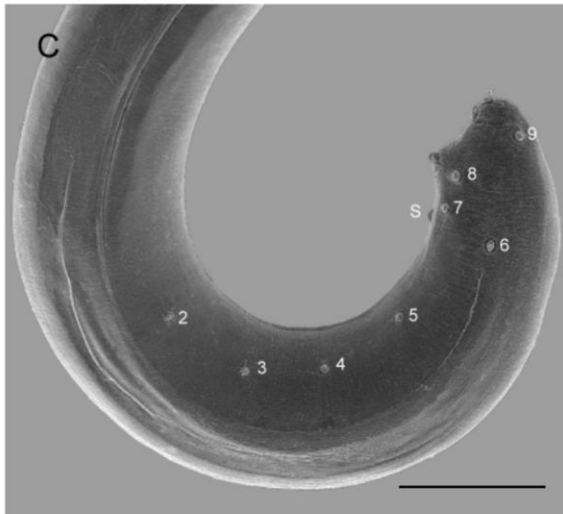
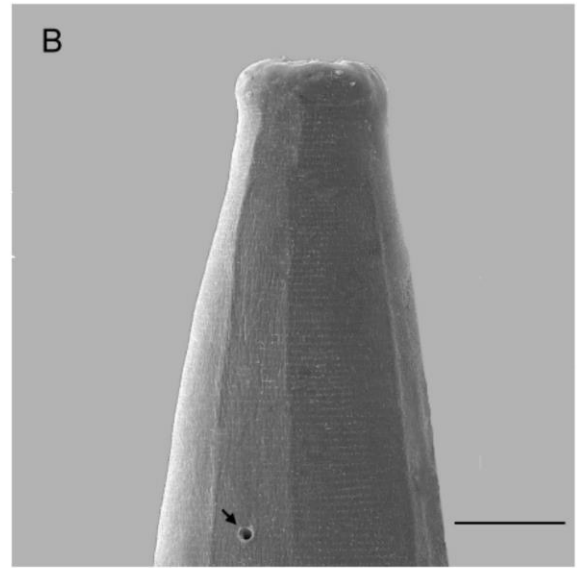
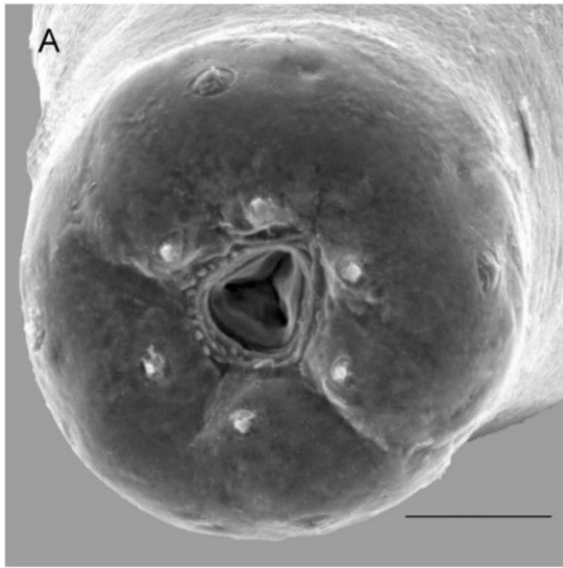


Figure 2. 3 Adult female of *Steinernema bertusi* n. sp. A: Pharyngeal region showing mouth and excretory pore (arrow); B: *En face* showing four cephalic papillae and six labial papillae; C, D: Vulva with double flapped epiptygmata; E, F: Anal region of first generation female showing mucron. (A, C, E = 50 μ m; B, D, F = 20 μ m.)

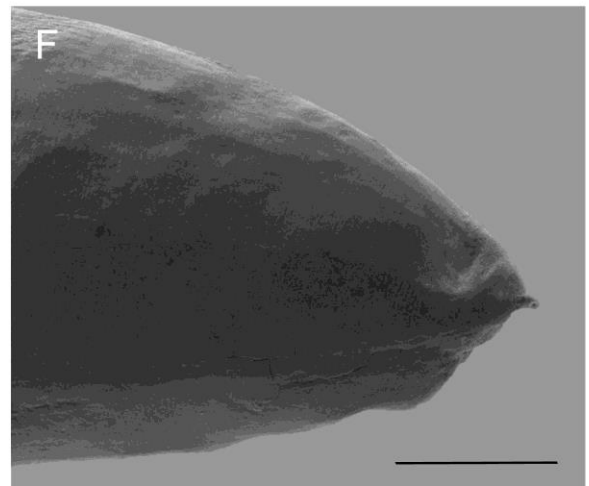
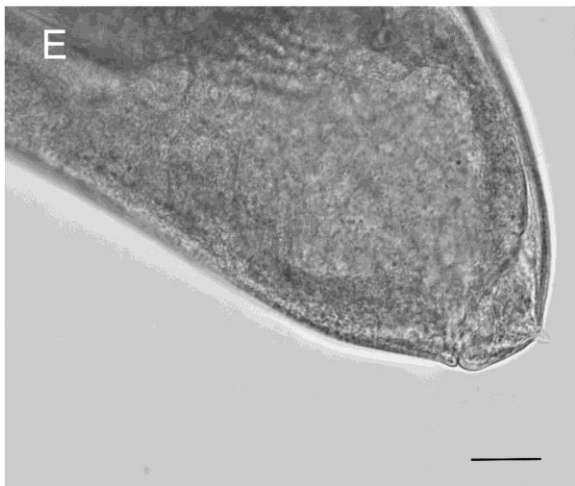
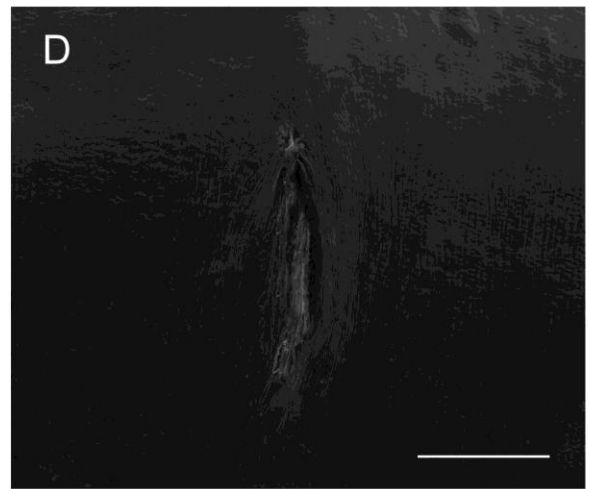
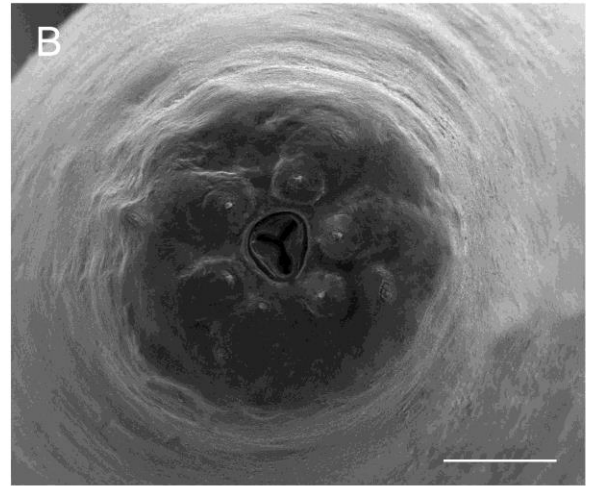
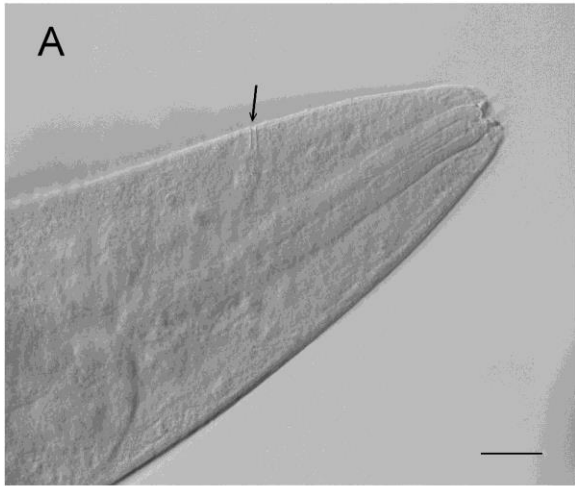


Figure 2. 4 Infective juvenile of *Steinernema bertusi* n. sp. A: *En face* view; B: Bacterial chamber (arrow); C-E: Lateral field; F, G: Tail region showing excretory pore (arrow). (A = 5 μ m, B, C, D, F, G = 20 μ m, E = 50 μ m.)

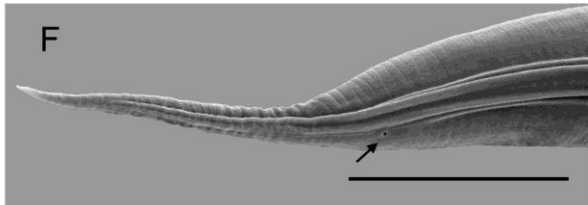
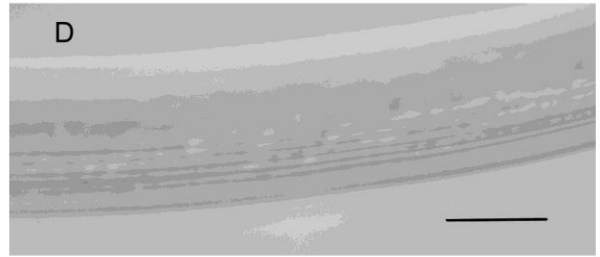
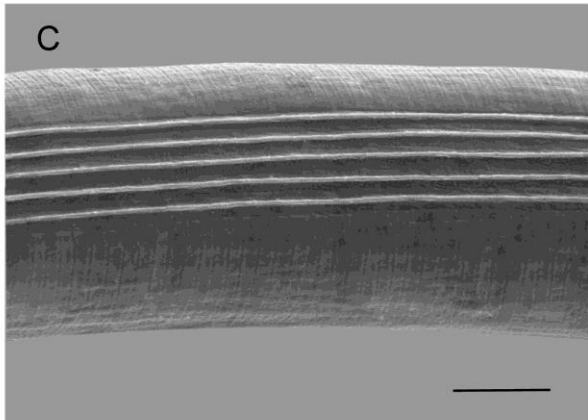
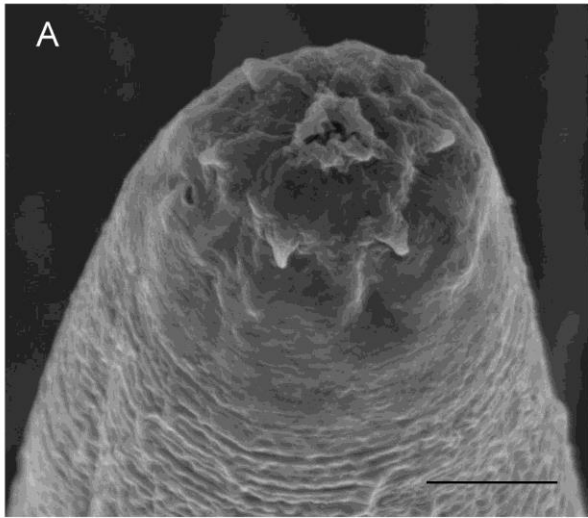


Figure 2. 5 Phylogenetic relationships of *Steinernema bertusi* n. sp. with other *Steinernema* spp. as inferred from analysis of the ITS rDNA region using *Caenorhabditis elegans* as the outgroup. Maximum Parsimony analysis, values at the nodes represent bootstrap proportion of 50% or more.

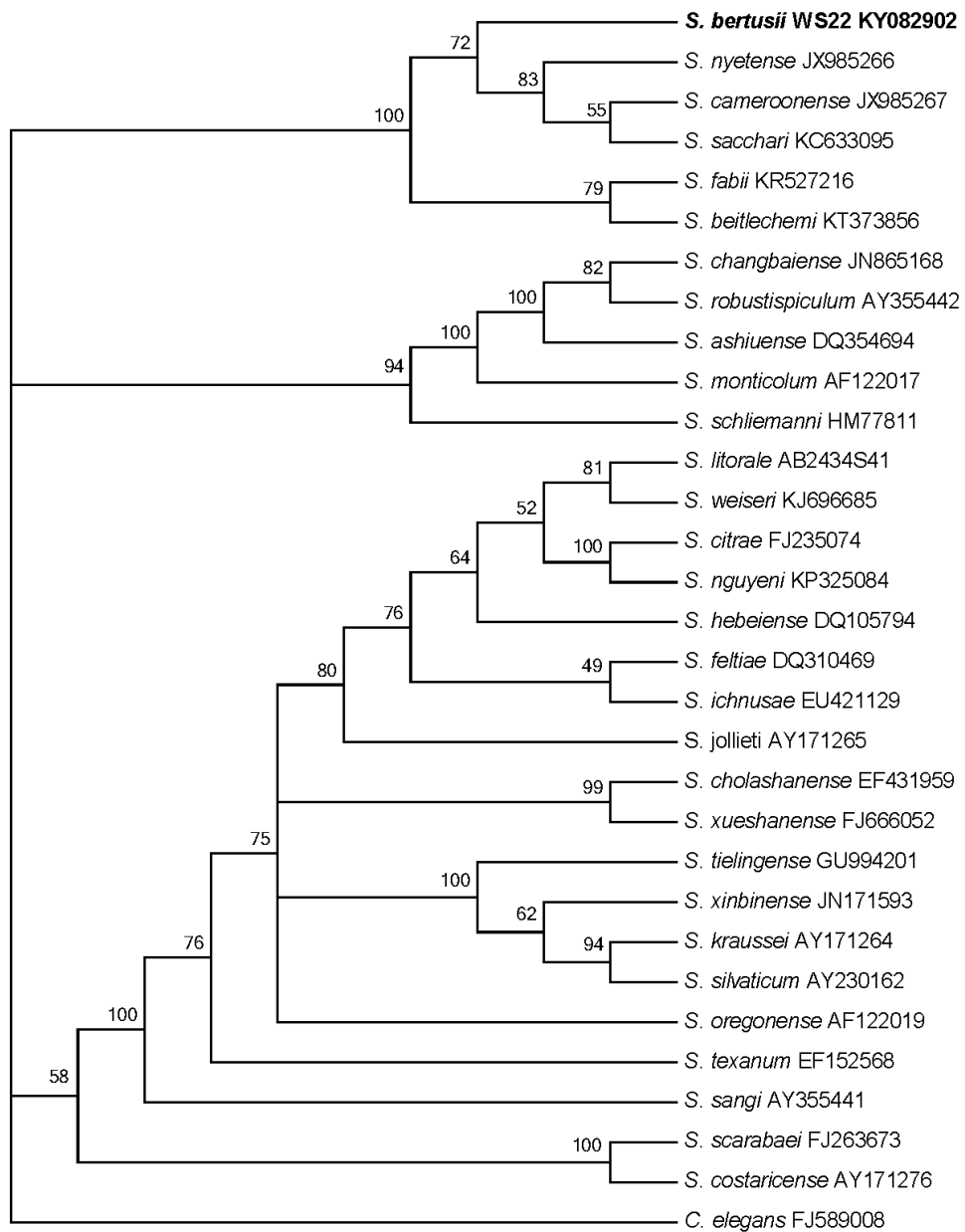
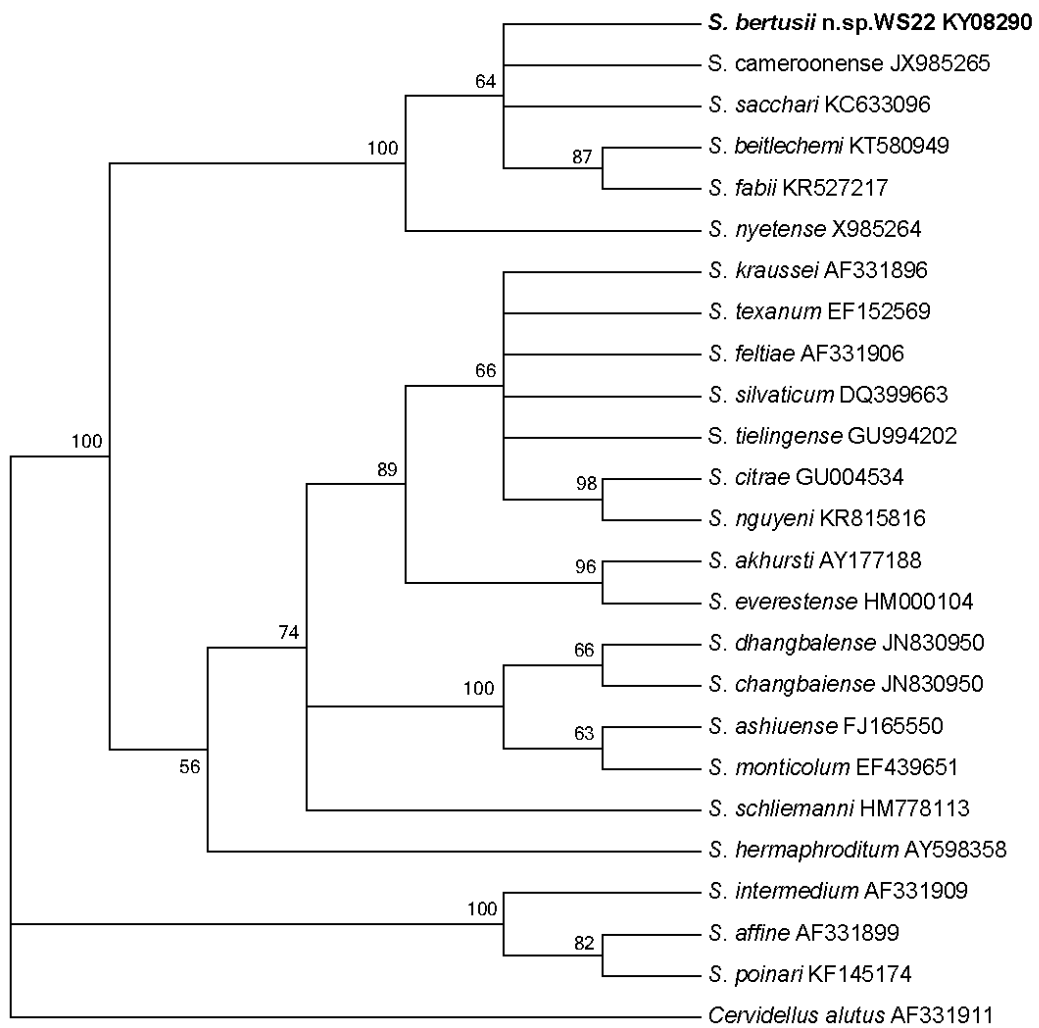


Figure 2. 6 Phylogenetic relationships of *Steinernema bertusi* n. sp. with other *Steinernema* spp. as inferred from analysis of the D2-D3 rDNA region using *Cervidullus alutus* as the outgroup. Maximum Parsimony analysis, values at the nodes represent bootstrap proportion of 50% or more.



