The Isolation, Characterisation and Granular Formulation of Native Entomopathogenic Nematodes in South Africa

by

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Abstract

Entomopathogenic nematodes (EPNs) are obligate pathogens in nature and are used in the biological control of insect pests of agricultural crops. Nematodes are simple, colourless, unsegmented, bilaterally symmetrical, pseudocoelomic, triploblastic, worm-like animals, enclosed within a tough, elastic, and flexible, chitin containing cuticle. Entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with insect pathogenic bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus*, respectively. The focuses of this research was to isolate, identify, phylogenetically analyse, and formulate entomopathogenic nematodes indigenous to South Africa. The entomopathogenic nematodes were isolated from soil samples originally collected in Brits and Walkerville. Subsequent to their collection the soil samples had been stored for a prolonged period (> 2 years) in a dehydrated state. To isolate EPNs the soil samples were rehydrated and baited with *Galleria mellonella* larvae. The methods used for the identification and phylogenetic analysis of the isolated nematodes involved genomic DNA extraction, PCR amplification of 18S rDNA and Sanger sequencing of the 18S rDNA amplicons. The entomopathogenic nematodes that were isolated included *Heterorhabditis bacteriophora* isolate 56-C and a new previously uncharacterised *Steinernema* species.

Another focus of the research was to isolate and identify the bacterial symbionts of the isolated entomopathogenic nematodes. The methods used for the isolation of the bacterial symbionts involved haemolymph extraction from the infected larvae and homogenisation of sterilized infective juveniles. The methods used for the identification and phylogenetic analysis of the isolated entomopathogenic bacterial species involved total genomic DNA extraction, PCR amplification of 16S rDNA, and Sanger sequencing of the 16 rDNA amplicon. The isolated bacteria were identified as *Xenorhabdus sp VP* and *Photorhabdus luminescens subspecies sonorensis Carbonca*.

The study also showed that the isolated entomopathogenic nematodes had survived in soils that had been kept in a state of complete dehydration for a prolonged period. The survival of infective juveniles in the desiccated soil could have been due to the induction of anhydrobiosis or dehydration tolerance. Thus, the aim of this study also involved an investigation into the possible induction of anhydrobiosis or dehydration toleration in formulated infective juveniles by regulating the rate of moisture loss from various formulation media used. In the study, the *Heterorhabditis bacteriophora* isolate 56-C was formulated in different hydroscopic or water-

absorbing powders which included diatomaceous earth, crystalline cellulose and clay. Results showed that the rate of moisture loss from the formulation media had a significant impact on the viability of formulated infective juveniles. The finding was interpreted as evidence supporting the hypothesis that the induction of anhydrobiosis or dehydration tolerance depends strongly on the rate of dehydration.

Both the comparative morphometric characterization and phylogenetic analysis of the previously uncharacterised *Steinernema sp* confirmed that it was a new species of *Steinernema*. The results showed that the average length of the infective juveniles was 975µm with a standard deviation of 72µm, therefore, the species fell under the glaseri-group of *Steinernema*. Phylogenetic analysis showed that the new species did not form a clade with any of the local *Steinernema* species, therefore, confirming that the species isolated from Brits was, in fact, a new species.

Declaration

I, Linda Tabile Didiza, declare that this dissertation is my own unaided work. It is submitted in fulfilment of the Master of Science degree in Microbiology and Biotechnology at the University of the Witwatersrand, Johannesburg. The dissertation has not been submitted for any other degree at the University.

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Dedication

To my amazing family. Thank you Tata and Mama for providing more than I needed to be the success that I am today. My beautiful sisters, thank for joy and happiness you always give even in the darkest times. The milestones I have been able to reach and are still working towards have been made easier with your constant support and guidance. Truly heaven sent. Thank you!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BLAST – Basic Local Alignment Tool

DNA – Deoxyribonucleic acid

EPN – Entomopathogenic nematodes

IJ – Infective Juveniles

ITS – Internal Transcribed Spacer

LSU RNA - Large Subunit Ribonucleic Acid

MALDI – Matrix-assisted laser desorption/ionisation

MEGA – Molecular Evolutionary Genetics Analysis

MUSCLE – Multiple Sequence Comparison by Log-Expectation

NBTA – Nutrient bromothymol blue agar

PCR – Polymerase Chain Reaction

rRNA – ribosomal Ribonucleic Acid

sd – standard deviation

SSU RNA – Small Subunit Ribonucleic Acid

StdDev – Standard Deviation

TAF - Triethanolamine formalin

1. Chapter One. Literature Review

1.1. Entomopathogenic Nematodes

Within the phylum, Nematoda, certain species of insect parasitic nematodes such as those falling within the genera *Steinernema, Heterorhabditis,* and *Oscheius* have been collectively referred to as entomopathogenic nematodes (EPNs) (Dillman et al., 2012; Lephoto and Gray, 2019). EPNs have evolved a symbiotic relationship with insect pathogenic bacteria, and are able to act as insect host locating vectors for their bacterial symbiotic partners. *Steinernema*, *Heterorhabditis,* and *Oscheius* are found to be mutually associated with the insect parasitic bacteria belonging to the genera *Xenorhabdus Photorhabdus* and *Serratia* respectively (Gozel and Gozel, 2016; Lephoto and Gray, 2019). The entomopathogenic bacteria are Gram-negative bacteria belonging to the family Enterobacteriaceae (Chaston et al., 2011). The bacteria are contained internally within the nematodes in bacterial reservoirs during the non-feeding and developmentally arrested juvenile or dauer stage of the EPN life cycle (Dillman et al., 2012). It has been speculated that the species of both EPN genera evolved independently through a process of convergent evolution from separate bacterivore or microbivore ancestors. The fact that EPNs have a broad insect host range has been one of the motivations for their commercial development as biocontrol agents against insect crop pests. Commercialization of EPNs as biocontrol agents has been made possible through the development of bioreactors for their mass production (Inman et al., 2012). Following their mass production, nematodes need to be formulated in the non-feeding and developmentally arrested infective juvenile (IJ) stage of the EPN life cycle. However, an effective formulation of EPN infective juveniles which can extend their shelf-life or longevity in the formulated state by maintaining both their viability and infectivity remains an important economic constraint on the EPN biocontrol industry (Ramakuwela et al., 2016). It is well established that like most soil dwelling nematodes, many other micro-invertebrates can survive in dehydrated soils in an either an anhydrobiotic or desiccation tolerate state particularly at the dauer state of the life (Tyson et al., 2012). It would seem that if EPNs could be formulated in an anhydrobiotic or desiccation tolerate state this would increase the IJ shelf-life and thereby make the management of EPN product inventories more efficient and effective in terms of supply and demand. Moreover, the EPN based biocontrol industry can increase its biocontrol product range, effectiveness and reliability through tailored EPN species specific application programmes for the control of targeted insect pests. However, this means that continued isolation and characterization of EPNs remains an

essential activity for biocontrol product development and expansion in the industry. Thus, keeping these factors in mind, the framework and focus of this study includes the isolation of EPNs from dehydrated soil samples, their identification, their morphometric characterization, the identification of their bacteria endosymbionts, and also their formulation as biocontrol agents.

On the basis of their unique body plan, the nematodes fall into the phylum Nematoda, which in terms of the number of species is the third most specious phylum in the metazoa after the phyla Arthropoda and Mollusca (Zhang, 2013). In summary, nematodes are unsegmented, bilaterally symmetrical, pseudocoelomic, triploblastic animals, enclosed within a tough, elastic and flexible, chitin containing cuticle. Their overall body shape is cylindrical, fusiform, or filiform, generally tapering at the anterior and posterior ends. The cuticle or outer integument covering the epidermis which consists of a multi-layered matrix of chitin and protein polymers is more or less transparent allowing for visualization of internal structures under the dissecting and compound microscope. The flexible chitinized integument facilities the maintenance of a positive turgor or hydrostatic pressure which is necessary for nematode mobility. Beneath the integument, the layer of longitudinal muscle forms an outer tube. The pseudo-coelom or body cavity is enclosed between the outer longitudinal muscular layer and the inner alimentary tract. The haemolymph or fluid within the body cavity also functions as a hydrostatic skeleton. The usual model for the underlying anatomical pattern, when based on a typical free-living soil dwelling microbivore such as those presented in nematology manuals, are usually modelled on an idealized rhabditid worm (Kaya and Stock, 1997). However, the wide-ranging morphological or anatomical diversity of nematodes within the phylum is based on a flexible and continuous variation of the basic simple underlying rhabditid body plan (De Ley, 2006). The gross morphology of steinernematids and heterorhabditids share many similarities with an idealized rhabditid worm belonging to the monophylic order Rhabditida (Kiontke and Fitch, 2013). In EPNs, the alimentary tract extending from a terminally situated mouth to the anus, which is situated near the posterior end, consists of a number of diagnostic anatomical features. From the anterior end, the foregut or pharynx consists of the mouth opening, the stoma or buccal cavity, procorpus, metacorpus, isthmus, and a muscular basal bulb. The pharynx is separated from the intestine by the pharynx-intestinal valve. Nematodes do not have a respiratory or blood circulatory system. Gas exchange occurs through the integument. With regard to osmoregulation and excretion, there exists a simple excretory system consisting of two lateral longitudinal tubes that open into two excretory pores at the anterior end. The

reproductive system consists of paired or single testes or ovaries (Blaxter and Koutsovoulos, 2015). Nematodes are found in most habitats on earth including Antarctica. They range between 0.2mm to 6m in length and can be free-living bacterivores, fungivores, parasites, and predators. In terms of biomass, the nematodes are the most abundant metazoa on the planet, accounting for 50% of the total multicellular animal biomass (Maule and Curtis, 2011). Given the fact of their basic and simple body plan, which in many respects has remained fairly invariant with regard to many anatomical features, they have still managed to have undergone an astonishing degree of evolutionary species radiation and ecological biodiversity (Blaxter, 2011).

1.2. Nematode Taxonomy

As multicellular triploblastic pseudo-coelomic bilateral animals, the phylum Nematoda falls into the kingdom of Animalia. They have recently been included in the super phylum Ecdysozoa with the arthropods on the basis of the following evidence: small subunit (SSU) ribosomal DNA (SSU rDNA or 18S rDNA) based phylogenetic affinities; possession of chitin; and having to undergo ecdysis or moulting (shedding of their integument) at the different development stages of their life cycle (Bert et al., 2011; Blaxter and Koutsovoulos, 2015). According to Blaxter and Koustovoulos (2015), the Ecdysozoa can be further subdivided into the following two groups, the Panarthropoda, and the Cycloneuralia. Included in the Pararthropoda are the following phyla: Tardigrada, Onychophora, and Arthropoda. Included in the Cycloneuralia are the following phyla, Nematoda, Nematomorpha, Priapulida, Kinorhyncha, and Loricifera. The Nematomorpha, on the basis of morphological and molecular evidence, has been defined as a sister group with regard to the Nematoda (Dunn et al., 2008). Originally the phylum Nematoda was divided into two classes Phasmida and Aphasmida (Ivashkin, 1961). Later, Chitwood and Chitwood (1937, 1958) renamed the two classes as Secernentea and Adenophorea, respectively, which in classical nematology became the two major taxonomic divisions of the phylum (Chitwood, 1958, 1937). The classical division of the Nematoda into the two classes Adenophorea and Secernentea is no longer supported by a phylogenetic system of classification based on the molecular evidence of the 18S rDNA sequence data (Blaxter, 2011; Blaxter et al., 1998; De Ley, 2002).

In Table 1.1, a revised phylogenetic classification of the higher level taxons in the phylum Nematoda based on 18S rDNA nucleotide sequences have been given. Moreover, the

relationships between various taxons at the levels of classes, subclasses, orders, suborders, families, genera, and species in terms of affinities and distance have been phylogenetically reconstructed using the molecular evidence 18S rDNA sequence data. Furthermore, nematode evolutionary and ecological diversity with respect to nematode environmental adaptations, niches, functional guilds, trophic relationships and ecological relations with other species nematodes, bacteria, fungi, plants, invertebrate and vertebrates have been superimposed upon these higher-level 18S rDNA based phylogenetic classifications thereby showing nematode species radiation and convergent evolution especially in terms of plant, vertebrate and invertebrate parasitism (Blaxter, 2011; Blaxter and Koutsovoulos, 2015). Blaxter (2011) has resolved the phylum into 5 phylogenetic lineages or clades: Dorylaimia – clade I, Enoplia – clade II, Spirurina – clade III; Tylenchina IV, and Rhabditina – clade V (Table 1.1). Holtermann et al., (2006) using SSU rDNA sequence data resolved the phylum into 12 clades (Holterman et al., 2006). According to Blaxter (2011), nematodes which are invertebrate parasites fall into the following phylogenetic clades Rhabditina (Rhabditomorpha and Diplogasteromorpha), Tylenchina (Panagrolaimorpha, Cephalobomorpha and Tyenchomorpha), Spirurina (Rhigonematomorpha and Oxyuridomorpha) and Dorylaimia (Mermithida) (Blaxter, 2011). The majority of vertebrate and invertebrate nematode parasites fall within the Chromadoria (Table 1). The remaining vertebrate and invertebrate parasites fall into the Dorylaimia lineage. It appears that no animal parasites fall into the Enoplia lineage. Nematodes which are microbivores or predators fall into all their major lineages. Nematodes which are plant parasites fall into the following lineages Tylenchina (Rhabditina), Diphtherophorina (Enoplia) and Dorylaimida (Dorylaimia). (Table on the following page).

Table 1.1. A summarized version of the major lineages of clades that form the backbone of the phylogenetic classification of the phylum Nematoda based on the small subunit (SSU) rDNA sequences (De Ley and Blaxter, 2002; De Ley, 2006; Holterman et al., 2006; Blaster, 2011).

Phylum Nematoda				
Class Enoplea			Class Chromadorea	
Sub-Class Enoplia (II)		Sub-Class Dorylaimia (I)	Sub-Class Chromadoria	
Enoplida	Triplonchida		Order Rhabditida	
			Sub-Orders falling under Rhabditida	Remaining Orders in Sub-Class Chromadoria
Enoplina	Tripylina	Trichinellida	Rhabditina (V)	Plectida
Tefusiina	Tobrilina	Dioctophymatida	Brevibuccidae	Araeolaimida
Oncholaimina	Diphtherophorina	Monochida	Tylenchina (IV)	Monhysterida
Ironina		Mermithida	Myolaimina	Desmodorida
Campydorina		Dorylaimida	Spirurina (III)	Chromadorida
Tripyloidina			Teratocephalidae	
Alaimina				

Briefly, nematodes falling into the Class Chromadorea have been characterized by the following diagnostic features: “1. Pore-like or slit-like amphid apertures vary from labial pores or slits to post-labial elaborate coils and spirals. 2. Cuticle usually annulated, sometimes ornamented with projections and setae. 3. Phasmids present or absent, generally posterior. 4. Oesophagus usually divided into bulbs, with 3 to 5 oesophageal glands. 5. The excretory system is glandular or tubular. 6. Female with one or two ovaries. 7. Caudal alae present or absent.” (Source quoted from: http: // nemaplex.ucdavis.edu / Taxadata /Chromadorea.htm).

The two most prominent EPN families, Steinernematidae and Heterorhabditidae, both fall into class Chromadoria under the order Rhabditida (Hazir et al., 2004). Within the broad Rhabditida clade, species belonging to the family Steinernematidae fall into the sub-clade or sub-order Tylenchina and superfamily Strongloidoidea and species belonging to the family Heterorhabditidae fall into the sub-clade Rhabditina (Table 1). This indicates an interesting example of the independent convergent evolution of a similar partnership consisting of the tripartite nematode vector/bacterial insect pathogen symbiont/insect host association. The EPN genus *Steinernema* is the most speciose with a 100 species reported thus far (Bhat et al., 2020). In the case of the EPN genus *Heterorhabditis* 17 species have so far been reported (Bhat et al., 2020).

Nematodes in the Chromadorea class are found in marine and terrestrial habitats. Entomopathogenic nematodes are obligate pathogens in nature and are used in the biological control of insects (Kaya and Gaugler, 1993). The identification of the first entomopathogenic nematode was in 1923, initially named *Aplectana kraussei* by Steiner, and later renamed *Steinernema kraussei* in 1923 (Steiner, 1923). The geographical distribution of the nematodes indicates that they existed during the period of the supercontinent, Pangaea (Poinar Jr and Grewal, 2012).

1.3. Identification of EPNs

The identification of EPN species has been done using morphological and molecular techniques. The morphological differences between genera and species within genera allow for classification and/or identification using techniques such as light and electron microscopy (Eyualem and Blaxter, 2003). Using light and electron microscopy, a number of anatomical features having specific diagnostic features have been used to identify nematode genus and species. The overall structure of the buccal cavity and pharyngeal morphology in terms of the size, shape, and number, or the presence or absence of fusion, of the various pharyngeal or oesophageal anatomical structures, can be used as diagnostic phenotypic features for nematode identification. In a generalized rhabditid, the oesophagus consists of the following component parts: anterior corpus, a swollen metacorpus or median bulb, a narrow isthmus, and an enlarged glandular posterior bulb. Thus oesophagus can be diagnostically characterized in terms of the number of parts, their presence or absence. Also, the stoma or buccal cavity be characterized

in terms of the presence, number, and nature of rhabdions which are the sclerotized segments in the stroma (Basyoni and Rizk, 2016). The presence, position and number of oesophagus glands are also diagnostic features. Other diagnostic traits include cuticle surface features, lip morphology, sense organs and tail morphology, the female reproductive system, including the number of ovaries. The male reproductive apparatus and other associated structures such as the spicule, capitulum, gubernaculum and bursae are also diagnostic in genus and species-specific. In addition to the use of the above anatomical characters for species identification, classical nematode taxonomy also makes use of traditional morphometrics for nematode species identification (Kaya and Stock, 1997). In this regard, the application of traditional morphometrics in classical nematode taxonomy involves applying the De Mann Formulae or the De Mann indices of anatomical or morphological measurements (Cobb, 1914; Kaya and Stock, 1997; Thorne, 1949). Morphological characters have been shown to be unreliable with regard to phylogenetic inference or phylogenetically constructed nematode systematics. Reporting a new EPN species convention requires that an anatomical characterization and morphometric measurements be submitted. However, advances in molecular phylogeny have shown that a more reliable metric in the form of nucleotide sequences is required for identifying nematodes and establishing unambiguously its phylogenetic affinities (Abebe et al., 2011). A range of genetic markers can be used as metrics for nematode identification and for the construction of nematode phylogenetic affinities and evolutionary relationships.

The earlier molecular techniques that have been used for the identification of entomopathogenic nematodes involved electrophoretic isoenzyme patterns, total protein patterns, immunological techniques, and restriction fragment length polymorphism (RFLP) (Burnell and Stock, 2000). A more reliable refinement of these earlier molecular methods for the identification of larger nematodes samples involved the application of RFLP analysis of polymerase chain reaction (PCR) amplified products for specific regions of the genome (Reid et al., 1997). In more recent years, the PCR amplification of the ribosomal cistron (rDNA) encoding ribosomal RNA (rRNA) has been successfully used for generating a highly reliable taxonomic or phylogenetic metric for the identification and taxonomic classification of EPNs (Holovachov et al., 2015). Ribosomal cistrons are polycistronic consisting of multiple tandem repeats or copies (> 50) per nematode genome thereby making it one of the most reliable metrics for nematode identification (Subirana and Messeguer, 2018). Each ribosomal cistron is comprised of the following sequential set of nucleotide sequence units: the small subunit gene (SSU) also referred to as 18S, internal transcribed spacer 1 (ITS1), the 5.8 gene, internal

transcribed spacer 2 (ITS2) and the large subunit gene (LSU) or 28S. An external non transcribed spacer (NTS) separates each transcribed cistron. Ribosomal DNA or 18S rDNA sequences can be treated as an operational taxonomic unit (OTU) in nematode identification or phylogenetic tree building. The SSU (18S), 5.8S, and LSU (28S) nucleotide sequences highly conserved, whereas the ITS1, ITS2, and NTS nucleotide sequences are highly variable (Torres-Machorro et al., 2010). Using universal PCR primers, amplicons consisting of partial 18S sequences, complete ITS1, 5.8 and ITS2 sequences, and partial 28S sequences can be generated. The regions that are of importance for the identification of entomopathogenic nematodes are the hypervariable or polymorphic internal transcribed spacers (ITS) sequences and the hypovariable or conserved sequences corresponding the 18S and 28S flanking regions of the PCR amplicon (Carta and Li, 2019). Together the hypervariable and hypovariable nucleotide sequences of the PCR amplicon constitute the unique genetic fingerprint for the identification of a given species of EPN. Identification of nematode species at the strain level has been an important area of study for population biology and the discovery of EPN species in survey studies. Ribosomal RNA has been used for the identification and classification of known and unknown species because it has a high copy number of regions which have both conserved and variable sequences (Porras-Alfaro et al., 2014). As already mentioned the highly repetitive or polycistronic rDNA with its internal transcribed spacer (ITS) regions flanked by the 18S and 28S ribosomal DNA genes, makes rDNA a suitable genetic marker. These conserved SSU and LSU regions are ideal candidates for molecular taxonomic purposes as these highly conserved regions allow for the construction of universal primers for polymerase chain reaction (PCR) amplification. The preferred marker for the identification of unknown nematodes has been the 18S rDNA sequence (Liu et al., 2000). The SSU rDNA as the most conserved region of the ribosomal RNA encoding cistron allows for the elucidation of deep phylogenetic relationships between EPN species (Holterman et al., 2006). In recent years the number of steinernematid and heterorhabditid SSU rDNA sequences available on public databases such as the National Centre for Biotechnology Information (NCBI) GenBank nucleotide sequence data base has grown exponentially. The Basic Local Alignment Search Tool (BLAST) facilitates the accessing of the nucleotide sequence data held in the GenBank library in order to compare the degree or level similarity of unknown entomopathogenic nematode SSU rDNA sequence data with the known EPN sequences in the data base. Such a BLAST search facilitates a comparison between a submitted nucleotide sequence (called the query) with known sequences held in the GenBank library of nucleotide sequences. The

BLAST algorithm will identify those SSU rDNA sequences in the library which resemble the query SSU rDNA sequence above a certain threshold.

1.4. Evolution of Entomopathogenic Nematodes

Nematodes exist in several forms of symbiotic associations with other species ranging from commensalism to mutualism to parasitism (Blaxter, 2011; Dillman et al., 2012; Murfin et al., 2012; Blaxter and Koutsovoulos, 2015). Nematodes also occupy a diverse range of trophic levels in the food chain. However, it has been speculated that the original ancestor of the phylum Nematoda was a parasite (Blaxter and Koutsovoulos, 2015). Parasitic species within the phylum Nematoda share similar infectious and free-living stages within their life cycle as is the case with the parasitic species falling within the phylum Nematomorpha which phylogenetically is the sister phyla of the nematodes. Like the Nematomorpha, the Nematoda had a marine origin (Blaxter and Koutsovoulos, 2015). Within the Nematoda, the sub-call Enoplia consists mainly of marine species and most of the species in this sub-class are free-living (Blaxter, 2011; Blaxter and Koutsovoulos, 2015; Kiontke and Fitch, 2013). The Dorylaimia is a sub-class comprised of both freshwater and terrestrial dwelling species (Blaxter, 2011; Blaxter and Koutsovoulos, 2015; Kiontke and Fitch, 2013). Major groups of plant and animal parasites also fall with the sub-class Dorylaimia (Blaxter, 2011). A large number of marine species of nematodes also fall within the class Chromadoria (Blaxter and Koutsovoulos, 2015). Also within the Chromadoria, a major terrestrial species radiation into diverse ecological niches and ecosystem trophic levels within the terrestrial food chain has occurred, with nematode species filling a diversity of ecological functional guilds as microbivores (bacterial and fungi feeders), predators, plant parasites, invertebrate parasites and vertebrate parasites (Blaxter, 2011). Within the terrestrial dwelling Chromadoria lineage of nematodes, entomopathogenic nematodes belonging to the genus *Heterorhabditis* fall within the Rhabditina clade and those belonging to the genus *Steinernema* fall within the Tylenchina clade. Here we have an example of the independent co-evolution of similar terrestrial entomopathogenic or invertebrate parasitic life cycles. In the phylum Nematoda, plant parasitism has evolved independently in three different phylogenetic lineages (Dorylaimida, Triplonchida, and Tylenchomorha) and animal parasitism (vertebrate and invertebrate) have evolved independently several times in two evolutionary separate phylogenetic clades, Dorylaimia and Chromadoria (Blaxter, 2011). The fact that the evolution of plant and animal parasitism has taken place in most cases in a terrestrial environment has necessitated the evolution of adaptations that facilitate survival under conditions of desiccation or dehydration

(Tyson et al., 2012). Furthermore, while the basic nematode life cycle consists of several stages that include egg, larval and adult stages, the evolution of parasitism in nematodes generally requires various kinds of adaptations with regard to the functional roles performed with respect to each developmental stage or life cycle stage within a parasite's life cycle (Maule and Curtis, 2011). This adaptations include responses to signals which trigger the switching from a free-living state to a parasitic state. In the case of steinernematids and heterorhabditids, there has been convergent evolution for the switching from a bacterial-feeding and reproductive life cycle state to a non-feeding developmentally arrested infective juvenile (IJ). In the IJ stage, the nematode can persist for lengthy periods in the soil even when the soil becomes completely dehydrated. In addition, during this developmentally arrested stage, the IJs act as 'temporary storage reservoirs' for a colony of insect pathogenic bacteria and also act as the insect host-locating vector for these bacterial when environmental conditions permit. In short, both steinernematids and heterorhabditids feed on their bacterial symbiotic partners during the developmental and reproductive stage of the EPN life cycle and act as developmentally arrested 'storage reservoirs' for their bacterial endosymbionts during the non-feeding stage of the life cycle. It needs to be emphasized with respect to their life cycle, that EPNs have co-evolved with their bacterial insect pathogenic symbiotic partners a twofold relationship involving a trophic phase, where the nematodes feed on the bacteria and a cooperative commensal phase where they act as a reservoir and vector for the bacteria (Dillman et al., 2012). The parasitic lifestyle of many nematodes can be divided into pre-parasitic and parasitic phases. In all plant and animal parasitic nematodes, including EPNs such as steinernematids and heterorhabditids, there has also been co-evolution and convergent evolution of adaptations which facilitate the evasion of the innate and/or acquired immune systems of their hosts (Maule and Curtis, 2011).

The different kinds or even sequence of nematode host association events that could lead to the evolution of a nematode-host parasitical relation has been considered by Dillman et al. (2012). The different kinds of relationships that nematodes have developed with various kinds of hosts may be classified into four groups (Dillman et al., 2012): phoretic (where the assistance of another species is required for dispersal); necromenic (where nutrients are derived from the cadavers of hosts); facultative (is not completely reliant on the host for completion of its life cycle) and obligate parasitism (completely reliant on the host for the completion of its life-cycle) (Dillman et al., 2012). Phoresy is defined as the interaction of the phoront attaching itself to the host for the means of dispersal (White et al., 2017). The phoresy of the nematode and the host may be very or less specific (Blaxter and Koutsovoulos, 2015). Those that have a less

phoretic relationship with the host, their dispersal stage is seen to be associated with several different transport hosts. An example of this kind of dispersal occurs with third stage juveniles. With regard to the evolution of the parasitic lifestyle of EPNs, the evolutionary pathway can be hypothetically envisaged in terms of a sequence of changing adaptations that drove the trophic transition from a pre-parasitic microbivore to a parasitic insect lifestyle. With reference to the original conceptual framework of Dillman et al., (2012) the following three modifications for the evolution nematode-insect associations can be formulated: 1) Free-living → Phoretic association with an insect→ Necromenic association with insect cadavers → Parasitism of insects by infecting insect hosts and feeding on living insects. 2) Free-living → Phoretic association with an insect→ Necromenic association with insect cadavers → Entomopathogeny, whereby a living insect or an insect cadaver is invaded and killed by bacteria associated endophytically with free-living bacterivore nematode. 3) Entomopathogenic nematode → free-living bacterivore → feeds on bacteria saprophytically associated with the insect cadavers → develops an endophytic or mutualistic or commensal association with one saprophytic bacteria which released bactericidal agents → evolution of two-phase trophic and commensal association → bacteria evolves into a broad host range insect pathogen → Entomopathogenic nematode – acts as a vector for the insect pathogenic bacteria and actively seeks out insect hosts.

The main common events in the evolution of parasitism in nematodes involve modifications and transitions in the basic stages of the life cycle. The stage in the life cycle in which parasites transition from a free-living state to a parasitic state is typically at the J3/L3 stage which in addition also represents the life cycle stages when phoretic associations occur (Sudhaus, 2010). Furthermore, in rhabditid parasites, nematodes usually infect their hosts during the J3 stage of the life cycle (Sudhaus, 2010). The J3 stage also corresponds to the diapausing stage of the free-living non-parasitic rhabditid nematode *Caenorhabditis elegans* during which time it is resistant or tolerant to desiccation stresses experienced during soil dehydration, a feature also shared with the infective J3 stages of parasitic nematodes.

To sum up, the phylogenetic lineages of EPNs can be outlined as follows: Invertebrate parasitic nematodes belonging to the genus *Heterorhabditis* (family Heterorhabditidae) fall within the Strongylomorpha (Strongylomorphs) clade (in the Rhabditina) as a sister group to major vertebrate parasites (Blaxter and Koutsovoulos, 2015). Invertebrate parasitic nematodes belonging to the genus *Steinernema* (family Steinernematidae) fall within the Strongyloidoidae

(Strongyloidoids) clade (Tylenchina; Panagrolaimomorpha) as a sister group to the mammal-parasitic nematodes (Blaxter and Koutsovoulos, 2015). The entomopathogens belonging to the Strongylomorphs and Stronguloidoids have independently evolved convergent parasitic lifestyles in which the following features are shared in common: formation of symbiotic associations with insect pathogenic bacteria; 'free-living' desiccation tolerate or resistant non-feeding developmentally-arrested J3s (IJs) invade the haemocoels of insect hosts; the IJs acting a vectors release their endosymbiotic associated insect pathogenic bacteria into the haemocoel of the insect host. In the next section, the different stages of the EPN life cycle will be described in more detail.

1.5. Life Cycle of Entomopathogenic Nematodes

The life cycle is made up of an egg stage, three juvenile stages, and a sexually reproductive adult stage. The third stage infective juvenile (IJ) represents the only free-living, non-feeding stage that can survive in the soil in a developmentally arrested state (Ciche et al., 2006). Using various chemical and environmental cues the IJs locate, attack, and infect its target insect host. Infective juveniles invade the haemocoel of a host and release the symbiotically associated bacteria (Dubey et al., 2011). The bacteria multiply rapidly in the host haemolymph, at the same releasing insecticidal toxins which cause the death of the host by septicaemia, typically within 24–48 hours. The bacteria also release antimicrobial agents which suppress the opportunistic invasion and colonization of the insect cadaver by microbial saprophytes. A phenomenon referred to as endotokia matricida involves the intra-uterine development and release of juveniles within adult females (Baliadi et al., 2009). The process occurs when the food supply in the insect cadaver has been depleted and allows for the release of infective juveniles (IJs) with sufficient energy reserves, to facilitate long term survival in the soil under challenging environmental conditions, including desiccation as the soil undergoes dehydration. During the process of endotokia matricida, a population or reservoir of symbiotic bacteria becomes preserved within the digestive tract of the IJs (heterorhabditids) or in a specialized vesical associated with the digestive tract of the IJs (steinernematids). This facilitates or preserves the entomopathogenic capacity of the IJs towards of insect hosts once conditions favourable for insect infection have been restored. On infection of the insect host, the IJs transition from the non-feeding developmentally-arrested state into bacterial-feeding third-stage juveniles. These juveniles start feeding on the bacteria which have been released into the insect haemocoel and develop into the fourth stage larvae. The fourth larval stage of the life

cycle represents the gateway to the development of the nematodes into the first generation of reproductive adult females and males. These adults' mate and the females lay eggs that hatch as first-stage juveniles. The first-stage juveniles moult successively to the second, third, and fourth stages. The life cycle continues for two to three generations until resources within the cadaver are depleted. Once the resources have been depleted, the nematodes cease feeding, and in response to possible bacterial signalling gravid females undergo endotokia matricida, releasing IJs in which bacteria have already entered bacterial storage reservoirs within the IJ digestive tract or in specialized chambers. Thereafter, these juveniles depending on environmental conditions either transition into desiccation-tolerant quiescent pre-infective states or revert to infective stages. If conditions are conducive the infective juveniles emerging from the cadaver search or forage for new insect hosts. The life cycle takes place over a period of about six to 11 days for steinernematids and 12 - 14 days for heterorhabditids (Chitra et al., 2017).

The advantages and biocontrol significance of using EPNs as biocontrol agents lie or rest within the IJ life cycle stage. In the non-feeding and developmentally-arrested stage of the EPN life cycle, IJs possess the physiological and morphological capacity to persist in the soil or in a formulated state. Formulated biocontrol agents are usually also non-polluting. In addition, they can infect a broad insect host range and can be applied to field crops and soils by spraying with standard pesticidal spray equipment, which is equipment that is ordinarily well-suited for applying pesticides to field crops. In nature, like other soil living nematodes and soil micro-fauna, entomopathogenic nematodes can survive in unfavourable soil environments in an inactive state, which significantly extends their life span and allows them to endure environmental fluctuations (Koppenhöfer et al., 2002).

The soil-dwelling IJ inactive phase of the life cycle can be divided into the following two alternative states: diapause and quiescence (Kaya and Gaugler, 1993). Diapause is a stage of arrested development where development does not continue until certain conditions have been fulfilled, such as when appropriate environmental conditions relating to temperature and moisture have been re-established (Moens and Perry, 2011). Quiescence is a facultative state that involves the lowering of nematode metabolism in response to unpredictable and unfavourable environmental conditions (Moens and Perry, 2011). The quiescence state is easily reversible when appropriate conditions are re-established. The disadvantages regarding the use of EPN as biocontrol agents include: possible detrimental effects regarding their ability to infect a broad host range which may include helpful insects; limited tolerance to environmental

conditions such as ultraviolet (UV) radiation, desiccation, temperature; poor storage capabilities; reduced field resistance and relatively high storage and transportation costs (Lacey and Georgis, 2012).

The bacterial symbionts of *Steinernema* and *Heterohabditis* are *Xenorhabdus* and *Photorhabdus* respectively (Bertrand et al., 2015). These bacteria belong to the family Enterobacteriaceae and are part of the γ-subclass of Proteobacteria (Boemare and Akhurst, 2006). Both bacterial symbionts are not able to reduce nitrate but *Xenorhabdus* is negative for catalase (Grewal et al., 2006). They are rod-shaped bacteria, motile, Gram-negative, facultative anaerobes, and non-spore forming (Burnell and Stock, 2000). The optimal temperature is typically 28C or less for *Xenorhabdus*. *Photorhabdus* are motile using peritrichous flagella (Boemare, 2002).

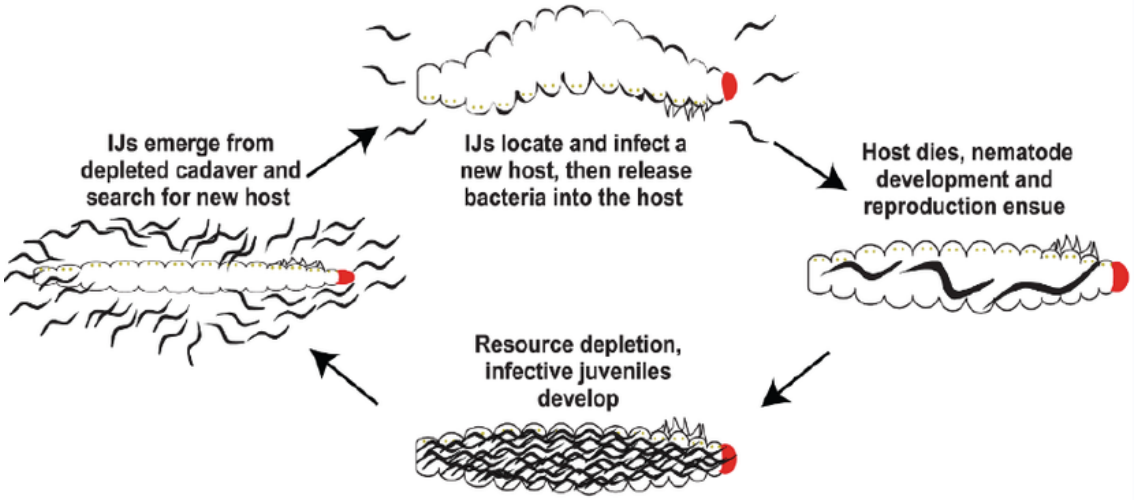

Figure 1.5.1. Life Cycle of Entomopathogenic Nematodes (Dillman et al., 2012).

The bacteria have two distinct physiological stages in their life cycle. The first stage is the symbionts' phoretic stage where *Xenorhabdus* is found in the intestinal vesicle of steinernematids infective juveniles (Boemare and Akhurst, 2006), whereas *Photorhabdus* is found in the anterior part of heterorhabditids infective juvenile guts. The second stage is where the symbionts reproduce within the host after entering the haemolymph of the host (Sicard et al., 2004). The life cycle of the bacterial symbionts begins when the third stage infective juveniles move into the insect, using natural points of entry such as the mouth, anus, and spicule (Goodrich-Blair and Clarke, 2007). The IJs release the bacteria into the host's body cavity and begin to develop into the fourth stage and eventually into adults. The development of the IJs

into adult nematodes is reliant on the presence of the bacteria in phase I (Goodrich-Blair and Clarke, 2007). In phase I, the bacteria release antimicrobial agents to ensure the specificity of the symbiosis by inhibiting the presence of microbial competitors. Phase I bacteria also release enzymes and other agents to assist in metabolizing the host's tissues (Forst and Nealson, 1996). Phase II seems to produce little or no antimicrobial agents (Kaya and Koppenhöfer, 1996). *Xenorhabdus* and *Photorhabdus* strains can be identified by their unique 16S rDNA sequences. *Xenorhabdus* and *Photorhabdus* have TTCG and TGAAAG sequences, respectively, for identification (Boemare, 2002).

The three-way partnership of the bacterial symbionts with its insect host, entomopathogenic nematode, and its attack on the secondary host, the target pest is one that requires greater understanding. The entomopathogenic nematode provides the bacteria with protection from other competing organisms in the surrounding environment and receives nutrients from the haemolymph of the insect. The nematode in return can use the bacteria for its pathogenic abilities to kill the host and feed on the bacteria and the nutrients supplied for its growth, development during the life cycle and the bacteria also prevent recolonization of the host haemolymph by other opportunistic microorganisms.

1.7. Entomopathogenic Nematode Foraging Behaviour

EPNs can be found either deep within the soil or on the surface depending on the type or species of nematode. For steinernematids and heterorhabditids, foraging strategies vary along a continuum cruising to ambushing (Campbell et al., 2003; P. S. Grewal et al., 1994; Lewis et al., 2006). There are five types of movement that EPNs can employ when searching for hosts and these are (1) cruising, (2) ambushing, (3) combination of both, (4) nictating and (5) jumping (Bal and Grewal, 2015; Van Zyl and Malan, 2014). Nematodes that are cruisers are highly motile and are effective against stationary or slow-moving insect pests in the soil. Ambushers are generally found on the surface and are less motile than cruisers. They find their host by waiting on the surface for a host to pass by. Nictating occurs where the nematode lifts its body from the surface and displays a waving motion while upright on its tail. Jumping allows for the nematode to adhere to passing host and this is known as external phoresis (Yeates et al., 2004). *Heterorhabditis* are normally seen as cruisers and search for their host deep within the soil. *Steinernema* is generally seen as ambushers and therefore are found either on the surface of the soil or just beneath the surface (Gozel and Gozel, 2016).

1.8. Insect Host Range and Biological Control of Crop Pests

The selection of a suitable EPN species from either genus for pest control is dependent on the susceptibility of the target pest for infection (Kaya et al., 2006; Lacey and Georgis, 2012). The host range of entomopathogenic nematodes has been often described as broad and surveys have shown that EPNs have been able to infect about 200 insect species (Kaya et al., 2006; Van Zyl and Malan, 2014). Laboratory-based host range infectivity studies need to be also verified with field-based trails against target crop pests.

Biological control involves the application of natural enemies to regulate the pest population down to manageable and non-damaging levels in the crop environment (Poinar, 1979). The two approaches to biological control include classical and augmentation control (Van Lenteren, 2012). Classical biological control has been based on the practice of using naturally present enemies or predators to reduce native pests. The environment is inoculated with the natural enemy and allowed to develop population levels which facilitate the control of targeted insect crop pests and this control can be maintained for a long period of time (Van Driesche et al., 2010). Augmentative biological control has been based on the repeated large release of natural enemies that have been mass-produced (Van Lenteren, 2012). This creates a situation where the number of natural enemies is significantly larger than that of the pest. The continual inundation of the natural enemy ensures that there is an immediate reduction in the pest population as well as a natural growth of the natural enemy in the environment through the generations. The criteria for the selection of potential biocontrol agents include the ability to successfully locate and identify prey populations; more rapid generation time compared to the prey; persistence in the event of low pest numbers and being highly specific towards the target pest (Bale et al., 2007).

A stable increase in the market value of biocontrol agents indicates a steady move away from classical forms of pest management to more green and effective methods of pest control. The convention on biological diversity dictates that countries have control over their genetic resources and arrangements, agreements governing the access to these resources, and the sharing of benefits that may come from their implementation between involved parties (Cock et al., 2010). Therefore, the identification and use of biological controls for pests in South Africa require in-depth research into various potential agents. Another contributing factor to the growth of the biological control market depends on the improvement of formulations.

Improved formulations will provide more effective biocontrol agents, more convenient application methods, and management practices to customers.

South Africa is one of the largest importers of pesticides in Southern Africa (Quinn et al., 2011). The legal use of certain pesticides in South Africa requires constant monitoring in terms of the use and the effect it has on the surrounding environment and the end consumer. There is a constant need for improving and updating policies and legislature on the application of synthetic or non-natural chemical insecticides for crop protection. In addition, education and training of farmers, farm labourers, and agricultural extension officers on the risks and the appropriate practices regarding insecticide use. Environmental impact monitoring, hazard, and risk assessment should be implemented by government agencies so that a reliable regulatory environment is maintained with regard to the use of pesticides in agro-ecosystems. The harmful effects of pesticides in the environment are biomagnification within the food chain and bioconcentration in predator species. Biomagnification of pesticides is the rise in the concentration of the pesticides and pesticidal residues within the food web where the pesticide is found to accumulate in the higher trophic levels (usually in predators) than what was initially released into the environment (Borga K., 2013). Bioconcentration of pesticides is the increased accumulation of the chemical within the tissues of the pest or insect predator than what has been found in a pesticide-free environment (Carvalho, 2017). A serious concern is the overexposure of pesticides to farmworkers as they are in contact with the pesticides from packaging, storage, and application (Damalas and Koutroubas, 2016). The harmful effects that pesticides have on humans include increased risk regarding the development of cancers, developmental defects of young children, infertility, and injury to cardiovascular tissues (Nicolopoulou-Stamati et al., 2016). The negative environmental effects are poor water quality and a decrease in the biodiversity of ecosystems. The use of pesticides has been known to cause harm to non-target organisms and also leads to the development of pesticide resistance in the target pests (Özkara et al., 2016). Different kinds of pesticides, for example, atrazine, terbuthylazine, simazine, have been detected when runoff water was monitored during the growing seasons (Du Preez et al., 2005). The non-specificity of most pesticides leads to a decline in non-target populations (Gill and Garg, 2014).

Therefore, there is a need for a change from a single-minded approach of pest control to integrated pest management. This would require the increased use of natural enemies of pests such as the use of entomopathogenic microorganisms such as fungi, bacteria, and nematodes

(Mazid et al., 2011). The objective of integrated pest management has been the utilisation of several pest control measures in a manner that is both economic and ecologically friendly (Ehler, 2006).

1.9. Temperature

The stress factors that greatly influence the persistence of EPNs in the soil environment are temperature and desiccation. Temperature plays an important role in the survival, infectivity, and persistence of nematodes in the soil (Sharmila et al., 2018). When exposed to the extreme temperatures, nematodes undergo desiccation which leads to shorter generation times, decreased infectivity, and lower virulence (Griffin, 1993). Establishing the ideal temperatures for the application of EPNs as biocontrol agents in agriculture is also dependent on finding the right strain which has the appropriate physiological adaptations for survival and infectivity in the selected or specific agro-ecological environments. In general, soil temperatures between 25°C and 28°C are ideal for applying all entomopathogenic nematode species (Grewal et al., 1994). Temperatures higher than 30°C lead to a decrease in the efficacy of nematode species and soil temperatures less than 10°C immobilize EPNs on the soil surface. It is well known that the surface temperature of exposed soils can become exceeding high. The prolonged exposure to UV light is detrimental to EPN persistence in the soil.

1.10. EPN Desiccation Tolerance and Anhydrobiosis

Survival within the fluctuating soil environment necessarily entails the evolution of adaptations to avoid or tolerate or resist drought stress. Nematodes, rotifers, and tardigrades as representatives of the soil micro-fauna are exposed to repetitive cycles of drought and soil desiccation. To survive soil desiccation nematodes, rotifers, and tardigrades have independently evolved desiccation tolerance capacities or the capacity to enter into a state of anhydrobiosis (Tyson et al., 2012). Soil micro-fauna given its minute size and corresponding high surface area to volume ratios has little capacity to avoid water loss as the soil environment undergoes dehydration, especially during the winter in summer rainfall regions. Nematodes including EPNs have little ability to intrinsically control the loss of water and therefore require a moist environment with high relative humidity that will slow down water loss so that they can complete their life cycles (O'leary et al., 2001). With regard to the IJs of EPNs dispersal and host finding within the soil environment requires a critical percentage of soil moisture content, sufficient anyway for there to be thin films of water adhering to the surface of soil

particles and high relative humidity within the interstitial soil spaces. The presence of sheaths on IJs helps IJs to survive desiccation. When the soil environment becomes increasingly dehydrated due to moisture evaporation, the sheath dries first and becomes a barrier between the IJ and the environment (Perry et al., 2012). The barrier slows down the rate of water loss and the IJ can survive in the environment until the condition changes. The differences in the sheaths between steinernematids and heterorhabditids show their differences in being able to tolerate desiccation. Steinernematids have loose-fitting sheaths and this is easily lost during foraging and heterorhabditids have tight-fitting sheaths. Therefore, heterorhabditids have the ability to survive desiccation better than steinernematids. It has been proposed that the induction of anhydrobiosis or dehydration tolerance in soil micro-fauna, possibly including the 'free-living' IJs of EPNs, is an adaptive response strictly facilitated by the gradual or slow loss of moisture from the soil environment under conditions of high relative humidity (Askary and Ahmad, 2017; Poinar Jr and Grewal, 2012; Tyson et al., 2012; Womersley et al., 1998). The induction process of anhydrobiosis or desiccation tolerance which is strictly dependent on a gradual loss of soil moisture at high relative humidity rather than a rapid rate of soil moisture loss is representative of an adaptive strategy typical of 'slow dehydration strategists' (Womersley et al., 1998).

1.11. Formulation of Entomopathogenic Nematodes

The difficulties experienced with regard to the development of entomopathogenic nematodes as biocontrol agents has been their formulation in media, which would ensure nematode or IJ viability and infectivity for extended periods of time (Askary and Ahmad, 2017; Heriberto et al., 2017; Poinar Jr and Grewal, 2012). Several methods have been used for the formulation of entomopathogenic nematodes (Askary and Ahmad, 2017; Heriberto et al., 2017). Examples of these formulations are the use of inert carriers and physical trapping of IJs within inert media. The advantages of inert carriers are their simplicity and ease of production. The disadvantages of the carrier-based formulations have been their requirement for refrigeration during storage and transport, therefore, making it expensive.

Carriers or media used in EPN IJ formulations include solids, gels, liquids, and even larval cadavers. The various components of an EPN formulation have can have different functions such as absorbents, adsorbents, emulsifiers, surfactants, thickeners, humectants dispersants, antimicrobials UV-ray protectors (Grewal, 2002). Examples of formulation carriers include

polyether-polyurethane sponge-based carriers (Strauch et al., 2000), gels (Bedding et al., 2002; Chen and Glazer, 2005; Hussein and Abdel-Aty, 2012), clays (Strauch et al., 2000), larval cadavers (Shapiro-Ilan et al., 2001) and various kinds of powdered formulations including diatomaceous earth and activated carbon (Grewal, 2002; Heriberto et al., 2017; Kagimu, 2018; Yukawa and Pitt, 1985). Gels comprised of different kinds of water absorbent or hydroscopic materials such as polyacrylamide (Bedding et al., 2000) and calcium alginate (Chen and Glazer, 2005; Hussein and Abdel-Aty, 2012; Vemmer and Patel, 2013) have encapsulated nematodes. Chen and Glazer (2005) encapsulated IJs in calcium alginate after exposing them to an osmotic pre-treatment. Calcium alginate encapsulated IJs of *Steinernema feltiae* had a survival rate of 98.8% survived after 6 months when stored at 23 ℃ and 100% relative humidity. In another case, encapsulation of Heterorhabditis bacteriophora and S. carpocapsae in calcium alginate had survival rates above 50% after 40 days (Hussein and Abdel-Aty 2012). In the case of an IJ formulation on polyether-polyurethane sponge carriers, a dewatered nematode cream containing 1.0 to 1.5 million IJs/g for *H. bacteriophora* and 1.3 million *for H. indica* were mixed with sponge cubes in an IJ to sponge mass ratio of 2:1 w/w (Strauch et al., 2000). The IJ formulate sponge cubes were packaged in 5 L plastic containers. In the case of formulating IJs in clays, a dewatered nematode cream containing 1.0 to 1.5 million IJs/g for *H. bacteriophora* and 1.3 million for *H. indica* were mixed with attapulgite and bentonite clays in IJ to clay mass ratio of 5:6 w/w (Strauch et al., 2000). The formulated IJs were stored at 5 and 25 ℃.

The physical trapping of EPNs within media includes the use of thin sheets of calcium alginate placed over plastic screens to trap EPNs in the alginate between layers of plastic (Grewal, 2002). EPNs are released from the trapping by dissolving the alginate sheets in sodium citrate. Issues that arise from this formulation has been the lengthy time required for preparation and then the removal of the EPNs from the screen and containers. Optimal temperature and moisture conditions are necessary for the continued viability of the nematodes as these facilitate reproduction, foraging, pathogenicity, and longevity in the soil (Askary, 2010). The induction of partial anhydrobiosis before formulation has seen to provide promising results especially in the context of various types of formulation strategies that have been based on media such as anhydrous polyacrylamide gel, powders, granules, and water-dispersible granules (Shapiro-Ilan et al., 2012). A promising development in formulation strategies has been the use of infected insect cadavers (Hiltpold et al., 2012). Anhydrobiotic induction of nematodes can be achieved by the formulation of IJs in gels, powders, and granules. Gel formulations involve

gels where the gelling medium, such as anhydrous polyacrylamide, has been mixed together with the nematodes. The gel allows for the nematodes to remain in an environment that has a water activity of about 0,995 (Grewal, 2002). Formulation with the use of powders requires that the nematodes to be mixed as a paste with powders, where the powders may be a moist or dry state (Maru et al., 2013). The powders strip the nematode of their surface moisture and facilitate their entry into a partially anhydrobiotic state. The granular formulation of nematodes requires them to be encapsulated by a layer of granules (Grewal, 1998). These granules may consist of media such as silica, clays, cellulose, lignin, and starches.

It is thought that EPNs can undergo partial anhydrobiosis when the environment becomes unfavourable, for example, extreme temperature, reduced moisture levels, and lack of hosts (Tyson et al., 2012; Campos–Herrera et al., 2012). The amount of energy consumed by the nematodes is reduced when in a state of anhydrobiosis or partial anhydrobiosis. It has been proposed that EPNs are only able to achieve partial anhydrobiosis because they still require a small amount of water to remain viable and are therefore considered as being drought tolerant rather being true anhydrobiotes (Heriberto et al., 2017). Changes in the environment, particularly the loss of water, cause nematodes to undergo physical and physiological. The physical changes include the clumping and coiling of the nematodes (Perry et al., 2012). Clumping and coiling reduce the amount of surface area exposed to the environment and thereby reduces water loss. The physiological changes in response to dehydration stress which reduce metabolic processes also conserve lipid reserves. Metabolic responses to dehydration stress which result in an increase in the production of trehalose also favour survival under conditions of dehydration stress. These responses can be included in the suite of drought stress adaptations which result in dehydration tolerance in EPNs (Tyson et al., 2012).

To sum up, the overriding objective of EPN formulation is to ensure or guarantee the long term preservation of IJ viability and infectivity. The capacity to induce IJ anhydrobiosis or dehydration tolerance in the actual formulation process will go a long way in achieving this commercially important goal. Moreover, formulated EPN biocontrol products should be user-friendly for the end-user, have a long term shelf life, simple preparation, and handling requirements. The process of formulation requires a detailed understanding of several factors: drought or dehydration stress responses, the biology of the biocontrol, the pathogen and host relationship, the effect of environmental factors such UV radiation and temperature, and the interactions with other soil organisms. According to Heriberto et al., (2017) the following

media for EPN formulations have been used by existing EPN biocontrol business enterprises can be summarized as follows: polymer and clay (BASF, Germany); polymer and clay (E-NEMA GmbH, Germany); clay (Andermatt Biocontrol, Switzerand); polymer and clay (Koppert, Netherlands); clay (Asa Jung Laboratory, USA); dispersible granules, sponge (BioLogic, USA); sponge (Hydro-Gardens, USA); sponge (M & R Durango, USA) (Heriberto et al., 2017).

1.12. Mass Production of EPNs

Mass production of EPNs as biocontrol agents of insect crop has expanded and achieved economies of scale over the past few decades (Askary and Ahmad, 2017; Heriberto et al., 2017). The development of suitable large scale bioreactor technologies for mass production remains one of the basic conditions for the successful commercialization of EPNs as biocontrol agents. Solid (Ramakuwela et al., 2016) and liquid phase bioprocess technologies (Shapiro-Ilan et al., 2012) have been used for the mass production of EPN IJs. Most recent reports indicate that the airlift bioreactors are preferred IJ mass production bioprocess technologies (Shapiro-Ilan et al., 2012). In these bioreactor systems, the IJ yields were influenced by the initial IJ inoculum density, initial bacterial densities, medium composition (carbohydrates, oils, and proteins) and aeration rates (Strauch and Ehlers, 2000). Scale ups for the mass production of *Heterorhabditis* species in 20 and 500 L bubble column bioreactors have been achieved by Surrey and Davies (1996). Their medium consisted of 1.25% (w/v) spray-dried egg yolk (SDEY), 2.30% (w/v) yeast extract, 0.23% (w/v) sodium chloride and 4.0% (v/v) corn oil (medium 1) or 1.25% (w/v) SDEY, 3.55% (w/v) whole milk powder, 0.50% (w/v) yeast extract, 0.23% (w/v) sodium chloride, 4.0% (v/v) corn oil (medium 2) (Surrey and Davies, 1996). Yields of 105 000 IJs /ml where achieved with medium 1 within between 15 to 20 days. Almost all bioreactor nutrient media that have been developed for EPN production are variations or modifications of the above media. The specific volumetric production capacity of the specific bioreactor system is the most fundamental factor with regard to assessing the economic viability of the technology for EPN production. For example, the minimum economically acceptable bioreactor volumetric production capacity measured in terms of IJs/ ml or IJs ml for the mass production of EPNs will be dependent on the minimum IJ application dose to field crops necessary for effective crop pest control. The minimum effective EPN IJs application dose is 2.5 x 109 IJs / ha (Shapiro-Ilan and Gaugler, 2002). Some of the bioreactor specific volumetric production capacities for different EPN species that have achieved include the

following results (Shapiro-Ilan and Gaugler, 2002): *H. indica* 450 000 IJs/ml; H bacteriophora 300 000 IJs/ml; *S carpocapsae* 320 000 IJs/ml; *H. megidis* 138 000 IJs/ml; *S. feltiae* 71 400 IJs/ml. Medium supplement with glucose and canola gave yields for *H bacteriophora* ranging from 360 00 to 425 000 IJs/ml. Most of the high bioreactor specific volumetric production capacities have been achieved for small laboratory airlift bioreactors.

1.13. Research Motivation

The isolation and identification of native entomopathogenic nematodes from South African soils is an essential step in establishing species distribution and prevalence in different regional environments and biomes such as grassland versus bushveld. It is also an essential step in the development of EPN based insect pest biocontrol agents. Surveys and sampling of various biomes will assist in the determination of the EPN species diversity, biogeographical distribution of EPN species, and also the potential insect host range of native EPN species in South Africa. The use of local EPNs could potentially provide EPNs better adapted to South African climate and local insect pests as opposed to foreign entomopathogenic nematodes. The formulation of EPNs involves knowledge of the biology of local EPNs in terms of their ability to enter a state of anhydrobiosis or dehydration tolerance when formulated. This in turn represents a fundamental requirement for improving the long term longevity of EPNs in the formulated state.