Chapter 3

Pathogenicity of entomopathogenic nematodes against white grubs from forestry and sugarcane plantations in South Africa

Abstract

White grubs are important pests of sugarcane and forest plantations in South Africa. Their control is difficult due to their cryptic nature and resistance to chemicals. In this study, we evaluated the potential use of entomopathogenic nematodes (EPNs) as an alternative method of control. Laboratory bioassays were performed using eleven locally isolated EPNs for their mortality effect on the third instar larvae of *Schizonycha affinis*, *Pegylis sommeri*, *Monochelus* sp. and *Maladera* sp. 4. Concentration trials to determine lethal dosages for three of the white grub species were performed using *Heterorhabditis zealandica* SF41. Subsequent bioassays were performed to determine the ability of the nematodes to grow in the cadavers of *S. affinis* and *P. sommeri*. The results showed that mortality percentage of the white grubs varied significantly but was low for most of the EPN species, except for *H. zealandica*. Overall, *Maladera* sp. 4. was the most susceptible and *P. sommeri* the least. The highest percentage mortality was observed at four weeks after inoculation, but the rate of mortality was highest in the first week. Increasing the EPN concentrations significantly reduced the LD₅₀ for all the white grub species. *Schizonycha affinis* had the lowest LD₅₀ at 38 IJs per larva after 28 days compared to *Maladera* sp4. with 284 and *P. sommeri* with 1035. Dissection of insect cadavers revealed possible limiting factors for low susceptibility as the inability of EPNs/ EPN symbiotic bacteria to infect the insect haemocoel since no EPNs or dead EPNs were found inside the cadavers.

Introduction

White grubs (Coleoptera: Scarabaeidae) are the root feeding larvae of Scarabid beetles (Ritcher, 1938; Jackson and Klein, 2006). They are sporadic pests of various crops, mainly feeding on plant roots and resulting in significant root damage. Their adults, known as chafer beetles, feed on plant leaves or bore into underground stems, creating beetle galleries (Harrison and Wingfield, 2016; Jackson and Klein, 2006).

In South Africa, and the neighbouring Kingdom of Eswatini, white grubs are the main insect pests of sugarcane and are also important establishment pests in plantation forestry (Echeverri-Molina and Govender, 2016a, b; Sivparsad *et al.* 2018). Govender (2007; 2014) reported that white grubs were responsible for 13 % of the damage in black wattle plantations in the KwaZulu-Natal province, while McArthur and Leslie (2004) reported an average of 23–55 % reduction in sugarcane yield in the KZN Midlands North area. The forestry plantations are found in close proximity with the sugarcane and there is an overlap of white grub species between these crops (Sivparsad *et al.* 2018). In terms of abundance of the pest species, *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Maladera* sp., *Adoretus ictericus* Burmeister and some other unidentified morphospecies have been found to be the most dominant establishment pests in commercial forestry and sugarcane plantations in the KwaZulu-Natal province (Way *et al.* 2011; Sivparsad *et al.* 2018; Echeverri-Molina and Govender, 2019).

The control of white grubs is difficult mainly because of their soil dwelling nature, resistance to chemical insecticides and the nocturnal lifestyle of their adults (Grewal *et al.* 2005; Jackson and Klein, 2006). While white grub control efforts have been dominated by the use of chemical insecticides, research using entomopathogenic nematodes (EPNs) has in general offered a greater control efficacy (Grewal *et al.* 2005). EPNs belong to the order Rhabditida and are known to parasitize various below- and aboveground insects (Grewal *et al.* 2005; Lacey and Georgis, 2012). The two families Steinernematidae and Heterorhabditidae are used worldwide

in biological control of various insect pests (Kaya and Koppenhöfer, 2004; Lacey and Georgis, 2012; Shapiro-Ilan *et al.* 2010).

EPNs kill their insect hosts using symbiotic bacteria, which they carry in their digestive system and sometimes in specialized bacterial chambers (Nobuyoshi, 2002). Death of the insect normally occurs within 48 h after infection (Kaya *et al.* 1993; Nobuyoshi, 2002). The advancement in *in vitro* mass production and formulation technology has allowed for reduction in production costs, prolonged shelf life and ease of transportation over long distances (Georgis *et al.* 2006; Cruz-Martínez *et al.* 2017). Thus, EPN-based products are available on the market and labelled for biocontrol of many prominent insect pests such as white grubs, fungus gnats, weevils, codling moth, cutworms and others (Lacey & Georgis, 2012). Compared to insecticides, EPNs are considered to provide a more effective and environmentally safer option for controlling white grubs (Grewal *et al.* 2005).

South Africa has a diversity of native EPNs, with a total of 17 *Steinernema* and seven *Heterorhabditis* species reported by 2017 (Malan & Hatting, 2015; Malan & Ferreira, 2017). A recent description of *Steinernema bertusi* Katumanyane, Tiedt, Malan & Hurley, brought the total of *Steinernema* species described from South Africa to 12, which is 12 % of the described species of this genus in the world (Katumanyane *et al.* 2020). During the survey for EPNs in plantations and natural forestry, Abate *et al.* (2018), recovered 4 % positive samples from the soil, which shows a high potential for existence of natural populations of EPNs in these habitats in South Africa.

Despite the diversity of native EPNs in South Africa and their potential as biological control agents for white grubs, there has currently only been one study to evaluate the potential use of EPNs to control white grubs in South Africa. Abate *et al.* (2019) evaluated the efficacy of native and non-native EPNs to control the white grub, *Heteronychus licas* Klug, a pest in sugarcane plantations. The results of the study showed that the local strain of *Heterorhabditis bacteriophora* Poinar provided better control for *H. licas*, compared to an imported strain of

the same EPN species and the native species *Steinernema feltiae* Filipjev. There have been no studies to evaluate the use of EPNs on white grub pests in plantation forests in South Africa, however, Abate *et al.* (2016, 2018) did isolate EPNs from soil collected in plantation forest areas, and showed their potential role as natural biological controls agents in that environment.

The aim of this study was to screen locally isolated EPNs for their biocontrol potential against native white grubs in South Africa. Eleven EPN species were screened against four of the most dominant white grub species from forestry and sugarcane plantations and mortality was recorded over a period of 28 days. The lethal dosage of the most effective EPN species from those bioassays was determined for three of the white grub species. This was followed by a qualitative study to examine the potential for different EPNs to infect, kill and reproduce in two of the most resistant white grub species.

Materials and methods

Source of white grubs

Four white grub species, namely *S. affinis* Boheman, *P. sommeri* Burmeister, *Maladera* sp. 4 and an unidentified *Monocheilus* sp. (Fig 3.1) were used in this study. Insect identification was done by means of a LUCID key, previously developed for white grubs in sugarcane plantations in southern African countries: https://keys.lucidcentral.org/keys/v3/sugarcane_white_grubs/sugarcane_white_grubs.html.

The white grubs were collected over different seasons in 2018 and 2019, from various geographical locations in wattle and sugarcane plantations in the KwaZulu-Natal province of South Africa (Table 5.1). White grubs were dug up from the soil at sites where patchy sugarcane growth was observed or in matured wattle plantations with a thick topsoil layer as prior monitoring had indicated these conditions to favour the highest number of grubs. Each white grub was placed in a 30 ml plastic vial, filled with moist autoclaved peat moss (Hygrotech

sustainable solutions) and provided with a fresh carrot disc to feed on. The vials containing white grubs were placed in cooler boxes for transport and later rearing at the Forestry and Agricultural Biotechnology Institute(FABI) Biocontrol Centre at the University of Pretoria, South Africa, at 23 °C and 60 – 70 % humidity. The autoclaved peat and carrot discs were replaced weekly until the white grubs were at their third instar growth stage, when they were used for experiments. White grubs that showed signs of pupation were not used in the study.

Source of EPNs

Twelve EPN species (Table 3.1) were used in this study. The nematode isolates were obtained from the nematode collections at the FABI Biocontrol Centre and the Department of Conservation Ecology and Entomology at Stellenbosch University. All nematodes were reared using third instar larvae of the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), reared in the laboratory. Modified White's traps were used to harvest the Infective Juveniles (IJs), for a period of one week after emergence. Harvested IJs were stored in distilled water at 12 °C, in horizontally placed culture flasks (Woodring and Kaya, 1988). The stored EPNs were shaken periodically for ventilation and used within three weeks after EPN harvest to ensure nematode viability. A fresh batch of nematodes was used for each experimental repetition.

Screening bioassays

A. Soil bioassays

The methods used by Koppenhöfer and Eugene (2003), An *et al.* (2012) and Wu *et al.* (2014) were modified and used in this experiment. A total of five EPNs including *H. bacteriophora*, *H. zealandica*-MJ2C, *S. fabii*, *S. jeffreyense* and *S. yigalemense* (Table 3.1) were separately tested for their potential to kill third instar larvae of *P. sommeri*, *Monochelus* sp., *Maladera* sp.

4. Two additional species, namely *S. sacchari* isolated from natural vegetation (Abate *et al.*

2018) and a strain of *S. fabii* (*S. fabii*-SCH10), isolated from *S. affinis* larvae during this study (Table 3.1), were further tested against the larvae of *S. affinis*.

For the set-up, plastic cups of 30 ml were filled with approximately 30 g of autoclaved soil. A white grub was placed on top and allowed to dig into the soil, before being inoculated with the EPNs. From a nematode suspension, 400 IJs were sprayed on the surface of the soil in each cup and the soil moisture was adjusted to 18% v/w. This was done by drying 100 g of the soil sample to determine its water content, then adjusting the soil water to 18% v/w. The control experiment was sprayed with distilled water only. A carrot disc was placed on top of the soil for the white grubs to feed. The vials were placed in a plastic container lined with moist tissue paper to maintain 100 % moisture and stored at 25 °C in a dark room. Ten white grubs were used for each treatment and the experiment was repeated three times, each repeat on a different test date, using a different batch of nematodes. Cumulative percentage mortality for each treatment was recorded over a month, at weekly intervals.

B. Petri dish bioassays

Based on the preliminary results of the soil bioassays, the most resistant white grubs, namely *S. affinis* and *P. sommeri*, were selected and subjected to further tests in Petri dishes. The objective of these tests was to investigate any changes in susceptibility of the white grub species if exposed directly to the EPNs. The susceptibility of the two white grub species to nine EPNs, namely *H. bacteriophora*, *H. baujardi*, *H. indica*, *H. noenieputensis*, *H. safricana*, *H. zealandica* MJ2C, *H. zealandica* SF41, *O. myriophila* and SCH10 (*S. fabii*) was tested. The susceptibility of *S. affinis* to *S. bertusi* was also tested.

For the experimental set-up, a single white grub was placed in a cell of a 12-well-bioassay plate lined with a filter paper disc and inoculated with 200 IJs suspended in 50 µl of distilled water. The method used to calculate the concentration of the nematodes was according to Glazer and Lewis (2000). Ten grubs of each white grub species were used for each of the treatments (EPN species), as well as for the control. The controls received water only. The

plates were placed in a plastic container lined with wet paper towels (100 % moisture), and kept in a growth chamber, at 25 °C for a period of 24 h.

Mortality reading was taken daily for up to seven days for *S. affinis* and up to 10 days for *P. sommeri*, as the mortality for *P. sommeri* was observed to take longer. Any dead larvae were removed, rinsed with distilled water and placed in a new 12-well plate lined with moist filter paper. Dead larvae were returned to the growth chamber to allow the EPNs, if present, to grow. All dissections of the cadavers were done on the 10th day post inoculation to confirm the presence of EPNs. Only cadavers with the presence of EPNs in them were counted in the mortality data. The experiment was repeated three times, for each nematode species on a different test date, using a fresh batch of nematodes.

Concentration trial

Based on the preliminary results of both the soil and Petri dish bioassays (Fig 3.2 – 3.5), *H. zealandica*-MJ2C was selected for the concentration bioassays because it showed consistent efficacy to kill all the tested white grubs. *Heterorhabditis zealandica*-MJ2C was the most effective EPN species for two of the white grub species, *P. sommeri* and *S. affinis*, while it showed the second highest percentage mortality for *Maladera* sp. 4 and an average mean mortality for *Monochelus* sp. Thus, a probit test was performed using *H. zealandica*-MJ2C to determine the lethal dosages (LD₅₀ and LD₉₀) of this EPN on the third instar larvae of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. *Monochelus* sp. was not included in this experiment as the larvae were not available in sufficient numbers. Similar methods as in the soil bioassay were used for this experiment. However, the treatments included different concentrations of *H. zealandica*-MJ2C, namely 0, 100, 200, 400, 800 and 1600 IJs per vial. Ten white grubs were used for each treatment (EPN concentration x 10 white grub species) and the experiment was repeated three times, each repeat on a different test date, using a different batch of nematodes. Cumulative percentage mortality was recorded over a month, at weekly intervals.

Examining EPN survival and growth inside insect cadavers

The ability of EPNs to grow in the insect cadavers of *S. affinis* and *P. sommeri* was tested using ten EPNs. These included *H. safricana*, *H. noenieputensis*, *H. indica*, *H. zealandica* MJ2C, *H. baujardi*, SCH10 (*S. fabii*), *H. bacteriophora*, *H. zealandica* SF41, *O. myriophila* and *S. bertusi* (Table 3.1). The objective of this experiment was to test the survival and growth of the nematodes so as to shed more light on the mechanisms of resistance in the two most resistant grub species, namely *S. affinis* and *P. sommeri* as observed from the soil and Petri dish bioassays. Similar methods as in the Petri dish bioassay were used for this experiment. Grubs were individually placed in Petri dishes and inoculated with 200 IJs of each EPN treatment. Ten grubs each of *S. affinis* and *P. sommeri* were used per EPN treatment. Inoculated insects were incubated at 25 °C in containers fitted with moist tissue paper. Insect mortality was recorded daily for a period of 10 days. Irrespective of the day of insect death, all dissections of dead insects were performed on the 10th day post inoculation to observe and record the status of the EPNs growing inside the haemocoel. The parameters measured included presence or absence of EPNs, stage of growth of EPNs, presence or absence of EPN symbiotic bacteria and general state of the cadaver.

Statistical analysis

All statistical analyses were performed using the R statistical software version 4.00 (R Development Core Team 2020). Mortality rates are proportion data bound between 0 and 1 and were analysed using a GLM with a binomial distribution (Zuur *et al.* 2009) to assess the overall effect of time and EPN species on percentage mortality. For each interval of time (7, 14, 21 and 28 days), the variation in mortality among treatments (EPN species) was analysed and represented using a bar plot. The effect of time and nematode concentration, on the mortality percentage of the different white grub species in the screening bioassays was determined by modelling the mortality data (percentage of white grubs that died during the experiment) using the two-parameter log-logistic function. The lower limit was fixed at 0 and

the upper limit was fixed at 1 as mostly suitable for binomial responses (Ritz *et al.* 2015). The two-parameter log-logistic function is given by the expression:

$$M(t) = \frac{1}{1 + \exp(b \cdot \log(t) - \log(DL_{50}))} \quad (\text{Eq 1})$$

$M(t)$ is the expected mortality rate at time t (weeks); b is proportional to the slope of M , at time t ; and DL_{50} is the dose at which 50% of the population would have died. The utility in using the two-parameter log-logistic function is that it offers the possibility to estimate the DL_{50} . In Eq. (1), it is assumed that $M(t)$ is bounded between 0 and maximum value of 1, which is convenient with mortality data. The two parameters of Eq. (1) were estimated using the `drc` function of the package `drc` (Ritz & Streibig 2005) in the R statistical software (R Development Core Team 2020). Data for the Petri dish-bioassays were also analysed using GLM with binomial distribution.

Results

Screening bioassays

Schizonycha affinis

The results of the soil bioassays showed that mortality percentage of *S. affinis* varied significantly amongst EPN species and over time ($p < 0.001$; Table 3.2). *Schizonycha affinis* mortality increased with time for all treatments (Fig 3.2A). Over time, the highest percentage mortality was constantly observed for *H. zealandica* MJ2C (Fig 3.2A). The maximum percentage mortality of 70 ± 1.2 % was obtained after 21 days and was significantly different from the next closest efficient species, *S. jeffreyense*, at 43 ± 1.2 % and *S. yirgalemense* at 30 ± 1.2 . Overall, *H. bacteriophora*, *S. fabii*, *S. sacchari*, and an isolate of *S. fabii* (SCH10) showed very low mortality rates of less than 20 % after 28 days. For all the nematodes, the mortality graph levelled off after 21 days (Fig 3.2A). Similar to the results obtained in the soil bioassays, the highest percentage mortality for the Petri dish-bioassays was obtained from *H.*

zealandica MJ2C at 63 % \pm 3.3 %, 7 days post-inoculation. *Heterorhabditis zealandica* SF1 gave the second highest mean mortality at 46.7 % \pm 3.3 % followed by *Oscheius myriophila* Poinar at 40.0 % \pm 1.5 %. The rest of the tested nematodes resulted in less than 30 % mortality, seven days post-inoculation (Fig 3.2B).

Pegylis sommeri

The results of the soil bioassay showed that mortality percentage varied significantly amongst EPN species ($p < 0.001$; Table 3.2), but not over time ($p = 0.872$; Table 3.2), suggesting that the mortality did not increase with time. At 28 days, mortality rates of *P. sommeri* were < 5 % across all EPNs, except *H. zealandica* MJ2C (Fig 3. 3A). *Heterorhabditis zealandica* MJ2C showed the highest percentage mortality, although only 30 % \pm 5.8 %. *Steinernema jeffreyense* and *S. yirgalemense* did not cause any mortality. In the petri dish bioassays, *P. sommeri* still maintained a very high resistance towards the tested EPNs (Fig 3.3B). The highest mortality achieved was 26.7 % \pm 1.2 % by *H. zealandica* SF41

Maladera sp. 4

The results of the soil bioassay showed that mortality rates increased significantly with time for all treatments/EPN species ($p < 0.001$; Table 3.2). At 28 days post inoculation, *H. bacteriophora* exhibited the highest percentages of mortality 93.3 % \pm 6.7 %, followed by *H. zealandica* MJ2C with a percentage mortality of 76.7 % \pm 6.7 % (Fig 3.4). *Steinernema fabii* and *S. jeffreyense* resulted in 63.3 % \pm 15 and 60 % \pm 10 % percentage mortality, respectively, while the lowest mortality rate (40.0 % \pm 15 %) was recorded for *S. yirgalemense* (Fig 3.4).

Monochelus sp.

The results of the soil bioassays showed that mortality increased with time but varied considerably among treatments (Fig 3.5). The highest mortality after 28 days of inoculation was recorded from *H. bacteriophora* (60.0 % \pm 11.5 %) and *S. jeffreyense* (60.0 % \pm 15.3 %) treated grubs. *Heterorhabditis zealandica* MJ2C caused an average mortality of 50 \pm 10 %

and was closely followed by *S. yirgalemense* at 47 % \pm 3.3 %. The lowest level of mortality was caused by *S. fabii* with a 30 % \pm 0 % mean mortality of 28 d post inoculation.

Concentration trial

Heterorhabditis zealandica MJ2C was used in the trial, because it was the most efficient EPN for three of the white grub species, namely *S. affinis*, *P. sommeri* and *Monochelus* sp. in the screening bioassays. The parameters estimated from the log-logistic equation (Eq. 1) are presented in Appendix 1. From the results of the study, the LD₅₀ was generally lowest for *S. affinis* at 38 IJs per grub, medium for *Maladera* sp. 4 at 284 IJ per grub and highest for *Pegylis sommeri* at 1035 IJs per grub at 28 days post inoculation (Table 3.3).