1.14. Aims and Objectives

1.14.1. Aims

The underlying aim of the study includes a) Isolation and identification of native South African EPN species and their bacterial symbionts from stored soil samples that have been in a state of dehydration for several years. b) Identification and description of any new species of EPNs recovered from the stored soil samples. c) Investigating the impact that the rate of dehydration has on the viability and infectivity of IJ formulated in hydroscopic or water absorbing powdered materials. The aim in the formulation studies focusing on how moisture status in the formulation media constrains or promotes long term storage of EPNs in the formulated state.

1.14.2. Objectives and Scope:

A statement of objectives and the scope of the study can be summarized as follows: a) Isolation of native EPNs from stored soil samples which have had previously been collected from grasslands in Gauteng and bushveld in North West Provinces using soil-larval baiting and

White trap techniques. b) Identification and phylogenetic analysis of native South African EPNs recovered from soil samples based on the small subunit ribosomal DNA (SSU rDNA or 18S DNA) sequence data. c) Isolation, culturing, identification, and phylogenetic analysis of the entomopathogenic symbiotic bacteria associated with the EPNs recovered from the soil samples. d) Morphometric description and phylogenetic analysis of any new EPN species recovered from the soil samples. e) Testing of the hypothesis that the rate of formulation dehydration effects the long-term viability of IJs in the formulation medium and by implication the rate of hydration influences the induction of anhydrobiosis or dehydration tolerance.

1.14.3. Summary of Experimental Approach

Figure 1.14.1 summarizes in outline the experimental design underlying this project. The activities undertaken in this project have summarized as follows: Soil samples were originally collected from Walkerville, Johannesburg, and Brits, North West province, South Africa, and subsequently stored after being for more than two years in a dehydrated state. Soil stored in plastic tubs were randomly selected and subjected to rehydration and larval baiting. It should be noted that these soils were previously used for the isolation of nematodes. The fact that EPNs could be recovered from these soils can be treated as a proof of concept that IJs can indeed survive for extended periods in dehydrated soils. Isolation of EPNs from stored soil samples with the use of *Galleria mellonella* insect baiting technique and recovery of IJs from larval cadaver using the White trap technique. Confirmation of Koch's postulates. Maintenance of pure culture of isolated EPNs. Molecular identification of isolated EPNs with the use of PCR amplification and sequencing of the 18S rDNA region. Isolation of bacterial symbionts from the haemolymph of cadavers and homogenate of IJs using NBTA plates. Molecular identification of isolated bacterial symbionts with the use of PCR amplification and sequencing of the 16S rDNA region. Morphological identification of possible new EPN species that had been isolated. Application of different powder-based formulations for the storage of EPNs and test viability and pathogenicity of IJs following storage in the powder-based formulations.

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2. Chapter Two: Isolation and Identification of Native Entomopathogenic Nematodes

2.1. Introduction

Nematodes represent a species-rich phylum, second only to the arthropods and molluscs. More than 25 000 identified species have been described (Kiontke and Fitch, 2013; Zhang, 2013). It is currently thought that the phylum may contain more than a million species that have yet to be described (Kiontke and Fitch, 2013; Lambshead and Boucher, 2003). Nematodes are ubiquitous, occupying a wide diversity of ecological niches, and also playing multiple roles within a broad range of different ecosystems (Kiontke and Finch, 2013; Bulgheresi, 2016). They also constitute a trophically diverse group, playing a significant role at different trophic levels within the soil web as omnivores, bacterivores, fungivores, plant parasites, vertebrate parasites, invertebrate parasites and also predators of other nematodes. They can be either be beneficial or detrimental to humans, animals, and agricultural crops depending on the species, especially species belonging to a parasitic genus. Previously, the phylum was thought to be composed of two classes, initially named Phasmidia and Aphasmidia and later renamed as Secernentea and Adenophorea, respectively (Chitwood, 1958, 1937; Holterman et al., 2006). However, molecular phylogenetics based on the conserved and hypervariable nucleotide sequences within the highly repetitive copies of 18S rDNA or SSU rDNA has resulted in a major revision of the phylogenetic relationship of classes, orders, families and species within the phylum (Blaxter and Koutsovoulos, 2015; De Ley and Blaxter, 2004; Holterman et al., 2006; Kiontke and Fitch, 2013). The phylum Nematoda is now comprised of three large monophyletic clades namely, Chromadoria, Enoplia, and Dorylaimia (De Ley, 2006; Kiontke and Fitch, 2013). In a similar fashion, the sequences of the SSU rDNA has been used as an unbiased molecular tool or ‘measurement’ or metric to identify nematodes and to establish the phylogenetic relationship between nematode species (Holterman et al., 2006). The 18S rDNA sequence has also been successfully applied as an unbiased and objective metric in the reporting and phylogenetic analysis of new species of EPNs. As stated previously, EPNs are obligate pathogens of insects in nature and have been used as biological control agents for the control of insect crop pests. Both steinernematids and heterorhabditids share a similar and relatively simple rhabditid-like body plan and they are also colourless or transparent, unsegmented, and lacking in appendages (Sharma et al., 2011). Thus their gross anatomy is

relatively easy to characterize morphometrically under the light microscope (Harry K. Kaya and Stock, 1997).

Steinernema and *Heterorhabditis* are found to be mutually associated with the insect parasitic bacteria belonging to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Gozel and Gozel, 2016). The entomopathogenic bacteria are Gram-negative bacteria belonging to the family Enterobacteriaceae. The life cycle of EPNs consists of an egg stage, three juvenile stages, and an adult stage (Harry K. Kaya and Stock, 1997). The third stage infective juvenile (IJs) is the only free-living (external to host), non-feeding and developmentally-arrested stage that is able to survive in the soil environment, even under desiccating conditions and is the life cycle stage in which insect host location and infection take place (Ciche et al., 2006). The mutualistic insect pathogenic endosymbiotic bacteria are carried in the intestinal region of the IJs and are released or escape from the IJs once the IJs have entered the haemocoel of the host (Koppenhöfer, 2010). On release, the bacteria reproduce rapidly within the nutrient rich haemocoel and they cause the death of the host by septicaemia, typically within 24–48 hours. Once the host has been infected by the IJs, the nematodes transition from infective juveniles to bacteria-feeding third-stage juveniles and develop subsequently into fourth stage juveniles that mature into the reproductive adults. The fourth-stage juveniles develop into adult females and males of the second generation. This life cycle continues for two to three generations until the nutrient resources in the cadaver are depleted. Once the nutrient resources derived from the bacterial digestion of the host tissues have been depleted, the nematodes cease feeding and within the gravid female following endotokia matricida bacteria within the body of female are ingested bacteria by IJs an become integrated into pelleted colony or population comprised of 50 or more individual bacteria cells which are housed within a specialized vesicle within the nematode or within a region in the nematode's intestine (Baliadi et al., 2016). Thereafter, the juveniles transform from pre-infective to infective stage juveniles which escape from the ruptured body of the adult reproductive female (Baliadi et al., 2016). The infective juveniles emerging from the host cadaver migrate into the surrounding soil environment where they search or forage for new insect hosts and this takes place after 6 – 11 days for steinernematids and 12 – 14 days for heterorhabditids (Chitra et al., 2017).

As already mentioned, the identification of EPNs can be done with the use of morphological and molecular techniques. The use of morphological characteristics makes identification between different isolates extremely difficult because of the close anatomical similarities which may have arisen as a direct consequence of convergent evolution resulting in the development

of similar anatomical features and traits for nematodes which have evolved (Blaxter et al., 1998). Phenotypic plasticity could result in the same species developing morphological variations in response to environmental gradients. Molecular identification of EPNs makes use of molecular techniques that involve total genomic DNA extraction, PCR amplification of conserved, and hypervariable genomic regions such as the highly repetitive ribosomal RNA or SSU rDNA nucleotide sequences. Identification of existing species or new species can be preliminarily and also fairly rapidly established through 18S rDNA nucleotide sequence BLAST alignment against the GenBank 18S rDNA sequence data base. As already discussed, the 18S rDNA with it conserved and variable regions makes it a powerful and reliable metric or even an unbiased OTU for placing the unknown nematode isolate in its genera or family and also making it possible to establish its phylogenetic affinities with other species of EPNs which have already been described (Půža et al., 2015). The variable internal transcribed spacer region (ITS) of EPNs is flanked by the repeating copies of conserved nuclear 18S and 28S ribosomal DNA genes. The rDNA genes occur in high copy numbers, therefore, making this a suitable genetic marker for molecular-based identification purposes (Blaxter et al., 1998; Dorris et al., 1999; Stock, 2002).

EPNs are naturally found in a range of soil environments and are well suited as biocontrol agents of insect pests, especially for those that have their larval stage in the soil (Van Zyl and Malan, 2014). They have been observed to infect a variety of species of insect crop pests, which fall within the lepidopteran, coleopteran, and dipteran orders, under laboratory and field conditions (McMullen II and Stock, 2014). Currently, most of the isolated and identified entomopathogenic nematodes have been found to fall mainly into the following two families, the Steinernematidae and Heterorhabditidae. The EPN genera which fall under Steinernematidae are *Steinernema* and *Neosteinernema*. In one report the genus *Steinernema* was observed to be comprised of over fifty species, and the genus *Neosteinernema* containing only one species, *N. longicurvicauda* (Gozel and Gozel, 2016). However, as already mentioned, the EPN genus *Steinernema* remains the most speciose of the two EPN genera, possibly containing a hundred species (Bhat et al., 2020). *Heterorhabditis* is the only genus found in the Heterorhabditidae. So far the genus *Heterorhabditis* has been stated to be comprised of 17 known species (Bhat et al., 2020).

In South Africa, several EPN surveys have been conducted (Abate et al., 2018; Malan and Ferreira, 2017). So far, in South Africa, a total of 17 *Steinernema* and 7 *Heterorhabditis* species have been isolated from soil surveys conducted throughout the country (Malan and Ferreira,

2017; Malan and Hatting, 2015). The two ubiquitous heterorhabditid species, *H. bacteriophora* and *H. zealandica* have also been found to be prevalent in South Africa (Addison et al., 2006). With regard to the isolation of new EPN species, the following steinernematid species have been recovered from South African soil samples: *S. khoisanae* (Malan et al., 2006), *S. citrae* (Malan et al., 2011), *S. sacchari* (Nthenga et al., 2014), *S. tophus* (Cimen et al., 2014), *S. innovationi* (Çimen et al., 2015), *S. jeffreyense* (A. P. Malan et al., 2016), *Steinernema nguyeni* (Malan et al., 2016), *S. beitlechemi* (Cimen et al., 2016), *S. fabii* (Abate et al., 2016) and *S. biddulphi* (Cimen et al., 2016). With regard to the isolation of new species of heterorhabditids, the following species have been isolated and described: *H. safricana* (Tiedt et al., 2008), *Heterorhabditis noenieputensis* (Malan et al., 2014).

With regard to the prevalence, distribution and diversity of EPNs collected in soil samples across the four provinces in South Africa, in one survey a total of 1506 samples were collected, 79 (5.2%) were EPN-positive, 44 of samples (55.7%) contained steinernematids and 35 of samples (44.3%) contained heterorhabditids (Hatting et al., 2009). In this survey, fewer EPNs were recovered from semiarid zones (13%) compared to the relatively higher numbers recovered in the subtropical regions (87%). In addition, three new species of *Steinernema* were recorded in the above survey. The cosmopolitan *Heterorhabditis bacteriophora* was found to be ubiquitous in the following provinces: Western Cape, Orange Free State, KwaZulu-Natal, and Mpumalanga.

2.2. Aims and Objectives

2.2.1. Aim

To isolate and identify native South African EPNs species from previously collected soil samples that have been in storage in a dehydrated state for prolonged periods of time (> 3 years).

2.2.2. Objectives

This study focused on the following objectives: a) Isolation of EPNs from soil samples previously collected from Brits and Walkerville using the *Galleria mellonella* larval soil baiting and White trap methods. The samples were collected from these areas as they were surrounded by active agricultural farming. b) Application of Koch's Postulates to confirm whether or not the nematodes recovered from the White traps were indeed entomopathogenic

nematodes. c) Extraction of genomic DNA from the successfully isolated entomopathogenic nematodes. d) Generation of suitable 18S rDNA based OTU nucleotide sequences for the molecular identification and phylogenetic characterization of EPNs recovered following the rehydration and *G. mellonella* larval baiting of soil samples which had previously been subjected to prolonged desiccation. For this objective PCR amplification of the hypervariable and hypovariable regions within the flanking boundaries of 18S and 28S rDNA using selected universal PCR primers was undertaken. e) Sanger sequencing of the 18S and 28S rDNA PCR generated amplicons. D) MEGA7 software-based molecular phylogenetic analysis of the nematode isolates based on their SSU RNA sequences.

2.3. Methods and Materials.

2.3.1. *In vivo* Rearing of *Galleria mellonella* Larvae

Galleria mellonella, the greater wax moth, was bred and maintained for the production of larvae for the isolation of nematodes from the soil samples and also for the *in vivo* culturing of the isolated EPNS. *G. mellonella* has always been eminently suitable as a model insect host for EPNs research because they are extremely vulnerable to infection by most EPNs, and are easy to culture and maintain in the laboratory, and also because they have rapid reproduction rates (Xuejuan and Hominick, 1991). The female and male moths were placed in 3-litre CONSOL bottles to allow for the mating process to occur. Crumpled wax paper strips were placed in the jar as the substrate for the moths to lay their eggs on. The eggs adhere to the wax paper making it easy for their collection. *Galleria* medium was continuously supplied to maintain the growth and development of the larvae. The medium consisted of Pronutro, honey, and glycerol (refer to Appendix for the recipe). The metal screw lids of the glass jars were modified by incorporating disks of stainless-steel mesh which were fitted into the inside of the lids so as to facilitate efficient air exchange and to ensure that the larvae or moths did not escape from the jars. The jars were incubated in the dark at 25 ^{o}C.

2.3.2. Isolation of Entomopathogenic Nematodes

2.3.2.1. Collection of Soil Samples

The soil samples had been originally collected from Brits (25.6100° S, 27.7960° E) and Walkerville (26° 25' 0" South, 27° 58' 0" E), areas to the north and the south of Johannesburg, respectively. The soil samples were collected in 1-litre plastic ice cream containers. Once in the laboratory, the soil samples which been previously in storage for a number of years in a

dehydrated state were rehydrated by the addition of 200ml of tap water to the containers. Thereafter the soil samples were kept hydrated for the facilitation of nematode resuscitation, survival, and mobility with regard to larval host location and infection.

2.3.2.2. Insect Baiting Technique

The technique for soil larvae baiting for the isolation of EPNs from soil samples was adapted from methods described by Kaya and Stock (1997). The EPNs were isolated from the collection of soil samples by baiting the soil in the containers with *G. mellonella* larvae. The plastic tubs of soil containing larvae were incubated at 25 ^{o}C to allow for larval infection by the infective juveniles which had managed to survive desiccation in the soil samples after long term storage. The plastic tubs were observed daily after baiting for the presence of dead larvae. Initial confirmation of infection by EPNs was determined by the change in colour of the infected or dead larvae. Larvae infected by heterorhabditids were expected to turn red or purple in colour and those infected by steinernematids were expected to turn brownish or blackish in colour (Shapiro-Ilan et al., 2002).

2.3.2.3. Isolation of EPNs from Infected Larvae by the White Trap Method

The IJs were isolated from infected larvae using a modified White trap method (White, 1927). The infected larvae or larval cadavers were briefly surface sterilised with 70% v/v ethanol to prevent the contamination of the larval cadavers and the infective juveniles trapped in the surrounding moat by other microorganisms. The lid of a smaller Petri dish (50 mm) was positioned in the centre of the larger Petri dish plate (90 mm). The smaller lid was covered with a 54 mm Whatman Number 1 filter paper disc and the infected cadaver was placed on it (one cadaver per disc). Distilled water was added into the larger Petri dish plate until the level of the moat had reached the edges of the filter paper so that the paper was kept constantly hydrated. The White traps were incubated at 25°C. The IJs emerged from the cadaver when the cadavers had collapsed.

2.3.2.4. Confirmation of the Infectivity and Virulence of IJs

The IJs were collected from the White traps into 50 ml sterile Falcon tubes and surface sterilized with 0.03% v/v sodium hypochlorite to prevent the contamination of IJ with other microorganisms. Sterile moist coarse river sand (40 g) was placed into 90 mm Petri dishes as the substrate to facilitate the infection of larvae which were placed on top of the sand. The moist sand also facilitated the movement of IJs. Before using the sand was first autoclaved to

ensure that no other microbial contaminants were present in the sand. The river sand was maintained at 8% moisture w/v. The autoclaved river sand was inoculated with the surface sterilised IJs. Ten *G. mellonella* larvae were placed on top of the sand and larval dissection was carried out to confirm the presence of infective juveniles within the haemocoel.

2.3.2.5. *In vivo* Maintenance of EPNs

In vivo culturing and maintenance of EPNs was carried out by White trap recovery of IJs from larval cadavers and re-infection of fresh larvae as described in section 2.3.2.3. (White, 1927; Xuejuan and Hominick, 1991).

2.3.3. Molecular Characterisation of EPNs

2.3.3.1. Genomic DNA Extraction

Total genomic DNA was extracted using the Puregene® DNA Purification Kit, following the instructions as per the Kit. For the kit protocol instructions refer to the Appendix.

2.3.3.2. PCR Amplification of 18S rDNA

The 18S rDNA nucleotides sequences in the extracted genomic DNA were amplified by means of the polymerase chain reaction. The PCR amplicon was sequenced in order to identify or establish the phylogenetic affinities of the nematode species via NCBI BLAST alignment. The Sanger sequenced 18S RNA nucleotide sequences uploaded in the form of FASTA files were subjected to a GenBank National Centre for Biotechnology Information (NCBI) BLAST search based on the application of the BLAST search algorithm parameters. The genomic DNA was submitted to Inqaba Biotechnological Industries Pty (Ltd) for PCR amplification and Sanger sequencing of the 18S rDNA PCR generated amplicon. For the PCR reagent mixture has been provided in Table 2.3.1. The oligonucleotides selected were ITS amplification specific universal forward primers (TW81) and reverse primers (AB28) as described by Joyce et al (1994) (Joyce et al., 1994). The concentration of the forward and reverse primers was 10 µM and the PCR reagents used have been given in Table 2.3.1. The amplification consisted of 35 thermo-cycles. The complete PCR amplification protocol has been described in Table 2.3.2. Information regarding the nucleotide sequences for the TW81 (forward primer) and AB28 (reverse primer) have been presented in Table 2.3.3.

Table 2.3.1. The reaction volumes and reagents of PCR.

Reagent	Volume (25µl)
Econo®TaqMM	12.5
TW81	1
AB28	1
Template DNA	1
Nuclease-free water	9.5

Table 2.3.2. The amplification procedure used for the amplification of the 18S rDNA sequence in the nematode genomic DNA.

Amplification steps	Temperature (℃)	Duration
Denaturation	95 ℃	60 seconds
Annealing	64 ℃	60 seconds
Extension	72 ℃	120 seconds
Final extension	72 ℃	10 minutes

Table 2.4.3. The nucleotide sequences of the universal primers used for the amplification of the ITS regions between the repetitive 18S rDNA and 28S rDNA region within the nematode genomic DNA.

Primer	Primer sequence	Tm (℃)	Ta (℃)
Forward primer (TW81)	5'-GCGGATCCGTTTCCGTAGGTGAACCTGC -3'	71.94	66.4
Reverse primer (AB28)	5'-GCGGATCCATATGCTTAAGTTCAGCGGGT -3'	68.87	63.87

2.3.3.3. Sequencing of the 18S rDNA Amplicon

The tandem repetitive rDNA which is comprised of contiguous nucleotide sequences arranged in the following sequential order: 18S, ITS1, 5.8S, ITS2, 28S, was PCR amplified using the rDNA universal primers TW81 (forward primer) and AB28 (reverse primer), which bind to sites within the 18S and 28S, respectively, which in turn flank the two ITS regions. The PCR generated rDNA amplicon underwent Sanger sequencing at Inqaba Biotechnical Industries. The Sanger DNA sequence chromatograms that were generated for the different EPN isolates were edited with the aid of the FinchTV 1.4.0 chromatogram viewer software (http://www.geospiza.com/Products/finchtv.shtml) which provides editing tools for generating consensus sequences. The edited 18S rDNA FASTA sequence files were uploaded onto the National Center for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLASTn) via the FinchTV Edit menu which provides a BLAST sequence option, following the selection of Nucleotide and BLASTn menu options. This FinchTV application makes it possible to communicate online with NCBI's BLASTn computational tool. FinchTV loads FASTA format versions of the sequences into the BLAST query box, following which the BLAST application searched and aligned the submitted query sequences against the sequences in the GenBank sequence database. The BLAST alignment algorithmic tool generates data on the statistical significance of the query sequence matches with the sequences in the GenBank database. Levels of statistical similarity vary from extremely high to extremely low matches. From this data provisional impressions regarding phylogenetic affinities or degree of species relatedness can be approximately inferred with regard to the query sequence as a prelude to phylogenetic trees building based on rigorous statistical inferences supported by neighbour joining, maximum likelihood or maximum parsimony criteria and analysis (Dorris et al., 1999).

2.3.3.4. Phylogenetic Analysis using MEGA7

Phylogenetic analysis was conducted using the PCR generated ITS containing 18S rDNA sequences of the unknown isolated species as the operational taxonomic units (OTUs) which were compared through sequence alignment with the OTU sequences of similar or related species. For the construction of phylogenetic trees known or identified EPN 18S rDNA sequences were downloaded as FASTA files from the nucleotide sequence GenBank database provided by the National Center for Biotechnological Information (NCBI). The bioinformatics software tool used for the alignment and phylogenetic analysis of the known and unknown

sequences was Molecular Evolutionary Genetics Analysis 7 (MEGA7) (Kumar et al., 2016). The FASTA files of all the selected EPN 18S rDNA sequences were loaded onto MEGA 7 and aligned amongst each other with *Caenorhabditis elegans* included as an outgroup. The Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool was used to align the sequences. The aligned sequences were used to construct phylogenetic trees and determine evolutionary divergence between the different EPN 18S rDNA aligned sequences. The phylogenetic relationships between the aligned sequences were determined using the maximum likelihood method (Nei et al., 1983). The construction of the trees was based on the clustering of taxa based on 1000 replicates in the bootstrap statistical test. The evolutionary distances of the aligned sequences were determined using the Tajima-Kei model (Kumar et al., 2016).

The following selected taxa were used for the construction of the heterorhabditid phylogenetic tree: *Heterorhabditis bacteriophora isolate 56-C* (FJ217351.1), *H. argentinensis* (AF029706.1), *H. indicus* (AF029710.1), *H. georgiana* (EU099032.1), *H. indica* (AY321483.1), *H. zealandica* (AY321481.1), *H. downesi* (AY321482.1), *H. megidis* (AY321480.1), *H. brevicaudis strain TG01* (DQ020278.2), *H. hawaiiensis* (AF029707.1*), H. pakistanense strain NBAIIH05* (KX954218.1*), H. baujardi* (AF548768.1), *H. taysearae* (EF043443.1), *H. amazonensis* (DQ665222.1), *H. safricana* (EF488006.1), *H. marelatus* (AY321479.1), *H. mexicana* (AY321478.1), *Caenorhabditis elegans* (NR 000054.1)

The following selected taxa were used for the construction of the steinernematid phylogenetic tree*: Steinernema khoisanae isolate BMMCB* (KT027382.1), *S. khoisanae* (KM275351.1), *S. khoisanae strain 106-C* (EU683802.1), *S. innovationi isolate SGI-60* (KJ578793.1), *S. arenarium* (DQ314288.1), *S. jeffreyense strain J194* (KC897093.1) *S. karii* (AY230173.1), *S. brazilense* (FJ410325.1), *S. akhursti* (DQ375757.2), *S. kraussei* (AY230174.1), *S. monticolum* (AF331914.1), *S. poinari strain 1160* (KF241752.1), *S. beddingi* (AY603397.1), *S. affine* (AY230159.1), *Caenorhabditis elegans* (NR_000054.1)

2.4. Results

2.4.1. Isolation of Entomopathogenic nematodes

The larvae infected by the entomopathogenic nematodes, which had been isolated from the stored soil samples, displayed the typical symptoms induced by heterorhabditid and steinernematid infections. The larval symptoms that were taken as indicative of steinernematid infection were the brown-black colour change and the softening of the cadaver (Figure 2.4.1.,

A.). The larval symptoms that were indicative of heterorhabditid infection had purple colour changes and the cadaver had a gummy texture (Figure 2.4.1., B). The White trap method facilitated the trapping and isolation of the emerging infective juveniles (Figure 2.4.2). The testing of Koch's Postulates confirmed that the death of cadaver was due to the isolated EPNs. It took four to five days for steinernematids to emerge from the cadaver and seven to 14 days for heterorhabditids.

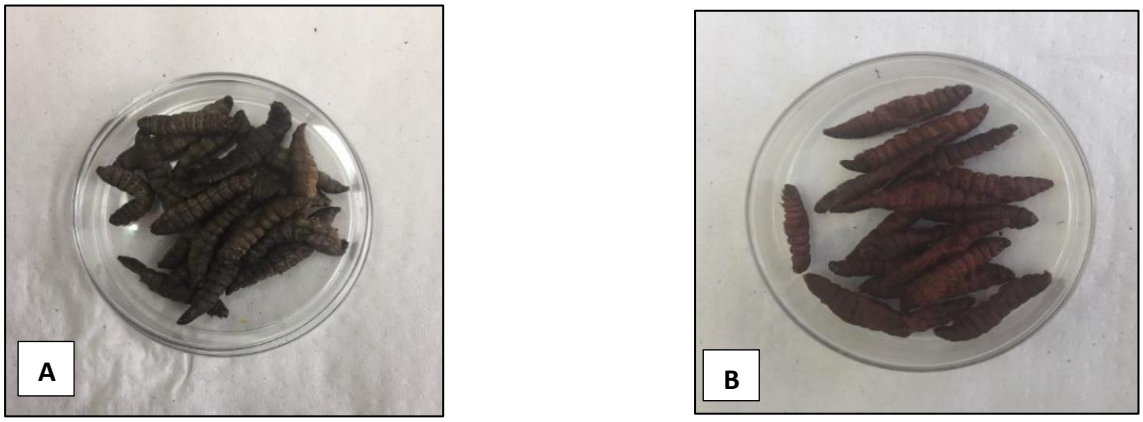

Figure 2.4.1. Infected Galleria mellonella. The larvae that indicated initial entomopathogenic nematode infection due to colour change. A) Larvae are dark brown to black colour which is indicative of infection by steinernematids. B) Larvae are maroon to dark red colour which is indicative of infection by heterorhabditids.

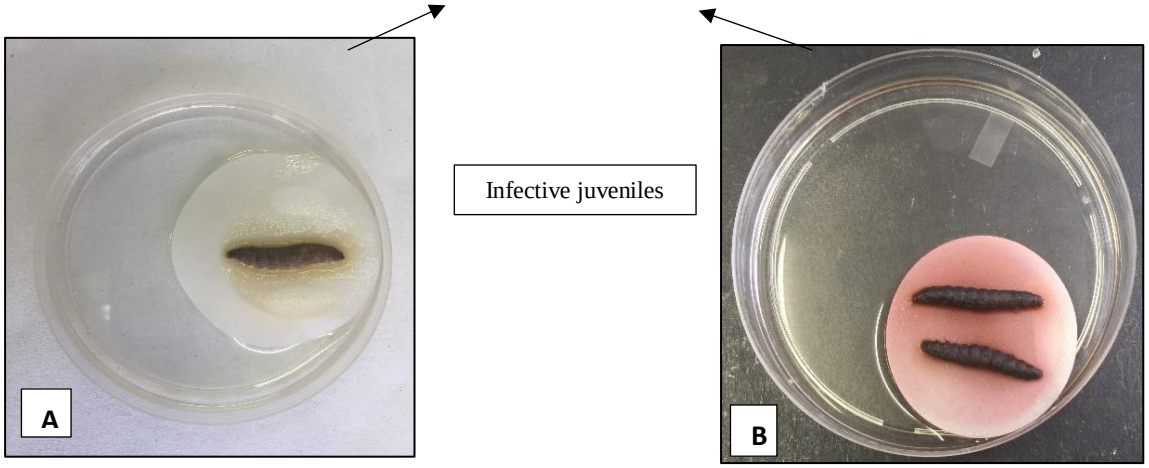

Figure 2.4.2. The White trap method used to isolate EPNs from a larval cadaver. A) Infective juvenile emergence is seen on the Whatman No 1 filter paper. B) Infective juvenile emergence is seen in the surrounding water moat.

2.4.2. Molecular Characterisation of EPNs

2.4.2.1. Genomic DNA Extraction

The sequences for the PCR amplicons of the 18S rDNA regions were received in the form of chromatograms. The chromatograms were viewed for errors and edited using FinchTV, and

saved as FASTA formatted files. The 18S rDNA sequences of unknown isolates A and B are illustrated (Figure 2.4.3. and Figure 2.4.4) (Sequences on the next page).

CCCTACACATTATGGCTTTTGATAGACTGAAACGGCGCAGGTTGCGTTTCTAAGT
GTCGATTTCGGTCATGAACGGCTTTTAATGGTTTCTATAGGTGTCTGGAGCAGCT
GTATGAGCGTGGCTGTTATGATGGACATTTAACATCATTTTACCGTGCGTTTGCG
CAGTTTCTAGAACGTTCGGTGATGAGAATTAAAGAGGTCAGTCGGAGACCCGCC
ATTGACAAACCACTATTAACTTTTTTACTTGATTATGCTCCTGGTATGGACGAAAC
ACAATCTTTATCAAGTCTTATCGGGGATCACTCGTCTATTCATGAAAAACGGGGA
AAAACCGTATTTGGGGAATTGCAACTATTGAACGCTAAATTTTGAACCAATGGAC
TATCAGGTTATATCTGATATATGTTGGTGAGGCATTAACTATTACTTGCGTCGCTT
GGACTGTTTTTCATGACTACTCTGCAAATACCTTTTCGGGATTGCCTTTTGGCATA
TTTAATGGGCGCATTTCATTCTTGCACGTTTCTTTCCGAGATTGCTCTCTGTGCGC
TGCTATCATATCGGTTCGTGCGTTATGGTTTTGGCGTGCTCTTGCCACTGACTTGA
CTAAGCTTCTGCTTTGTGCGTAATCGTTTCTTGAAGATCGGTAACCATGTCATTGA
TTTAACACGTTTCTTGATCAGCGGACGCATTGTGACTTTAATCGATGTTTTCGATT
ACACCTCACTCAAGCAAGACTACCCGCTGACTTAAACTGTG

Figure 2.4.3. The 18S rDNA sequence of unknown isolate A. The NCBI BLASTn results revealed a high affinity to *Steinernema* species, *Steinernema khoisanae* (KM275351.1).

GGCTTCGAGAAGAGTGGAGACTGCTGTATTGGGGCTTTCGGGCTCTGGTATGATG
GAAACCATTTTAATCGCAATGGCTTGAACCCGGGCAAAAGTCGTAACAAGGTAT
CTGTAGGTGAACCTGCAGATGGATCATCGCCGAAACYTTATGGGTAATGCTTTGA
TCACGAGAGATCGGTACCAATGGAATCAGGCTTGTTCTTGATTTCAATCGGTTTC
TCACCCCATCTAAGCTCATGGAGAGGTGTCTAGTCCCAATTGGAGTCGCTTTGAG
TGACGGCTATGAAAATTGGGTATGTTCCCCGTGAGGGTCGAGCATAGACTTTATG
AACAGTGCTGGAGCTGTCGCCTCACCAAAAAATCATCGATAACTGGTGGCTATGT
GTGACATTAGTCACATAGGTATCTGCTGATGCAGAGAGCCTTAATGAGTTGTTCG
TGTCATCTGACCTACAACCGCCACTATCGGTAAATCAAACCAATTAACTTGTTTC
TTGTGTCGTGTTAATACATACTGGCAAAGTGTATTAGCTTTAGCGATGGATCGGT
TGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCA
GACGCTTAGAGTGGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGG
CACGTCTGGCTCAGGGTTGTTTAATAAGCGAAAGTGTTGAAAGTTCATTAAACGA
GAGTTCGGTGATACTGACAACACTGCGTCGATCGGTGTACTGTTGAAAGTACCCC
GTTCAAGTATCTTTATGGGGCAACATGTCTTCTATACGGAGACATGAAAGATATT
AAGAGTATATACCTGTGGATGCCCACGTATGAAATATGACGTGTCGTATACATGG
CTAGGAGGTATGTCTCAGATGAATTTGTTTATGCAACCTGAGCTCAGTCGTGATT
ACCCGCCGAACTTAAGCATATCCCTTTTC

Figure 2.4.4. The 18S rDNA sequence of unknown isolate B. The NCBI BLASTn results revealed a high affinity to *Heterorhabditis* species, *Heterorhabditis bacteriophora* (FJ217351.1).

2.4.3. Phylogenetic Analysis

Phylogenetic analysis of the isolated EPNs was performed using the MEGA7 and the Maximum Likelihood Method was used for the identification or establishing phylogenetic relatedness of the isolates at the species level. The phylogenetic tree shows that the unknown isolate A clustered on the same clade with *S. khoisanae isolate BMMCB* and *S. khoisanae* which was isolated by Boiphelo Motupi and Vincent. M Gray in Johannesburg in 2016, South Africa, and Lee Ann Soobramoney and Vincent M. Gray in Johannesburg in 2016, respectively (figure 4.3.1). However, the latter two steinernematids may have been incorrectly identified as *S. khoisanae.* The clustering of these two species with unknown isolate A shows that they shared common ancestry with *S. khoisanae*, and hence the likelihood is high that they are sister species which have radiated from a common ancestor shared with *S. khoisanae*, the slight difference in the branch lengths of unknown isolate A and *S. khoisanae BMMCB* indicates that *S. khoisanae BMMCB* (KT027382.1) has undergone a degree of genetic variation over a period of time which has given rise to a new strain or new species.

The phylogenetic tree shows that the unknown isolate B clustered on the same clade with *H. bacteriophora strain* 56-C which was isolated by Malan et al. (2011) in Western Cape, South Africa (figure 4.3.2). The clustering of these two species showed that they shared a common ancestor and had a bootstrap percentage of 100%. The branch lengths of unknown isolate B and *H. bacteriophora strain* 56-C indicates they are both the exact species the unknown isolate B was identified to be *H. bacteriophora* strain TEL because it has a 100% similarity to the query. *H. bacteriophora TEL* was isolated by T.E Lephoto and V.M Gray in Johannesburg, South Africa.

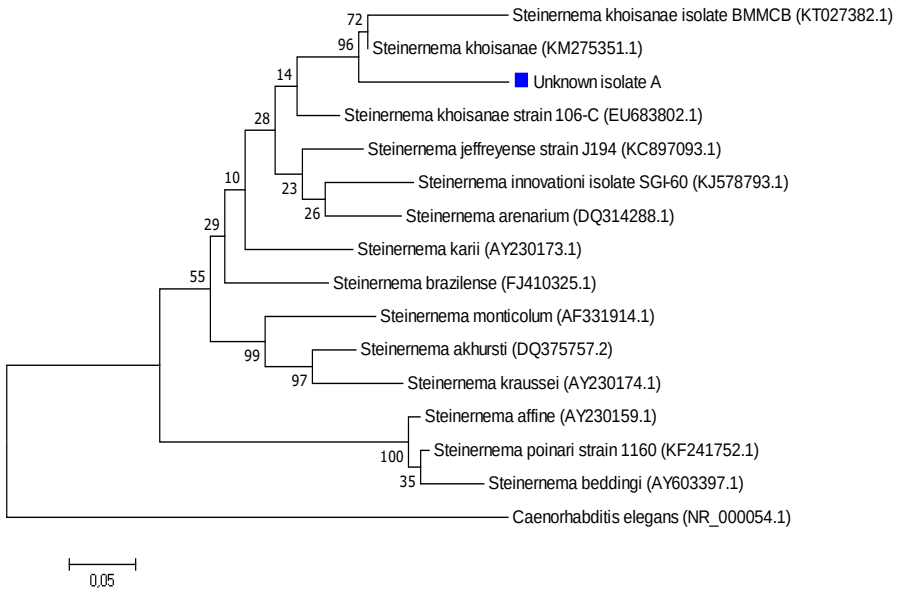

Figure 2.4.5. Phylogenetic relationships of 15 *Steinernema spp* based on analysis of the ITS rDNA regions. The outgroup is *Caenorhabditis elegans*. The unknown isolate A is indicated by the blue box. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the number of substitutions per site. The accession numbers are indicated in the brackets. The numbers at the nodes represent the bootstrap percentage of that specific node.

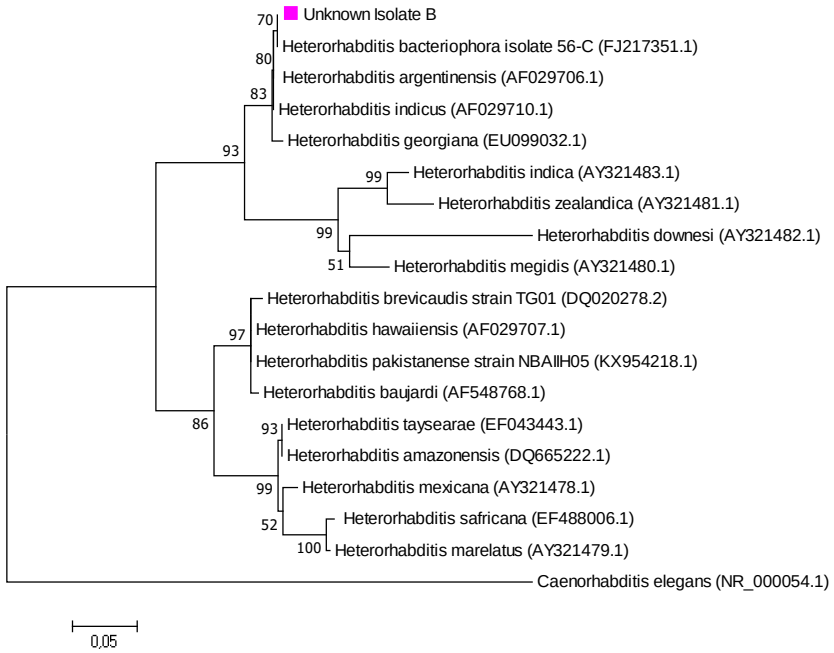

Figure 2.4.6. Phylogenetic relationships of 18 *Heterorhabditis* spp based on analysis of the ITS rDNA regions. The outgroup is *Caenorhabditis elegans*. The unknown isolate B is indicated by the purple box. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the

number of substitutions per site. The accession numbers are indicated in the brackets. The numbers at the nodes represent the bootstrap percentage of that specific node.

2.4.4. Evolutionary Divergence

The evolutionary divergence between EPN species can be estimated in terms of genetic or evolutionary distances between 18S rDNA sequences. Genetic distance, in turn, can be estimated or measured using MEGA7 software which computes the proportion of nucleotide differences between each pair of aligned 18S rDNA sequences, where the 18S rDNA sequences in turn also represent the OTUs corresponding to the different EPNs species and also represent 'measurable' or 'metric' genetic markers to which statistical significance can be assigned to the values used for measuring the degrees of similarities or differences between EPN species. Evolutionary divergence amongst the aligned species was determined using MEGA7 pairwise distance. The lowest evolutionary divergence is shown between unknown isolate A and *S. khoisanae* (0,120) which indicated that these species are closely related and have acquired genetic variations that make the isolates different (Table 2.4.4.). The lowest evolutionary divergence is between unknown isolate B and *H. bacteriophora strain* 56-C (0,000) which indicated that these species are identical (Table 2.4.5.).

Table 2.4.4. Estimates of Evolutionary Divergence between the sequences of 15 *Steinernema* species. The number of base substitutions per site from between sequences is shown. The number of base substitutions per site between sequences is shown in black. Standard error estimate(s) are shown above the diagonal (in blue) and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Unknown isolate A		0,026	0,036	0,024	0,060	0,017	0,024	0,037	0,026	0,025	0,029	0,030	0,027	0,036	0,022	0,029
2. KT027382.1 Steinernema khoisanae isolate BMMCB	0,239		0,033	0,025	0,057	0,016	0,024	0,034	0,025	0,027	0,029	0,030	0,027	0,033	0,023	0,029
3. KF241752.1 Steinernema poinari strain 1160	0,362	0,320		0,031	0,064	0,027	0,031	0,011	0,032	0,032	0,031	0,034	0,032	0,007	0,030	0,034
4. KJ578793.1 Steinernema innovationi isolate SGI-60	0,216	0,214	0,300		0,056	0,017	0,017	0,032	0,017	0,020	0,022	0,022	0,021	0,031	0,016	0,025
5. NR_000054.1 Caenorhabditis elegans	0,659	0,656	0,678	0,622		0,051	0,051	0,067	0,055	0,058	0,054	0,060	0,053	0,065	0,052	0,063
6. KM275351.1 Steinernema khoisanae	0,120	0,104	0,247	0,118	0,575		0,016	0,028	0,018	0,020	0,021	0,023	0,019	0,027	0,014	0,022
7. KC897093.1 Steinernema jeffreyense strain J194	0,214	0,215	0,318	0,123	0,586	0,121		0,032	0,016	0,019	0,020	0,022	0,020	0,031	0,014	0,022
8. AY603397.1_Steinernema_beddingi	0,383	0,345	0,054	0,320	0,705	0,265	0,329		0,033	0,035	0,032	0,035	0,032	0,012	0,030	0,036
9. DQ314288.1_Steinernema_arenarium	0,232	0,226	0,322	0,120	0,624	0,123	0,111	0,327		0,017	0,022	0,025	0,020	0,032	0,016	0,024
10. FJ410325.1_Steinernema_brazilense	0,239	0,248	0,303	0,153	0,636	0,153	0,154	0,335	0,128		0,022	0,025	0,020	0,031	0,018	0,023
11. DQ375757.2_Steinernema_akhursti	0,265	0,278	0,297	0,188	0,598	0,171	0,164	0,307	0,181	0,179		0,015	0,021	0,031	0,020	0,019
12. AY230174.1_Steinernema_kraussei	0,283	0,304	0,336	0,201	0,670	0,204	0,195	0,352	0,224	0,220	0,100		0,024	0,034	0,023	0,021
13. AY230173.1_Steinernema_karii	0,257	0,237	0,318	0,173	0,606	0,142	0,157	0,322	0,154	0,156	0,179	0,221		0,032	0,019	0,023
14. AY230159.1_Steinernema_affine	0,368	0,323	0,024	0,307	0,682	0,253	0,318	0,063	0,329	0,296	0,297	0,345	0,319		0,030	0,034
15. EU683802.1 Steinernema khoisanae strain 106-C	0,190	0,186	0,295	0,111	0,577	0,085	0,090	0,301	0,113	0,139	0,162	0,205	0,144	0,289		0,023
16. AF331914.1_Steinernema_monticolum	0,281	0,290	0,341	0,230	0,701	0,191	0,202	0,376	0,227	0,206	0,146	0,165	0,203	0,351	0,213	

Table 2.4.5. Estimates of Evolutionary Divergence between the sequences of 18 *Heterorhabditis* species. The number of base substitutions per site between sequences is shown. The number of base substitutions per site between sequences is shown in black. Standard error estimate(s) are shown above the diagonal (in blue) and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Unknown isolate B		0,073	0,023	0,025	0,028	0,026	0,000	0,022	0,029	0,023	0,006	0,003	0,026	0,021	0,029	0,027	0,024	0,003	0,023
2. NR_000054.1 Caenorhabditis elegans	0,637		0,071	0,074	0,070	0,071	0,073	0,081	0,099	0,088	0,072	0,072	0,071	0,079	0,071	0,075	0,073	0,072	0,071
3. KX954218.1 Heterorhabditis pakistanense strain NBAIIH05	0,163	0,618		0,005	0,020	0,016	0,023	0,032	0,036	0,034	0,023	0,023	0,016	0,030	0,019	0,018	0,004	0,023	0,000
4. DQ020278.2 Heterorhabditis brevicaudis strain TG01	0,175	0,644	0,009		0,021	0,017	0,025	0,034	0,038	0,035	0,025	0,025	0,017	0,032	0,020	0,019	0,006	0,025	0,005
5. EF488006.1_Heterorhabditis_safricana	0,226	0,634	0,113	0,124		0,012	0,028	0,036	0,039	0,036	0,028	0,028	0,012	0,034	0,005	0,012	0,020	0,028	0,020
6. EF043443.1_Heterorhabditis_taysearae	0,195	0,629	0,082	0,093	0,046		0,026	0,035	0,038	0,036	0,025	0,026	0,000	0,032	0,011	0,007	0,017	0,026	0,016
7. FJ217351.1 Heterorhabditis bacteriophora isolate 56-C	0,000	0,637	0,163	0,175	0,226	0,195		0,022	0,029	0,023	0,006	0,003	0,026	0,021	0,029	0,027	0,024	0,003	0,023
8. AY321483.1_Heterorhabditis_indica	0,145	0,716	0,262	0,276	0,297	0,279	0,145		0,025	0,012	0,023	0,022	0,035	0,017	0,037	0,034	0,032	0,022	0,032
9. AY321482.1_Heterorhabditis_downesi	0,233	0,840	0,322	0,337	0,341	0,331	0,233	0,192		0,026	0,029	0,029	0,038	0,022	0,039	0,037	0,036	0,029	0,036
10. AY321481.1_Heterorhabditis_zealandica	0,169	0,748	0,290	0,305	0,312	0,299	0,169	0,052	0,201		0,024	0,023	0,036	0,020	0,037	0,035	0,034	0,023	0,034
11. EU099032.1 Heterorhabditis georgiana	0,012	0,632	0,160	0,171	0,218	0,187	0,012	0,152	0,233	0,176		0,005	0,025	0,021	0,029	0,026	0,023	0,005	0,023
12. AF029706.1 Heterorhabditis argentinensis	0,003	0,630	0,160	0,171	0,222	0,191	0,003	0,145	0,233	0,169	0,009		0,026	0,021	0,029	0,026	0,023	0,000	0,023
13. DQ665222.1 Heterorhabditis amazonensis	0,195	0,629	0,082	0,093	0,046	0,000	0,195	0,279	0,331	0,299	0,187	0,191		0,032	0,011	0,007	0,017	0,026	0,016
14. AY321480.1_Heterorhabditis_megidis	0,135	0,674	0,238	0,251	0,276	0,255	0,135	0,096	0,165	0,117	0,138	0,131	0,255		0,035	0,032	0,030	0,021	0,030
15. AY321479.1_Heterorhabditis_marelatus	0,231	0,642	0,110	0,121	0,009	0,043	0,231	0,302	0,341	0,317	0,223	0,227	0,043	0,281		0,012	0,020	0,029	0,019
16. AY321478.1_Heterorhabditis_mexicana	0,199	0,655	0,093	0,103	0,049	0,018	0,199	0,274	0,322	0,293	0,191	0,195	0,018	0,254	0,046		0,018	0,026	0,018
17. AF548768.1_Heterorhabditis_baujardi	0,163	0,633	0,006	0,015	0,120	0,089	0,163	0,262	0,322	0,290	0,160	0,160	0,089	0,238	0,117	0,099		0,023	0,004
18. AF029710.1 Heterorhabditis indicus	0,003	0,630	0,160	0,171	0,222	0,191	0,003	0,145	0,233	0,169	0,009	0,000	0,191	0,131	0,227	0,195	0,160		0,023
19. AF029707.1_Heterorhabditis_hawaiiensis	0,163	0,618	0,000	0,009	0,113	0,082	0,163	0,262	0,322	0,290	0,160	0,160	0,082	0,238	0,110	0,093	0,006	0,160	

2.5. Discussion

In this study, experimental and molecular-based techniques were used in the isolation, identification, and phylogenetic analysis of the unknown isolates A and B that were originally collected from Walkerville and Brits, Gauteng. In this study, it was also confirmed that both the new *Steinernema* species and *H. bacteriophora* were capable of surviving in completed dehydrated soils that had been stored in plastic ice cream tubes for lengthy periods of time. Their capacity to survive in dehydrated soils may be due either to their capacity to undergo anhydrobiosis or they have evolved desiccation tolerance adaptations (Tyson et al., 2012). In any event, it appears possible that EPNs could be formulated as biocontrol agents within an appropriately dehydrated formulation medium or substrate with interstitial spaces that simulate a soil environment. Within this artificial environment, it may be possible to induce the IJs to enter either a state of anhydrobiosis or desiccation tolerance. This possibility will be considered later in the dissertation.

The EPNs, *Steinernema* and *Heterorhabditis*, complete their life cycles primarily in soil environments (Hazir et al., 2004). They have been used as biocontrol agents of insect pests that have their larval stage in the soil. Examples of such insects include those falling into lepidopteran, dipteran and coleopteran species. Interestingly, EPNs have been isolated and identified in various parts of the world, from ocean seafronts to arid deserts except for

Antarctica (Campos–Herrera et al., 2012). The various soil habitats that EPNs have been known to survive indicate that there may be significant differences in host range, reproduction, infectivity, and survival (Griffin, 2012). The complex soil environment that EPNs inhabit provides many challenges that they have successfully overcome. The survival hardships that EPNs encounter in the soil are temperature and moisture. These hardships impact EPNs ability to locate host species, effectively kill their host and persistence in the soil (Labaude and Griffin, 2018). At low temperatures, EPNs become inactive due to a decrease in mobility and metabolic processes for the conservation of energy (Griffin, 1993). EPNs require a film of free water for movement, therefore low moisture levels also cause the EPNs to become inactive (Radová and Trnková, 2010). The presence of many soil pathogens means that the death of the host may be due to these organisms and not EPNs. Therefore, phenotypic identification of death caused by EPNs allows for initial confirmation of their presence. An initial phenotypic identification is the change of colour of the infected larvae. Death caused by *H. bacteriophora* is seen by a maroon-purple with a maroon, brick red and green colour change of the dead cadaver and by *Steinernema* species, a dark brown-black, brown and beige colour change of the dead cadaver (Shapiro-Ilan et al., 2002). The White trap technique allowed for the isolation of potential entomopathogenic nematodes. The cause of death of the *G. mellonella* larvae was confirmed to be caused by the isolated entomopathogenic nematodes by the confirmation of Koch's Postulates of isolation, propagation, re-infection, and re-isolation of the IJs.

Insects have well developed innate immune responses but lack or have poorly adapted acquired immune response (Hoffmann, 1995). Insects have multiple levels of protection against invasion of microorganism pathogens such as physical barriers (e.g. cuticle), humoral immune responses, and cellular immune responses (Marmaras et al., 1996). EPNs are successful natural enemies because their method of invasion is rapid. They gain access through natural openings or for heterorhabditids, through the cuticle. Death of the insect takes place within 24 – 48 hours (Stuart et al., 2006). The presence of the entomopathogenic mutualistic bacteria allows for the constant supply of food and protection from secondary opportunistic microorganisms present in the environment.

The identification of nematodes has previously been done using methods of morphology and morphometric. In terms of *Steinernema*, this is relatively simple as they are amphimictic and cross-breeding between females and males is adequate in the identification of biological species (Shapiro-Ilan and Gaugler, 2002). Problems are experienced in the analysis of

Heterorhabditis as the species have alternating automictic and amphimictic stages (Shapiro-Ilan and Gaugler, 2002; Strauch et al., 1994). Therefore, the need for detailed morphological diagnostics has allowed for molecular techniques to lead the movement in nematode identification. Nucleotide sequence analysis is used to distinguish the different levels of taxonomy and give a better understanding of phylogeny in EPNs (Stock, 2002). The molecular techniques that have been employed are PCR and restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) (Stock, 2002). The use of RAPD allows researchers to determine the genetic variation of a new isolate (Hashmi et al., 1996). RFLP is used in the initial evaluation of EPNs but not for the evaluation of large samples of DNA and this technique is laborious and time-consuming (Liu et al., 2000). The regions of DNA that are amplified are the internal transcribed spacer (ITS) of the ribosomal RNA (Powers et al.,1997). The ITS region produces sufficient information about the variation of EPNs and provides a detailed phylogenetic analysis (Půža et al., 2015).

The analysis of phylogeny within and between species provides knowledge in the evolutionary biology of EPNs. A better understanding of the evolution of entomopathogenic nematodes further allows for the successful use of EPNs in agriculture as part of an integrated pest management programme. The differences in survivorship in different regions of South Africa affect the infectivity of the entomopathogenic nematode for insect pests. The construction of phylogenetic trees assists in the assessment of phylogenetic relationships that exist between unknown EPN isolates and known EPN species.

The phylogenetic analysis of the ITS rDNA region indicated that the unknown isolate A is an isolate of the species *Steinernema*. Unknown isolate A formed a clade with *S. khoisanae* BMMCB (KT027382.1) and *S. khoisanae* (KM27535.1). Unknown isolate A clustered with *S. khoisanae*. The phylogenetic analysis of the ITS rDNA region indicated that the unknown isolate B is an isolate of the species belonging to the genus *Heterorhabditis*. Unknown isolate B formed a clade with *H. bacteriophora TEL* (MH453898.1) and *H. bacteriophora strain* 56-C (FJ217351.1). The lack of branching within the clade shows that all three isolates are identical.

Evolutionary distances are the calculated pairwise distances between multiple aligned sequences. These distances are determined by the number of substitutions per 100 bases. There are several models that can be used to determine evolutionary distances and the type of model used is dependent on the parameters of the study as well as whether amino acids or nucleotide

sequences are being used. The evolutionary distances for this study were calculated using the Tajima-Nei model. The Tajima-Nei model assumes that the rate of substitution between the bases is the same whether it is from A to T or G to T. The evolutionary distances between the three possible *Steinernema* species show that the significant differences in the branch lengths are due to several genetic changes that have occurred that have led to the development of new species. The lowest evolutionary distances between unknown isolate A and other *Steinernema spp* isolates are seen between unknown isolate A and *S. khoisanae*. The evolutionary distance between the two isolates is 0,120 nucleotide substitutions per 100 bases. Therefore, the two isolates sequences share a high percentage of similarity but are genetically different. The lowest evolutionary distances between isolate B and other *Heterorhabditis spp* isolates are seen between unknown isolate B and *H. bacteriophora* isolate 56-C. The evolutionary distance between the two isolates is 0,000 nucleotide substitutions per 100 bases. Therefore, the two isolates are identical because their sequences did not undergo any substitutions.

2.6. Conclusion

The methods of soil baiting and White traps allowed for the isolation and the early identification of potential EPNs. Accurate identification of the isolates required extraction, amplification, and sequencing of 18S ribosomal RNA. The isolates were identified as belonging to the *H. bacteriophora* and *S. khoisanae* species. Phylogenetic analysis provided information on the evolutionary relationships of the isolated species and other known species from South Africa and the wider world. The bioinformatics analysis of the isolates indicated the unknown isolate A was likely a new *Steinernema* isolate and requires further characterization. The new isolate was deposited into GenBank and the accession number received was MH697401.2. Bioinformatics analysis also revealed that unknown isolate B was likely *H. bacteriophora* isolate 56-C and *H. bacteriophora* isolate TEL. Further developments in molecular identification of EPNs have included the use of cytochrome oxidase I (COI) and 12S mitochondrial genes.