Examining EPN growth inside insect cadaver

Heterorhabditis zealandica MJ2C showed a high mortality potential for *S. affinis*, with most successful infections and ability to complete the life cycle inside the haemocoel of the grubs (Table 3.4). It was also observed to be the only nematode species that was able to protect its cadavers from attack by unidentified mite species (Fig 3.6A). *Oscheius myriophila* showed a comparatively high mortality for *S. affinis*, killing on average four out of ten individuals and was able to complete its lifecycle within a week (Table 3.4). However, *O. myriophila* was unable to protect any of its cadavers; they were mostly putrid and full of mites. Similarly, whenever *H. indica* colonised and killed *S. affinis*, EPNs were unable to develop inside the cadavers, as only bacteria were observed at the end of the experiment (Table 3.4). For *H. bacteriophora* and *H. zealandica* SF41, there was mostly ESB colonisations observed but no surviving EPNs in the cadavers. *Steinernema bertusi* showed a low percentage mortality towards *S. affinis*; all the infections recorded were successful. However, *S. bertusi* was unable to keep clean cadavers. The rest of the EPN species tested showed very low mortality rates. For *H. safricana*, *H. noenieputensis* and SCH10 (*S. fabii*), no ESBs/EPNs were observed in the dead cadavers (Table 3.4)”

Heterorhabditis bacteriophora killed about 20 % of the *P. sommeri* grubs (Table 3.5) and kept clean cadavers that were observed not to be attacked by mites (Fig 3.6B). For most of the cadavers, the red symbiotic bacteria but no EPNs were observed for *H. bacteriophora*. In two of the grubs, the symbiotic bacteria of *H. bacteriophora* only colonised the upper body of *P. sommeri* (Fig 3.6D). However, the rest of the EPNs used were mostly unable to infect *P. sommeri*. *Heterorhabditis zealandica* SF41 also infected about 20 % of the grubs (Table 3.5), but the EPNs were unable to complete their lifecycles. Stunted and dead nematodes (Fig 3.6C) were observed.

It was generally observed that most EPN-associated bacteria were unable to protect the *P. sommeri* and *S. affinis* cadavers from colonisation by other bacteria, possibly through interference from the insect's own gut bacteria. That was seen by the colour changes in the cadaver, from the one typical of the symbiotic bacteria to dark colours hours after infection, coupled with a foul smell after a few days.

Discussion

The results of this study showed varying degrees of susceptibility of the white grubs *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and *Monocheilus* sp. to South African EPNs. Generally, *Maladera* sp. 4 was the most susceptible, while *P. sommeri* was the least susceptible. *Schizonycha affinis* and *Monocheilus* sp. were moderately susceptible. Other than *P. sommeri*, all the tested white grubs were moderately susceptible to the EPN *H. zealandica* MJ2C. From the probit test, the LD₅₀ values obtained using *H. zealandica* MJ2C were relatively high for *P. sommeri* (1035 IJs/larva), followed by *Maladera* sp4. (284 IJs/larva) and lastly *S. affinis* (38 IJs/larva) at 28 days post inoculation.

The varying degrees of susceptibility observed for different white grubs and EPN species/ isolates in our study are typical of what has already been shown for white grub susceptibility to EPNs elsewhere (Grewal *et al.* 2005). It has generally been agreed that the efficacy of

EPNs against white grubs can be difficult to predict, as the control efficacy recorded from previous experiments is inconsistent (Grewal *et al.* 2005). The consensus is that these inconsistencies can be explained by biotic factors such as differences in host attractiveness, differences between EPNs in their dispersal rates and ability to penetrate through the cuticle/gut wall of the grubs (Georgis & Gaugler, 1991; Koppenhöfer *et al.* 2007), and/or environmental factors such as moisture, temperature and soil physical properties (Kaya *et al.* 1993). In our experiments, only *H. zealandica* MJ2C was able to provide consistency in mortality potential across the tested white grub species. Even then, the percentage mortality varied with repetitions and *H. zealandica* MJ2C was not able to effectively kill third instar larvae of *P. sommeri*.

The obtained LD₅₀ values gradually decreased over time. This was as expected, as the mortality of the white grubs increased with time for all the species, with the exception of *P. sommeri*, where the mortality remained constant after seven days post inoculation in the vial experiments. At 28 days post-inoculation, we obtained the LD₅₀ of 1035 IJs/larva for *P. sommeri*, 284 IJs/larva for *Maladera* sp. 4 and 38 IJs/larva for *S. affinis*. The obtained LD₅₀ are high, but comparable to those obtained by other studies on white grubs, further emphasizing the high resistance of white grubs to EPNs. For example, Sankaranarayanan *et al.* (2018) used 24-well plates to test different strains of *H. indica* on the third instars of the white grub, *Holotrichia serrata* F. (Coleoptera: Scarabaeidae) and obtained mean lethal dosage between the range 2015 IJs/grub and 7359/grub. However, Pokhrel *et al.* (2018) obtained rather lower LD₅₀ after exposing second instar larvae of *Chiloloba acuta* (Coleoptera: Scarabaeidae), *S. abbasi* and *S. siamkayai* in 40 g of silt-loam soil. The resulting LD₅₀ values was 44.9 IJs/ml and 50 98.1 IJ/ml, respectively, 14 days post-inoculation. These were very low values in comparison to what we obtained during our probit experiment 14 days post-inoculation, namely 238, 497 and 1369 IJs/larva for *S. affinis*, *Maladera* sp. 4 and *P. sommeri*, respectively. It is important to keep in mind that the low LD₅₀ of Pokhrel *et al.* (2018) were obtained with

second instar larvae which are generally known to be less resistant than the third instars (Alvandi *et al.* 2017).

Observing the EPN growth in the haemocoel of *S. affinis* and *P. sommeri* revealed a number of factors that may be causing the low susceptibility of these two white grubs to EPNs. These may include the inability of the EPNs to penetrate the insect's cuticle and the gut cavity, as there were no EPNs observed in the haemocoel of some of the grubs when dissected. Additionally, the inability of the EPN associated symbiotic bacteria to grow and protect the EPNs and the cadaver could have hindered otherwise successful infections.

Stokwe & Malan (2017) observed that EPN symbiotic bacteria was unable to grow on the haemolymph of the woolly apple aphid, *Eriosoma lanigerum* and ultimately the nematodes were unable to effect successful infections. For white grubs, possible interference to symbiotic bacteria growth can include antagonistic effect from the white grubs' gut bacteria (Skowronek *et al.* 2020). For example, *H. zealandica* MJ2C was observed to be able to protect its cadaver from mites and the cadavers did not obtain a foul smell. The fact that *H. zealandica* SF41 did not yield similar results suggests that *H. zealandica* MJ2C's unique characteristic may be attributed to its different symbiotic bacteria. *Heterorhabditis zealandica* SF41 has been found to be associated with the symbiotic bacteria *Photorhabdus heterorhabditis* Ferreira, Reenen, Endo, Tailliez, Pagès, Spröer, Malan, Dicks (Ferreira *et al.* 2014), whereas *H. zealandica* MJ2C is known to be associated with *Photorhabdus temperata* subsp. *tasmaniensis* (Tailliez *et al.* 2010, Maneesakorn *et al.* 2011).

Previously, Skowronek *et al.* (2020) found that bacteria from the midgut of the common white grub, *Melolontha melolontha* L. larvae, exhibited antagonistic activity against EPN symbiotic bacteria. Thus, it is possible that the symbiotic bacteria of *H. zealandica* MJ2C can counteract the white grubs' antagonistic bacteria, but this requires further investigation. Similar to our observations, Karagoz *et al.* (2007) noted that when the white grubs were killed by EPNs, the associated *Sancassania* sp. (Acari: Acaridae) mites moulted to the adult stage and began

feeding on the host tissues and/or microbes associated with the cadavers, as well as on exiting infective juveniles (IJs). This could further emphasize *H. zealandica* MJ2C's use of its symbiotic bacteria to protect its cadaver.

Conclusion

Our results are consistent with results from previous studies that have shown that white grubs have varying degrees of susceptibility to EPNs. Variations in susceptibility cut across different EPN species and strains of the same EPN species. The implication of this pattern on the biological control of white grubs using EPNs is that EPN screening is required for each white grub species. Furthermore, different strains of the same EPN species may be required to be tested for different geographical regions as inconsistency in efficacy has been shown in this regard.

For South African white grub species, it was shown that a considerable mortality can be achieved using *H. zealandica* MJ2C. However, *H. zealandica* MJ2C showed limited capacity to control *P. sommeri*. Further tests are required to test for the resistance mechanisms of *P. sommeri*. Most EPNs were unable to grow in the haemocoel of the two most resistant grubs, *P. sommeri* and *S. affinis*. This implies that such EPNs, even when capable of penetrating the white grubs, lack the biological compatibility required to kill the white grubs. Such incompatibility may be because of the immunological defences presented by the white grubs. Future studies should focus on identifying specific pathways in which the white grubs defend themselves against the EPNs, but also further screening studies can be done to identify more efficient EPN species.

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Table 3. 1 *Heterorhabditis* and *Steinernema* isolates used in the study, with their associated host plants, origin, GenBank accession number, and the length and width of the infective juveniles (IJ) in micron (μm).

Species	Isolate	Associated host plant/insect	Origin (town/province)	Genbank accession number (ITS region)	Length of IJ (μm)	Width of IJ (μm)	Reference (Isolation)
<i>H. bacteriophora</i>	SF351	Indigenous forest	KwaZulu-Natal	MF535289	588 (512-671)	23 (18-31)	Abate <i>et al.</i> 2018
<i>H. baujardi</i>	Birhan strain	Indigenous forest	KwaZulu-Natal	MF535521	551 (497-595)	20 (18-22)	Abate <i>et al.</i> 2018
<i>H. indica</i>	SGS	Grapevine	Bonnievale, Western Cape	GQ377411	528 (479-573)	20 (19-22)	-
<i>H. noenieputensis</i> *	SF669	Fig tree	Noenieput, Northern Cape	JN620538	528 (484-563)	21 (19-23)	Malan <i>et al.</i> 2014
<i>H. safricana</i>	SF281	Peach tree	Western Cape	EF488006	600(550-676)	21 (18-23)	Malan <i>et al.</i> 2008
<i>H. zealandica</i> (green)	MJ2C	Citrus orchard	Hex River Valley, Western Cape	MF370073	685 (570-740)	27 (22-30)	James <i>et al.</i> 2018
<i>H. zealandica</i> (blue)	SF41	Natural vegetation	Patensie, Eastern Cape	EU699436	685 (570-740)	27 (22-30)	Malan <i>et al.</i> 2006
<i>Oscheius myriophila</i>	OM18*	White grub	KwaZulu-Natal	-	564 (504-611)	23 (19-26)	Katumanyane unpubl.
<i>S. bertusi</i>	SB19*	Natural vegetation	Mpumalanga	KY082902	716 (628-814)	32 (28-36)	Katumanyane <i>et al.</i> 2020
<i>S. fabii</i>	ML15	Wattle	Mpumalanga	KR527216	641 (590-697)	28 (26-31)	Abate <i>et al.</i> 2018
<i>S. jeffreyense</i>	J194	Guava tree	Jeffrey's Bay, Eastern Cape	KC897093	924 (784-1043)	35 (23-43)	Malan <i>et al.</i> 2016
<i>S. sacchari</i>	DUK	Indigenous forest	Gingindlovu, KwaZulu-Natal	MF538659	680 (630-722)	37 (30-47)	Abate <i>et al.</i> 2018

<i>S. yirgalemense</i>	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295	685 (570-740)	29 (24-33)	Malan <i>et al.</i> 2011
<i>S. fabii</i>	SCH10*	White grub	KwaZulu-Natal	-	641 (590-697)	28 (26-31)	Katumanyane unpubl.

*isolated during this study (Refer to Chapter 5, Table 5.1)

Table 3. 2 The effects of EPNs and time on the mortality rate (GLM testing) of *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and *Monochelus* sp. for the soil bioassays.

		<i>Schizonycha affinis</i>	<i>Pegylis sommeri</i>	<i>Maladera</i> sp. 4	<i>Monochelus</i> sp.
EPNs	F value	22.58	45.83	20.55	10.93
	Pr(>F)	<0.001	<0.001	<0.001	<0.001
Time	F value	34.00	0.23	13.29	9.18
	Pr(>F)	<0.001	0.872	<0.001	<0.001

Table 3. 3 Probit analysis test results showing the LD50's obtained for different white grubs after inoculation with different concentrations of *Heterorhabditis zealandica* MJ2C; 0, 100, 200, 400, 800 and 1600 IJs.

		<i>Schizonycha affinis</i>		<i>Pegylis sommeri</i>		<i>Maladera</i> sp. 4	
		Est.	P	Est.	P	Est.	P
Slope	7 days	-0.65	<0.001	-0.99	<0.001	-0.86	<0.001
	14 days	-0.38	0.029	-1.21	<0.001	-1.10	<0.001
	21 days	-0.39	0.031	-1.23	<0.001	-1.04	<0.001
	28 days	-0.53	0.011	-1.30	<0.001	-1.29	<0.001
DL ₅₀	7 days	762	0.001	2226	0.009	1152	<0.001
	14 days	238	0.044	1369	<0.001	497	<0.001
	21 days	74	0.241	1136	<0.001	319	<0.001
	28 days	38	0.269	1035	<0.001	284	<0.001

Table 3. 4 Dissection observations for *Schizonycha affinis*. The table shows the number of white grubs that were found dead over a period of 10 days post-inoculation, the status of EPNs and symbiotic bacteria inside the haemolymph as well as the status of the insect cadaver

EPN species	Repetition	Day post inoculation							Notes
		1	2	3	4	5	6	7	
<i>O. myriophila</i>	1	5	5	5	5	5	5	5	Mostly successful colonisations but could not keep clean cadavers. The life cycle was completed within a short period of time - that is, less than 7 days.
	2	0	1	1	1	1	1	1	
	3	0	4	5	6	6	6	6	
<i>H. safricana</i>	1	0	0	0	1	1	1	1	No ESB/EPNs observed for any of the dead grubs.
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
<i>H. noenieputensis</i>	1	0	1	1	1	1	1	1	No ESB/EPNs observed for any of the dead grubs.
	2	0	0	1	1	1	1	1	
	3	0	0	1	2	2	2	2	
<i>H. indica</i>	1	0	2	2	3	3	3	3	All life cycles unsuccessful except for one, ESB unable to develop in the haemocoel.
	2	0	1	1	1	2	2	2	
	3	0	1	2	2	3	3	3	
<i>H. zealandica MJ2C</i>	1	0	4	6	6	6	6	6	All successful colonisations and kept clean cadavers.
	2	0	2	4	6	6	6	6	
	3	0	3	6	7	7	7	7	
<i>H. baujardi</i>	1	0	0	0	0	0	0	0	Three successful EPN cycles. No ESB/EPNs observed for the rest of the dead grubs.
	2	0	0	1	2	3	3	3	

	3	0	0	0	2	2	2	2	
<i>SCH10 (S. fabii)</i>	1	0	0	1	1	1	1	1	No ESB/EPNs observed for any of the dead grubs.
	2	0	0	0	0	0	0	0	
	3	0	0	0	1	1	1	1	
<i>H. bacteriophora</i>	1	0	2	3	3	3	3	3	Mostly ESB present but no successful EPNs.
	2	0	4	4	5	5	5	5	
	3	0	1	1	1	1	1	1	
<i>H. zealandica SF41</i>	1	0	4	4	4	5	5	5	There was mostly ESB colonisations and dead EPNs or no EPNs at all. Only two completed their lifecycle. ESB was not able keep a clean cadaver. Cadavers semi-decomposed and full of mites.
	2	0	1	2	3	4	4	4	
	3	0	0	1	5	5	5	5	
<i>S. bertusi</i>	1	0	3	3	3	3	3	3	All Successful infections, however, ESB was not able keep a clean cadaver. Cadavers semi-decomposed and full of mites.
	2	0	0	0	0	0	0	0	
	3	0	2	3	3	3	3	3	
Control	1, 2,3	0	0	0	0	0	0	0	No mortality recorded.

Table 3. 5 Dissection observations for *Pegylis sommeri*. The table shows the number of white grubs that were found dead over a period of 10 days post-inoculation, the status of EPNs and symbiotic bacteria inside the haemolymph as well as the status of the insect cadaver.

EPN species	Repetition	Day post inoculation										Notes
		1	2	3	4	5	6	7	8	9	10	
<i>H. zealandica</i> SF41	1	0	0	0	2	3	4	5	5	6	6	Mostly ESB present but dead or stunted adult EPNs observed. ESB unable to protect some of the cadavers.
	2	0	0	1	1	1	1	1	1	1	1	
	3	0	0	0	2	2	2	2	2	2	2	
<i>H. bacteriophora</i>	1	0	0	0	1	1	2	2	2	2	2	EPNs mostly able to complete lifecycle in cadavers, however ESB present in some but adult EPNs dead or absent. ESB able to maintain clean cadaver.
	2	0	1	1	1	1	1	1	1	1	1	
	3	0	0	0	2	3	3	3	3	3	3	
<i>H. noenieputensis</i>	1	0	0	0	1	1	1	2	2	2	2	Mostly ESB present but dead or stunted adult EPNs observed. No ESB/EPNs present in the cadavers that died after 7 th day.
	2	0	0	0	0	0	0	1	1	1	1	
	3	0	0	0	0	1	1	1	1	1	1	
<i>H. baujardi</i>	1	0	0	0	0	1	1	1	1	1	1	No ESB / EPNs observed in the cadavers.
	2	0	0	0	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	1	1	1	1	
<i>H. zealandica</i> MJ2C	1	0	0	0	0	0	0	0	0	0	0	Life cycle completed in two of the cadavers, ESB was not able keep a clean cadaver. Cadavers semi-decomposed and full of mites. ESB present in one cadaver but no EPNs.
	2	0	0	0	0	0	0	0	0	0	0	
	3	0	1	2	2	3	3	3	3	3	3	

<i>H. indica</i>	1,2,3	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.
SCH10 (<i>S. fabii</i>)	1,2,3	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.
<i>H. safricana</i>	1,2,3	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.
<i>O. myriophila</i>	1,2,3	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.
<i>S. bertusi</i>	1	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.
Control	1, 2,3	0	0	0	0	0	0	0	0	0	0	0	No mortality recorded.

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Figure 3. 1 The raster patterns of white grub species used in this study and their corresponding adult beetles. A, E – *Schizonycha affinis*, D, F – *Pegylis sommeri*, C, G – *Maladera* sp. 4 and D, H – *Monochelus* sp.

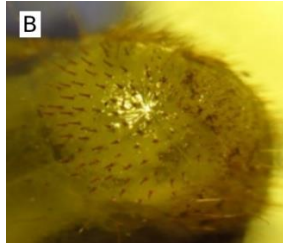


Figure 3. 2 Effects of different EPN treatments and time (7 – 28 days) on mortality rate of the white grub *Schizonycha affinis* placed in vials containing soil (A) and Petri dish bioassay mortality recorded after 7 days (B).