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3. Chapter Three: Isolation and Identification of Bacterial Endosymbiont

3.1. Introduction

EPNs species belonging to the genera, *Steinernema* and *Heterorhabditis*, have evolved mutualistic relationships with insect pathogenic or entomopathogenic bacterial endosymbionts belonging to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Chaston et al., 2011; Sajnaga and Kazimierczak, 2020). These bacteria belong to the family Enterobacteriaceae and are part of the γ-subclass of Proteobacteria (Brachmann, 2009). The characteristic features shared by the bacteria include the following attributes: Gram-negative, rod-shaped, facultative anaerobes, negative for oxidase, asporogenous, chemo-organic heterotrophs (Boemare, 2002). Both bacterial symbionts are unable to reduce nitrate. *Xenorhabdus* is negative for catalase (Boemare, 2002), whereas *Photorhabdus* is catalase-positive. In contrast to *Xenorhabdus*, several species of *Photorhabdus* are bioluminescent. *Xenorhabdus* and *Photorhabdus* have different physiological states in their life cycle. This would reflect the metabolic and physiological coordination of the microsymbiont's life cycle with the EPN life cycle. For example, the symbiotic bacteria have the following life cycle phases (Chaston et al., 2011; Sajnaga and Kazimierczak, 2020): A dormant non-feeding phoretic phase within the infective juvenile (IJ) which is both host and vector. An actively growing pathogenic phase within the insect host. A saprophytic feeding phase within the insect host. In the phoretic phase, the bacteria is a state in which the bacterial colony population appears to remain constant within the non-feeding developmentally-arrested infective juveniles. This state will be referred to as the first state. Once the bacteria are released from the IJs into the host haemolymph, the bacteria enter the metabolically active and dividing state, which will be referred to as the second state. In addition to these two states, the bacterial also exist in two different physiological phases or phase variants, known as phase I and phase II, especially when grown in culture, bacterial change from phase I state to phase II state (Forst et al., 1997). Usually, bacteria are most infective in phase I physiological state. Phase II states can be induced by repetitive subculturing, long term storage as cultures, and by nutrient stress. In summary, phase II usually arises abruptly when the bacteria are maintained over prolonged periods on artificial culture, and seldom occurs within the insect host during the later or feeding and reproductive stages of the nematode life cycle. The development of the IJs into adult nematodes is reliant on the presence of the bacteria in the phase I state as in this state the bacteria produces antimicrobial compounds that prevent the colonization of fungi, yeasts and other bacteria from the external

environment (Gulcu et al., 2017). The so-called first physiological state, previously mentioned, also corresponds to the bacterial symbionts' phoretic stage during which time in the case of *Xenorhabdus*, the bacteria are located in the intestinal vesicle of steinernematid (IJs), whereas with *Photorhabdus*, the bacteria are found in the anterior part of the gut of heterorhabditid IJs (Griffin et al., 2005). Once the IJs have entered the haemocoel of the insect host, the bacteria are released from the IJs. Following their release into the haemolymph the bacteria undergo a transition from the first physiological state (dormant phoretic stage of the microsymbiont life cycle) in the second physiological state (pathogenic stage of the microsymbiont life cycle), which is a more metabolically active state, and a state in which they begin to divide rapidly (saprophytic stage of the microsymbiont life cycle). While in the second physiological state they also begin to release antimicrobial and insecticidal agents, and other compounds which suppress the host innate immune system, into the haemolymph thereby also facilitating the creation of a monoxenic environment (Hinchliffe et al., 2010). With regard to the two phases previously mentioned, it is while in the phase I state that bacteria are most infective or virulent, and are usually able to synthesis and release antimicrobial compounds, proteins inhibitors, and enzymes to assist in metabolizing the tissues of the insect cadaver (Boemare, 2002). Also, the release of insecticidal compounds causes the death of the host by septicaemia. The presence of bacteria is generally a necessary requirement for the successful survival of entomopathogenic nematodes. The bacteria have been seen to play three vital roles: to be an effective pathogen to the insect host; to facilitate nematode growth and development and to re-colonise the infective juvenile stage of the life cycle which occurs once all the nutrients in the insect cadaver have been depleted, and at this specific stage of the EPN life cycle the bacteria also possibly play a role in the induction of the non-feeding and developmentally arrested state of the infective juveniles (Emelianoff et al., 2008).

The insecticidal compounds released by the bacteria damage the insect host's tissues and interfere with the innate immune system of the host. The antimicrobial agents produced by the *Xenorhabdus* include benzylideneacetone, nematophin, xenocoumacins xenortides, xenematide, bicornutins and the antimicrobial agents produced by *Photorhabdus luminescens* strains have been identified as 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3- diol, 3,5, -dihydroxy-4-isopropyl-stilbene and the β-lactam carbapenem. Although the bacterial symbionts produce these antimicrobial symbionts as well as other compounds that protect the cadaver from colonization of other saprophytic opportunistic microorganisms, the insect cadaver also requires protection from colonization of other potential insect pathogens. It has

been reported that bacterial symbionts are able to resist the colonization of these insects by the production of unidentified compounds (Hinchliffe et al., 2010). The breakdown of the insect tissues provides nutrition for the growth of the bacteria and nematodes in turn feed on the bacteria and also on the nutrients derived from the host. While feeding on the bacteria, the nematodes undergo development into reproductive adults and once the nutrient supply has been exhausted the nematode life cycle enters into the non-feeding developmentally arrested state or infective juvenile state, as has already been mentioned (Ciche et al., 2006; Ferreira and Malan, 2014).

The multiple tandem 16S rDNA repetitive regions of the ribosomal genes are the most conserved genomic sequences in the eubacteria and archaebacterial kingdoms. The signature sequences found in this region are uniquely conserved sequences and are generally 5-10 bases long. *Xenorhabdus* and *Photorhabdus* strains can be identified by their unique 16S rDNA signature sequences. *Xenorhabdus* and *Photorhabdus* have TTCG and TGAAAG sequences respectively that allow for their identification (Boemare, 2002; Ferreira, 2013).

3.2. Aim and Objectives

3.2.1. Aim

To isolate and identify endosymbiotic bacteria of EPNs species isolated from South African soils that have been stored for prolonged periods in a dehydrated state in plastic tubs following their collection.

3.2.2. Objectives

The objectives have been summarized: a) Isolation of endosymbiotic bacteria from homogenised EPN IJs and also from the haemolymph extracted from infected larvae. b) Culturing of the isolated bacteria for selection on NBTA plates. c) Extraction of genomic DNA extraction from putative insect pathogenic endosymbiotic bacteria growing on the NBTA plates. d) PCR amplification of the hypervariable internal transcribed spacer (ITS) sequences located within the multiple tandem 16S rDNA repetitive regions of the bacterial genome. e) Sanger sequencing of the PCR amplified ITS sequences within the repetitive 16S rDNA sequences. f) Molecular phylogenetic characterization of the bacterial isolates based on 16S rDNA sequences as the metric or operational taxonomic unit for identification by BLAST comparison against the 16S rDNA sequences on GenBank database.

3.3. Methods and Materials

3.3.1. Isolation of Bacteria from Infected *Galleria mellonella* Larvae

The bacterial symbiont was isolated from the haemolymph of EPN infected larvae. Larval cadavers were retrieved from soil inoculated with a pure culture of infective juveniles. The pure culture of IJs was originally isolated from White traps. The IJs were used to inoculate larvae placed on 40g of sterile moist river sand (8% v/w) within sterile 90 mm Petri dishes and subcultured under *in vivo* conditions via re-infection of fresh larvae, a process which was repeated several times to ensure that a pure EPN culture was isolated. The larval cadavers were surface sterilised with 70% ethanol and a further second sterilisation step was carried out by the submergence of the cadaver in 70% ethanol and thereafter the larval cadaver was flamed for 2-3 seconds. The haemolymph was drawn from the larval haemocoel using a sterile syringe and collected into a sterile Eppendorf tube that contained sterilised distilled water (Kaya and Stock, 1997).

3.3.2. Isolation from Infective Juveniles

The IJs were collected from White traps and placed in 50 ml sterile Falcon tubes. They were surface sterilised by incubating in 0.03% sodium hypochlorite for three hours to ensure the elimination of all external microbial contaminants adhering to the epidermis of IJs. Any viable bacteria adhering to the surface of IJs would result in contamination and would interfere with the isolation of pure bacterial cultures residing within the IJs. Following their sterilization, the IJs were then rinsed with sterile Ringer's solution (Woodring and Kaya, 1988). The IJs were crushed and homogenised with sterilized plastic pestles in sterile Eppendorf tubes. The homogenate was transferred into a sterile Eppendorf that contained 0.1ml sterilized Nutrient Broth and incubated at 25°C for 24 hours.

3.3.3. Nutrient Bromothymol Triphenyltetrazolium Agar (NBTA) Plates

Samples isolated from the infected larval haemolymph and sterile IJs were streaked onto NBTA plates (refer to Appendix) and were incubated at 25°C for 2-3 days. Blue-green colonies on the NBTA plates indicated the presence of *Xenorhabdus* phase I bacteria (Kaya and Stock, 1997). The presence of phase I bacteria for *Photorhabdus* was indicated by greenish colonies with brownish centres on the NBTA plate (Kaya and Stock, 1997). The phase I bacteria was sub-cultured several times to ensure pure culture. The plates will be stored at 4°C until required for further analysis.

3.3.4. Bacterial DNA extraction

Pure colonies isolated were from the NBTA plates and resuspended in sterile distilled water. The ZR Fungal/bacterial DNA kit (catalogue number D6005) was used for the isolation of bacterial DNA. In terms of what has now become standard routine practice in many laboratories, the PCR amplification and sequencing of the ITS region within the 16 rDNA was outsourced to Inqaba Biotechnological Industries Pty (Ltd), South Africa.

3.3.5. PCR amplification of 16S rDNA

The hypervariable species-specific ITS sequence located within the 16S rDNA was amplified by a polymerase chain reaction and the sequence of the 16S rDNA amplicon was used to identify the bacterial species. The reaction mixture consisted of forward and reverse primers, Master Mix, genomic DNA, forward primer, reverse primer, and nuclease-free water. The 16S rDNA region was amplified using the following bacterial universal primers: EUB968 forward primer (5'-ACGGGCGGTGTGTRC-3') and UNIV1382 reverse primer (5'-AACGCGAAGAACCTTAC-3') (Table 3.3.1.). The amplification cycle consisted of 35 cycles (Table 3.3.2.). The denaturation occurred at 95°C for 60 seconds. The annealing occurred at 57°C for 60 seconds. The extension occurred at 72°C for 120 seconds and the final extension after cycling occurred at 72°C for 10 minutes (Table 3.3.3.).

Table 3.3.1. The reaction volumes and reagents of PCR.

Reagent	Volume (µl)
Master Mix	12,5
Bacterial DNA	1
EUB968	1
UNIV1382	1
Nuclease-free water	9,5

Table 3.3.2. Forward and Reverse Primers Used for the Amplification of the 16S rDNA regions.

EUB968 Forward primer	5'-ACGGGCGGTGTGTRC-3'	62	57
UNIV1382 Reverse primer	5'-AACGCGAAGAACCTTAC-3'	66	61

Table 3.3.3. The amplification procedure used for the amplification of the ITS regions between the 16S rDNA.

Amplification steps	Temperature (℃)	Duration
Denaturation	94 ℃	30 seconds
Annealing	57 ℃	45 seconds
Extension	72 ℃	90 seconds
Final extension	72 ℃	7 Minutes

3.3.6. Sequencing of the 16S rDNA

The ITS containing PCR generated amplicons of the 16S rDNA were subjected to Sanger sequencing at Inqaba Biotechnological Industries Pty (Ltd) South Africa. The chromatograms of sequences that were received from Inqaba were viewed, edited, and corrected for errors using the software FinchTV 1.4.0. The FASTA files of the edited sequences were uploaded into the Basic Local Alignment Search Tool (BLASTn) on the National Centre for Biotechnology Information (NCBI), where the query sequences were aligned against all the rDNA sequences in the NCBI database, thereafter the BLASTn results outputs showed the levels of similarity amongst possible matches.

3.3.7. Phylogenetic Analysis using MEGA7

Phylogenetic analysis was conducted using the rDNA ITS sequence as the operational taxonomic unit (OTU). The sequence of the ITS based OTU of the unknown bacteria isolates was compared with the sequences of phylogenetically related bacterial species, which were referenced to the known OTU sequences. The sequences of known species were downloaded

from the NCBI nucleotide database. The FASTA file of the unknown and the files of known bacteria was uploaded onto MEGA7 (Molecular Evolutionary Genetics Analysis version 7) which was the software tool used for phylogenetic analysis of the unknown sequence (Kumar et al. 1994; Tamura et al. 2011). The sequences were first aligned with MUSCLE (multiple sequence alignment method) using the default parameters (Edgar, 2004). The outgroup that was used was *Escherichia coli* (NR024570.1). The aligned sequences were subjected to the maximum likelihood method to determine the phylogenetic relationships between the species. The method allowed for phylogenetic analysis of sequences with different base compositions. The phylogenetic trees were constructed by the clustering of associated taxa based on a bootstrap value of 1000 replicates in statistical tests. The evolutionary divergence between aligned sequences was analysed by MEGA7 pairwise distance. The evolutionary distances of the aligned sequences were determined using the Kimura-2 parameter.

The following 16S rDNA sequences downloaded from the NCBI GenBank database were used for the construction of the phylogenetic tree for analysing the phylogenetic relationships of the unknown *Photorhabdus* isolate:

(MK039075.1) *P. stackebrandtii* strain DSM 23271, (MK039076.1) *P.khanii* strain DSM 3369, (MK039072.1) *P.tasmaniensis* strain DSM 22387, (AY296252.1) *P. temperata* (Z76752.1) *P. asymbiotica*, (D78005.1) *P. luminescens*, (MK039087.1) *P. namnaonensis* strain PB45.5 (MK039085.1) *P. hainanensis* strain C8404, (MK039083.1) *P. caribbeanensis* strain HG29, (MK039081.1) *P. kayaii* strain DSM, (MK039080.1) *P. bodei* strain LJ24-63, (MK039079.1) *P. kleinii* strain DSM, (MK039084.1) *P. noenieputensis* strain AM7 (MK039069.1) *P. cinerea* strain DSM 19724, (NR 024570.1) *Escherichia coli* strain U5/41

The following 16S rDNA sequences downloaded from the NCBI Genbank database were used for the construction of the phylogenetic tree for analysing the phylogenetic relationships of the unknown o *Xenorhabdus* isolate:

(DQ211710.1) *X. griffinae* strain ID10, (D78010.1) *X. poinarii*, (AJ810295.1) *X. szentirmaii*, (AY278674.1) *X. nematophila*, (D78007.1) *X. bovienii*, (D78008.1) *X. japonica*, (HQ142625.2) *X. khoisanae* strain SF87, (JX623966.1) *X. khoisanae* strain SF80, (DQ211719.1) *X. hominickii* strain KE01, (AJ810294.1) *X. ehlersii*, (AJ810293.1) *X. budapestensis*, (AJ810292.2) *X. innexi*, (DQ202309.1) *X. stockiae* strain TH01, (NR 024570.1) *Escherichia coli* strain U 5/41

3.4. Results

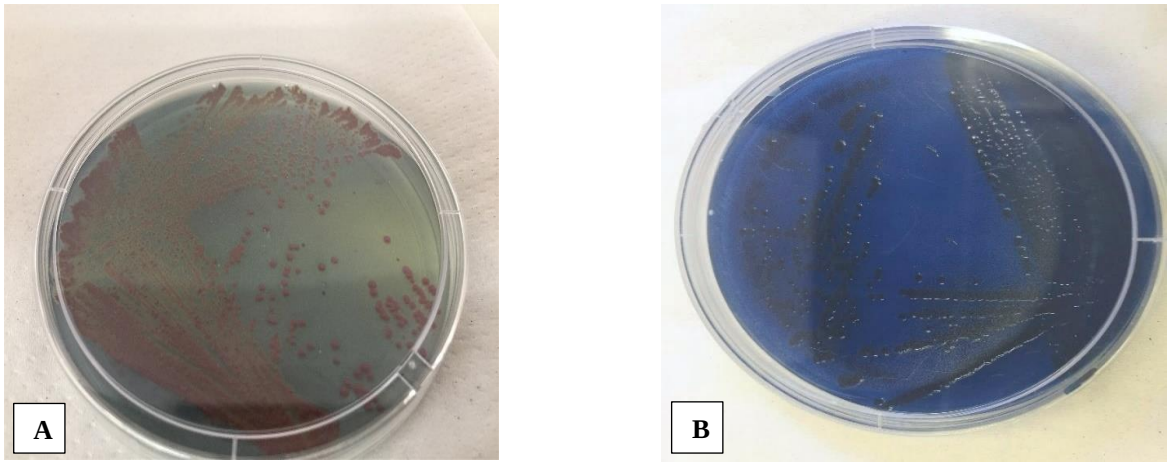

Figure 3.4.1. The Phase I colonies of the unknown isolates A and B cultured on Nutrient Bromothymol Triphenyltetrazolium Agar.

3.4.1. Isolated Bacteria

The isolated bacteria of unknown A and B were streaked onto the NBTA medium and incubated for 2-3 days at 25 ℃ (Figure 3.4.1). The colonies produced on the plates for the unknown isolate A were blue-green in colour, circular in shape, had a smooth texture, a diameter of approximately 0.3mm, slightly elevated and no spores were produced. The colonies produced on the plates for the unknown isolate B were brick-red in colour, circular in shape, had a smooth texture, a diameter of approximately 0.2mm, slightly elevated and no spores were produced (Table 3.4.1).

Table 3.4.1. The morphological characteristics seen on NBTA plates of Phase I bacterial colonies from unknown bacterial isolates A and B

Morphological Characteristics	Unknown Isolate A	Unknown Isolate B
Phase	Phase I	Phase I
Colony colour	Blue-green	Brick-red
Surface texture	Smooth	Smooth
Colony elevation	Slightly elevated	Slightly elevated
Shape	Circular	Circular
Spores	-	-

3.4.2. Sequencing of 16S rDNA

The chromatograms of the PCR amplified 16S and 23S rDNA intergenic spacer (ITS) sequences for the unknown bacterial isolates were uploaded onto FinchTV and edited. The edited sequences were uploaded from FinchTV onto NCBI BLASTn for the establishment of taxonomic affinities with 16S and 23S rDNA sequences registered on the NCBI Genbank database. The results that were received showed that unknown isolate A had a high similarity to the *Xenorhabdus* species (Figure 3.4.2.) and unknown isolate B had a high similarity to the *Photorhabdus* species (Figure 3.4.3.).

TGCAAGTCGGGCGGCAGCGGGAAGAAGCTTGCTTCTTTGCCGGCGAGCGGCGGA
CGGGTGAGTAATGTCTGGGGATCTGCCCGATGGAGGGGGATAACCACTGGAAAC
GGTGGCTAATACCGCATAACCTCGAGAGAGCAAAGTGGGGGACCTTCGGGCCTC
ACACCATCGGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCAC
CTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAG
TACTTTCAGTGGGGAGGAAGGCRCAGGGTTRAATACACCCTGTGATTGACGTTAC
CCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAA
GTTAGATGTGAAATCCCCGGGCTTAACCCGGGAATGGCATCTAAGACTGGTTGA
CTAGAGTCTCGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTC
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTG
TAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACG
CGTTAAATCGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTACTCTTGACATCCACGGAATTCTGCAGAGATGCGGAAGTGCCTT
CGGGACCCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCACGTGATG
GTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGA
TACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTCTGTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTA
GATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTGCAAAAGAAGTCGGTAGCTTAACCTTCGGGAGGGCGCTG
ACCACTT

Figure 3.4.2. The bacterial sequence of unknown isolate A. The NCBI BLASTn results revealed high affinity to *Xenorhabdus* species, *Xenorhabdus griffiniae* (DQ211710.1)

TTGAAGAGTTTTATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGC
AAGTCGAGCGGTAACAGGAAAGCGCTTGCGCTTTGGCTGACGAGCGGCGGACGG
GTGAGTAATGTCTGGGGATCTGCCCGAGGGCGGGGGATAACCACTGGAAACGGT
GGCTAATACCGCATAATGTCGCGAGACCAAAGTGGGGGACCTGAAAGGGCCTCA
CGCCATCGGATGAACCCAGATGGGATTAGCTAGTAGGTAGGGTAAAGGCCTACC
TAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CCAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGT
ACTTTCAGCGGGGAGGAAGGGTTCAGCTTGAACAGAGCTGGATTTTGACGTTAC
CCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG
TGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAA
GTTAGATGTGAAATCCCCGGGCTCAACCTGGGAATGGCATCTAAGACTGGTTGG
CTGGAGTCTCGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTC
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTG
TAAACGATGTCGATTTGGAGGTTGTGGCCTTGAGCTGTGGCTTCCGGAGCTAACG
CGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTACTCTTGACATCCAGAGAAGACCTCAGAGATGAGGTTGTGCCTT
CGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCGTGAAG
GCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGGCCCTGACGAGTAGGGCTACACACGTGCTACAATGGCGGA
TACAAAGTGAAGCGACCTCGCGAGAGCAAGCGGAACACACAAAGTCTGTCGTAG
TCCGGATTGGAGTCGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAG
ATCAGCATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACAC
CATGGGAGTGGGTTGCAAAAGAAGTCGGTAGCTTAACCGCAAGGAGGGCGCTGA
CCACTTTGTGGCTCATGACTGGGGTGAAGTC

Figure 3.4.3. The bacterial sequence of unknown isolate B. The NCBI BLASTn results revealed a high affinity to *Photorhabdus* species, *Photorhadus luminescens* (D788005.1).

3.4.3. Phylogenetic analysis

PCR-amplified generated 16S rDNA sequences can readily be used as unbiased metric operational taxonomic units (OTUs) for the analysis of phylogenetic and taxonomic similarity through alignment of the query 16S rDNA-based OTUs with 16S rDNA-based OTU sequences in reference databases such as NCBI Genbank. Soft applications such as MEGA7 can be used to infer likely taxonomic and phylogenetic relationships between the query sequences and the database reference sequences. In this instance, phylogenetic analysis was performed using MEGA7 software. The Maximum Likelihood Method was used for the construction of the trees which depicted the taxonomic affinities of unknown and known species (Nei et al., 1983) The ITS sequence of the unknown isolate A was compared with the sequences of strains of different species and subspecies of the genus *Xenorhabdus* (Figure 3.4.4). The sequence of unknown

isolate A clustered with *X. griffinae* strain ID10 (DQ211710.1) on the same clade. The clustering of the two isolates shows that they share a common ancestor and have a bootstrap percentage of 92%. The difference in the branch length of the two species indicates that *X. griffinae* strain ID10 has undergone a greater degree of genetic variation from its common ancestor over a period of time.

The ITS sequence of the 16S rDNA gene sequence of unknown isolate B was compared with the sequences of strains of different species and subspecies of the genus *Photorhabdus* (Figure 3.4.5). The unknown isolate B clustered with *P. luminescens* (MK039087.1) on the same clade. The clustering of the two isolates shows that they share a common ancestor and have a bootstrap percentage of 83%. The difference in the branch length of the two species indicates that *P. luminescens* has undergone a greater degree of genetic variation from its common ancestor over a period of time.

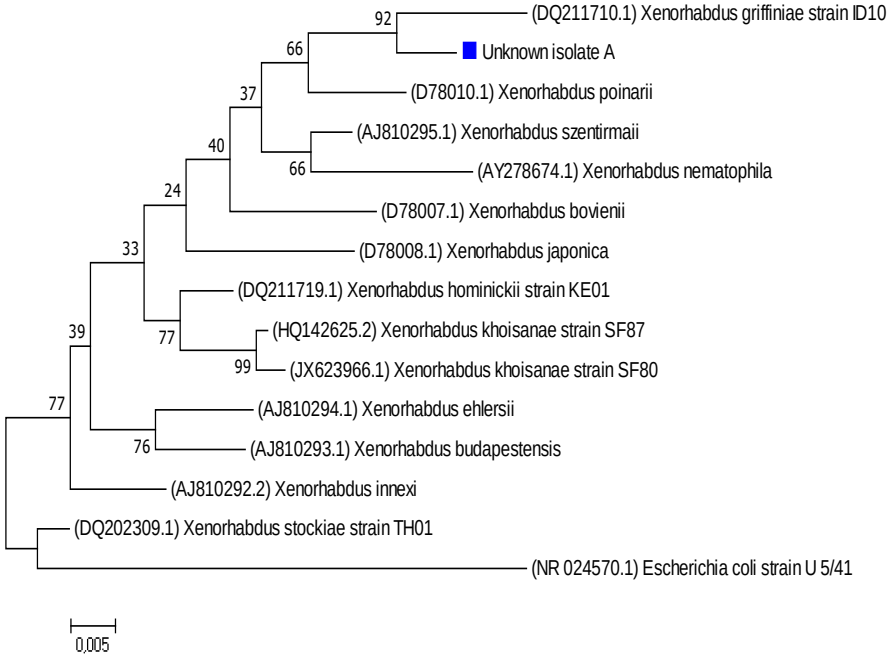

Figure 3.4.4. Phylogenetic relationships of 15 *Xenorhabdus spp* based on analysis of the ITS rDNA regions. The unknown isolate is indicated by the blue box. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the number of substitutions per site. The accession numbers are indicated in the brackets. The numbers at the nodes represent a bootstrap percentage of that specific node.

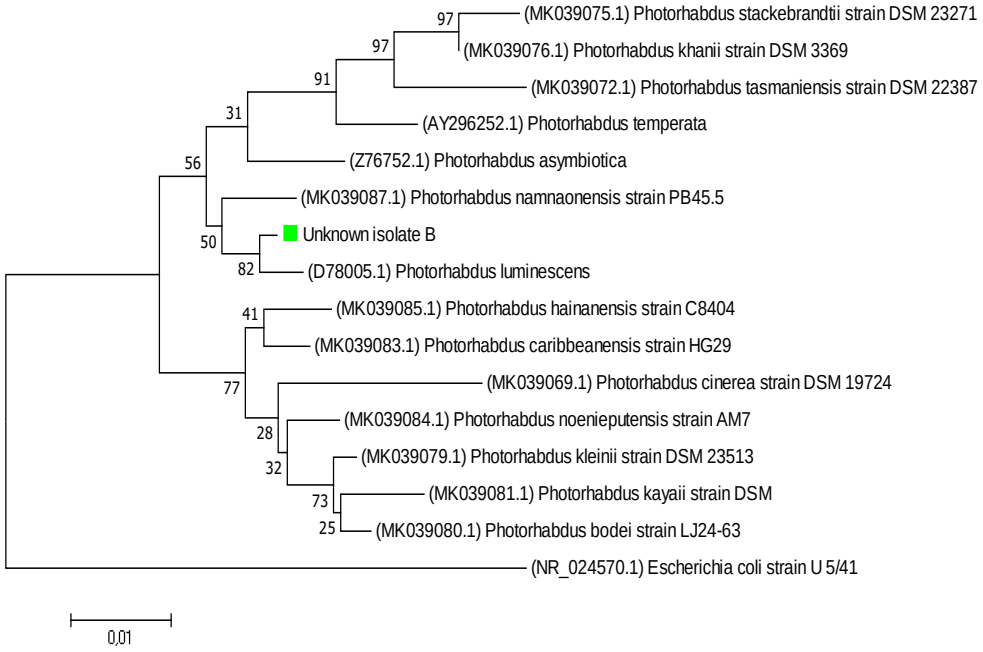

Figure 3.4.5. Phylogenetic relationships of 16 *Photorhabdus spp* based on analysis of the ITS rDNA regions. The unknown isolate is indicated by the green box. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the number of substitutions per site. The accession numbers are indicated in the brackets. The numbers at the nodes represent a bootstrap percentage of that specific node.

3.4.4. Evolutionary distances

Evolutionary divergence amongst the aligned 16S rDNA sequences of the query and reference species was determined using the MEGA7 pairwise distance software. The lowest evolutionary divergence is shown between unknown isolate A and *X.s griffinae* strain ID10 (0.21) which indicates that the two species are closely related (Table 3.4.2). The lowest evolutionary divergence is shown between unknown isolate B and *P. luminescens* (0.006) which indicates that the two species are closely related (Table 3.4.3). (Tables on the next page).