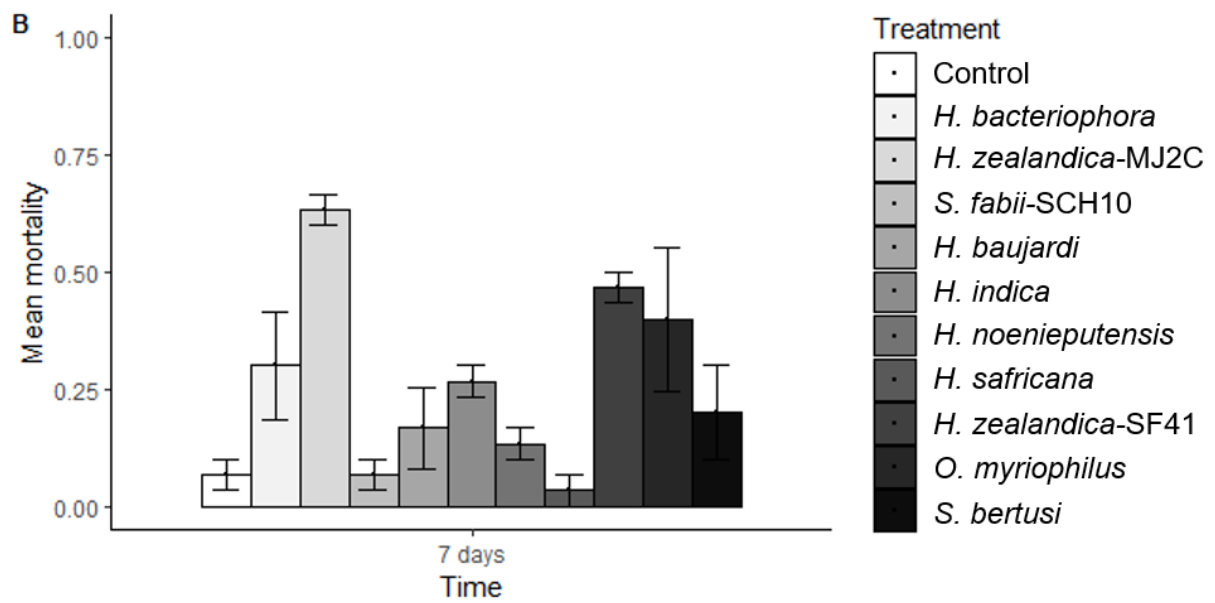
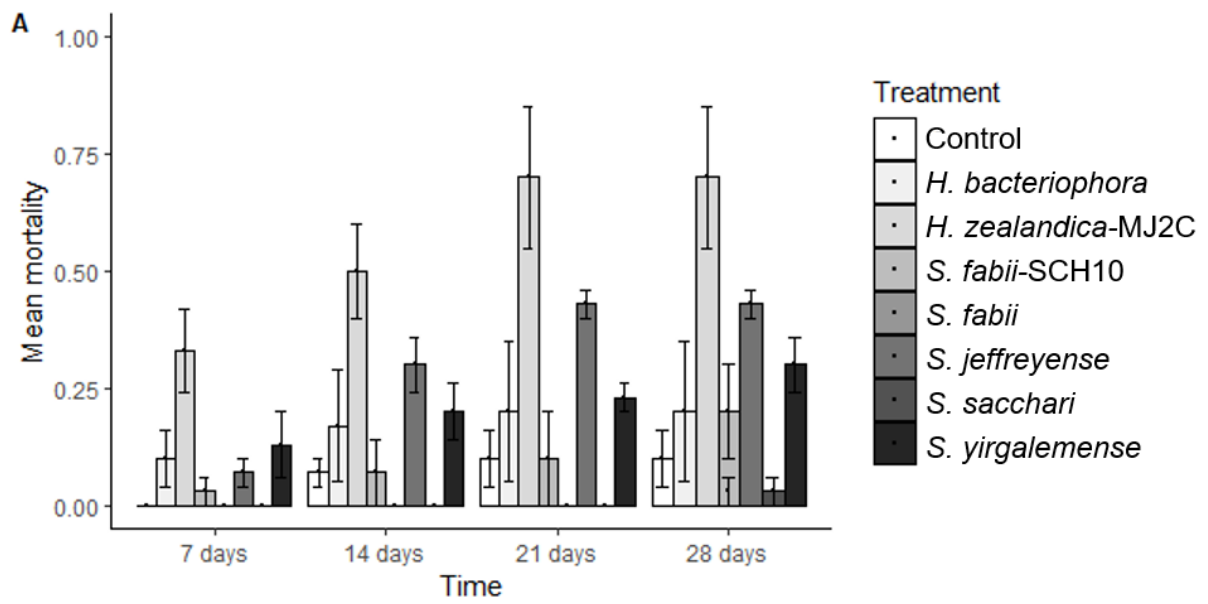


Figure 3. 3 Individual effects of different EPN treatments and time (7 – 28 days) on mortality rate of the white grub, *Pegylis sommeri*, placed in vials containing soil (A) and petri dish-bioassay mortality recorded after 10 days (B).

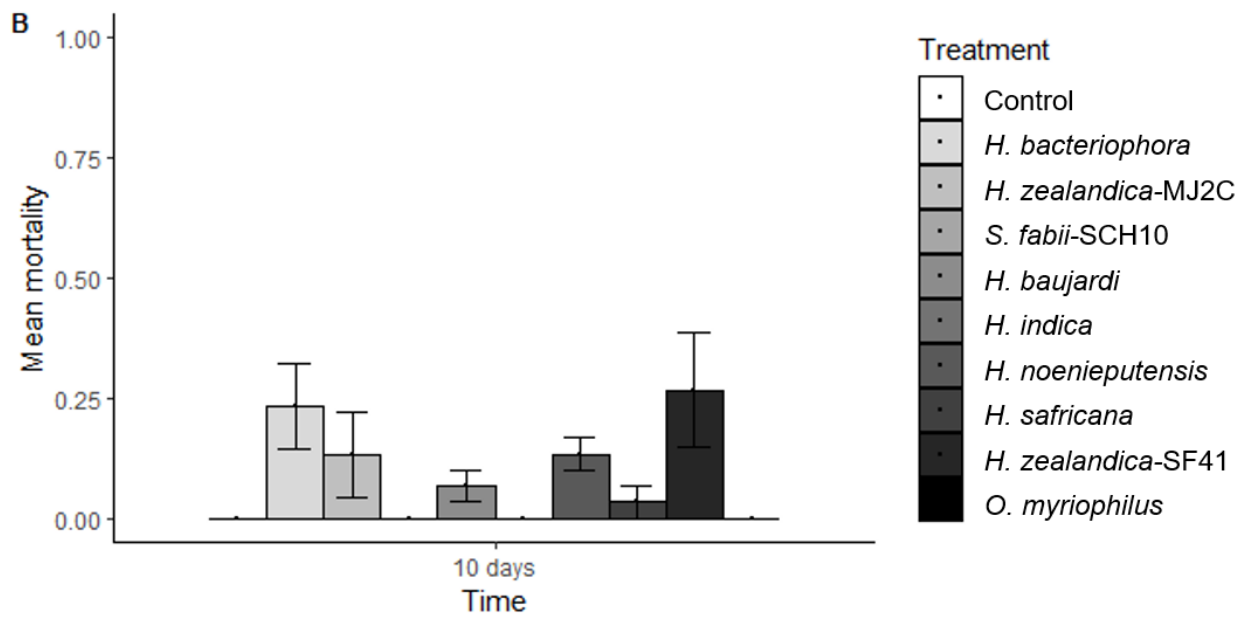
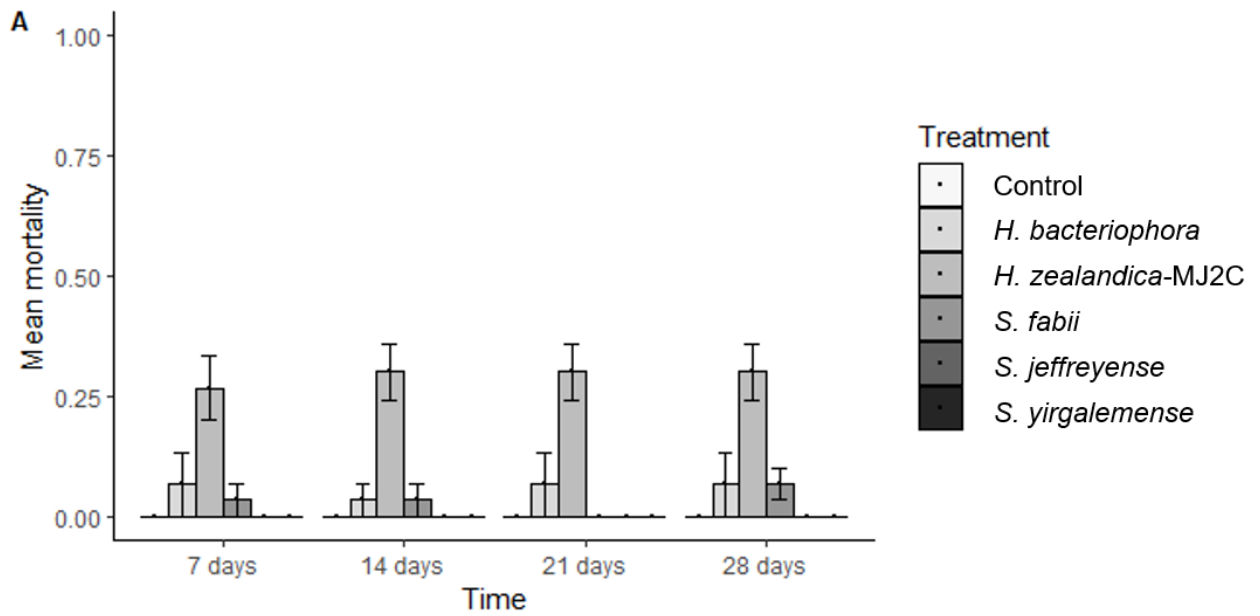


Figure 3. 4 Individual effects of treatments (EPN species) and time (7 – 28 days) on mortality rate of the white grub *Maladera* sp. 4

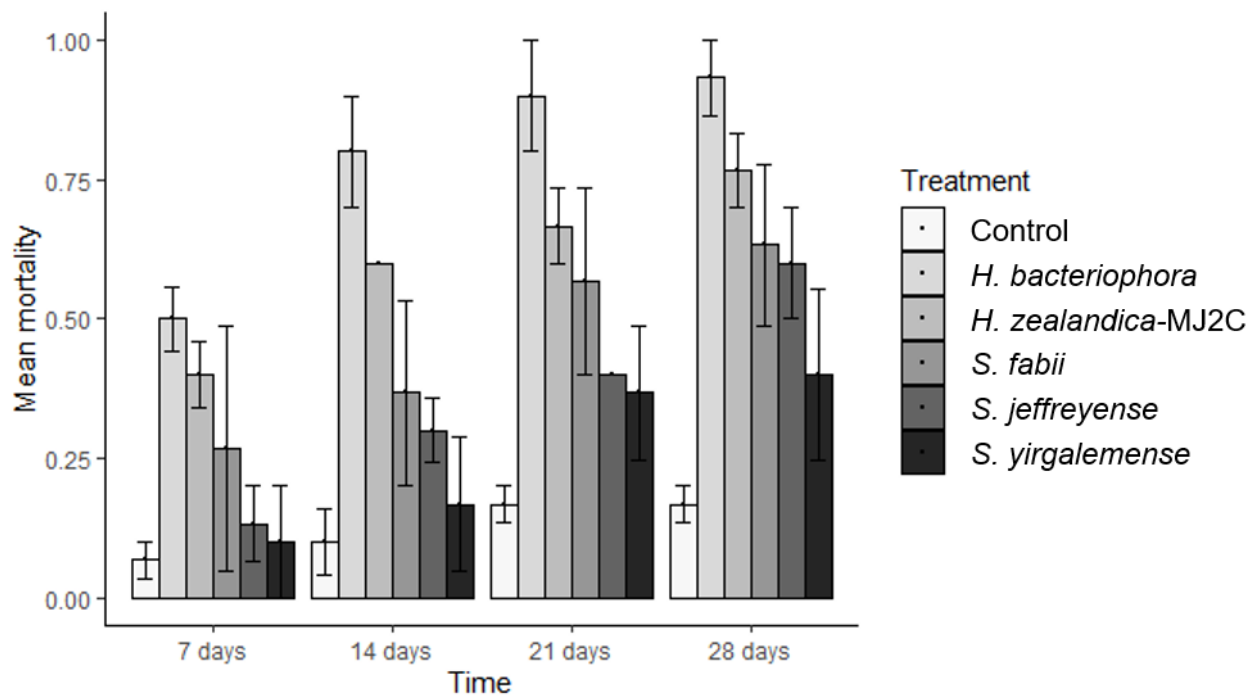


Figure 3. 5 Individual effects of treatments (EPN species) and time (7 – 28 days) on mortality rate of the white grub *Monochelus* sp.

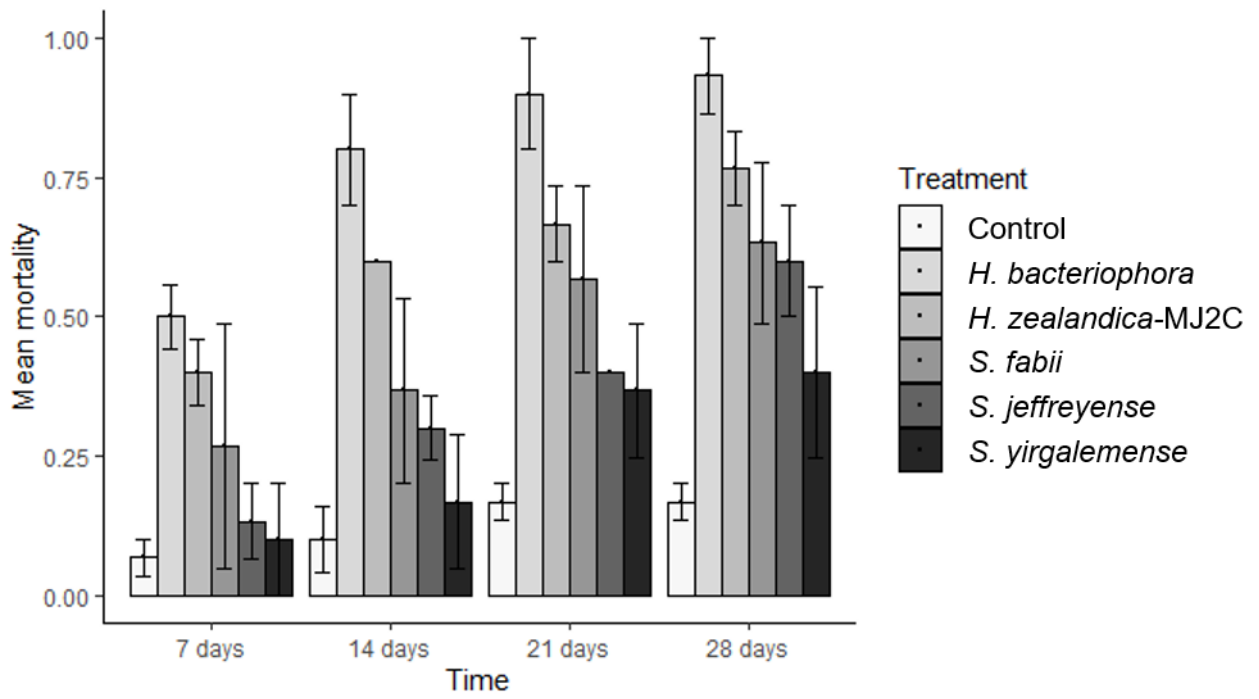
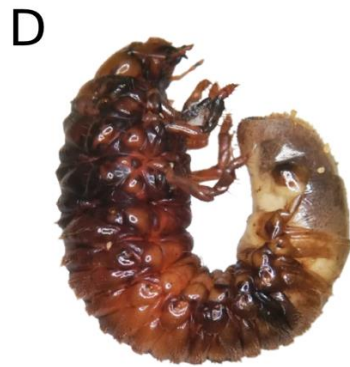
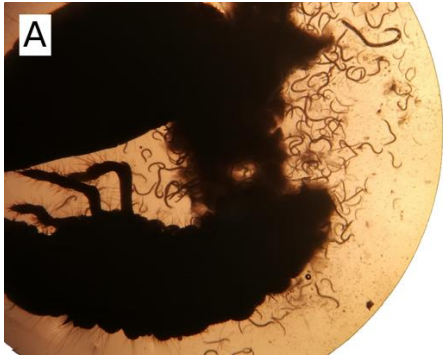


Figure 3. 6 A). *Heterorhabditis zealandica* MJ2C successfully infected and completed lifecycle in the cadaver of the white grub *Schizonycha affinis*. B). *Heterorhabditis bacteriophora* successfully infected completed life cycle in the cadaver of *Pegylis sommeri*. C). Dead and stunted adults of *Heterorhabditis noenieputensis* after infecting *Pegylis sommeri*. D). *Heterorhabditis bacteriophora* only infected upper body of *Pegylis sommeri*.



Chapter 4

Resistance mechanisms of white grubs (Coleoptera: Scarabaeidae) to entomopathogenic nematodes

Abstract

White grubs are sporadic root feeding larvae of scarabid beetles. Their control is difficult because of their cryptic nature and resistance to chemical insecticides. There is potential for the use of entomopathogenic nematodes (EPNs) for the control of white grubs, as their use in some previous studies have resulted in a higher control efficacy for white grubs, compared to chemical insecticides. However, the successful infection of white grubs by EPNs is limited by the white grubs' innate resistance mechanisms, which are classified as behavioural, morphological and physiological defences. The objective of this study was to determine the different physiological defence mechanisms of white grubs to attack by EPNs. The white grub species *Pegylis sommeri* and *Schizonycha affinis*, which were previously found to have a high level of resistance to locally isolated EPNs, and *Maladera* sp. 4., which had previously shown a relative high level of susceptibility, were examined. The ability of the EPNs to penetrate the white grubs and of the EPN symbiotic bacteria to grow in the insect haemolymph, the encapsulation rates, as well as the nematode growth inside the insect haemolymph, were determined through bioassays. The results showed that the ability of the nematodes to penetrate the white grubs, the encapsulation rates and growth in the haemolymph varied between EPN and white grub species. However, the different species of the EPN symbiotic bacteria were able to grow in the haemolymph of all the white grub species tested. The results indicate that the white grubs investigated tended to resist the initial penetration of most of the EPNs studied to avoid infection.

Key words: immunity, encapsulation, *Pegylis sommeri*, *Schizonycha affinis*

Introduction

White grubs (Coleoptera: Scarabaeidae) are the root feeding larvae of scarabid beetles (Ritcher, 1996). In South Africa and the neighbouring kingdom of Eswatini, white grubs are important insect pests in sugarcane and black wattle, *Acacia mearnsii* De Wild (Fabales: Fabaceae), plantations (Echeverri-Molina and Govender, 2016a, b). Their control is difficult because of their soil dwelling nature and resistance to chemical insecticides (Grewal *et al.* 2004). The adult beetles are nocturnal, further complicating the use of the available control options (Jackson and Klein, 2006). Entomopathogenic nematodes (EPNs) have been shown, in some cases, to be able to provide a higher control efficacy against white grubs, when compared to chemical insecticides (Grewal *et al.* 2004).

EPNs are soil dwelling microscopic round worms of the order Rhabditida that are known to parasitize various insects (Grewal *et al.* 2005; Lacey and Georgis 2012). They kill their insect hosts with the aid of symbiotic bacteria, which they carry in their digestive system and sometimes in a specialized bacterial vesicle (Nobuyoshi, 2002). Death of the insect hosts normally occurs within 48 h after infection (Kaya *et al.* 1993; Nobuyoshi, 2002).

The third instar larvae of the EPNs, known as the infective juvenile (IJ), is the only free-living stage of the EPN and can move in the soil and locate potential hosts, which it invades through their natural openings (Nobuyoshi, 2002; Lacey and Georgis 2012). Their soil dwelling activity partly accounts for the EPNs' suitability for the control of white grubs. This suitability is however sometimes limited by the white grubs' innate defence mechanisms to nematode infection which is a result of the co-evolution of white grubs and nematodes in the soil (Klein *et al.* 2007). The defence mechanisms of insects can generally be separated into primary, secondary and tertiary levels (Smilanich *et al.* 2009), but can also be classified into behavioural, morphological or physiological defences (Klein *et al.* 2007).

The primary defence mechanisms help the insects to avoid encounter with the pathogens. In the case of white grubs, some of the documented primary defences include their cryptic nature

(Gaugler, 1988; Gross, 1993; Gaugler *et al.* 1994), association with nematode predatory organisms such as mites (Karagoz *et al.* 2007), evasive and aggressive behaviour such as grooming (Gaugler *et al.* 1994, Alvandi *et al.* 2017) and changes in the respiratory patterns. This involves releasing the respiratory gas in bursts or infrequently rather than continuously, which assists white grubs to avoid detection by the EPNs (Gaugler, 1988, Kaya *et al.* 1993; Gaugler *et al.* 1994, Grewal *et al.* 2005).

The secondary defence mechanisms reduce the energy costs required to get rid of an infection. For white grubs, these include possession of spines, hairs, brushing off invading nematodes, thrashing or dropping, regurgitating and copious defecating (Smilanich *et al.* 2009), as well as the gut's dense peritrophic membrane, which delays EPN penetration through the gut, while food passage removes nematodes from the vulnerable alimentary tract (Forschler and Gardner, 1991; Gaugler *et al.* 1994; Grewal *et al.* 2005). The hard exo-skeleton and impenetrable sieve plates covering the spiracles (Gaugler *et al.* 1994; Forschler and Gardner, 1991; Brivio and Mastore, 2018) also limit EPN entry into the white grubs' body which further complicates the infection process.

The tertiary defence is the innate immunity and is aimed at providing resistance after an infection. The tertiary defence includes the cellular responses, such as phagocytosis, nodulation, and encapsulation mediated by haemocytes (Strand, 2008) and humoral responses, which includes production of soluble effector molecules; antimicrobial protein complexes and the activation of phenoloxidase cascades. (Hoffmann, 2003; Kanost *et al.* 2004; Strand, 2008; Tsakas and Marmaras, 2010; Alvandi *et al.* 2017). Various tertiary resistance mechanisms in white grubs have been identified, such as encapsulation and melanization in *Popillia japonica* Newman (Li *et al.* 2007; An *et al.* 2012) and encapsulation and phenyl oxidase activity in *Polyphylla adspersa* Motschulsky (Alvandi *et al.* 2017). These

studies indicated the importance nematodes escaping initial encapsulation for a successful infection.

In previous studies, the efficacy of various locally isolated EPN species were evaluated against five of the predominant white grub species in South Africa (Abate *et al.* 2019; Chapter 3). The white grub species included *Schizonycha affinis* Boheman, *Pegylis sommeri* Burmeister, *Maladera* sp. 4 and *Monochelus* sp. (Chapter 3), and *Heteronychus licas* Klug (Abate *et al.* 2019). *Pegylis sommeri* and *S. affinis* were the most resistant to the EPNs. *Pegylis sommeri* was resistant to most of the tested EPNs with the highest percentage mortality of 30 %, obtained with *Heterorhabditis zealandica* Poinar MJ2C after 14 days of exposure. Mean percentage mortality of *S. affinis* of >50 % was achieved with six of the seven tested EPN species, but only *H. zealandica* MJ2C achieving a mean percentage mortality of 70 % at 21 days post infection. The observed level of resistance of white grubs to EPNs was expected, as they are known to be generally resistant to EPNs based on previous studies (Abate *et al.* 2019; Chapter 3). However, the resistance mechanisms used by these white grub species and consequently what traits, which differ between white grub species, determine the level of resistance to EPNs, is not known.

The aim of this study was to identify some of the tertiary or physiological defence mechanisms shown by the white grubs to nematode infection. We examined the ability of five strains of *H. zealandica*, *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams to grow and complete their lifecycle in the haemolymph of the white grubs *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and *Galleria mellonella* (Lepidoptera: Pyralidae). The ability of these nematodes to infect the third instar larvae of *S. affinis* and *P. sommeri* were also determined. Furthermore, we determined the rates of encapsulation of *H. zealandica*, *S. fabii* and *S. yirgalemense* by larvae of *S. affinis*, *P. sommeri* and *Maladera* sp. 4. The ability of the symbiotic bacteria, *Photorhabdus heterorhabditis* Ferreira, Reenen, Endo, Tailliez, Pagès, Spröer, Malan, Dicks, *Photorhabdus temperata* Fischer-Le Saux, Viillard, Brunel, Normand, Boemare,

Xenorhabdus indica Somvanshi, Lang, Ganguly, Swiderski, Saxena, Stackebrandt and *Xenorhabdus bovienii* (Akhurst) Akhurst & Boemare to grow in the sterile haemolymph of *S. affinis*, *P. sommeri* and *Maladera* sp. 4 was also determined.

Materials and methods

Source of insects

Four white grub species, namely *S. affinis*, *P. sommeri*, *Maladera* sp. 4 and an unidentified *Monocheilus* sp. were used in this study. Insect identification was done by using LUCID key, previously developed for white grubs in sugarcane plantations in southern African countries: https://keys.lucidcentral.org/keys/v3/sugarcane_white_grubs/sugarcane_white_grubs.html.

The white grubs were collected over different seasons in 2018 and 2019, from various geographical locations in wattle and sugarcane plantations in the KwaZulu-Natal province of South Africa (Table 4.1). White grubs were dug up from the soil at sites where patchy sugarcane growth was observed or in mature wattle plantations with a thick topsoil layer, as prior monitoring indicated these conditions favour higher number of grubs. Each white grub was placed in a 30 ml plastic vial, filled with moist autoclaved peat moss (Hygrotech Sustainable Solutions) and provided with a fresh carrot disc as a food source. The vials containing white grubs were placed in cooler boxes for transport. The grubs were reared at the Forestry and Agricultural Biotechnology Institute (FABI) Biocontrol Centre at the University of Pretoria, South Africa, at 23 °C and 60 – 70 % humidity. The autoclaved peat and carrot discs were replaced weekly, and third instar grubs were used for experiments. White grubs that showed signs of pupation were excluded from the study.

Source of nematodes

Five EPN species (Table 4.2) were used in this study. The nematode isolates were obtained from the nematode collections at the FABI Biocontrol Centre and the Department of Conservation Ecology and Entomology at Stellenbosch University. All nematodes were reared

using third instar larvae of the greater wax moth, *G. mellonella*, from a laboratory culture. Modified White traps were used to harvest the infective juveniles (IJs), for a period of one week after emergence. Harvested IJs were stored in distilled water at 12 °C, in horizontally placed culture flasks (Woodring and Kaya, 1988). The stored EPNs were shaken periodically for ventilation and used within three weeks after EPN harvest to ensure nematode viability. A fresh batch of nematodes was used for each experimental repetition.

EPN symbiotic bacteria isolation

We used the methodology in Stokwe and Malan (2017) to isolate the EPN symbiotic bacteria species used in this study. For the bacteria isolation, petri dishes containing five wax moth larvae were each inoculated with IJs of the different nematode species at a rate of 400 IJs per plate. When the infected wax moth larvae were moribund, the hind leg of each insect was pricked with a sterile syringe needle, and a drop of haemolymph was streaked on separate NBTA plates (8.0 g nutrient agar; 25 mg bromothymol blue; 40 mg 2,3,5-triphenyltetrazolium chloride per litre). The plates were incubated at 28°C for two days. From each NBTA plate, a single bacterial colony was isolated with a loop and suspended in 30 ml tryptic soy broth (TSB) in an Erlenmeyer flask, plugged with non-absorbent cotton. The flasks were incubated on an orbital shaker at 28°C for 48 h. The bacterial culture (1 ml) was pipetted into 1.8 ml cryogenic tubes for preservation at -80 °C in 30 % glycerol.

Nematode growth in insect haemolymph

Four EPNs, namely *H. zealandica MJ2C*, *H. zealandica SF41*, *S. fabii* and *S. yirgalemense*, were tested for their ability to grow and reproduce in the haemolymph of the white grubs *S. affinis* and *P. sommeri*. Haemolymph of *G. mellonella* and *Maladera* sp. 4 were used as the positive control, as *Maladera* sp. 4 had shown a higher level of susceptibility to the EPNs in previous experiments (Chapter 3). The insects were surface sterilized with 75 % ethanol and patted dry with a paper towel. The insects were pierced on the hind leg with a sterile insulin needle to release a drop (about 50 µl) of the haemolymph onto a sterile Petri dish. Ten IJs of

each nematode were transferred to an individual drop of the insect haemolymph and moist cotton wool was added to the petri dish to keep it at a 100 % relative humidity. The Petri dishes were gently placed in a plastic container, lined with moist tissue paper and incubated at 25 °C for 10 days.

Nematode development was checked and recorded daily for the developmental status of the nematodes. Observations were made for the presence of infective juveniles (IJs), RIJs = recovered IJs (RIJs), adult (A), dead adults (DA), recovered first generation infective juveniles (R1GIJs) and second generation IJs (2GIJs), in the drops of sterile insect haemolymph. The presence of second generation IJs in the haemolymph indicated that nematodes were able to complete their life cycle and consequently a low level of resistance from the white grub. Ten replicates (haemolymph drops) were used for each white grub – EPN combination and the experiment was repeated twice, each repetition on a different test date, using a different batch of nematodes.

Infection bioassay

An infection bioassay was used to determine the ability and rate of penetration of the EPNs *H. bacteriophora*, *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense* into the haemocoel of *Shizonyca affinis* and *P. sommeri* over a period of 72 hours. Thirty white grubs were used for each white grub - EPN combination and thirty were used for the control experiment which was inoculated with distilled water. For each species, single white grubs were placed in cells of 12-well-bioassay plates lined with filter paper discs. Each grub was inoculated with 200 IJs of the EPNs suspended in 50 µl of distilled water. The white grubs in the control experiment were inoculated with distilled water. The plates were placed in a plastic container lined with wet paper towels (100 % moisture), and kept in a growth chamber, at 25 °C for a period of 6-72 h. For each treatment, five grubs were randomly removed after 6, 12, 24, 48, 72 and 120 h, washed with a stream of distilled water and frozen at -40 °C, until dissection. Dissections were made with the aid of a light microscope, to determine the presence or absence of nematodes.

The experiment was repeated twice for each grub species, each on a different test date, using a fresh batch of nematodes. Data for percentage of infected grubs was analysed using the statistical analysis program R (R Core Team, 2014). The R function (AOV) was used to perform one-way ANOVA test to identify group means differences and standard errors (SE). For multiple pairwise-comparison, the Tukey Honest Significant Differences, R function: Tukey (HSD) was used to determine if the mean difference between specific pairs of groups were statistically significant. The Tukey (HD) function accepts the fitted ANOVA as its argument.

Encapsulation test

The method employed by Li *et al.* (2007) was modified and used to determine the rate of encapsulation of three nematodes by the white grubs *S. affinis*, *P. sommeri* and *Maladera* sp. 4. The nematodes used included *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense*. Ten grubs were used for each white grub – EPN species combination. Ten IJs of each nematode species were injected laterally to the base of a foreleg, in the haemocoel of the insect, using a sterile insulin needle. The inoculated grubs were placed in filter paper lined bioassay plates and incubated at 25 °C.

The nematodes were recovered at 10 h post injection. The duration of incubation was determined from the nematode growth in haemolymph and the melanization observed to occur after 10 h during a pilot study. To recover nematodes, the grubs were dissected in a Petri dish filled with Ringer's solution. Care was taken not to damage the insect's digestive system, which was removed before recovering the nematodes in the haemolymph. The rest of the tissues were further examined for presence of nematodes. Encapsulation was measured by counting the proportion of nematodes with haemocytes attached at 400x magnification. Encapsulated nematodes only included cellular, but not melanotic encapsulation and was defined as nematodes with attached blood cells (Li *et al.* 2007). The experiment was repeated twice, with each experiment using a different batch of nematodes. Data for proportion of encapsulated nematodes was analysed using the statistical program R.

The R function (AOV) was used to perform one-way ANOVA test to identify group means differences. For multiple pairwise-comparison, the Tukey Honest Significant Differences, R function: Tukey (HSD) was used to determine if the mean difference between specific pairs of groups were statistically significant. The Tukey (HD) function accepts the fitted ANOVA as its argument.

Humoral immunity measurement using inhibitory zone assays.

The procedure by Stokwe and Malan (2017), Roy and Kim (2020) and Skowronek *et al.* (2020) were modified and used in this experiment to determine the ability of four EPN symbiotic bacteria species to grow in the haemolymph extracts of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. Four bacteria species were isolated and used in this experiment, namely *Photorhabdus heterorhabditis* and *P. temperata*, and *Xenorhabdus indica* was isolated from from *S. feltiae* *S. yirgalemense*, *H. zealandica* MJ2C and *H. zealandica* SF41 respectively. (Table 4.3). The insect haemolymph was obtained by sterilising white grubs with 75 % alcohol, after which they were pricked on the hind leg using a sterile needle to obtain the insect haemolymph. A metal loop was flamed and used to scrape the surface of the frozen bacteria stored at $-80\text{ }^{\circ}\text{C}$ in Eppendorf tubes and streaked onto NBTA media. The agar plates were sealed with Parafilm and incubated in the dark at $28\text{ }^{\circ}\text{C}$ for 48 h. A single colony was taken with a sterile loop and inoculated on TSB in an Erlenmeyer flask placed on an orbital shaker at $28\text{ }^{\circ}\text{C}$ in a growth chamber for 48 h. All available isolates of bacteria were evaluated visually for growth using the spotting technique suggested by Berkvens *et.al.* (2014). A sterile glass spreader was used to evenly distribute 50 μl of 48 h old TSB bacterial culture from the Erlenmeyer flask onto an NBTA plate. Five filter paper discs (of 3mm diam.) were then placed in a line configuration on the inoculated medium. Four discs were each spotted with 10 μl of the insect haemolymph, while 10 μl of 5 % sodium hypochlorite (JIK) was used to spot the middle disc as a control. The bacterial proliferation or inhibition around the filter discs was visually observed and

recorded after 2-4 days post-treatment. The experiment was repeated three times, each using a different batch of bacteria.

Results

Nematode growth in insect haemolymph

The results showed varying ability of the nematodes to grow and reproduce in the insect haemolymph (Table 4.4). *Maladera* sp. 4 showed the least level of resistance to the tested nematodes, as all the tested EPNs species were able to grow in the haemolymph of *Maladera* sp. 4. *Steinernema fabii* particularly preferred the haemolymph of *Maladera* sp. 4. and the nematode completed its life cycle faster in the haemolymph of the *Maladera* sp. 4. even when compared to the positive control experiment using the haemolymph of *G. mellonella*. The second generation IJs of *S. fabii* was observed at 4 DAI (days after inoculation) (Fig. 4.1C) when inoculated in the haemolymph of *Maladera* sp. 4.

Pegylis sommeri showed a moderate level of resistance to the growth of EPNs. Three of the four tested EPNs namely *H. zealandica* MJ2C, *S. fabii* and *S. yirgalemense* were able to complete their lifecycles in the haemolymph of *P. sommeri*. However, *H. zealandica* SF41 was unable to grow on the haemolymph of *P. sommeri*, as the IJs were observed to either die or not enter the recovery phase (Fig 4.1A).

Schizonycha affinis was the most resistant to the growth of EPNs. Only *S. fabii* and *H. zealandica* MJ2C (Fig. 4.1B) were able to complete their lifecycle in the haemolymph of *S. affinis*. The IJs of *H. zealandica* SF41 did not recover from the infective juvenile stage, while some IJs of *S. yirgalemense* were observed to recover but died as adults, thus unable to complete their cycle.

Infection bioassay

The ability and rate of nematode infection differed between EPN species and insect host. For *S. affinis*, the percentage of infected grubs increased over time for *H. zealandica* MJ2C and *S. yirgalemense* ($p < 0.001$). The percentage of infected grubs varied significantly across EPN species ($p < 0.001$) (Fig. 4.2A). The highest percentage of infected grubs was obtained by *H. zealandica* MJ2C at 120 hours post infection (HPI) (mean percentage infection of grubs = 80 ± 0.0). Both *H. bacteriophora* and *S. yirgalemense* moderately infected *S. affinis*. A mean percentage infection of grubs equal to 50 ± 10.0 was achieved by *H. bacteriophora* at 48 HPI. This infection rate reduced with time. *Steinernema yirgalemense* achieved a moderate percentage infection of grubs, namely 50 ± 10.0 at 120 HPI. The EPN *S. fabii* was not found to infect *S. affinis* throughout the trial period except for a very low infection percentage of 10 ± 10.0 at 72 HPI (Fig. 4.2A).

For *Maladera* sp. 4, the percentage of infection was generally high for all the EPN species and increased over time for all the EPN species ($p > 0.001$). The highest mean percentage infection of 80 ± 0.0 was achieved by *H. bacteriophora* at 120 HPI. *Heterorhabditis zealandica* MJ2C resulted in a mean percentage infection of 70 ± 10.0 at 120 HPI. *Steinernema yirgalemense* and *S. fabii* were both able to cause a moderate mean percentage infection to *Maladera* sp. 4 (Fig. 4.2B).

For *P. sommeri*, the results of the analysis showed *S. fabii* and *S. yirgalemense* species were unable to infect the white grub during the trial period. *Heterorhabditis zealandica* MJ2C and *H. bacteriophora* caused low percentage infection during the trial period. The highest percentage infection for *P. sommeri* of 40 ± 0.0 was from *H. zealandica* MJ2C at 48 HPI (Fig. 4.2C).

Encapsulation test

The analysis of the results of this experiment showed varying degrees of rates of nematode encapsulation for the different white grub species (Fig. 4.3). *Schizonycha affinis* achieved a high encapsulation of *S. yirgalemense*, namely $60 \pm 11.5\%$ (Fig. 4.4), but only $23 \pm 3.3\%$ and $3.3 \pm 3.3\%$ for *S. fabii* and *H. zealandica* MJ2C, respectively. *Pegylis sommeri* achieved encapsulation of $43 \pm 3.3\%$ for *S. yirgalemense*, which was slightly higher but not significantly different from that of *H. zealandica* MJ2C at $40 \pm 10.0\%$ ($p = 0.95$). *Maladera* sp. 4. obtained 50 % encapsulation for the same nematode. *Maladera* sp. 4 achieved the highest encapsulation rate for *S. yirgalemense* at $46 \pm 3.3\%$ followed by *H. zealandica* MJ2C at $30 \pm 5.7\%$ while the lowest was achieved for *S. fabii* at $13 \pm 3.3\%$.

Humoral immunity measurement using inhibitory zone assays.