Table 3.4.2. Estimates of Evolutionary Divergence between the sequences of 15 *Xenorhabdus* spp. The number of base substitutions per site between sequences is shown. The number of base substitutions per site between sequences is shown. The number of base substitutions per site between sequences is shown in black. Standard error estimate(s) are shown above the diagonal (in blue) and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Unknown isolate A		0.005	0.005	0.004	0.006	0.006	0.004	0.005	0.005	0.004	0.005	0.005	0.007	0.005	0.005
2. D78008.1 Xenorhabdus japonica	0.042		0.005	0.005	0.006	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.008	0.004	0.004
3. DQ211719.1 Xenorhabdus hominickii strain KE01	0.034	0.032		0.005	0.004	0.005	0.004	0.004	0.005	0.005	0.004	0.005	0.007	0.003	0.004
4. DQ211710.1 Xenorhabdus griffiniae strain ID10	0.021	0.046	0.042		0.006	0.005	0.005	0.004	0.005	0.004	0.005	0.006	0.008	0.005	0.005
5. DQ202309.1 Xenorhabdus stockiae strain TH01	0.038	0.040	0.026	0.045		0.004	0.005	0.005	0.005	0.005	0.006	0.006	0.007	0.005	0.005
6. AJ810292.2 Xenorhabdus innexi	0.038	0.033	0.027	0.041	0.024		0.005	0.004	0.005	0.005	0.005	0.006	0.008	0.005	0.005
7. AJ810295.1 Xenorhabdus szentirmaii	0.023	0.031	0.027	0.036	0.030	0.033		0.005	0.005	0.004	0.004	0.004	0.007	0.004	0.004
8. AJ810294.1 Xenorhabdus ehlersii	0.032	0.030	0.029	0.030	0.032	0.024	0.036		0.004	0.005	0.005	0.006	0.008	0.004	0.005
9. AJ810293.1 Xenorhabdus budapestensis	0.033	0.033	0.030	0.042	0.030	0.027	0.033	0.021		0.005	0.005	0.006	0.008	0.004	0.005
10. D78010.1 Xenorhabdus poinarii	0.027	0.041	0.031	0.030	0.036	0.038	0.023	0.033	0.037		0.005	0.005	0.007	0.005	0.005
11. D78007.1 Xenorhabdus bovienii	0.034	0.033	0.027	0.042	0.041	0.037	0.026	0.038	0.038	0.030		0.005	0.007	0.005	0.005
12. AY278674.1 Xenorhabdus nematophila	0.038	0.036	0.037	0.044	0.035	0.048	0.023	0.050	0.046	0.036	0.039		0.008	0.005	0.005
13. NR_024570.1 Escherichia coli strain U 5/41	0.073	0.078	0.070	0.082	0.058	0.076	0.068	0.080	0.080	0.067	0.071	0.069		0.008	0.008
14. HQ142625.2 Xenorhabdus khoisanae strain SF87	0.032	0.028	0.015	0.039	0.030	0.030	0.023	0.029	0.026	0.033	0.030	0.033	0.074		0.002
15. JX623966.1 Xenorhabdus khoisanae strain SF80	0.036	0.030	0.018	0.041	0.032	0.033	0.027	0.031	0.030	0.036	0.034	0.037	0.078	0.004	

Table 3.4.3. Estimates of Evolutionary Divergence between the sequences of 16 *Photorhabdus* spp. The number of base substitutions per site between sequences is shown. The number of base substitutions per site between sequences is shown. The number of base substitutions per site between sequences is shown in black. Standard error estimate(s) are shown above the diagonal (in blue) and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Unknown isolate B		0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.002	0.004	0.003	0.005	0.005	0.004	0.005	0.007
2. Z76752.1 Photorhabdus asymbiotica	0.017		0.005	0.005	0.005	0.005	0.005	0.005	0.004	0.004	0.004	0.005	0.005	0.004	0.005	0.008
3. MK039085.1 Photorhabdus hainanensis strain C8404	0.023	0.035		0.003	0.003	0.003	0.003	0.003	0.004	0.005	0.004	0.005	0.006	0.005	0.004	0.008
4. MK039084.1 Photorhabdus noenieputensis strain AM7	0.028	0.031	0.014		0.003	0.003	0.003	0.003	0.005	0.005	0.004	0.005	0.006	0.005	0.004	0.008
5. MK039083.1 Photorhabdus caribbeanensis strain HG29	0.024	0.028	0.011	0.014		0.004	0.003	0.003	0.004	0.005	0.004	0.005	0.006	0.005	0.004	0.007
6. MK039081.1 Photorhabdus kayaii strain DSM	0.027	0.032	0.018	0.015	0.020		0.003	0.003	0.004	0.005	0.004	0.005	0.006	0.006	0.005	0.008
7. MK039080.1 Photorhabdus bodei strain LJ24-63	0.026	0.032	0.012	0.014	0.013	0.011		0.002	0.004	0.005	0.004	0.005	0.006	0.005	0.004	0.008
8. MK039079.1 Photorhabdus kleinii strain DSM 23513	0.028	0.031	0.014	0.012	0.016	0.011	0.006		0.004	0.005	0.004	0.005	0.006	0.005	0.004	0.008
9. D78005.1 Photorhabdus luminescens	0.006	0.018	0.023	0.030	0.025	0.030	0.029	0.031		0.004	0.003	0.005	0.006	0.005	0.005	0.008
10. AY296252.1 Photorhabdus temperata	0.024	0.026	0.036	0.038	0.035	0.042	0.037	0.039	0.025		0.005	0.004	0.005	0.004	0.004	0.008
11. MK039087.1 Photorhabdus namnaonensis strain PB45.5	0.013	0.020	0.021	0.024	0.026	0.029	0.028	0.028	0.014	0.028		0.005	0.005	0.005	0.005	0.008
12. MK039075.1 Photorhabdus stackebrandtii strain DSM 23271	0.031	0.031	0.036	0.035	0.035	0.043	0.039	0.039	0.032	0.023	0.033		0.004	0.002	0.005	0.008
13. MK039072.1 Photorhabdus tasmaniensis strain DSM 22387	0.034	0.034	0.044	0.047	0.043	0.046	0.048	0.051	0.035	0.027	0.034	0.024		0.004	0.006	0.008
14. MK039076.1 Photorhabdus khanii strain DSM 3369	0.028	0.024	0.039	0.038	0.035	0.043	0.042	0.043	0.029	0.020	0.032	0.006	0.019		0.005	0.008
15. MK039069.1 Photorhabdus cinerea strain DSM 19724	0.040	0.036	0.030	0.025	0.027	0.028	0.026	0.027	0.041	0.031	0.041	0.035	0.044	0.037		0.007
16. NR_024570.1 Escherichia coli strain U 5/41	0.075	0.082	0.080	0.081	0.079	0.080	0.082	0.083	0.077	0.079	0.073	0.079	0.080	0.081	0.079	

3.5. Discussion

The isolation, identification, and phylogenetic analysis of the endosymbiont bacteria of the two isolated entomopathogenic nematodes were undertaken in this study. There are various bacterial culture media that can be used for isolation and identification of bacteria. Solid media contains a gelling agent such as agar, which allows for the growth of non-fastidious bacteria. The physical structure of solid media allows for bacteria to grow in a manner that allows for the determination of colony characteristics. An example of solid media is NBTA. The morphological characteristic of *Xenorhabdus* on NBTA is that the colonies appear blue to blue-green in phase I (Akhurst, 1983). The morphological characteristics of *Photorhabdus* on NBTA are that colonies appear brick/dark red-purple in phase I (Boemare, 2002). The bacterial colonies of the unknown isolate A appeared blue-green on NBTA. The colonies were circular in shape and had a diameter of approximately 0.3mm. The colonies were slightly elevated and opaque in appearance. These morphological characteristics indicated that the unknown isolate may be *Xenorhabdus*. The bacterial colonies of the unknown isolate B appeared brick-red on NBTA. The colonies were circular in shape, had a diameter of approximately 0.2mm and there was no formation of spores. These morphological characteristics indicated that the unknown isolate may be *Photorhabdus*.

The identification of bacterial species is done by the analysis of ribosomal DNA. The 16S rDNA has been most commonly used as it is relatively large (approximately 1500bp) and has remained conserved over time (Patwardhan et al., 2014). The hypervariable regions of the 16S rDNA gene account for differences amongst related sequences. These differences are due to random changes that have no functional relevance or consequences and the hypervariable regions can be treated as a molecular clock for tracking genetic divergences over time and in this sense can be used as a measure of time (Tshikhudo et al., 2013). The hypervariable regions are flanked by conserved regions, the sequence of the conserved has been used for the design of universal primers for the PCR amplification of both conserved and hypervariable regions within the 16S rDNA sequence.

The colonies of the NBTA plates of the isolated bacterial species were also sent to Inqaba Biotechnical Industries Pty (Ltd) South Africa for amplification and sequencing of the 16S rDNA region. The received sequences were entered onto the BLASTn tool on NCBI for comparison to other known sequences. *Xenorhabdus* sp. VP-2016a (KU578109.1) was

identified as unknown isolate A. The unknown bacterial isolate B had a high similarity to *P. luminescens* subsp. *sonorensis* strain Caborca (JQ912644.1). *Xenorhabdus* sp. VP-2016a clustered with *X. griffinae* ID10 (D78010.1). *Xenorhabdus* sp. VP-2016a and *X.s griffinae* ID10 shared common ancestry but the difference in branch length shows that *X. griffinae* ID10 has undergone a degree of genetic variation over a period of time. The unknown isolate B clustered with *P. luminescens* (D78005.1) with a good bootstrap percentage of 82%. The evolutionary distance between the unknown isolate A and *X. griffinae* strain ID10 was 0.210 which indicates a divergent evolution from a common ancestor. The evolutionary distance between unknown isolate B and *P. luminescens* was 0,006 which indicates a divergent evolution from a common ancestor.

3.6. Conclusion

The methods of bacterial isolation on NBTA plates and molecular techniques allowed for the identification of unknown isolates as belonging to either *Photorhabdus* or *Xenorhabdus* species. The distinct colour change of the bacterial isolates on NBTA plates gave an early indication of which species was present. The unknown isolate A produced blue-green colonies while unknown isolate B produced brick-red colonies. Molecular techniques were used to give an accurate identification of the unknown isolates. This was done by the amplification and sequencing of the 16S rDNA region and this revealed that unknown isolate A was *Xenorhabdus* sp VP-2016 and unknown isolate B was *P. luminescens* subspecies Carborca. Phylogenetic analysis provided knowledge on the relationship of the unknown isolates and known species. Unknown isolate A shared a clade with *X. griffinae* ID10 while unknown isolate B shared a clade with *P. luminescens*. These relationships indicate that they share a common ancestor but are genetically different due to genetic variations that have taken place over a long period of time. Although the use of 16S rDNA for bacterial isolation has been a common practice and has its numerous advantages, there are shortfalls such as incorrect identification of species that share the same sequence at this gene, often due to the misnaming of one or more species. Another method that may be used to avoid this situation is the use of DNA-DNA re-association assay or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). DNA-DNA re-association assay measures the degree of genetic similarity between pools of DNA sequences and therefore determines genetic distances between two organisms (Rosselló-Mora, 2006). The shortfall for these techniques is that it is time-consuming and there is no central database(s) for referencing and comparison. Matrix-assisted laser

desorption/ionisation time-of-flight mass spectrometry is the use of a laser energy absorbing matrix that creates ions from large molecules (Schumann and Maier, 2014). These techniques have two main advantages: identify isolates rapidly and reliably at a low cost and a rapid turn-around time because of the absence of purification of isolates.

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4. Chapter Four: An Investigation into the Formulation Potential of *Heterorhabditis bacteriophora*

4.1. Introduction

Increasing the shelf-life of IJs remains the overriding practical and economic goal of EPN formulation. It is not an over-exaggeration to claim that the commercial viability of all biocontrol enterprises based on the sale of EPNs depends critically on the effectiveness of its formulation of IJs. The main ingredient in nematode formulation is the carrier medium or substrate in which the IJs are carried or contained or embedded. The overriding purpose of the carrier medium is the long-term pre-sale preservation of the viability and infectivity of IJs following their mass production (Askary and Ahmad, 2017; Strauch et al., 2000). Preservation or extension of product shelf-life requires the development of formulations that possess the properties and capacities for maintaining long-term longevity of IJs in a viable and infectious state. Attempts have been made to formulate infective juveniles in various carrier media such as clays, activated charcoal, sponge, vermiculite, peat, alginate gels, and water-dispersible granules (Askary and Ahmad, 2017; Georgis, 1990; Kagimu et al., 2017; Strauch et al., 2000). Moreover, the formulation of infective juveniles in the infected cadavers of insect larvae such as *G. mellonella* has also been considered (Shapiro-Ilan et al., 2001). Formulations may be either wet or dry. However, dry or dehydrated formulations of IJs still remains in the developmental stages and has not quite yet been realized as a reliable technology. Most current formulations are either wet or have reduced moisture content rather been completely dehydrated formulations.

Increasing the shelf-life of formulated IJs also depends strongly on decreasing their metabolic rate so that their storage reserves of lipids and fats are maintained and not depleted. The depletion of storage reserves can be prevented by reducing IJ activity and metabolic rates by reducing temperature and/or by the induction of a state of dormancy or quiescence. The latter would be preferable as maintaining formulated IJs at low temperatures could become an unsustainable cost factor. Under natural conditions in the non-feeding and developmentally-arrested state of the EPN life cycle in the case of both steinernematids and heterorhabditids, the IJs are subjected to dehydration stresses as the level of soil moisture declines. In fact, IJs do indeed survive for long periods in soils that have become completely dehydrated especially during the dry winter periods in the summer rainfall regions of South Africa. This capacity for seasonal survival in dehydrated or completely desiccated soils indicates that not only the IJs of

EPNs but also many other species of invertebrate micro-fauna, including many species of soil-dwelling nematodes occupying a diversity of trophic niches, all share metabolic and physiological adaptations which allows them to enter into a state of long-term dormancy under drought conditions (Tyson et al., 2012). Panagrolaimid nematodes capable of anhydrobiosis have been shown to express a great diversity of stress response genes. Transcriptome sequencing, assembly, and annotation have been used to discover putative candidate genes in panagrolaimid nematodes which encode proteins linked to processes facilitating the induction of anhydrobiosis (Tyson et al., 2012).

Development of formulations that ensure the longevity of IJs under long- term storage has been the single most critical constraint restricting the development and application of entomopathogenic nematodes as biocontrol agents of insect pests of agricultural crops. In nature the non-feeding developmentally-arrested IJs of EPNs are able to withstand low soil moisture conditions, or even completely dehydrated or desiccated soils, for prolonged periods of time, possibly stretching for years. However, it has not yet been possible to replicate this phenomenon in the laboratory using various formulation media in which IJs can be stored under low moisture conditions or under completely dehydrated states. It seems that IJs cannot be maintained in a viable state for lengthy periods of time under conditions where they appear to be susceptible to high oxygen levels or high ambient temperatures (Poinar Jr and Grewal, 2012). The stresses that affect the viability and storage potential of EPNs include temperature extremes, oxygen deprivation, the water potential of the surrounding medium, shear stress, desiccation, and microbial contamination. All of these factors influence nematode quality and viability leading to reduced shelf-life. EPNs are made up of 60% lipids and this represents an essential energy storage reserve. Therefore, energy conservation or maintenance of storage lipid reserves remains a vital factor in prolonging the survival of IJs and extending the shelf-life of EPN-based bioinsecticides. To repeat, temperature and moisture are the two major factors that affect IJ longevity, survival, infectivity, and pathogenicity, especially when in the formulated state (Rohde et al., 2010). At low temperatures, EPNs experience reduced metabolic activity, and low moisture causes or induces EPN IJs to become dormant in soils (Kagimu et al., 2017). Anhydrobiosis is a reversible, physiologically arrested state of dormancy induced by dehydration, and the successful induction of the state of anhydrobiosis in IJs within a suitable formulation medium would represent the achievement of an essential strategic goal for the improvement of EPN storage stability for prolonged periods, and especially at ambient temperatures, as this would be a significant cost saving factor. It has been established that

entomopathogenic nematodes can undergo partial anhydrobiosis, hence theoretically speaking, the formulation of IJs in a dehydrated state could be achieved (Grewal, 2000; O'leary et al., 2001; Tyson et al., 2012). Being in the physiological and metabolic state of anhydrobiosis also allows for a reduction in the consumption of energy reserves by nematodes, and possibly, thereby increases their vigour and infectivity when released into the soil as biocontrol agents. Therefore, formulations that are able to induce states of anhydrobiosis or dehydration tolerance in nematodes need to be developed. Such developments could be based on the utilization of formulation media which can facilitate induction of low moisture tolerance, that is, media such as hydroscopic or water-absorbing gels, powders, and granules (Kagimu and Malan, 2019). What is meant by granules is the coating of a droplet of IJs in a shell of hydroscopic powdered media such as clays or gelling material or diatomaceous earth or a mixture of these substances. An example of gel formulation is where the nematodes are mixed with anhydrous polyacrylamide which acting as a gelling agent that allows for water activity of between 0.800 and 0.995 to be attained (Grewal, 2002). Powder-based formulation of IJs involves mixing nematodes in the form of sludge with moist or dry clays or diatomaceous earth or mixtures of clays, diatomaceous, and other hygroscopic materials or gelling agents. The mixing of nematodes suspended in water or as a hydrated sludge with clays or diatomaceous earth or mixtures of various hygroscopic materials, whether in a dry or a moist state, can facilitate the slow or rapid removal of surface moisture or moisture in the surrounding environment, thereby inducing partial desiccation of the nematodes and also of the immediate spatial environment surrounding the nematodes. It is assumed that the rate of moisture removal is critical in the process of inducing nematode adaptations to an environment undergoing dehydration.

As mentioned, the granular formulation is where the nematodes are encapsulated initially in a shell of hygroscopic material or media for example in Lucerne meal and wheat flour. However, the material used in this method was later replaced by water-dispersible material which allowed for granular formulations of nematodes (Grewal and Georgis, 1999). These are some of the various methods based on hygroscopic materials that have been used for nematode formulation.

To repeat, by way of definition, formulation methods involve the use of inert carriers as the medium in which IJs are embedded or encapsulated. Inert carriers or media are thus integral to nematode formulation. Moreover, one of the important properties of inert carriers appears to be their moisture-holding and moisture absorbing capacities. As has already been noted, the rate of dehydration or moisture loss from the environment surrounding the IJs appears to be critical for the induction of anhydrobiosis or desiccation tolerance (Kagimu and Malan, 2019;

Tyson et al., 2012). Inert carriers may also be selected on the basis of facilitating IJ mobility, meaning that nematodes are able to remain fully active as they are able to move with ease in or on the carrier (Grewal, 2002). Examples of these carriers are polyether-polyurethane sponge-based and vermiculite. These carriers make formulation easy and cost-effective in terms of production, but the disadvantage is that they require refrigeration and maintenance of moisture content within critical boundaries during storage and transport which makes this method expensive. The energy reserves of nematodes within such formulations can become quickly depleted. One way of resolving this problem would involve limiting the activity of nematodes by methods of physical trapping or confinement or by employing metabolic inhibitors (Chen and Glazer, 2005). One option would be a formulation in which nematodes are physically trapped between thin sheets of calcium alginate, the nematodes being placed on plastic screens, and trapped between calcium alginate gels (Umamaheswari et al., 2006). In this formulation approach, the nematodes are freed from the alginate gel by dissolving the gel in water that contains sodium citrate (Grewal, 2000). The problems that arise with this type of formulation is time consumption in terms of preparation, application, and effective disposal of plastic screens and containers. Therefore, at the level of large-scale production, this mode of IJ formulation is not deemed economical. The inert carrier formulations allow for maintenance of high viability, but they are unable to be packaged at high densities which in turn limits their usefulness in a large-scale application. Nematodes in many kinds of gel formulations are not able to survive at room temperature and also due to their formulation ingredients, some gels are difficult to dissolve and can also clog the sprayers. However, hydroscopic powder-based formulations in which critical moisture levels are maintained has the advantages of ease of application and the physical properties which facilitate nematodes storage for up to three months at 22°C without significant reductions in viability (Kagimu and Malan, 2019). The fundamental requirements of a successful entomopathogenic nematode formulation would be those which not only maintain nematode viability but also maintain nematode pathogenicity. The pathogenicity of the entomopathogenic nematodes can be tested with one-on-one bioassays (Ricci et al., 1996). This allows for a determination of whether the nematodes have retained their pathogenicity, and also it gives an indication of the virulence of the formulated nematode IJs it terms of how rapidly it causes the death of its insect host. It has been noticed that the longer the nematodes remain in formulation the greater the likelihood of their pathogenicity decreasing, and also the likelihood in the decline in the level of their lipid energy reserves, resulting in turn in a reduction in their vigour.

It should be noted that with respect to the soil environment both plants and soil-dwelling invertebrates are subjected to regular and fluctuating extremes of temperature and low moisture stresses. Most animals including nematodes die when losing between 15 and 20% of their body water content (Barrett, 1982). However, many animals, especially soil-dwelling micro-fauna have evolved the capacity to survive conditions of extreme soil dehydration by entering into an anhydrobiotic state of dormancy or suspended animation (Keilin, 1959; Tyson et al., 2012).

Hypothesis

In the study, the effects of moisture loss on the survivorship of IJs in different formulation media were undertaken. The nematode used in the investigation as the model EPN were isolated from soil samples that had been previously subjected to prolonged dehydration.

4.2. Aims and Objectives

4.2.1. Aim

Investigation of the efficacy of different hydroscopic or water-absorbing powder-based formulations with regard to promoting EPN infective juvenile survivorship, viability, and infectivity at ambient temperatures in response to different rates of evaporative moisture loss from the formulation media in which the IIs were embedded.

4.2.2. Objectives

Evaluation of the efficacies of hydroscopic or water-absorbing powdered formulations based on different combinations of diatomaceous earth, crystalline cellulose, and clay on IJs survivorship, viability and infectivity/pathogenicity at ambient temperatures in response to different rates of evaporative moisture losses under two regimes of relativity humidity.

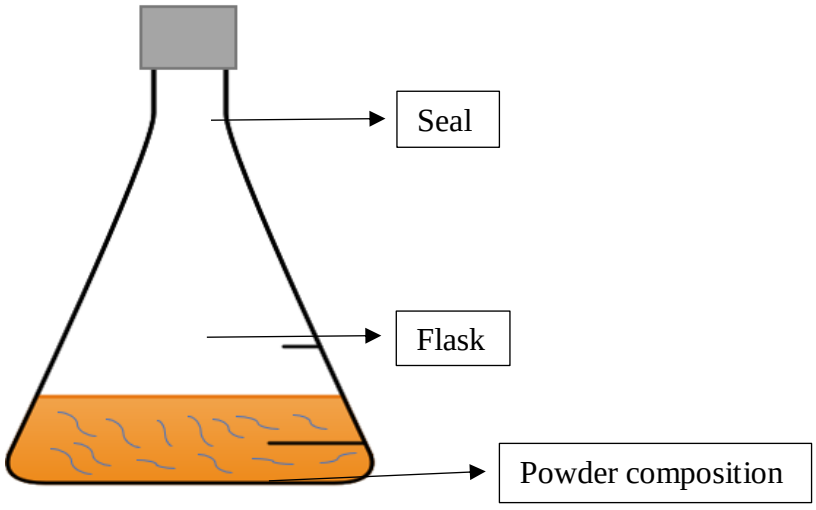
4.3. Methods and Materials

4.3.1. Model Entomopathogenic Nematode

In the study, *H. bacteriophora* was isolated from soil stored in 1 litre plastic tubs which had previously been collected from an undisturbed grassland south of Johannesburg. The soil had been completely dry for more than two years. The soil in the tub was rehydrated for EPN isolation. *H. bacteriophora* IJs were isolated from the soil by baiting with last-instar larvae of *G. mellonella* according to the procedures described in Woodring and Kaya (1988) (Woodring and Kaya, 1988). The infected cadavers were be placed on White traps in order to recover the IJs as previously described in Chapter 2. All procedures were carried out at 25°C.

4.3.2. Formulation Treatments

The hydroscopic powders that were used for the formulation were diatomaceous earth (DE), crystalline cellulose (CE), and clay (CA). After mixing the IJs with water-saturated formulation media, the viability, survivorship, and virulence of the IJs in response to moisture loss from the formulation medium was monitored. At set time intervals the formulation medium was rehydrated to its original water content and the viability and pathogenicity or virulence of the



IJs was evaluated. Moisture loss was mor rically. In each formulation treatment, the combined initial mass (formulation medium + water + 40 000 IJs) was 10 g. The underlying

Figure 4.3.1. Graphical representation of experimental setup.

hypothesis was that with a gradual loss of moisture the IJs would adapt to the loss of moisture in the same fashion as would be the case in the natural soil environment. It was also hypothesized that the interstitial nature of the formulation medium would simulate that of the natural soil environment to varying degrees. The aim of these experiments was to ascertain whether anhydrobiosis or desiccation tolerance could be induced in a controlled manner. The nematode and formulation mixtures were added to the Erlenmeyer flasks (Figure 4.3.1). The experiments were conducted in triplicates and kept at 25°C. Nematode viability was determined at set time intervals by direct counting under a dissecting microscope. Statistical analysis was conducted using repeated-measures ANOVA analysis with MaxStat®. Repeated measures ANOVA was used to compare the data of identical samples/populations observed certain

time/observational points (Thomas and Zumbo, 2012). The null hypothesis of the experiment is that there are no differences between the population means.

In the first experiment, IJs were subjected to the following four formulation treatments: diatomaceous earth (DE) only, cellulose (CE) only, clay (CA) only, and a mixture of diatomaceous, cellulose and clay in a mass ratio of 1:1:1. Suspension of IJs in water was used as the control. The experiment was conducted under the prevailing ambient relative humidity. A moistened cotton-wool ball was used to close the opening of the flask to slow down the rate of evaporative moisture loss from the Erlenmeyer flask. However, the cotton-wool plug was allowed to dehydrate so as not to restrict moisture loss from the formulation media, thereby facilitating the induction of IJ anhydrobiosis or moisture stress tolerance.

In the second experiments the formulation treatments were made up as follows: diatomaceous earth (DE), cellulose (CE) and clay (CA) were mixed in a 1:1:1 ratio (1CE: 1DE: 1CA) and 1:2:1 ratios (1CE: 2DE: 1CA; 1DE: 2CE: 1CA; 1CE: 2CA: 1DE) on a mass basis with a total formulation mass (formulation medium + water + nematodes) per Erlenmeyer flask being 10 g. As with experiment one, the moisture loss and dehydration treatment were conducted under ambient relative humidity conditions. However, the Erlenmeyer cotton-wool plug was kept hydrated so that the relative humidity of the atmosphere within the flask remained higher than the ambient relativity in the environment external to the flask. As in experiment one, the concentration of IJs used for all the treatments was 40 000 IJs per 10 g of hydrated formulation medium.

4.3.3. Pathogenicity of formulated nematodes

The IJ pathogenicity bioassay experiments were performed in sterile 24 well plates. Sterilized river sand with a moisture content of 8% w/v was placed in each well. The formulated IJs were surface sterilized with 0.03% (v/v) sodium hydrochloride solution. The sterilised IJs were used to inoculate each well. A single larva was placed in each well and the pathogenicity of the formulated nematodes was determined by monitoring larval mortality over time. The experiments were conducted at 25°C. Observations on mortality were done at 24 intervals until complete mortality was reached. Statistical analysis was conducted using repeated-measures ANOVA on MaxStat®.

4.4. Results

4.4.1. Formulation

Experiment one: Infective juvenile viability in various unmixed or pure hydrated powdered formulations and in a mixed formulation consisting of equal parts of cellulose, diatomaceous earth and clay (1CE: 1DE: 1CA) were initially recorded by counting the number of viable IJs at the start the experiment and then counting again at days 4 and 8 (Figure 4.4.1.).

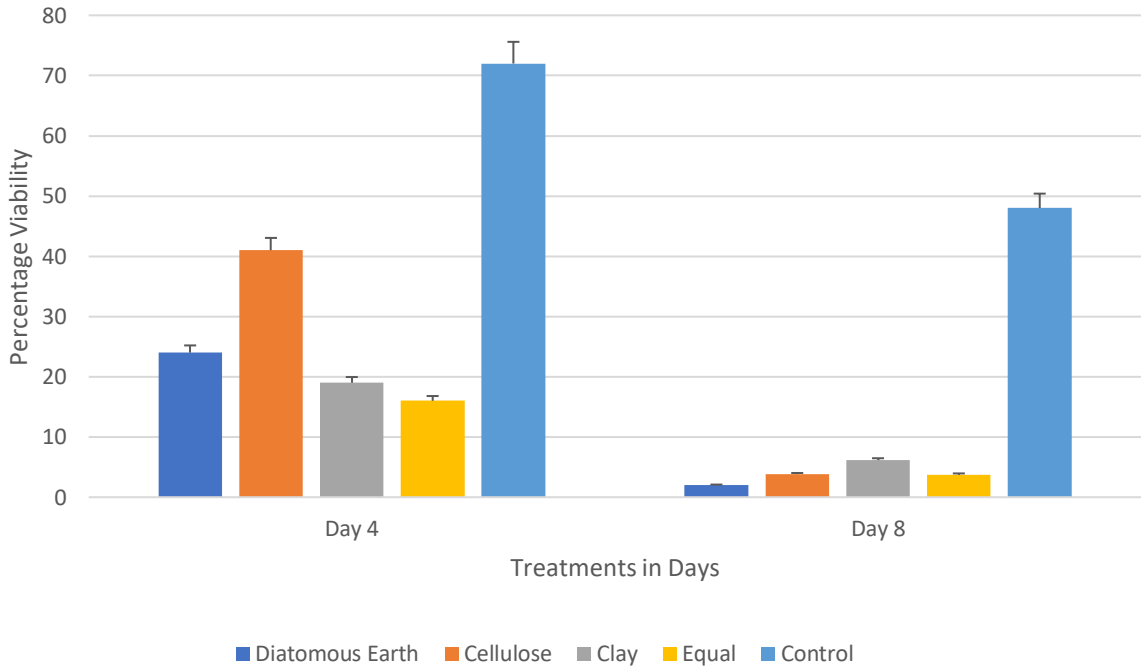


Figure 4.4.1. The mean percentage of viable *Heterorhabditis bacteriophora* infective juveniles recovered (95 % confidence level) from formulations that had undergone moisture loss. The infective juveniles were formulated in diatomaceous earth, cellulose, and clay. The infective juveniles were also formulated in a mixture consisting of equal proportions of diatomaceous earth, cellulose, and clay. Infective juvenile viability was estimated after 4 and 8 days after the flasks had been allowed to equilibrate in an external environment of ambient humidity at 25°C ($F = 16.947$; $p < 0.05$). Error bars show standard error of the mean. The length of the bars indicates how far from the true mean value the samples deviate.