The results of the humoral immunity using inhibitory zone bioassays, showed that all the tested symbiotic bacteria including *P. heterorhabditis*, *P. temperata*, *X. indica* and *X. bovienii* were able to grow in the sterile haemolymph of *P. sommeri*, *S. affinis*, *Maladera* sp. 4 as well as the susceptible control experiment (*G. mellonella*) (Fig. 4.5) By using visual observation, we did not record any significant differences in the ability of the different bacteria colonies to colonise the haemolymph, neither did we observe any differences in the colonization times.

Discussion

This study showed that the white grubs *S. affinis* and *P. sommeri* are the most resistant to EPN infection. This is in agreement with the results of the pathogenicity study in Chapter 3. The resistance was reflected in the low percentage mortality obtained in the screening bioassays, limited ability of the EPNs to penetrate the grubs, limited capacity of the EPNs to grow in the haemolymph of the grubs and the high rates of encapsulation of the nematodes by the grubs. Comparably, *Maladera* sp. 4 was more susceptible to attack by EPNs.

The experiment to determine the ability of the EPNs to grow in the haemolymph of the grubs resulted in mixed results, where some EPNs were able to grow in the haemolymph, while others were not. For example, for *S. affinis*, only *S. fabii* and *H. zealandica* MJ2C were occasionally able to complete their life cycle in the insect haemolymph. This growth inhibition can be attributed to the activation of the immunomodulation process of the insect. Various active compounds, as well as prophenoloxidase are known to occur in the haemolymph of insects and can mediate the insect's humoral defence (Söderhall and Cerenius, 1998). For example, Roy and Kim (2020) observed that the phenoloxidase activity and the expression of two antimicrobial peptides was higher in the more resistant *Tenebrio molitor* Linnaeus than in the more susceptible *Spodoptera exigua* Hübner. El Sadawy *et al.* (2020) observed additional protein bands in nematode infected *Rhynchophorus ferrugineus* Olivier and attributed this to the ability of the host to release a protein as a form of defence against the parasite. Since all the four species of EPN symbiotic bacteria used in the current tests were able to separately grow in the sterile haemolymph of all the tested insects, it suggests the presence of an inhibitory factor to the recovery of the EPN from its infective juvenile stage and the subsequent secretion of its symbiotic bacteria. This inhibitory factor did not affect *S. fabii* as this nematode was able to reproduce in the haemolymph of all the tested insects.

The results of the infection bioassay correlated with the results of the pathogenicity studies in Chapter 3. The nematodes *H. zealandica* MJ2C and *H. bacteriophora* that resulted in a high mortality effect for the grubs showed a higher ability to infect the white grubs in the current study than other species. The nematode *S. fabii* that had shown little to no ability to infect any of the white grubs, showed very little ability to penetrate the white grubs. The penetration rate of the nematode plays an important role towards the EPNs virulence, and the rate of penetration is directly proportional to the virulence rate (Hominick and Reid, 1990; Roy and Kim, 2020). Roy and Kim (2020) reported that the penetration rate of the IJs into the haemolymph of *S. exigua* was much higher when compared to *T. molitor* which was found to be more resistant to the EPNs. However, other researchers have found no correlation between

penetration rate and mortality. For example, Batalla-Carrera *et al.* (2014) reported that the penetration rate of EPNs into Hazelnut Weevil, *Curculio nucum* Linnaeus rather varied based on the life stage and increasing nematode dosage did not necessarily result in higher mortality.

Encapsulation was observed for all the nematode species and by all the white grub species and was highest for *S. affinis*. *Schizonycha affinis* also showed the most inhibition to nematode growth in the haemolymph. This indicates that this white grub uses encapsulation as a means of resistance to EPN attack. Encapsulation involves the use of multiple haemocytes (granulocytes and plasmatocytes) to attach to the invading EPN (Schmidt *et al.* 2001; Strand, 2008) and aggregate on the surface coat of the nematode's cuticle. Finally, the encapsulated nematode will be killed by the by-products of phenoloxidase cascade (e.g., quinone and melanin) within a cellular capsule entrapped within a necrotic internal layer of haemocytes (Strand, 2008; Kanost and Gorman, 2008; Alvandi *et al.* 2017). Granulocytes and plasmatocytes have been reported to be the most abundant haemocytes in the white grub, *Polyphylla adspersa* Motschulsky (Coleoptera: Scarabaeidae), (Alvandi *et al.* 2017) and are involved in the cellular encapsulation that leads to capsule formation (Lavine & Strand 2002).

For the humoral immunity measurement using inhibitory zone assays, all the tested EPN symbiotic bacteria including *P. heterorhabditis*, *P. temperata*, *X. indica* and *X. bovienii* was able to grow in the sterile haemolymph of *P. sommeri*, *S. affinis*, *Maladera* sp. 4. These results indicate that the resistance mechanisms that the tested grubs use against EPNs might also lie in the inability of the EPNs to penetrate the grubs, since the bacteria are able to grow in the insect haemolymph. These results differ from those obtained by Stokwe *et al.* (2015) and Javal *et al.* (2019) in whose experiments the haemocoel of the woolly apple aphid *Eriosoma lanigerum* Hausmann (Hemiptera: Aphididae) and the longhorned beetle, *Cacosceles newmannii* Thompson (Coleoptera: Cerambycidae), respectively, were shown to suppress the growth of symbiotic bacteria of some EPNs. Inhibition of EPN bacterial growth would in turn inhibit EPN growth and reproduction. For example, when Javal *et al.* (2019) tested the three locally isolated *Steinernema* species against the larvae of *C. newmannii*, a very low level of

pathogenicity was observed, and this was attributed to the suppression of symbiotic bacteria of EPNs in the insect's haemocoel and thus inability of the IJ to access their main nutrient source. This mechanism of suppression of the bacteria, however, does not appear to play a role in the interactions we tested.

It is important to understand the patterns of susceptibility and resistance mechanisms specific to each white grub species as this will inform decisions regarding the application of EPNs for the biological control of white grubs. Our results attempt to explain some of the resistance mechanisms that South African white grubs use against attack by EPNs. However, our study did not exhaust all the possible resistance mechanisms. Importantly, future studies should focus on such aspects as the humoral and cellular defence mechanisms that the white grubs use to defend when attacked by EPNs.

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Table 4. 1 Geographical location of sites for white grub (Coleoptera: Scarabaeidae) species collected in the KwaZulu-Natal province of South Africa, indicating the host plant and most dominant grub species collected at the site.

Site*	Dominant grub species	Host plant	GPS coordinates
Dalton1	<i>Pegylis sommeri</i>	Wattle	29°12'40.4"S 30°38'09.0"E
Dalton2	<i>S. affinis</i>	Sugarcane	29°12'52.8"S 30°37'55.5"E
Dalton3	<i>Maladera</i> sp4.	Sugarcane	29°12'52.8"S 30°37'55.5"E
Dalton4	<i>Maladera</i> sp4.	Wattle	29°12'52.8"S 30°37'55.5"E
Dolphin coast1	<i>S. affinis</i>	Sugarcane	29°12'35.0"S 31°28'06.0"E
Dolphin coast2	<i>S. affinis</i>	Sugarcane	29°12'35.0"S 31°28'06.0"E
Hilton1	<i>Maladera</i> sp4., <i>S. affinis</i>	Wattle	29°29'30.4"S 30°18'35.9"E
Hilton2	<i>Maladera</i> sp4., <i>S. affinis</i>	Wattle	29°28'33.3"S 30°18'42.2"E
Mbalenhle	<i>Schizonycha affinis</i>	Sugarcane	29°19'22.7"S 30°48'52.9"E
Piet Retief	<i>Maladera</i> sp4.	Wattle	27°07'20.0"S 30°57'04.5"E
Wartburg1	<i>S. affinis</i>	Sugarcane	29°25'51.6"S 30°39'21.6"E
Wartburg2	<i>S. affinis</i>	Sugarcane	29°14'50.7"S 30°39'55.7"E
Wartburg3	<i>P. sommeri</i>	Sugarcane	29°28'47.4"S 30°39'36.3"E

*Site name is based on the nearest city

Table 4. 2 *Heterorhabditis* and *Steinernema* isolates used in the study, with their associated host plants, origin, GenBank accession number, and the length and width of the infective juveniles (IJ) in micron (μm).

Species	Isolate	Associated host plant/ insect	Origin (town/ province)	GenBank accession number (ITS region)	Length of IJ (μm)	Width of IJ (μm)	Reference
<i>Heterorhabditis bacteriophora</i>	SF351	Indigenous forest	KwaZulu-Natal	MF535289	588 (512-671)	23 (18-31)	Abate <i>et al.</i> (2018)
<i>H. zealandica</i> (green)	MJ2C	Citrus orchard	Hex River Valley, Western Cape	MF370073	685 (570-740)	27 (22-30)	James <i>et al.</i> (2018)
<i>H. zealandica</i> (blue)	SF41	Natural vegetation	Patensie, Eastern Cape	EU699436	685 (570-740)	27 (22-30)	Malan <i>et al.</i> (2006)
<i>Steinernema yirgalemense</i>	157-C	Citrus orchard	Friedenheim, Mpumalanga	EU625295	685 (570-740)	29 (24-33)	Malan <i>et al.</i> (2011)
<i>S. fabii</i>	SCH10*	White grub	KwaZulu-Natal	MW618681	641 (590-697)	28 (26-31)	Katumanyane unpubl.

*Isolated during this study (Chapter 5, Table 5.1)

Table 4. 3 *Photorhabdus* and *Xenorhabdus* symbiotic bacteria associated with *Heterorhabditis* and *Steinernema* isolates used in the study, with their associated host nematode.

EPN symbiotic bacteria species	Associated nematode species/isolate	Associated host plant/insect	Origin (town/province)	Reference (EPN Isolation)
<i>Photorhabdus temperata</i>	<i>H. zealandica</i> MJ2C (green)	Citrus orchard	Hex River Valley, Western Cape	James <i>et al.</i> (2018)
<i>P. heterorhabditis</i>	<i>H. zealandica</i> SF41 (blue)	Natural vegetation	Patensie, Eastern Cape	Malan <i>et al.</i> (2006)
<i>Xenorhabdus indica</i>	<i>S. yirgalemense</i> 157-C	Citrus orchard	Friedenheim, Mpumalanga	Malan <i>et al.</i> (2006)
<i>X. bovienii</i>	<i>S. feltiae</i>	-	e-nema	-

Table 4. 4 Growth of *Heterorhabditis zealandica* MJ2C, *Heterorhabditis zealandica* SF41, *Steinernema yirgalemense*, and *S. fabii* in the sterile haemolymph of three white grub species, over a period of 10 days after inoculation (DAI).

EPN species	DAI	2		3		4		5		6		Observed 6 DAI
	Replicate	I	II	I	II	I	II	I	II	I	II	
<i>Schizonycha affinis</i>	<i>H. zealandica</i> MJ2C	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	A	1GIJs	A + 1GIJs	1GIJs	Successful cycle in one rep. IJs never recovered, No ESB observed
	<i>H. zealandica</i> SF41	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	IJs never recovered, No ESB observed
	<i>S. fabii</i>	1GIJs	1GIJs	A	R1GIJs	A + 2GIJs	A	2GIJs	A + 2GIJs	2GIJs	2GIJs	Successful cycle
	<i>S. yirgalemense</i>	1GIJs	1GIJs	R1GIJs	1GIJs	DA + IJs	1GIJs	1GIJs	1GIJs	1GIJs	1GIJs	Cycle unsuccessful
<i>Pegylis somerri</i>	<i>H. zealandica</i> MJ2C	1GIJs	R1GIJs	R1GIJs	A	A	DA	A	DA	A + 2GIJs	DA	Successful cycle
	<i>H. zealandica</i> SF41	1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	D1GIJs	Dead IJs
	<i>S. fabii</i>	RIJs	R1GIJs	A	A	A + 2GIJs	DA	2GIJs	DA	2GIJs	DA	Successful cycle in one rep.
	<i>S. yirgalemense</i>	1G IJs	1GIJs	R1GIJs	R1GIJs	A	A	2GIJs	2GIJs	2GIJs	2GIJs	Successful cycle
<i>Maladera sp. 4</i>	<i>S. affinis</i>	1GIJs	1GIJs	A	R1GIJs	A + 2GIJs	A	2GIJs	A + 2GIJs	2GIJs	2GIJs	Successful cycle
	<i>H. zealandica</i> MJ2C	1GIJs + A	1GIJs	A	R1GIJs	A	R1GIJs	A	R1GIJs	A + 2GIJs	DA	Successful cycle in one rep.
	<i>H. zealandica</i> SF41	RIJs	1GIJs	A	1GIJs	A + 2GIJs	1GIJs	2GIJs	1GIJs	2GIJs	1GIJs	Successful cycle in one rep.
	<i>S. fabii</i>	RIJs	A	A	A	A + 2GIJs	A + 2GIJs	2GIJs	2GIJs	2GIJs	2GIJs	Successful cycle
	<i>S. yirgalemense</i>	RIJs	A	A	A	A + 2GIJs	2GIJs	2GIJs	2GIJs	2GIJs	2GIJs	Successful cycle

EPN species	DAI	2		3		4		5		6		Observed 6 DAI
	Replicate	I	II	I	II	I	II	I	II	I	II	
<i>Galleria mellonella</i>	<i>H. zealandica</i> MJ2C	1GIJs	1GIJs	R1GIJs	R1GIJs	A	R1GIJs	A	A	A	2GIJs	Successful cycle
	<i>H. zealandica</i> SF41	1GIJs	R1GIJs	R1GIJs	A	A	A	A	A + 2GIJs	A + 2GIJs	2GIJs	Successful cycle
	<i>S. fabii</i>	1GIJs	1GIJs	R1GIJs	1GIJs	A	1GIJs	2G IJs	1GIJs	2GIJs	1GIJs	Successful cycle only in one rep.
	<i>S. yirgalemense</i>	1GIJs	1GIJs	R1GIJs	R1GIJs	A	A	A	A + 2GIJs	2GIJs	2GIJs	Successful cycle

IJs = Infective juveniles, RIJs = Recovered IJs, A = Adult, DA = Dead adult, R1GIJs = Recovered first generation infective juveniles, 2GIJs = Second generation IJs

Figure 4. 1 A: IJs of *Heterorhabditis zealandica* SF41 unable to grow on the sterile haemolymph of *Pegylis sommeri*; B: Adults of *H. zealandica* MJ2C growing in the sterile haemolymph of *Schizonycha affinis*; C: The second generation IJs of *Steinernema fabii* observed at five days after inoculation into the sterile haemolymph of *Maladera* sp. 4.

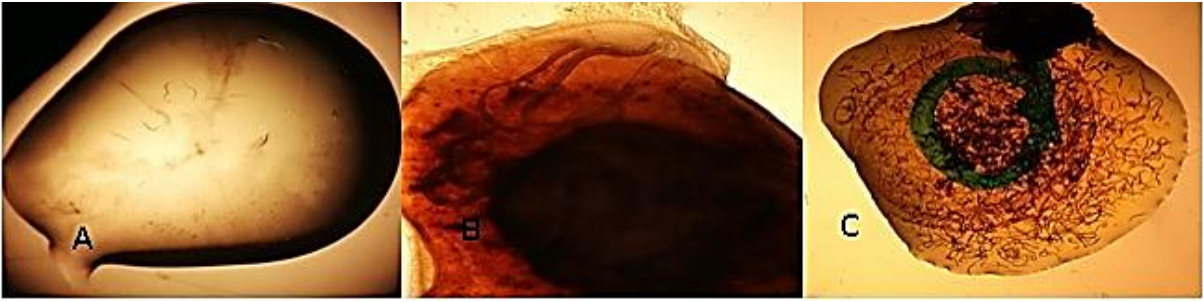
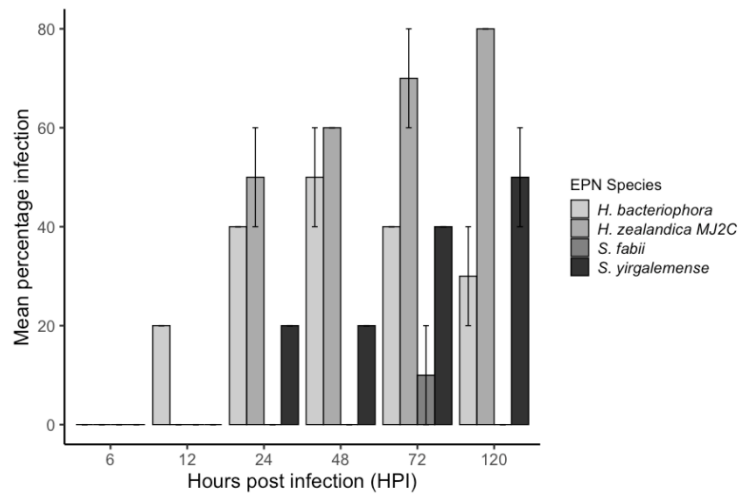
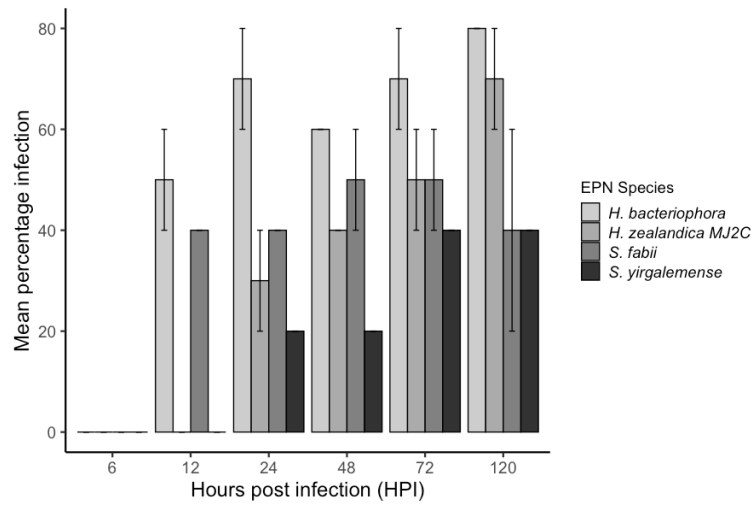


Figure 4.2. Results of the infection bioassay. Mean percentage of infected larvae of A. *Schizonycha affinis*, B. *Maladera* sp. 4 and C. *Pegylis sommeri*.

A *Schizonycha affinis*



B *Maladera sp. 4.*



C *Pegylis sommeri*

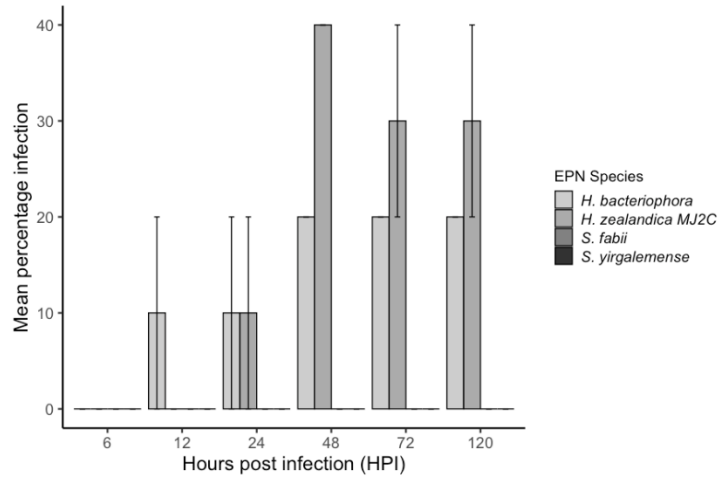


Figure 4. 3 Mean percentage of encapsulated nematodes of *Heterorhabditis zealandica* MJ2C, *Steinernema fabii* and *S. yirgalemense* in the haemocoel of *Maladera* sp. 4, *Pegylis sommeri* and *Schizonycha affinis*, at 10 hours post infection (HPI).