By day eight IJ viability decreased dramatically in all formulation treatments, that is, in diatomaceous earth and cellulose and clay, and finally, also in the 1:1:1 mixture of diatomaceous earth, cellulose, and clay, especially after the percentage moisture content of all formulations had fallen to below 50% of its original value (Figure 4.4.1). The control in which IJs were suspended in water gave the highest percentage viability after eight days at 25 °C. A higher percentage IJ survival was expected at least in the combined cellulose, diatomaceous and clay formulation on the premise that the interstitial spatial and volumetric geometries within the mixed formulation would have had sufficient surface-bound water (matrix water

potential) which would have been in equilibrium with the IJs internal water potential and that this should have been conducive to facilitating the induction of IJ dehydration stress tolerance. However this did not seem to be the case, and so the formulations did not effectively simulate the interstitial soil environment as was hypothesized. At this juncture, one needs to be reminded of the fact that on a routine basis in the laboratory, for the preservation and curating of the EPN collections, White trap isolated IJs have been regularly added directly to soils in 1 L tubes, and the IJs remained viable for lengthy periods of time even after the soils had become completely dehydrated. The original IJs could be recovered from the soils following the rehydrating and larval baiting of the soils. This could not be replicated in the above experiment. The null hypothesis of the experiment is that there are no differences between the population means. Therefore, the results of the statistical analysis indicated the rejection of the null hypothesis. Therefore, there are differences in the population means.

Experiment two: The viability results for the IJs which had been kept in mixed powder formulations maintained at higher relative humidity within the flasks are given in Figure 4.4.2. To reiterate, in this experiment there were three treatments with 1:2:1 proportion of CE, DE, and CA and one treatment with 1:1:1 proportion of CE, DE, and CA under relativity humidity conditions higher than the external ambient humidity. The control involved suspending IJs in water. Compared to the previous results (Figure 4.4.1) the IJs remained viable for a longer period of time when the humidity within the flask was maintained at a level higher than the external ambient humidity (Figure 4.4.2). Keeping the cotton-wool plug hydrated slowed down the rate of moisture loss from the formulation mixtures. The viability of IJs in the powder formulation of equal composition treatment 1CE:1DE:1CA) and in the treatment with higher cellulose content (1DE:2CE:1CA) dropped below 50% on day 12 compared to the other two formulation treatments in which IJ viability dropped below 50% only by day 20 (Figure 4.4.2 shown on next page). The results of the statistical analysis indicated that the rejection of the null hypothesis. Therefore, there are differences in the population means.

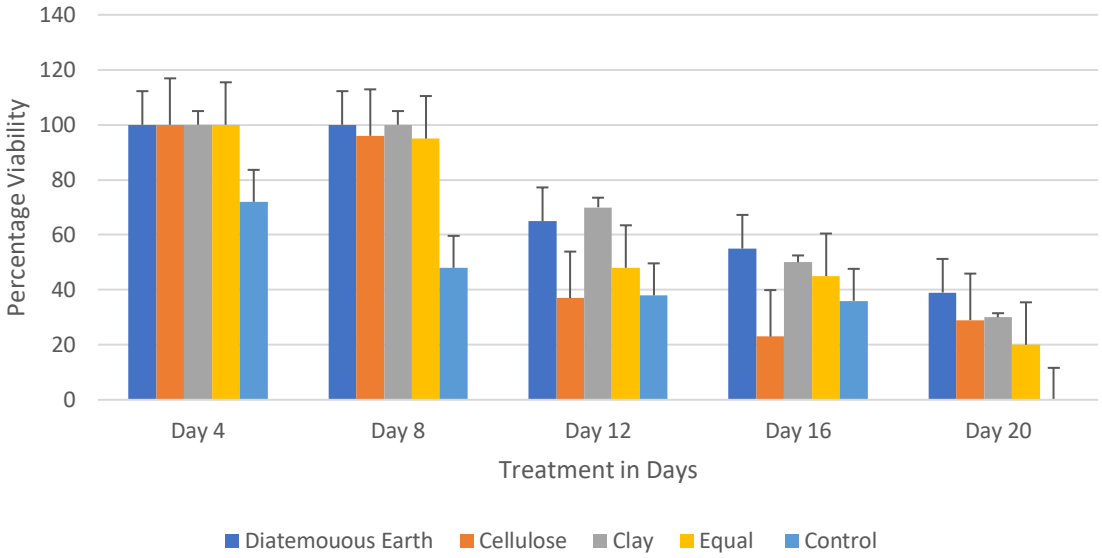


Figure 4.4.2. The mean percentage viability of *Heterorhabditis bacteriophora* infective juveniles recovered (95 % confidence level) from the different formulation mixtures at the different time intervals over a 20 day period. The histograms show the effects of proportionally higher clay content, higher diatomaceous earth content, and higher cellulose content compared to the formulation mixture which was comprised of equal amounts of CE, DE, and CA. ($F = 8,904$; $p < 0,05$). Error bars show standard error of the mean. The length of the bars indicates how far from the true mean value the samples deviate.

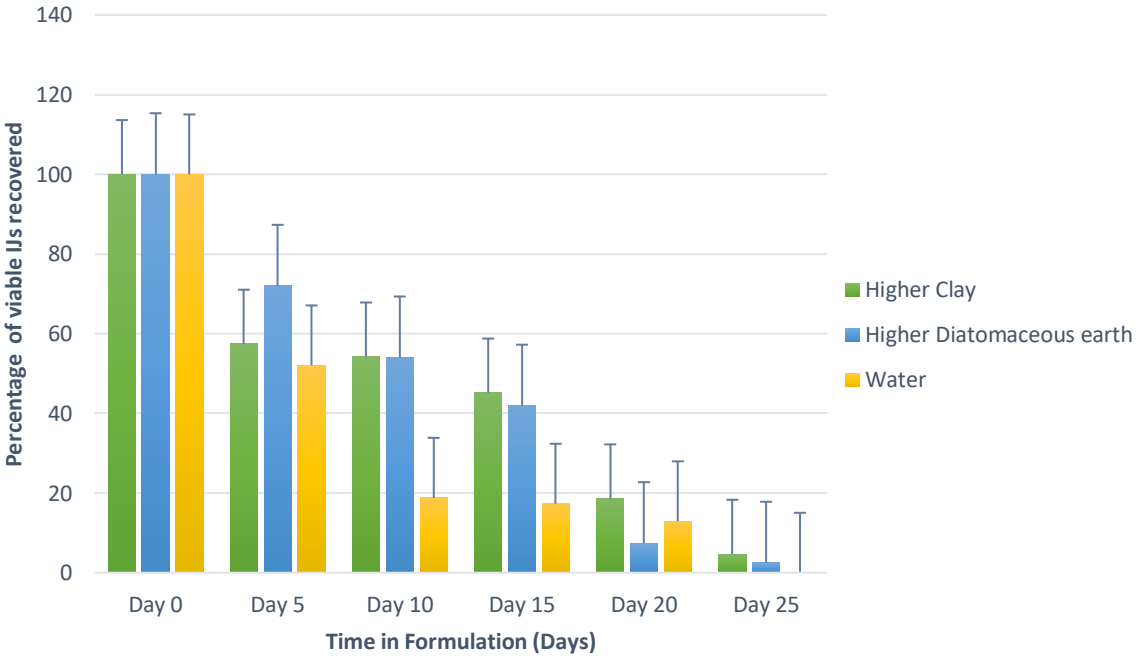


Figure 4.4.3. The mean percentage of viable infective juveniles recovered (95 % confidence level) of *Heterorhabditis bacteriophora* infective juveniles in higher clay content, higher diatomaceous earth content, and control over a period of 25 days ($F = 3.711$; $p > 0.05$). Error bars show standard error of the mean.

Experiment three: Further studies were conducted into IJ viability in the following formulation treatments 1CE:2DE:1CA, 1CE:2CA:1DE and 1CE:1DE:1CA, at the higher relative humidity with an ambient temperature of 25°C over a period of 25 days. Infective juvenile viability and infectivity in higher clay (1CE:2CA:1Da) and higher diatomaceous earth (1CE:2DE:1CA) formulations were observed given for *H. bacteriophora* (Figures 4.4.3). The recovery of viable IJs was recorded every five days for a period of 25 days. The recovery of IJs from the higher clay formulation was recorded as percentages. From day five to 25 days, there is a decrease from approximately 57% to less than 5%. Recovery from higher diatomaceous earth formulation was approximately 71% after five days to less than 3% on the last day. Therefore, survival in both formulations decreased with increasing time in the formulation.

The repeated-measures ANOVA was conducted to determine whether the average difference between infective juvenile viability in formulation compositions of higher clay, higher diatomaceous earth, and water was significant. Analysis of both experiments indicated a failure to reject the null hypothesis. There is no significant difference between higher formulation of clay (mean = 118.750; standard deviation = 12.5), higher composition of diatomaceous earth (mean = 1195.75; standard deviation = 2258.659) and water control (mean = 75.00; standard deviation = 50.00) with $F = 3.711$ and $p > 0.05$. Therefore, there is no significant difference between infective juvenile viability in formulation compositions of higher clay or higher diatomaceous earth.

4.4.2. Pathogenicity of formulated nematodes

The pathogenicity of the infective juveniles recovered from the different formulations was determined with the use of 24 well bioassays (Figure 4.4.4). Following the death of larvae, the cadavers were placed on White traps and the IJs retrieved from White traps within three days, confirming the larval death was due to IJ infection. The influence of formulation treatments on IJ infectivity was established. Complete larval mortality was observed after 120 hours following exposure to infective IJs recovered from higher diatomaceous earth formulation. Complete larval mortality was observed after 96 hours following exposure to IJs recovered from higher clay formulation. Larvae infected with IJs recovered from water controls showed complete mortality after 72 hours. The repeated-measures ANOVA was conducted to determine the average difference between infective juvenile pathogenicity in formulation compositions of higher clay content, higher diatomaceous earth content, higher cellulose content, and water. Analysis of both experiments indicated a failure to reject the null

hypothesis. There is no significant difference between all compositions. Therefore, there is no significant difference between in infective juvenile pathogenicity in formulation compositions of higher clay and higher diatomaceous earth.

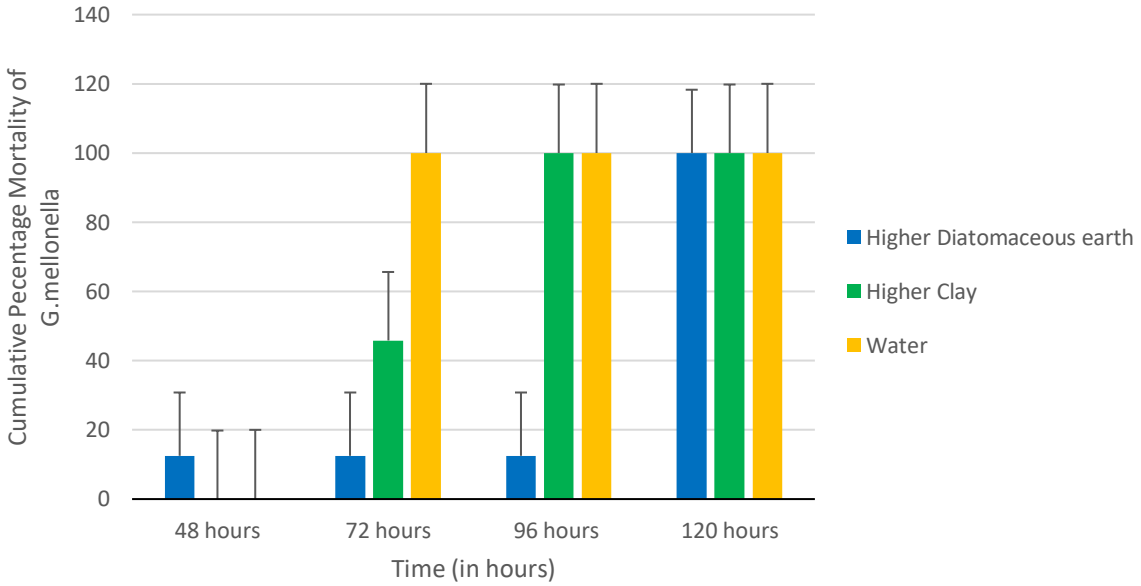


Figure 4.4.4. Mean percentage mortality (95% confidence level) of *Galleria mellonella* inoculated with the infective juveniles of *Heterorhabditis. bacteriophora* following formulation and storage in higher clay and higher diatomaceous earth formulation treatments, and compared to the water control ($F = 1.806$; $p > 0,05$). Error bars indicate the standard error of the mean. The length of the bars indicates how far from the true mean value the samples deviate.

4.5. Discussion

A reliable EPN formulation should make long term nematode survival and maintenance of infectivity within the specified formulated environments possible. The commercial use of entomopathogenic nematodes has encountered problems of stability in storage and shelf-life. It seems that the presence of water or a certain critical level of moisture within the formulation medium was an essential requirement for the long term viability of nematodes. The level of moisture content within the formulation medium, especially a commercial formulation medium, would need to be greater than the merely bound water or moisture only at the level of the matric potential. This would suggest that the interstitial spatial surfaces would be covered by a film of water sufficient for movement or mobility of Ifs within the formulation matrix. Usually, EPNs have been supplied in a moist gel-like formulation within a sealed sleeve by various vendors. The user instructions usually advise the customer that the product is kept refrigerated. The EPN formulations products which are currently commercially available from

a number of manufacturers and suppliers are generally packaged in moist gel-like formulations contained within the water and air impermeable sleeves have a limited shelf-life. However, their un-refrigerated or ambient temperature shelf-life is short relative to the refrigerated shelf-life (data not shown).

This observation regarding the lack of long term viability of nematodes within an artificial formulated environment at ambient temperatures is not consistent with the fact that IJs in nature are able to survive for long periods in soils that have become completely dehydrated and are subjected to extremes of high and low temperatures The survival of infective juveniles is also dependent on their energy reserves once environmental conditions have become detrimental (Perry et al., 2012).

It is therefore important that these two factors (induction of anhydrobiosis or desiccation tolerance and conservation of lipid reserves) are kept in mind with regard to IJ survival and pathogenicity of the nematodes in the formulation. An option for IJ formulation is to encapsulate or create an environment or matrix that avoids the stresses caused by changes in the biotic and abiotic factors such as low moisture, extreme temperatures, exposure to UV light, sudden desiccation and contamination by opportunistic microorganisms (Hazir et al., 2004). This will improve stability in storage and longer shelf life. Quantifying the viability of infective juveniles has been determined by the percentage moving/living infective juveniles versus those that are dead. This investigation determined the survivorship and infectivity of *H. bacteriophora* infective juveniles in two different wet absorbing or hydroscopic mixed powder formulations at room temperature. In this study, the survival of the infective juveniles of *H. bacteriophora* was observed to be dependent on the type of formulation, humidity, and formulation moisture content. The results show that infective juveniles remained viable for a longer period of time in an environment under higher than ambient relative humidity. The two types of formulations showed that they both performed equally well in maintaining the viability of the infective juveniles. The drop in viability after two weeks indicated that the IJs may not have properly entered the state of partial anhydrobiosis or dehydration stress tolerance. It seems that the controlled induction of anhydrobiosis or desiccation tolerance will remain a fundamental goal in the development of a formulation for EPN IJs. Therefore, further studies need to be conducted to improve *H. bacteriophora* storage capabilities. A similar study conducted by Kagimu reported similar findings to this study (Kagimu and Malan, 2019). Infective juveniles formulated in diatomaceous earth showed a steady decrease in viability and this may have been due to the consistency of the powder (Kagimu and Malan, 2019). Granule

structure, homogeneity of the EPNs and the resistances experience while in formulation plays a role in the efficacy of EPNs. Therefore, changes in the formulations are required to extend the infective juveniles' stability in storage. The results from this study suggest that the pathway towards the successful induction of IJ desiccation tolerance or dehydration resistance within the various formulation media requires the gradual loss of water in order to allow for the necessary metabolic and physiological changes to take place. The successful induction of partial anhydrobiosis or desiccation tolerance in infective juveniles such that their viability and infectivity be preserved on recovery following rehydration is also dependent on storage reserve stability (Grewal et al., 2006). Important metabolic or biochemical factors that also play a significant role in dehydration tolerance include trehalose accumulation. Trehalose is an example of a carbohydrate which plays an important physiological role in nematode desiccation tolerance. The mechanism responsible for the trehalose effects involves the substitution of structural water associated with the lipid bilayers, and thereby maintaining the lipid bilayer in a liquid crystalline form, thereby preventing vitrification, which involves the conversion of lipid liquid crystalline structures to amorphous glass-like structures which are not conducive for cell survival under desiccation stress or under freezing (O'leary et al., 2001). *H. bacteriophora* is known to require a slower rate of water loss compared to steinernematids species (Inman et al., 2012). This may be due to *H. bacteriophora* employing a cruiser type of foraging strategy and which results in the exploration of deeper soil layers where the level of humidity is higher and more constant.

4.6. Conclusions

Different formulations strategies are required for the development of an appropriate formulation for different entomopathogenic nematodes. Important considerations that need to be properly understood concern the physical nature of the natural environment in which nematodes exist and the nature of the biotic and abiotic factors that are encountered by the nematodes. The ranges in soil temperature, soil moisture, and soil humidity, and also the foraging strategies of the nematodes need to be known in order to fully understand the kind of environment in which the prospective formulation would be required to simulate. There are several areas that can be further investigated in future studies. Determination of the appropriate method for the induction of partial anhydrobiosis or desiccation tolerance of *H. bacteriophora* infective juveniles is one key area. The level of relative humidity that will lead to the improvement in the stability of the infective juveniles in the formulation and ultimately in long

term storage is another factor. An assessment of the possible microbial contaminants that the infective juveniles may encounter in a formulation is also an important consideration.

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5. Chapter Five: Study of a possible new Steinernema species in South Africa

5.1. Introduction

The discovery of EPNs as potential biological control agents has led to the isolation and identification of EPNs on every continent except for Antarctica (Kaya et al., 2006; Popiel and Hominick, 1992). Thus far, EPNs have been assigned to two families, Steinernematidae and Heterorhabditidae, with both families falling into the order Rhabditida (Kaya et al., 2006). These species are mutually associated with their insect pathogenic bacterial symbionts, *Xenorhabdus,* and *Photorhabdus*, respectively (Burnell and Stock, 2000). The life cycle of the entomopathogenic nematodes starts with the bacterial symbiont-colonized, non-feeding developmentally-arrested third-stage infective juveniles living within the soil. This is the only 'free-living' stage within the life cycle (Hazir et al., 2004). In *Steinernema* species, the entomopathogenic bacterial symbiont which colonizes the IJs, forming a mutualistic relationship with the nematode, are housed or carried by its nematode vector in a specialized receptacle structure associated with the digestive tract until an insect host is located and infected by the IJ (Chaston et al., 2011). The infective juveniles search for an insect host using either physical or chemical cues (Lewis et al., 2006). Once the host has been located, the infective juvenile enters the host through natural openings and releases its bacterial symbiont into the haemocoel. The multiplication of the bacteria within the host leads to the release of toxins, enzymes, insecticidal and antimicrobial compounds. The various toxins, proteases, and antimicrobial agents released by the bacteria overwhelm the insect host immune system, breakdown tissues, and prevent the opportunistic colonization by other microorganisms (Burnell and Stock, 2000). Following the infection of an insect host, the previously developmentally-arrested IJs undergo development into sexually reproductive adults and reproduction proceeds for two to three generations until the food supply is depleted. At this stage, the infective juveniles emerge from the insect cadaver and continue to search for another insect host or enters a state of dormancy if the environmental conditions are no longer conducive for host foraging**.**

The family Steinernematidae consists of two genera, *Steinernema,* and *Neosteinernema* (Nguyen and Smart Jr, 1994; Poinar Jr and Grewal, 2012). The genus *Neosteinernema* is comprised of one species, *N. longcurvicauda*. The amphimictic mode of reproduction for *Steinernema* requires the involvement of at least two infective juveniles, therefore, increasing the chances of invasion by both males and females (Rolston et al., 2006). The only exception

to this is *S. hermaphroditum*, whose infective juveniles develop into self-fertilising hermaphrodites (Chaerani and Stock, 2004). The host range of *Steinernema* is variable as most species have been isolated in the laboratory using *Galleria mellonella* as the baiting insect. Therefore, the determination of the natural host range will be biased towards the model insect hosts chosen for the bioassays of insect pathogenicity or for the isolation of putative entomopathogenic nematodes from soil samples. Nematodes belonging to the genus *Steinernema* are not easily identified solely by means of morphology or morphometric characterization. Accurate identification also requires the use of molecular techniques such as sequencing and base-pair sequence alignment of selected species-specific marker genes**.** Genes encoding small subunit ribosomal RNA (SSU rRNA) are usually present in the genomes of most metazoan. They are found in high copy numbers, have both conserved and variable regions and have consequently become one of the preferred marker genes for nematode identification and also for the phylogenetic analysis of phylum Nematoda (De Ley and Blaxter, 2004; Holterman et al., 2006). The two variable internal transcribed spacer (ITS) regions within the repetitive copies of EPN rDNA which are in turn flanked by the conserved nuclear 18S and 28S ribosomal DNA genes confirms the observation that the combination of the conserved sequences of the 18S and 28S regions and the polymorphic or highly variable ITS sequences confers on rDNA the attribute of being an ideal and unique species-specific genetic fingerprint and thus fulfils the role of a suitable and reliable species-specific genetic marker for most molecular-based identification of nematodes (Holterman et al., 2006; Liu et al., 2000).

In South Africa, the following new steinernematid species have been described: *S. khoisanae* (Malan et al., 2006), *S. citrae* (Malan et al., 2011), *S. sacchari* (Nthenga et al., 2014), *S. tophus* (Cimen et al., 2014), *S. innovationi* (Çimen et al., 2015), *S. jeffreyense* (A. P. Malan et al., 2016), *Steinernema nguyeni* (Malan et al., 2016), *S. beitlechemi* (Cimen et al., 2016a), *S. fabii* (Abate et al., 2016) and *S. biddulphi* (Cimen et al., 2016). The genus *Steinernema* remains the most speciose of the two EPN genera, possibly containing a hundred (Bhat et al., 2020). In this study, a putative new steinernematid species have been described. The nematode was isolated from a previously collected soil sample which has been stored in a dehydrated state for more than two years.

5.2. Aims and Objectives

5.2.1. Aims

Description of new uncharacterised *Steinernema* species.

5.2.2. Objectives

The objectives have been summed up as follows: a) Isolation of adults and infective juveniles from infected cadavers for the preparation of slides for each life cycle stage. b) Light microscopy-based description of the gross morphology of adults and infective juveniles under a light microscope. c) Light microscopy-based morphometric characterization of adults and infective juveniles.

5.3. Methods and Materials

5.3.1. Entomopathogenic nematode isolation source and culture

The soil samples that were used to isolate entomopathogenic nematodes were collected in Brits. The isolate was named *Steinernema sp LTV*. The locality was in the bushveld in the Brits area in the North West Province., South Africa. The coordinates were 25.6100° S, 27.7960° E. The annual average temperature ranged from 5°C - 29°C. The annual average precipitation was approximately 620 mm. The soil type sampled was dark brown (humus stained) sandy-loamy soil. The soil sample had been previously collected from the above location and had been kept in storage in 1-litre plastic tubes for more than two years. The soil from which the nematodes were isolated had been in a dehydrated or desiccated state for a prolonged period of time. This observation confirms that entomopathogenic nematodes and the species under study can survive extreme soil dehydration which is strongly indicative of their capacity to undergo anhydrobiosis or drought-tolerance as a survival strategy (Tyson et al., 2012).

5.3.2. Isolation of EPNs from infected larvae by dissection and the White Trap Method

First-generation adults were isolated from the cadaver, by dissection in sterile distilled water, three days post-infection. Second-generation adults and infective juveniles were retrieved from the White traps (White, 1927). Nematodes were allowed to settle in 50ml Falcon tubes filled with 0.03% v/v of sodium hypochlorite solution to surface sterilise the IJs for one hour.

5.3.3. Morphological characterisation

5.3.3.1. Heat-killing and fixing nematodes

The nematodes were heat-killed by suspension in 60 ºC distilled water for two minutes. The water was discarded after two minutes and replaced with single strength triethanolamine formalin (TAF) that had been heated at 60°C for ten minutes for the fixation process. The nematodes were then placed on ice for fifteen minutes to relax the structure of the nematodes. Concurrently, double strength TAF was heated at 65°C for fifteen minutes. The double-strength TAF was added to the nematodes and incubated for 48 hours at 25°C in the dark.

5.3.3.2. Light Microscopy

The quantification of morphometric parameters was done by the mounting of the fixed nematodes onto microscope slides. The prepared slides were viewed using an Olympus BX63/OFM microscope fitted with a Nikon camera for recording anatomical features of first- and second-generation adults and infective juveniles.

5.4. Results

5.4.1. Description

5.4.1.1. Infective juveniles

The shape of infective juveniles after heat killing was almost straight, slender, gradually tapering posteriorly. The head region was continuous with the body. The nerve ring was observed to encircle the isthmus region of the pharynx. The pharynx was long, narrow with slightly expanded procorpus. The excretory pore was located approximately mid-corpus. The tail region was conoid with pointed terminus.

5.4.1.2. First-generation females

The shape of the first-generation female after heat killing was C-shaped, or strongly spiralled.

The head region was rounded and continuous with the body. The body length was larger than second-generation females (average length = 7969 µm; Std Dev = 1012). The pharynx was muscular with cylindrical procorpus; swollen metacorpus; distinct isthmus. The excretory pore was located about the mid procorpus level or surrounding isthmus. The nerve ring surrounded

the isthmus, just anterior to the basal bulb. The tail was blunt and conoid without a mucron. The vulva was located near the middle of the body with slightly protruding lips and mostly symmetric.

5.4.1.3. Second generation females

The second-generation females had an open C-shaped body when killed. Smaller than first-generation females. Vulva on asymmetrical protuberance and situated at mid-body. Post anal swelling present. Tail tapering gently to a sharp point.

5.4.1.4. First-generation males

The shape of first-generation males after heat killing was J shaped and posterior was ventrally curved. The head was truncated to slightly round and continuous with the body. The body length was larger (average = 1953 µm; Std Dev = 157) than second-generation males. The nerve ring was located about mid isthmus level/anterior part of the basal bulb. The pharynx was muscular, cylindrical and isthmus was present. The excretory pore was posterior to the nerve ring located mostly in the vicinity of but sometimes posterior to the basal bulb. The tail region was conoid without mucron. The spicule was paired, symmetrical, curved with ochre brown colouration and the manubrium of the spicules was at times elongate. The gubernaculum was boat-shaped and 3/4 the length of spicules.

5.4.1.5. Second generation males

The second-generation males are similar to first-generation male except body length (121µl; Std Dev = 27) shorter and body diameter (81µl; Std Dev = 13) less.

5.4.2. Type host and locality

Steinernema sp LTV was collected from a soil sample in the Brits area, North West province, South Africa, by means of bait trapping using larvae of *Galleria mellonella*. The nematode was collected from an area in which bushveld vegetation was predominant, at 25.6100° S, 27.7960° E. The natural host is unknown.

5.4.3. Molecular characterisation

5.4.3.1. Phylogenetic Analysis

Phylogenetic analysis of the new isolate, *Steinernema sp LTV*, and various species belonging to the *Steinernema glaseri* group was performed by MEGA7 and the Maximum Likelihood method was used for the analysing the relationships of the phylogenetic relationships between

related species. The phylogenetic tree shows that the *Steinernema sp LTV* formed a separate branch with no clustering of other species. The closest species to *Steinernerma* sp *LTV* was *Steinernema khoisanae* (isolate SF87), *Steinernema innovation*, *Steinernema jefferyense*, and *Steinernema ethiopense* (Figure 5.4.1). See chapter 2 for the methods used.

5.4.3.2. Evolutionary distances

The evolutionary distances of the four *Steinernema spp* that have been identified in South Africa show that they are all separate species. The rate of base substitutions per site in relation to *S. sp LTV* and *S. jeffereysense*, *S. khoisanae*, and *S. innovationi* is 0.171, 0.189, 0186 respectively (Table 5.4.3.).

Table 5.4.1. Morphometrics of *Steinernema sp LTV*. Measurements are in μm and expressed in the form: mean ± sd (range).