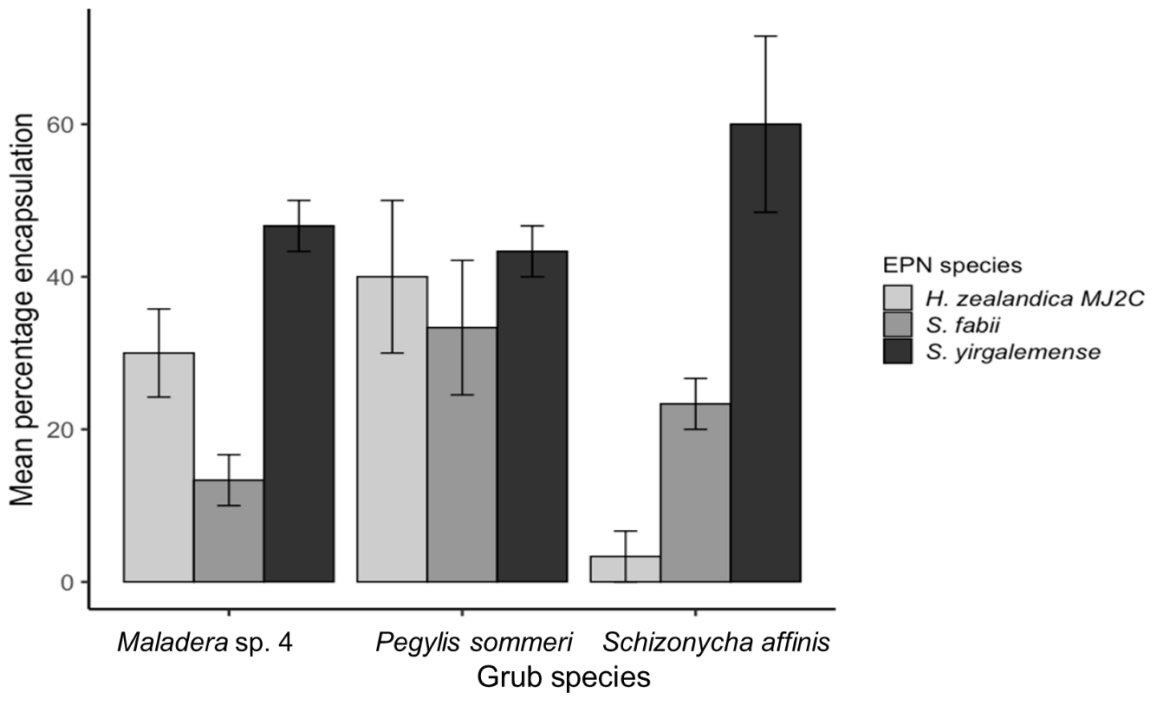
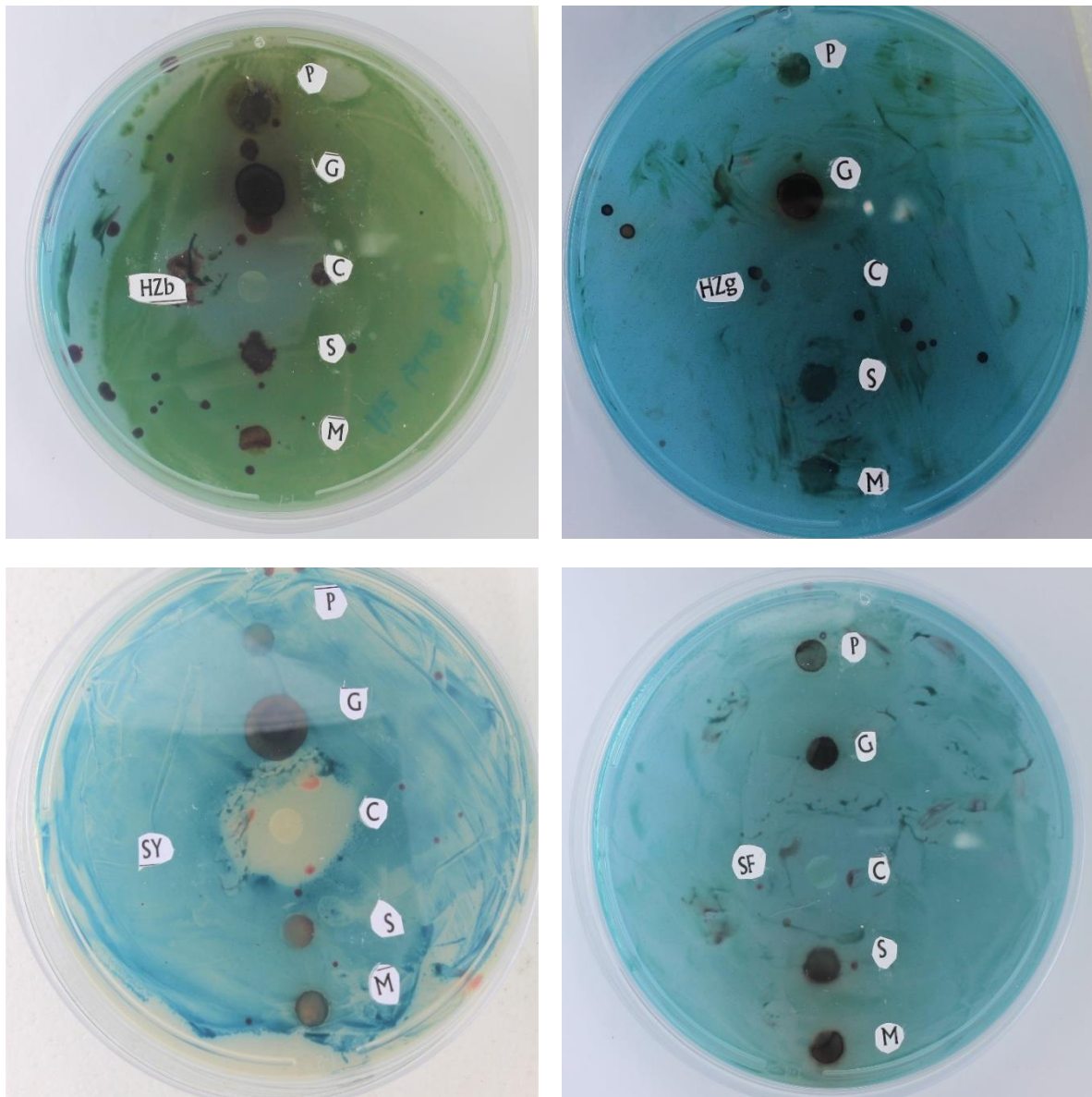


Figure 4. 4 Encapsulated entomopathogenic nematodes in the haemocoel of *Schizonycha affinis*.



Figure 4. 5 Entomopathogenic nematode symbiotic bacteria (Hzb = *Photorhabdus temperata*, Hzg = *Photorhabdus heterorhabditis*, SY = *Xenorhabdus indica* and SF = *X. bovienii*) growth on the discs stained with the haemolymph of P = *Pegylis sommeri*, G = *Galleria mellonella*, C = Control, S = *Schizonycha affinis* and M = *Maladera* sp. 4.



Chapter 5

Entomopathogenic nematodes isolated from white grubs (Coleoptera: Scarabaeidae) in the KwaZulu-Natal province of South Africa

Abstract

White grubs (Coleoptera: Scarabaeidae) are root feeding larvae of beetles that are sporadic pests in agriculture and can lead to economic damage. The grubs feed on the roots of plants, while the adult beetle can bore into underground stems, as well as cause defoliation of plants. During a survey for white grubs in the KwaZulu-Natal province in South Africa, sporadic incidence of larvae with symptoms of nematode infections were detected in wattle plantations and sugarcane. The larvae with infection symptoms were isolated, washed and put on water traps to collect infective juveniles of possible nematode infections. Three species of entomopathogenic nematodes (EPNs) were isolated from the white grub larvae. These included *Oscheius myriophila* and *Steinernema bertusi* isolated from *Maladera* sp. 4. and *Steinernema fabii* isolated from *Maladera* sp. 4., *Pegylis sommeri* and *Schizonchya affinis*. Limited success in laboratory infections were obtained using these EPN species to inoculate white grubs and visual observation of field collected grubs indicated that most white grubs infected were possibly those undergoing moulting. This is the first report of locally occurring EPNs naturally associated with white grub species in South Africa.

Key words: natural infections, *Steinernema fabii*, *Steinernema bertusi*, *Oscheius myriophila*

Introduction

EPNs are microscopic round worms of the order Rhabditida that under laboratory conditions, are known to parasitize nearly all insect orders (Grewal *et al.* 2005; Lacey and Georgis, 2012). For this reason and due to advances in mass production, storage technology and limited border restrictions, EPNs are available on the market for the biological control of various agricultural insect pests worldwide (Kaya and Koppenhöfer, 2004; Shapiro-Ilan *et al.* 2010). They are present in a variety of soil habitats (Kaya *et al.* 1993) where they infect soil-dwelling and litter insects. Little is known about their natural insect hosts, as they are mostly baited from the soil using larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) and mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). For example, in a review done by Peters (1996), information was lacking on natural hosts for seven out of the 18 recognized *Steinernema* species and three out of the six recognized *Heterorhabditis* species. To date, information regarding natural hosts of EPNs remains scant.

EPNs have been naturally isolated from white grubs. Chandel *et al.* (2018) provided a list of up to eleven different species of EPNs that have been naturally isolated from white grubs. White grubs (Coleoptera: Scarabaeidae) are the root feeding larvae of scarab beetles (Jackson and Klein, 2006; Ritcher, 1966). They are sporadic pests in agriculture and important insect pests in sugarcane and wattle plantations (Echeverri-Molina and Govender, 2016a, 2016b). High infestations of white grubs cause economic damage to farmers by feeding on plant roots, while adult beetles bore into underground stems, as well as defoliate plants (Jackson and Klein, 2006; Raodeo and Deshpande, 1987).

During a white grub sampling and collection trip in the KwaZulu-Natal province of South Africa (Chapter 3), white grubs with symptoms of nematode infection were recovered from the collected samples. This finding provided a rare opportunity to isolate and identify the nematode species naturally infecting the white grubs. Nematodes isolated from natural infections have the relative advantage of being naturally adapted to the host and environmental conditions

(Abate *et al.* 2017). This would be particularly advantageous for hosts such as white grubs that have been shown to have developed various resistance mechanisms to EPN infection (Grewal *et al.* 2004). The aim of this study was to identify the EPN species that were isolated from white grubs and to match them to their white grub natural hosts.

Materials and methods

Source of insects

White grubs with symptoms of EPN infection were collected from the soil over four seasons, from different geographical regions and two host plants – that is sugarcane and wattle in the KwaZulu-Natal province of South Africa (Table 1). The dug up white grubs were isolated in a 30 ml plastic vial with loose moist soil. The vials were placed in cooler boxes and transported to the insectary at the FABI Biocontrol Centre of the University of Pretoria. White grubs were washed with distilled water, identified to species level and those showing symptoms of possible nematode infection were each kept separately for incubation. Each insect was placed in a small tissue culture Petri dish lined with a moist filter paper. Incubation was done at 25 °C and 100 % relative humidity. Once the infective juveniles (IJs) were emerged from the cadavers, they were transferred to white traps for harvesting. Harvested IJs were stored in distilled water at 12 °C, in horizontally placed culture flasks (Woodring and Kaya, 1988). The IJs obtained from the White traps were inoculated in *G. mellonella* larvae and reared to obtain adult nematodes to use for DNA extraction.

Identification of EPN isolates

DNA extraction

From each isolate, DNA from a single young female nematode (Table 1) was extracted using the protocols outlined in Nguyen (2007) for single nematode extraction. Single nematode extraction was used because the initial infection of an insect host by an EPN induces the release of substances that suppresses subsequent penetration of other IJs (Glazer, 1997), so

it is normally expected to find one EPN species infecting a host. The lysis buffer used for DNA extraction consisted of 50 mM MgCl₂, 10 mM DTT, 4.5% Tween-20, 0.1% gelatine and 1 µl of proteinase K at 60 µg m⁻¹. The first-generation female was placed in 30 µl drop of the lysis buffer pipetted on the upper side of a 0.5 ml micro centrifuge tube. The nematode was cut into a few pieces, using a sterile insulin needle and the contents were immediately placed on ice and transferred to -80 °C for 20 min. For total lysis of the cells and digestion of the proteins, the tubes were incubated at 65°C for 1 h and at 95°C for 10 min in a thermocycler (GeneAmp 2720). The tube was cooled on ice and centrifuged at 11 600 g at 10°C for 2 min and 5 µl were pipetted from the supernatant and used in the PCR amplification.

PCR amplification

PCR amplification of the ITS region were conducted by following the protocol described in Nguyen (2007). The ITS region of the ribosomal DNA was amplified in a 25 µl reaction. The ITS region were amplified using the PCR primers TW81 [5'-GTTTCCGTAGGTGAACCTGC-3'] and AB28 [5'-ATATGCTTAAGTTCAGCGGGT-3']. PCR amplifications were carried out in tubes containing 5 µl nematode lysate, together with 0.5 µm of each primer and 12.5 µl KAPA2G™ Robust Hotstart ReadyMix (KAPA Biosystems). The final reaction volume was 25 µl. The cycling conditions were as follows: denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, with all conditions being repeated for 35 cycles. A 2-min incubation period at 72°C followed the last cycle in order to complete any partially synthesised strands.

The PCR product was then run on 1% agarose gel in a 1 × TBE buffer and visualised using Gel Red. Post-PCR purification was done using the NucleoFast Purification System (Macherey Nagel). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by electrophoresis on the 3730 × 1 DNA Analyser (Applied Biosystems) at the DNA Sequencing facility, University of Pretoria.

Sequence alignment and phylogenetic analysis

Sequences were assembled, analysed, and edited using the CLC Main Workbench ver. 8.1. The obtained sequences were compared with sequences on GenBank (NCBI) using BLASTn to determine the species identification. Sequences were then aligned using ClustalX 2.1 (Thompson *et al.*, 1997), while phylogenetic analyses of sequence data were done using the Maximum Parsimony (MP) method in MEGA5 (Tamura *et al.* 2011). Support for tree branches was evaluated statistically by means of a bootstrap analysis based on 1000 re-samplings of the dataset. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by means of the random addition of sequences (10 replicates).

Results

From the 30 infected white grubs from which single nematodes were isolated and sequenced, a total of 26 (87 %) of the nematodes were identified as *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers & Hurley. Three (10 %) were identified as *Oscheius myriophila* Poinar 1986, and one (3 %) as *Steinernema bertusi* Katumanyane, Malan, Tiedt & Hurley (Fig. 1 and Table 1). The white grubs found to be infected with EPNs were collected across different seasons in 2018, 2019 and 2020 (Table 1). From *Maladera* sp. 4., the EPNs *S. fabii*, *S. bertusi* and *O. myriophila* were isolated. From *Pegylis sommeri*, only *S. fabii* was isolated, while for *S. affinis* both *S. fabii* and *O. myriophila* were isolated. In wattle plantations all three EPN species were found in association with the three grub species, while in sugarcane all white grub species were associated with *S. fabii* and only one species, *S. affinis*, with *O. myriophila* (Table 1).

Discussion

Information on the natural hosts of entomopathogenic nematodes (EPNs) and their interaction with naturally occurring nematode and insect populations is not readily available, although most recorded naturally occurring nematode infections are from sampling of pest insects, in which case insects occur in high densities (Peters, 1996). In our study we isolated three EPN

species from naturally infected white grubs. The nematodes were isolated from the white grubs *P. sommeri*, *S. affinis* and *Maladera* sp. 4. These white grubs are pests of different crops including sugarcane and wattle (Echeverri-Molina and Govender, 2016a, 2016b) in the KwaZulu-Natal province. The sugarcane plantations are found in proximity with the wattle plantations and therefore there is an overlap of white grub species between these crops (Sivparsad *et al.* 2018).

The EPNs isolated in this study were *S. fabii*, *O. myriophila* and *S. bertusi*. *Steinernema fabii*, by far the most dominant EPN isolated from the white grubs, belongs to the *cameroonense*-clade. The original isolation and subsequent description of *S. fabii* was by trapping with *G. mellonella* larvae from the soil in an *Acacia mearnsii* plantation in the Mpumalanga province of South Africa (Abate *et al.* 2016). *Steinernema bertusi* also belongs to the *cameroonense*-clade and to date has been isolated twice; from an *Acacia mearnsii* plantation in Tito, Mpumalanga, and from an area with natural vegetation in Port Edward, KwaZulu-Natal, South Africa (Steyn *et al.* 2017; Abate *et al.* 2018; Katumanyane *et al.* 2020). Both isolations were done through baiting with *G. mellonella* larvae. The *cameroonense*-clade contains EPNspecies that have only been reported from the African continent. The species in this clade have their origins in the Americas (Spiridonov and Subbotin, 2016).

Oscheius myriophila has been isolated from various hosts to date (Demirbag, 2018; Ye *et al.* 2018; Del Rocio Castro-Ortega *et al.* 2020). The original isolation and description was from the garden millipede, *Oxidis gracilis* Koch (Polydesmida) in California, USA (Poinar, 1986). *Oscheius* spp. are divided into two groups which include the Dolichura and Insectivora groups (Ye *et al.* 2010; Liu *et al.* 2012). Species under the insectivora group are characterized by leptoderan bursa, crochet needle-shaped spicules and normal rectum, whereas the dolichura group has a peloderan bursa, probe head spicule tips and expandable rectum (Sudhaus, 1976).

The *Oscheius* nematodes were in the past not grouped under EPNs. However, as these nematodes have shown similar insect parasitism techniques as the Steinernematids and the Heterorhabditids, they are yet to be described as true EPNs (Dilman *et al.* 2012). For example, the recently described *Oscheius chongmingensis* Tumian (= *Heterorhabditoides*), *Oscheius rugaoensis* Zhang, Liu, Tan, Wang, Qiao, Yedid, Dai, Qiu, Yan, Tan, Su, Lai & Gao (= *Heterorhabditoides*) and *Oscheius carolinensis* Ye, Torres-Marragan, Cardoza show potential as EPNs (Ye *et al.* 2010; Torres-Barragan *et al.* 2011; Liu *et al.* 2012). Newly described species from South Africa, *Oscheius safricana* Serepa-Dlamini & Gray and *Oscheius basothovii* Lephoto & Gray have also been shown to share similar attributes with *Steinernema* and *Heterorhabditis* (Dillman *et al.* 2012; Lephoto *et al.* 2016; Serepa-Dlamini and Gray, 2018). In our study, *O. myriophila* grouped close to other *Oscheius* spp. and to the South African isolated new species of *Oscheius safricana* and *Oscheius basothovii* in the Insectivora group. The insect associated members of the genus *Oscheius* are associated with the insectivorous symbiotic bacteria of *Serratia* (Enterobacterales: Yersiniaceae) (Dillman *et al.* 2012). In terms of classification as EPNs, the above evidence shows that some *Oscheius* species fit the description of true EPNs, however an official classification as such is still lacking.

Some EPNs, such as *S. scarabaei*, have been determined to be very effective biocontrol agents for the insects with which that they are naturally associated (Cappaert and Koppenhöfer, 2003; Koppenhöfer and Fuzy, 2003). However, this was not supported by our laboratory bioassays for some of the white grub species (Chapter 4). *Steinernema fabii* did not cause any mortality to third instar larvae of *Schizonycha affinis* and *P. sommeri* in soil bioassays, while it only killed less than 5% of those tested in Petri dish bioassays. In the case of *Maladera* sp. 4, *S. fabii* provided 63 % mortality in soil bioassays using 30 ml vials. However, *S. fabii* was able to grow in the haemolymph of *P. sommeri*, *S. affinis* and *Maladera* sp. 4. Thus, *S. fabii* has a high ability to reproduce in the tested grubs, but a low ability to infect third

instar larvae. It is possible that *S. fabii* is able to infect other developmental stages more effectively.

Due to the consistent isolation of *S. fabii* from white grubs over seasons, the relationship between *S. fabii* and white grubs might be a relatively balanced nematode-host association in contrast to being an epizootic. However, due to the low biocontrol efficacy achieved in the laboratory, *S. fabii* might be an opportunistic EPN which attacks grubs when they are more susceptible. In our case, the susceptibility might be a result of the moulting of larvae from one instar to the other, as the highest number of isolated EPNs were from recently moulted, but rather healthy looking young third instar larvae. EPNs are known to opportunistically infect weak insects in the field (Kaya *et al.* 1993), and for this reason EPNs do not always have an exceptionally high biocontrol potential towards their natural hosts. However, the low virulence of *S. fabii* could have been a result of the stage of growth of the insect hosts as the virulence tests in Chapter 3. were performed using only third instar larvae of the white grub larvae. Third instar larvae of white grubs have previously been determined to be more resistant to EPN infection compared to the first and second instar larvae (Grewal *et al.* 2004; Alvandi *et al.* 2017).

Steinernema bertusi and *O. myriophila* were only isolated from white grubs on a few occasions. This could possibly be related to the sampling, and further collections would be needed to confirm the prevalence of the three EPN species, and possibly other EPN species, on naturally infected white grub species. During the Petri dish bioassays in Chapter 3, *O. myriophila* was able to kill an average of 40 % of *S. affinis*, while *S. bertusi* only killed 10 %. No mortality was recorded with either of the nematodes when exposed to *P. sommeri*. Due to the unavailability of white grubs, we were not able to grow these nematodes in the haemolymph of those white grubs.

Conclusion

Our study shows that various white grub species naturally host EPN species, thus demonstrating the potential for EPNs to be used as biological control agents for white grub pest species in forest plantations, sugarcane and other crops. *Steinernema fabii* was by far the dominant EPN isolated from white grubs and would be a good candidate to further investigate its potential as a biocontrol agent. This nematode reproduces fast in the haemolymph of white grubs but has a low infection potential on its own. We hypothesize that *S. fabii* is an opportunistic EPN in the field and attacks more susceptible grubs possibly those undergoing moulting. But *S. fabii*'s low virulence could also be a result of the more resistant targeted growth stage of the host. If this is the case, *S. fabii* can be targeted as a biological control agent during the seasons when white grubs are moulting.

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Table 5. 1 Entomopathogenic nematodes (*Steinernema* and *Oscheius* spp.) associated with white grub (Coleoptera: Scarabaeidae) species from the KwaZulu-Natal province in South Africa, with isolate number, location, host plant of the white grub species and the season of collection.

Isolate	Nematode ID	Grub species	GPS coordinates of collection site	Nearest city	Host plant	Month collected	GenBank ref.
SCH10	<i>Steinernema fabii</i>	<i>Schizonchya affinis</i>	29°26'04.9"S 30°38'30.6"E	Watburg	Sugarcane	June 2018	MW618681
AK02	<i>S. fabii</i>	<i>S. affinis</i>	29°19'22.7"S 30°48'52.9"E	Mbalenhle	Sugarcane	June 2018	MW618682
AK03	<i>S. fabii</i>	<i>Pegylis sommeri</i>	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	June 2018	MW618683
AK04	<i>S. fabii</i>	<i>P. sommeri</i>	29°12'40.4"S 30°38'09.0"E	Dalton	Wattle	June 2018	MW618684
AK05	<i>S. fabii</i>	<i>S. affinis</i>	29°29'30.4"S 30°18'35.9"E	Hilton	Wattle	June 2018	MW618685
AK06	<i>S. fabii</i>	<i>S. affinis</i>	29°28'33.3"S 30°18'42.2"E	Hilton	Wattle	June 2018	MW618686
AK07	<i>S. fabii</i>	<i>S. affinis</i>	29°28'33.3"S 30°18'42.2"E	Hilton	Wattle	June 2018	MW618687
AK08	<i>S. fabii</i>	<i>S. affinis</i>	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	June 2018	MW618688
AK09	<i>S. fabii</i>	<i>S. affinis</i>	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	May 2019	MW618689
AK10	<i>S. fabii</i>	<i>S. affinis</i>	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	May 2019	MW618690
AK11	<i>S. fabii</i>	<i>S. affinis</i>	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	June 2018	MW618691
AK12	<i>S. fabii</i>	<i>S. affinis</i>	29°12'35.0"S 31°28'06.0"E	Dolphin coast	Sugarcane	June 2018	MW618672
AK13	<i>S. fabii</i>	<i>Maladera</i> sp. 4.	29°29'30.4"S 30°18'35.9"E	Hilton	Wattle	Dec 2018	MW618693

AK14	<i>S. fabii</i>	<i>Maladera</i> sp. 4.	29°12'52.8"S 30°37'55.5"E	Dalton	Sugarcane	Dec 2018	MW618694
AK15	<i>S. fabii</i>	<i>S. affinis</i>	29°25'51.6"S 30°39'21.6"E	Wartburg	Sugarcane	Mar 2020	MW618695
AK16	<i>S. fabii</i>	<i>S. affinis</i>	29°14'50.7"S 30°39'55.7"E	Wartburg	Sugarcane	Mar 2020	MW618696
AK17	<i>S. fabii</i>	<i>S. affinis</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Mar 2020	MW618697
AK18	<i>S. fabii</i>	<i>S. affinis</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Dec 2018	MW618698
AK19	<i>S. fabii</i>	<i>S. affinis</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Dec 2018	MW618699
AK20	<i>S. fabii</i>	<i>P. sommeri</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Mar 2019	MW618700
AK21	<i>S. fabii</i>	<i>P. sommeri</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Mar 2019	MW618701
AK22	<i>S. fabii</i>	<i>P. sommeri</i>	29°28'47.4"S 30°39'36.3"E	Wartburg	Sugarcane	Mar 2019	MW618702
AK23	<i>S. fabii</i>	<i>S. affinis</i>	29°12'35.0"S 31°28'06.0"E	Dolphin coast	Sugarcane	Mar 2019	MW618703
AK24	<i>S. fabii</i>	<i>Maladera</i> sp. 4.	29°12'35.0"S 31°28'06.0"E	Dolphin coast	Sugarcane	Mar 2019	MW618704
AK25	<i>S. fabii</i>	<i>P. sommeri</i>	27°07'20.0"S 30°57'04.5"E	Piet Retief	Wattle	Mar 2019	MW618705
AK26	<i>S. fabii</i>	<i>P. sommeri</i>	27°07'20.0"S 30°57'04.5"E	Piet Retief	Wattle	Mar 2019	MW618706
AK27	<i>Steinernema bertusi</i>	<i>Maladera</i> sp. 4.	27°07'20.0"S 30°57'04.5"E	Piet Retief	Wattle	May 2019	MW618707
AK28	<i>Oscheius myriophila</i>	<i>S. affinis</i>	29°29'30.4"S 30°18'35.9"E	Hilton	Sugarcane	Mar 2019	MW618708
AK29	<i>O. myriophila</i>	<i>Maladera</i> sp. 4.	29°28'33.3"S 30°18'42.2"E	Hilton	Wattle	Dec 2019	MW618709
AK30	<i>O. myriophila</i>	<i>Maladera</i> sp. 4.	29°12'52.8"S 30°37'55.5"E	Dalton	Wattle	Dec 2019	MW618710

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Figure 5. 1 Maximum Parsimony derived phylogenetic relationships of the different *Steinernema* nematodes isolated from white grubs based on analysis of ITS rDNA regions, using *Caenorhabditis elegans* as the outgroup.

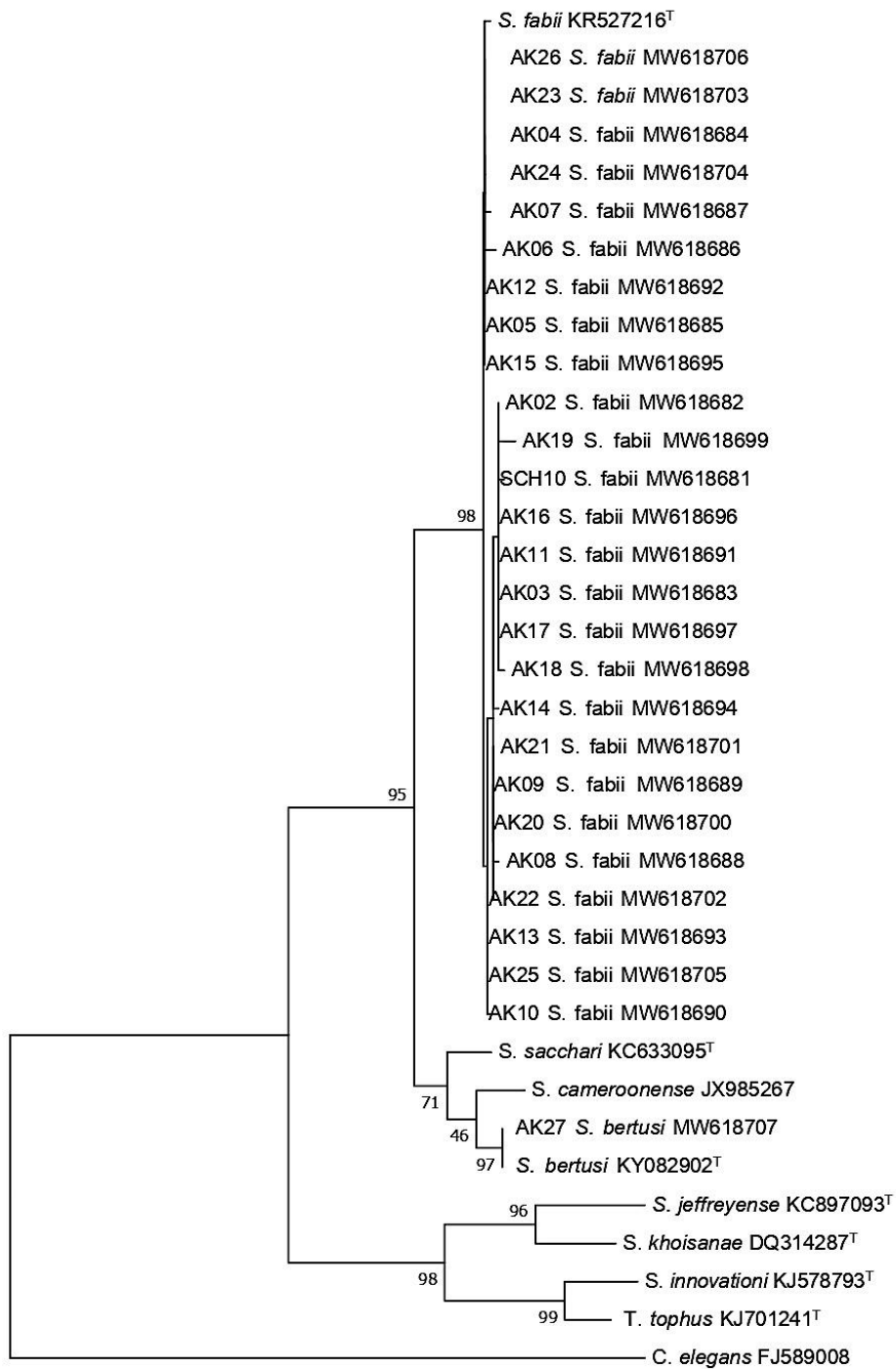
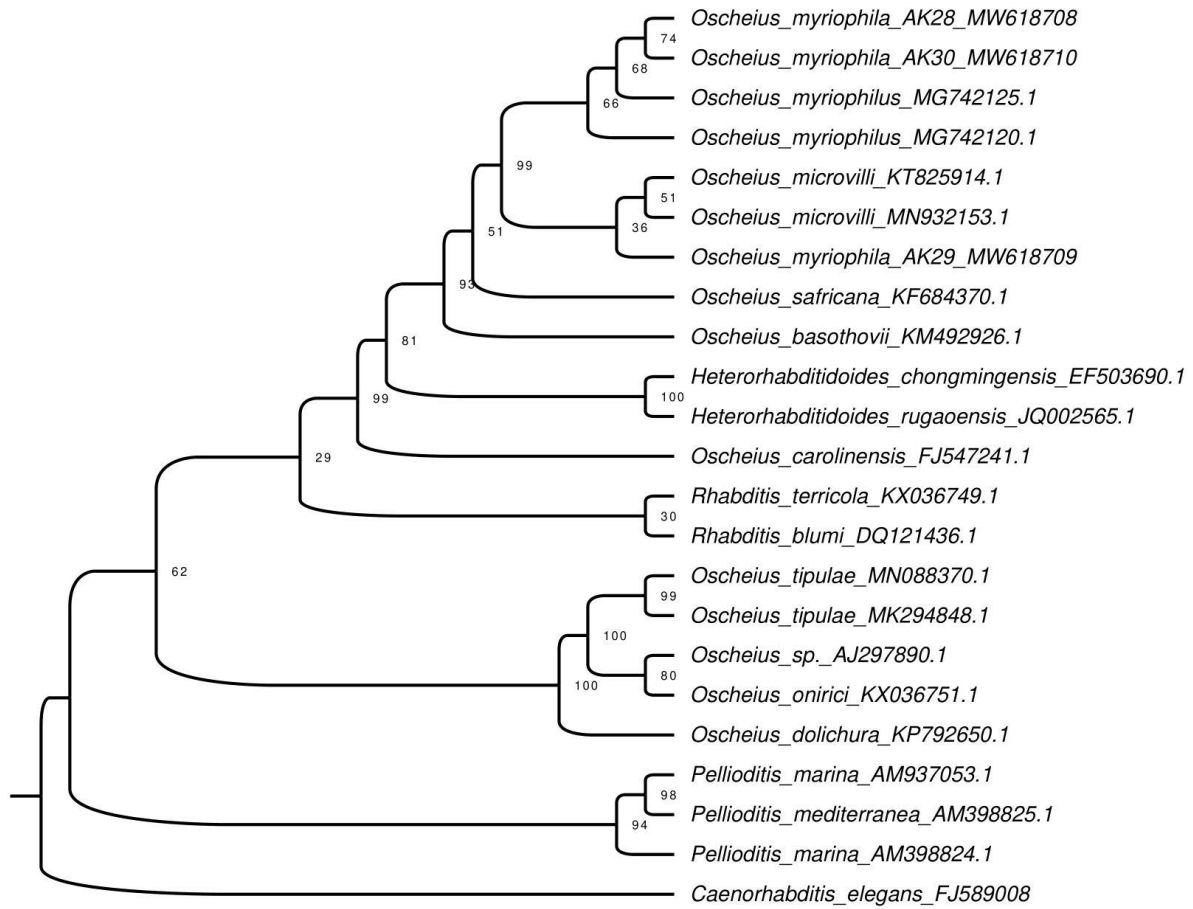


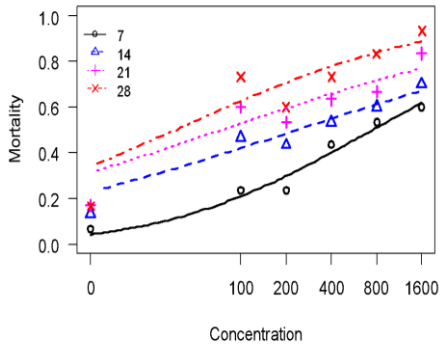
Figure 5. 2 Maximum Parsimony derived phylogenetic relationships of the *Oscheius myriophilus* nematodes isolated from white grubs based on analysis of ITS rDNA regions, using *Caenorhabditis elegans* as the outgroup.



2.0

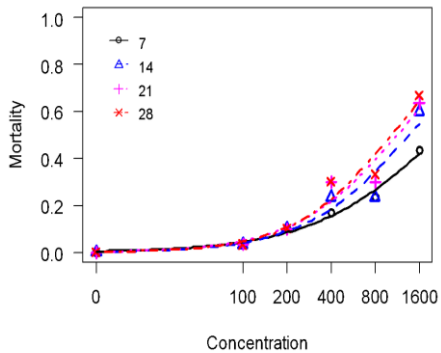
Appendix 1. Estimates of the two parameters (slope and LD50) of the log-logistic equation modelling the mortality rate of *Schizonycha affinis*, *Pegylis sommeri* and *Maladera* sp4. after inoculation with different concentrations of *Heterorhabditis zealandica* MJ2C; 0, 100, 200, 400, 800 and 1600 IJs per grub

S. affinis



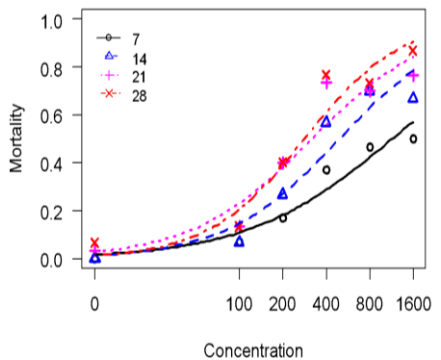
	Day	Estimate	Std. Error	t-value	p-value
Slope Estimate	7	-0.65	0.19	-3.51	0.0004
	14	-0.38	0.17	-2.18	0.0295
	21	-0.39	0.18	-2.16	0.0308
	28	-0.53	0.21	-2.54	0.0111
DL50 Estimate	7	761.61	235.39	3.24	0.0012
	14	237.59	117.98	2.01	0.0440
	21	74.49	63.47	1.17	0.2405
	28	37.61	34.08	1.10	0.2698

Pegylis sommeri



	Day	Estimate	Std. Error	t-value	p-value
Slope Estimate	7	-0.99	0.26	-3.79	0.000
	14	-1.21	0.26	-4.67	0.000
	21	-1.23	0.25	-4.93	0.000
	28	-1.30	0.25	-5.14	0.000
DL50 Estimate	7	2226.11	857.17	2.60	0.009
	14	1369.32	315.44	4.34	0.000
	21	1136.29	231.46	4.91	0.000
	28	1035.12	191.72	5.40	0.000

Maladera sp4.



	Day	Estimate	Std. Error	t-value	p-value	
Slope Estimate	7	-0.864	0.209	-	4.140	0.0000
	14	-1.100	0.209	-	5.269	0.0000
	21	-1.041	0.204	-	5.090	0.0000
	28	-1.295	0.227	-	5.703	0.0000
DL50 Estimate	7	1152.058	329.650	3.495	0.0005	
	14	497.122	84.583	5.877	0.0000	
	21	319.434	56.914	5.613	0.0000	
	28	283.863	43.230	6.566	0.0000	