Characterisation	**Holotype**	**Infective juvenile**	**First Generation**		**Second Generation**	
			Female	**Male**	**Female**	**Male**
Number of Specimens		20	20	20	20	20
Body Length (L)	2212	975 ± 72 (862 - 1120)	7969 ± 1012 (6295 - 9515)	1953 ± 157 (1609 - 2151)	2943 ± 330 (2375 - 3728)	1560 ±127 (1272 – 1761)
Body Width (W)	147	51 ± 3 (43 - 47)	255 ± 54 (186 - 357)	121 ± 27 (86 - 219)	195 ± 13 (173 - 221)	81 ± 13 (58 – 109)
Anterior end to Excretory Pore (EP)	130	115 ± 27 (88 - 142)	175 ± 54 (69 - 306)	135 ± 20 (94 - 166)	168 ± 12 (145 - 189)	125 ± 1 (124 – 126)

Characterisation	**Holotype**	**Infective juvenile**	**First Generation**		**Second Generation**	
			Female	**Male**	**Female**	**Male**
Number of Specimens		20	20	20	20	20
Pharynx Length (P)	110	108 ± 12 (83 - 126)	154 ± 15 (129 - 183)	123 ± 9 (114 - 132)	137 ± 13 (113 - 164)	115 ± 10 (87 – 129)
Tail Length (T)	35	19 ± 8 (10 - 38)	40 ± 12 (18 - 70)	33 ± 5 (23 - 39)	25 ± 7 (11 - 38)	24 ± 4 (16 - 35)
Anal Body Width (ABW)	98	50 ± 7 (43 - 58)	119 ± 19 (80 - 155)	94 ± 7 (83 - 112)	80 ± 10 (65 - 98)	164 ± 11 (133-181)
Spicule Length (S)	85	-	-	86 ± 4 (81 -93)	-	80 ± 12 (62 - 103)
Gubernaculum Length (G)	44	-	-	41 ± 5 (30 - 50)	-	38 ± 5 (31 – 49)
Anterior end to Vulva (V)	-	-	3148 ± 616 (2418 - 5341)	-	1356 ± 230 (1019 - 2025)	-
Anterior end to Nerve Ring (NR)	-	141 ± 8 (133 - 148)	227 ± 15 (207 - 267)	167 ± (147 - 187)	203 ± 17 (186 - 221)	164 ± 11 (133 – 181)
a = L/W		19 ± 23 (20 – 23)	31 ± 19 (27 - 34)	16 ± 6 (10 - 19)	15 ± 25 (17 - 182)	19 ± 10 (16 – 22)

Characterisation	Holotype	Infective juvenile	First Generation		Second Generation	
			Female	Male	Female	Male
Number of Specimens		20	20	20	20	20
b = L/ES		9 ± 6 (8 - 10)	52 ± 68 (49 - 52)	16 ± 18 (1 - 16)	21 ± 25 (21 - 23)	14 ± 12 (14 – 16)
c = L/TL		50 ± 9 (29 - 90)	200 ± 85 (135 - 341)	60 ± 32 (55 - 69)	116 ± 48 (98 - 218)	65 ± 32 (50 – 78)
c’ = L/ABW		19 ± 10 (19,95 - 19,41)	67 ± 54 (61 - 79)	20 ± 21 (19,34 - 19,09)	37 ± 34 (36 - 38)	10 ± 12 (9,56 - 9,71)

Table 5.4.2. Comparative morphometrics of the first-generation males and third-stage infective juveniles of *Steinernema* spp in the glaseri group.

	Infective Juveniles			First Generation Males		
Species	**Body Length**	**Excretory Pore**	**Body Width**	**Tail Length**	**Spicule**	**Gubernaculum**
***S. boemarei* (Lee et al., 2009)**	1,103 (1,005–1,3230	91 (82–111)	96 (70–122)	40 (32–46)	79 (64–96)	52 (43–65)
***S. diaprepesi* (Nguyen and Duncan, 2002)**	1,002 (880–1,133)	74 (66–83)	**113 (90–145)**	25 (20–32)	79 (71–90)	54 (45–61)

	Infective Juveniles			First Generation Males		
Species	**Body Length**	**Excretory Pore**	**Body Width**	**Tail Length**	**Spicule**	**Gubernaculum**
***S. hermaphroditum* (Chaerani and Stock, 2004)**	928.5 (700-1100)	65 (62.5-68)		**33.5 (28-35)**	68 (65-70)	48 (47-50)
***S. khoisanae* (Malan et al., 2006)**	**1089 (966-1214)**	**92 (84-100)**	**108 (88-125)**	**37 (30-40)**	**79 (68-95)**	**51 (40-87)**
***S innovationi* (Çimen et al., 2015)**	**1054 (1000-1103)**	**88 (82–91)**	**139 (100–171)**	**51 (42–76)**	**81 (74–91)**	**58 (54–63)**
***S. australe* (Nguyen et al., 2009)**	1316 (1162-1484)	110 (95-125)	75 (61-97)	29 (20-35)	72 (55-78)	45 (36-51)
***S. braziliense* (Nguyen et al., 2010)**	1054 (1000–1103)	88 (82–91)	139 (100–171)	51 (42–76)	81 (74–91)	58 (54–63)
***S. lamjungense* (Moens et al., 2011)**	832 (690-950)	68 (61-73)	154 (116-205)	31 (21-38)	87 (81-94)	57 (50-66)
***S. guangdongense* (Qiu et al., 2004)**	1055 (987–1145)	80 (71-85)	112 (90–135)	31 (24–38)	86 (80–94)	64 (47–73)
***S. hebeiense* (Moens et al., 2006)**	658 (610-710)	48 (43-51)	86 (74-98)	30 (24-35)	57 (51-63)	46 (38-50)

	Infective Juveniles			First Generation Males		
Species	**Body Length**	**Excretory Pore**	**Body Width**	**Tail Length**	**Spicule**	**Gubernaculum**
***S. phyllophagae* (Buss and Nguyen, 2011)**	1289 (1133-1395)	99 (84-120)	65 (58-79)	28 (25-34)	72 (65-77	51 (46-56)
***S. pui* (Qiu et al., 2011)**	1004 (900–1120)	85 (80–95)	137 (118–180)	32 (29–38)	84 (78–88)	62 (58–65)
***S. longicaudum* (Nguyen and Smart Jr, 1994)**	1040 (931-1194)	76 (71-85)	137,5 (98-191)	46 (28-48)	84 (75-97)	63 (55-68)
***S. apuliae* (Triggiani et al., 2004)**	1064 (945 – 1212)	95 ± 4.2 (86 – 102)	104 (86 – 124)	38 (33 – 41)	72 (64 – 80)	50 (46 – 54)
S.khoisanae strain LTV	**975 (862 - 1120)**	**115 (88 - 142)**	**121 (86 - 219)**	**33 (23 - 39)**	**86 (81 – 93)**	**41 (30 - 50)**
***S. scarabaei* (Koppenhöfer and Stock, 2003)**	918 (890–959)	77 (72–81.5)			75 (67–83)	44 (36–50)
***S.jefferyense* (Malan et al., 2016)**	**926 (784–1043)**	**87 (78–107)**	**139 (94–167)**	**27 (21–33)**	**88 (79–95)**	**57 (51–61)**
***S.ethiopiense* (Tamiru et al., 2012)**	**898 (768–1010)**	**78 (65–84)**	**49 (46–57)**		**73 (69–77)**	**73 (69–77)**

Table 5.4.3. Estimates of evolutionary divergence with respect to polymorphisms in the ITS regions and also in the conserved regions of partial 18S and 28S ribosomal rDNA gene sequences of 19 Steinernema spp that belonging to the glaseri-group. The number of base substitutions per site between sequences is shown in black. The standard error estimate(s) are indicated in blue. Standard error estimate(s) are shown above the diagonal (in blue) and were obtained by a bootstrap procedure (1000 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. MH697401.2 Steinernema sp LTV		0,063	0,021	0,022	0,022	0,026	0,024	0,021	0,020	0,025	0,024	0,026	0,023	0,024	0,022	0,021	0,029	0,021	0,021
2. FJ589008.1 Caenorhabditis elegans	0,729		0,058	0,059	0,054	0,062	0,059	0,057	0,060	0,061	0,056	0,062	0,056	0,061	0,058	0,055	0,069	0,059	0,058
3. MF663703.1 Steinernema hermaphroditum	0,176	0,660		0,014	0,013	0,019	0,014	0,009	0,009	0,019	0,016	0,021	0,014	0,015	0,014	0,014	0,022	0,010	0,009
4. KJ578793.1 Steinernema innovationi	0,186	0,679	0,084		0,014	0,020	0,015	0,015	0,015	0,022	0,018	0,021	0,018	0,017	0,015	0,015	0,027	0,015	0,015
5. KC897093.1 Steinernema jeffreyense	0,189	0,642	0,075	0,099		0,019	0,016	0,013	0,014	0,021	0,017	0,024	0,016	0,017	0,016	0,012	0,023	0,014	0,013
6. JN651414.1 Steinernema sp. TT-2011	0,233	0,704	0,134	0,148	0,143		0,021	0,019	0,019	0,026	0,020	0,024	0,019	0,022	0,019	0,019	0,027	0,018	0,018
7. HQ416968.1 Steinernema apuliae	0,222	0,679	0,080	0,104	0,102	0,166		0,016	0,016	0,022	0,019	0,023	0,017	0,014	0,014	0,017	0,026	0,017	0,015
8. HM000101.1 Steinernema lamjungense	0,180	0,654	0,040	0,096	0,075	0,133	0,100		0,011	0,019	0,016	0,023	0,016	0,017	0,013	0,014	0,023	0,010	0,009
9. GU395618.1 Steinernema sp. YNd393	0,169	0,674	0,040	0,097	0,090	0,136	0,107	0,051		0,018	0,016	0,022	0,015	0,016	0,014	0,014	0,024	0,011	0,009
10. FJ410327.1 Steinernema phyllophagae	0,236	0,712	0,141	0,169	0,169	0,221	0,167	0,141	0,133		0,019	0,025	0,019	0,020	0,019	0,021	0,030	0,020	0,020
11. FJ410325.1 Steinernema brazilense	0,219	0,662	0,110	0,125	0,130	0,149	0,144	0,118	0,115	0,146		0,023	0,013	0,017	0,015	0,017	0,027	0,016	0,016
12. FJ263673.1 Steinernema scarabaei	0,269	0,704	0,181	0,173	0,213	0,208	0,195	0,192	0,184	0,210	0,192		0,021	0,023	0,023	0,023	0,029	0,022	0,022
13. FJ235125.1 Steinernema australe	0,203	0,665	0,095	0,122	0,115	0,143	0,122	0,109	0,104	0,140	0,075	0,182		0,016	0,013	0,015	0,026	0,015	0,015
14. FJ152414.1 Steinernema sp. SS-2007a	0,218	0,699	0,095	0,112	0,114	0,172	0,080	0,108	0,098	0,142	0,118	0,196	0,109		0,015	0,018	0,027	0,017	0,016
15. AF122021.1 Steinernema diaprepesi	0,193	0,688	0,082	0,102	0,100	0,133	0,085	0,077	0,085	0,138	0,090	0,187	0,070	0,090		0,014	0,026	0,013	0,013
16. DQ314287.1 Steinernema khoisanae	0,171	0,653	0,080	0,095	0,063	0,144	0,110	0,085	0,083	0,164	0,126	0,199	0,110	0,111	0,095		0,024	0,014	0,014
17. DQ105794.1 Steinernema hebeiense	0,299	0,748	0,200	0,254	0,213	0,251	0,239	0,217	0,222	0,288	0,250	0,258	0,249	0,258	0,245	0,214		0,024	0,023
18. AY230177.1 Steinernema longicaudum	0,178	0,677	0,044	0,099	0,082	0,130	0,109	0,044	0,049	0,147	0,113	0,194	0,094	0,107	0,080	0,085	0,225		0,008
19. AY170341.1 Steinernema guangdongense	0,172	0,670	0,040	0,104	0,077	0,130	0,102	0,037	0,037	0,152	0,113	0,186	0,097	0,105	0,078	0,080	0,219	0,031	

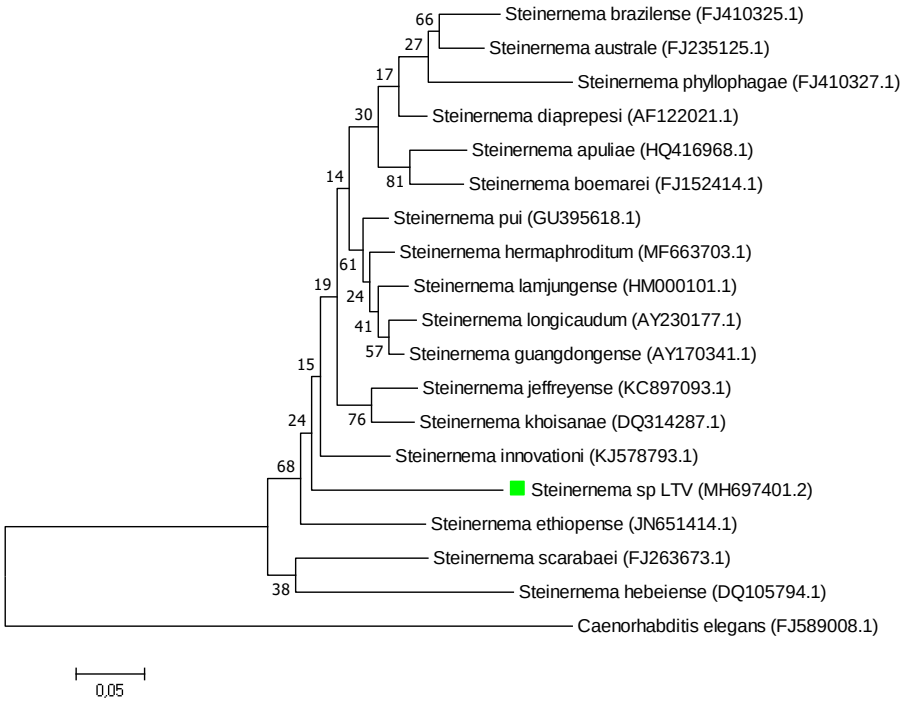

Figure 5.4.1. Phylogenetic relationships of 19 *Steinernema* spp based on analysis of the ITS rDNA regions. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The *Steinernema* sp LTV is indicated by the green box. The accession numbers are indicated in the brackets. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the number of substitutions per site. The numbers at the nodes represent the bootstrap percentage of that specific node. The scale bar for branch lengths is indicated.

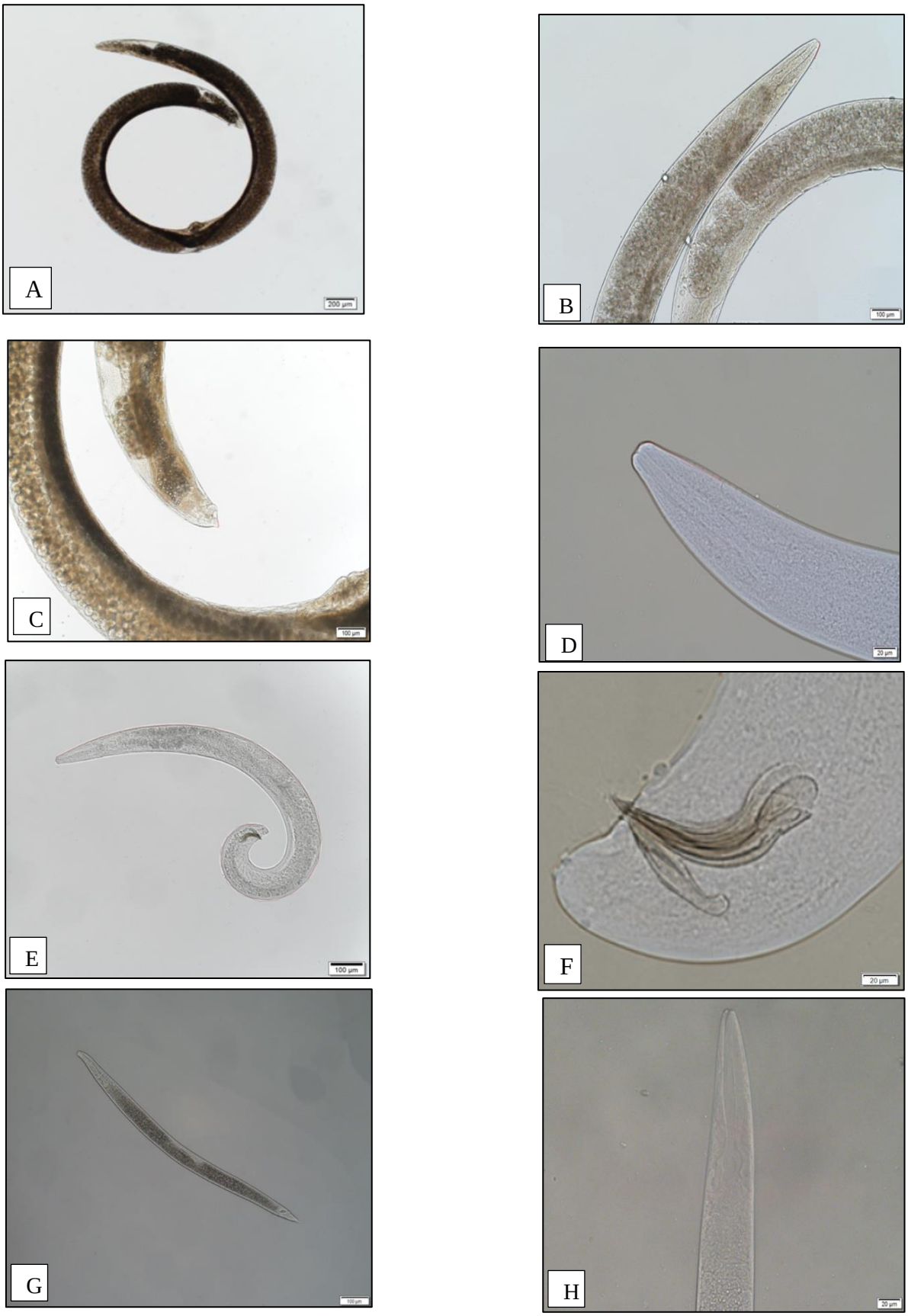

Figure 5.4.2. Morphology of *Steinenerma* sp LTV seen under a light microscope. A-C: First-generation female. A: Closed C-shaped body after heat killing, B: Anterior end and C: Posterior end. D-F: First-generation male. D: Anterior end. E: J-shaped body after heat killing. J: Posterior end. G and H: Infective Juvenile (whole body and anterior end). Scale bar indicated.

5.5. Discussion

The EPN that was isolated from the Brits area was determined to be a new species of *Steinernema,* distantly related to *S. khoisanae*. The morphometric traits data of the strain were compared to nine *Steinernema* species. The identification of *Steinernema* species requires the use of infective juveniles and first-generation males.

The new *Steinernema* species LTV was identified by the morphometrics of the infective juveniles. The body length and width are 975µm and 51µm respectively. The measurement of the excretory pore from the anterior end is 115µm. The length of the tail is 19µm. The strain is closely related to species in the *S. glaseri*, group which is characterised as the infective juveniles larger than 900µm. In comparison with other members of the *glaseri*-group, the infective juvenile of *Steinernema sp* LTV different from *S. khoisanae* by the smaller body; closer in length with *S. jefferyense*. The distance of the excretory pore is larger than the rest of the members of the group except for *S. australe*. The tail length of the first-generation males of the *Steinernema sp LTV* is similar in size to *S. hermaphroditum* and is mostly in range with the other species. The length of the spicule is similar to *S. guangdongense* and *S. braziliense* and is in range with most of the species. The gubernaculum length is similar to *S. australe*, *S. hebeiense, S. scarabaei*. *Steinernema sp LTV* is bigger than *S. ethiopiense* (898µm) but smaller than *S. khoisanae* (1075µm). The spicule is similar to *S. khoisanae* (85µm) and *S. guangdongense* (80µm) gubernaculum smaller than most but closest in length to *S. scarabaei* (54µm). Differences in body width may be due to differences in where the measurements were taken the excretory pore of the isolate is located more anteriorly than *S. khoisanae* (93µm) and has a similar distance to S. *australe* (110µm). The differences in the morphometric data may be due to the differences in habitat (Burnell and Stock, 2000). Different strategies of host-seeking are required for successful infection and persistence in the soil. Further morphological identification is required for a complete characterisation of the new isolate.

The main reason for analysing phylogeny of species using the Maximum Likelihood method is the ability to compare different trees and evolutionary models within a statistical framework (Guindon and Gascuel, 2003). The phylogenetic relationship of *Steinernema sp LTV* and *Steinernema spp* that belong to the glaseri group were assessed. *Steinernema sp LTV* did not

form a clade with another species, it out grouped on itself with a bootstrap percentage of 24%. The phylogenetic trees further confirm that *Steinernema sp LTV* is a new species.

5.6. Conclusion

The new species identified from the Brits area belong to the *S. glaseri* group. Further morphological analysis using scanning electron microscopy is required. Determination of the insect host range is required. Temperature range and desiccation capabilities are required for assessing possible use as a biocontrol agent of insect crop pests in the agricultural sector.

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6. Chapter Six: Conclusion and Future work

In this research, entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, and their bacterial symbionts, *Xenorhabdus*, and *Photorhabdus* were isolated. Isolation and identification of EPNs in South Africa are important for future prospects as biological control agents for the agricultural sector. The identification of the EPNs and their bacterial symbionts was done with the use of molecular identification techniques involving as genomic DNA extraction, PCR amplification of components of the ribosomal genes and Sanger sequencing of the PCR rDNA amplicons. The isolated EPNs were identified as *Heterorhabditis bacteriophora* isolate 56-C and a new uncharacterised *Steinernema* species. The bacterial symbionts were identified as *Xenorhabdus sp VP* and *Photorhabdus luminescens subspecies sonorensis Carbonca*. The use of bioinformatics computational tools such as BLASTn and MEGA7 was important for this study as it provided information about the phylogenetic relationships of the isolated EPNs to other known EPNs. The phylogenetic tree of *Steinernema* spp showed that the isolated species was a new species as formed no clade with other species. The phylogenetic tree of *Heterorhabditis* spp showed that the other isolated EPN was indeed *Heterorhabditis bacteriophora* isolate 56-C.

Formulation of EPNs is important for the commercialisation of the nematodes for use in larger markets such as agriculture. The requirements for a commercial biological control agent are that it must: be user-friendly; able to remain viable in formulation for extended periods and be cost-effective. The research looked at the use of powder formulations for *Heterorhabditis bacteriophora* isolate 56-C. The powders that were used were diatomaceous earth, cellulose, and clay. The results indicated that *Heterorhabditis bacteriophora* isolate 56-C has the potential for commercialisation as it was able to remain viable for 15 days in the formulation. A substantial decrease in viability was seen after 15 days and this may have been due to the fact that all the infective juveniles may have not successfully entered into a state of partial anhydrobiosis or into a state of dehydration tolerance or resistance. It was hypothesized that this may be due to the fact that the rate of dehydration was too rapid for the induction of anhydrobiosis or dehydration tolerance. Further studies will need to be conducted to determine the ideal conditions required for *Heterorhabditis bacteriophora* isolate 56-C to enter partial anhydrobiosis or dehydration tolerance. The role of humidity and temperature in the successful induction of anhydrobiosis or dehydration tolerance needs to be further investigated in EPN formulation development.

The new characterised *Steinernema sp* was entered into GenBank under the name *Steinernema sp LTV* (accession number: MH697401.2). Morphological and morphometrics descriptions were carried out on the new species. It appears that the new *Steinernema sp LTV* belongs to the *Steinernema-glaseri* group given that the infective juveniles measured longer than 900µm. The description of the morphometrics showed that *Steinernema sp LTV* was indeed a new *Steinernema* sp. Further work that is required for the full characterisation of *Steinernema sp LTV* will entail acquiring SEM images of the adults and infective juveniles.

Appendix

Media for symbiotic bacteria associated with entomopathogenic nematodes

NBTA (adapted from Akhurst, 1980)

1 litre nutrient agar

0.04g triphenyltetrazolium chloride (TTC)

0.025g bromothymol blue (BTB)

1. Mix nutrient agar and BTB.

2. Autoclave at 121˚C and 15 psi for 15 min.

3. Add TTC, just before pouring into petri dishes, however, ensure the autoclaved medium is less than 50˚C. TTC will break down if added when medium is too hot.

4. Swirl to mix.

5. Dispense into sterile Petri dishes and leave to solidify.

Nutrient broth (commercially available)

Composition (g/l)

1g Meat extract

2g Yeast extract

5g Peptone

8g Sodium chloride

6. Weigh out nutrient broth powder and suspend in 1000ml distilled water.

7. Mix well and dispense adequate amounts into volumetric flasks.

8. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation one

Nutrient Broth

4.0% (W/V) Canola oil

1. Weigh out nutrient broth powder and suspend in the desired volume of distilled water.

2. Mix well and dispense adequate amounts into volumetric flasks.

3. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth.

4. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation two

Nutrient Broth

4.0% (W/V) Canola oil

25mg/ml glucose

1. Weigh out nutrient broth powder and suspend in the desired volume of distilled water.

2. Add glucose.

3. Mix well and dispense adequate amounts into volumetric flasks.

4. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth.

5. Autoclave at 121˚C and 15 psi for 15 min.

0.1% jik solution for infective juvenile sterilization

34ml distilled water

1ml 3.5% jik

1. Autoclave distilled water.

2. Mix jik and distilled water in bottles.

3. Autoclave at 121˚C and 15 psi for 15 min.

Isolation of genomic DNA from bacterial cells(Protocol from ZR Fungal/Bacterial DNA Kit catalogue# D6005)

1) Pick an isolated bacterial colony from a previously streaked NBTA plate and suspend in a ZR Bashing Bead TM Lysis Tube.

2) Vortex at maximum speed for 5 minutes.

3) Centrifuge the ZR Bashing Bead TM Lysis Tube in a microcentrifuge at 10 000 x g (rpm) for 1 minute.

4) Transfer up to 400µl supernatant to a Zymo-Spin TM IV Spin Filter in a Collection Tube and centrifuge at 7000 rpm for 1 minute.

5) Add 1200µl of Fungal/ Bacterial DNA binding buffer to the filtrate in the Collection Tube from Step 4.

6) Transfer 800µl of the mixture from Step 5 to a Zymo-Spin TM II Column in a Collection Tube and centrifuge at 10000rpm for 1 minute.

7) Discard the flow through from the Collection Tube and Repeat Step 6.

8) Add 200µl DNA Pre-Wash Buffer to the Zymo-Spin TM II Column in a new Collection Tube and centrifuge at 10000rpm for 1 minute.

9) Add 500µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin TM II Column and centrifuge at 10000rpm for 1 minute.

10) Transfer the Zymo-Spin TM II Column to a clean 1.5 ml microcentrifuge tube and add 100µl DNA Elution Buffer directly to the column matrix. Centrifuge at 100000rpm for 30 seconds to elute the DNA.

Nematode Genomic DNA extraction (Protocol from Puregene® DNA Purification Kit, Gentra systems 2003)

1) Rinse infective juveniles three times using approximately 4ml distilled water per wash.

2) Pellet nematodes in a microfuge tube by spinning at 14000rpm for 10 minutes. Place on ice for 30 seconds. Remove excess water.

3) Re-suspend nematode pellet in 1 ml distilled water and transfer the nematode suspension to a 1.5 ml microfuge tube on ice.

4) Centrifuge at 13000-16000 rpm for 3 minutes than place the tube on ice for at least 30 seconds and discard the supernatant.

5) Add 600µl Cell Lysis Solution (from the kit) and invert several times.

6) Add 3µl Proteinase K solution (from the kit) and invert 25 times. Incubate at 55˚C for 3 hours to overnight until the tissue particulates have dissolved. Invert periodically.

7) Add 3µl RNaseA Solution (from kit) to the cell lysate, invert 25 times and incubate at 37˚C for 15-30 minutes.

8) Cool the sample to room temperature.

9) Add 200µl Protein Precipitation Solution (from kit) to the RNaseA treated cell lysate.

10) Vortex at high speed for 20 seconds.

11) Centrifuge at 13000-16000 rpm for 3 minutes. A tight protein pellet should form. If this pellet is not visible repeat step 10, followed by incubation on ice for 5 minutes,

than repeat step 11.

12) Pour the supernatant containing the DNA into a 1.5ml centrifuge tube containing 600µl 100% Isopropanol.

13) Invert gently 50 times.

14) Centrifuge at 13000-16000 rpm for 1 minute, the DNA will be visible as a white pellet.

15) Pour off the supernatant and drain the tube on clean absorbent paper.

16) Add 600µl 70% Ethanol and invert the tube to wash the pellet.

17) Centrifuge at 13000-16000 rpm for 1 minute and carefully pour off the ethanol. Pour slowly as the pellet may be loose.

18) Invert and drain the tube on absorbent paper again and allow to air dry for 10-15 minutes.

19) Add 100µl DNA hydration Solution (from the kit).

20) Rehydrate the DNA by incubating the sample 1 hour at 65˚C. Tap the tube to aid dispersing the DNA.

21) Store DNA at 4˚C.