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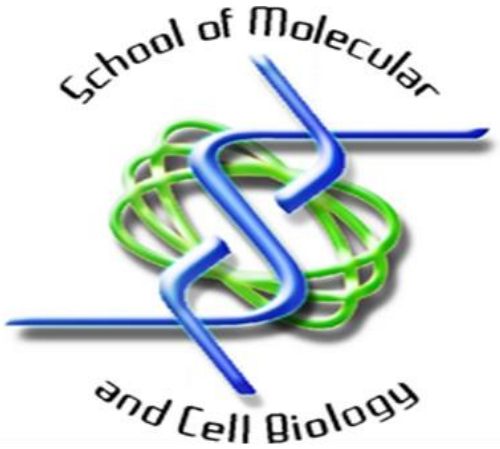
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GENOMICS OF ENTOMOPATHOGENIC BACTERIAL ENDOSYMBIONT SPECIES ASSOCIATED WITH DESICCATION TOLERANT ENTOMOPATHOGENIC NEMATODE

Dissertation by: Miss Boipelo Mothupi (456502)
Supervisor: Professor Vincent Myles Gray
Year: 2016

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

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DECLARATION

- I hereby declare that this Dissertation is my own, unaided work and material obtained from other sources have been referenced and cited.
- The experimental protocols, analysis of the results and write up of this dissertation were done by myself
- It is being submitted for the Degree of Master of Science with dissertation at the University of the Witwatersrand, Johannesburg.
- This work has not been submitted before for any degree or examination at any other University.

Boipelo Mothupi

Signature:.....

Date: 16 February 2016

DEDICATION

This work is dedicated to my beloved late mother Boikhutso Jeanette Mothupi (1969 – 1998). Special gratitude to my wonderful guardian parents Mr Gobona Albert Ramokga and Mrs Keatlaretse Jacqueline Ramokga, for their unending love and support, I would not be where I am if it was not for their support throughout my studies. I thank Mr Benny Elias Selebogo for he has always believed in me and nurtured my love for education, his encouragement and countless motivation have played a pivotal role in my growth both personally and professionally. Special thanks to my partner and pillar of strength Kealeboga Gladstone Moiloa for his love, support and motivation.

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- National Research Foundation Scholarship, Gauteng Department of Agriculture and Rural Development, and Postgraduate Merit Award Scholarship for financial assistance and for funding of the project.

- I would like to thank my family for their love and continuous support throughout project period.

- I thank my dear friend and colleague Stephanie Naidoo for support and encouragement.

- I thank God for his unending love, and providing me with the strength and wisdom to complete the project.

RESEARCH OUTPUTS

Publications

1. Mothupi B, Featherston J and Gray V (2015). Draft-whole genome sequence and annotation of *Xenorhabdus griffiniae* strain BMMCB associated with *Steinernema Khoisanae* strain BMMCB. *Genome announcements* ***3****(4):*e00785-15
2. Naidoo S, Mothupi B, Featherston J and Gray V (2015). Draft Genome Sequence and Assembly of *Photorhabdus heterorhabditis* Strain VMG, a bacterial symbiont associated with the entomopathogenic nematode, *Heterorhabditis zealandica. Genome announcements*, 3(5): e01279-15

Conference Output (Oral presentation/Poster presentation):

Conference 1: 7th Annual Gauteng Department of Agriculture and Rural Development Symposium- (2014)
Output: Poster Presentation
Poster title: Entomopathogenic nematodes as potential biocontrol agents of insects pests in agricultural industries: effect of dehydration and soil texture on infectivity and longevity
Authors: Mothupi B and Gray V

Conference 2: 8th Annual Gauteng Department of Agriculture and Rural Development Symposium- (2015)
Output: Poster Presentation
Poster title: Isolation and molecular characterization of entomopathogenic nematodes species in the Gauteng region
Authors: Mothupi B, Naidoo S and Gray V

Abstract:

Entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* have emerged excellent as non-chemical alternatives for control of insect pest population. They have a specific mutualistic symbioses with bacterial symbionts in the genera *Photorhabdus* and *Xenorhabdus*, respectively. Native EPN species that are able to tolerate environmental stress including desiccation are of great interest for application. The aim of this study was to isolate indigenous EPN species from soil samples collected from Brits, North West province in South Africa, and to investigate their ability to tolerate desiccation stress. The second aim was to isolate the bacterial symbiont and sequence, assemble and annotate its whole genomic DNA. Insect baiting technique and White trap method proved useful in the recovery of nematodes from collected soil samples and infected cadaver, respectively. Molecular identification based on the amplification of the 18S rDNA and phylogenetic relationships revealed high affinity of the unknown EPN isolate 10 to *Steinernema* species and due to variation in evolutionary divergence distance, the unknown isolate was identified as *Steinernema* spp. isolate 10 . Isolates 35 and 42 revealed high similarity to *Heterorhabditis zealandica* strain Bartow (accession number: GU174009.1), *Heterorhabditis zealandica* strain NZH3 (accession number: EF530041.1) and the South African isolate *Heterorhabditis zealandica* strain SF41 (EU699436.1). Both *Steinernema* spp. isolate 10 and *Heterorhabditis* species could tolerate desiccation. *Steinernema* spp. isolate 10 was tolerant up to 11 days of desiccation exposure in loamy sand and up to 9 days of exposure in river sand, causing 26, 6% and 13, 4% cumulative larval mortality after 96 hours, post resuscitation by rehydration, respectively. *Heterorhabditis* spp. could tolerate desiccation up to 13 days of exposure and induced 26.6% cumulative larval mortality on both loamy and river sand after 96 hours post resuscitation. Swarming, aggregation, coiling and clumping behavioural characteristics were observed when *Steinernema* spp. isolate 10 was exposed to desiccation and *Heterorhabditis* species displayed no similar behavioural characteristics associated with desiccation tolerance. Morphological characteristics of the unknown *Steinernema* spp. isolate 10 have been described, and the thick cuticle and sheath which are both associated with tolerance to desiccation stress have been noted. The bacterial symbiont was isolated from larval hosts infected with *Steinernema* spp. isolate 10 and molecular identification through NCBI Blastn based on the 16S rDNA revealed high affinity to *Xenorhabdus* bacterial species. Phylogenetic relationships and evolutionary divergence estimates

revealed genetic variation and the species was identified as *Xenorhabdus* bacterial isolate. The genome assembly *of Xenorhabdus* bacterial isolate using CLC Bio revealed a total length of 4, 183, 779 bp with 231 contigs (>=400bp), GC content of 44.7% and N50 of 57,901 bp. Annotation of the assembled genome through NCBI PGAAP annotation pipeline revealed 3,950 genes (3,601 protein coding sequences (CDS) and 266 pseudogenes), 12 rRNAs and 70 tRNAs. RAST annotation revealed 55 of virulence, disease and defense subsystem features which are involved in the pathogenicity of *Xenorhabdus* bacterial isolate. The ability of EPNs to tolerate environmental stress is highly crucial and one of the determining factors for biocontrol potential and successful application, thus the indigenous desiccation tolerant EPN isolate, *Steinernema* spp. isolate 10 holds great potential as a biological control agent. The genome sequencing and annotation reveals insight to behavioural and physiological attributes of bacterial symbionts and this study will contribute to the understanding of pathogenicity and evolution of the bacteria–nematode complex.

CHAPTER1: Literature Review

1.1 INTRODUCTION

1.1.1 Agriculture and chemical pesticides

Environmentally sustainable protection of agricultural crops from insect pests is highly essential for the production of food. The high demand of food is growing almost exponentially due to increasing world population. South Africa also plays a pivotal role in the global food supply chain through its agricultural exports. Agriculture contributes about 3% to South Africa's gross domestic product (GDP). Every crop has its complex of insect pests which have and will develop resistance to conventional chemical pesticides. Synthetic chemical insecticides also have a negative impact on the environment and human health, and without the application of synthetic chemical insecticides to crops, yield loses are more or less guaranteed (Kiniuki, 2001). The development of alternative pest control agents which are sustainable and environmentally friendly is a necessity. Different kinds of biocontrol agents have been developed as alternatives to the conventional chemical based pesticides. Chemical pesticides including fungicides, herbicides and insecticides have long been employed since 1818s to combat pests which feed on plants, however, for chemical pesticides to be effective they need to be biologically active and toxic (Kuniuki, 2001). Because they are toxic, they are potentially hazardous to human beings, animals and the environment.

Health effects can either be acute or severe, and includes irritation of eyes and skin, nervous system affection and mimicking of hormones causing reproductive problems and cancer. Association with pesticides has been reported to cause non-Hodgkin leukemia and lymphoma. Environmental effects include air, soil and water pollution (Kuniuki, 2001). Another major problem with pesticides is that insects develop resistance against them within a short period of time resulting in amplification of pest population and destruction of natural enemies, hence the need for the application of entomopathogenic nematodes (EPNs) as biocontrol agents of insect pests.

1.1.2 Biocontrol control

Biological control (BC) is the control of pests by disrupting their ecological status through the use of organisms that are natural predators, parasites, or pathogens (Pionar, 1979). The natural enemies include bacteria, fungi and nematodes. Sustainable pest management is an essential

input activity for successful crop production hence there is a need to make greater and more effective utilization of all the natural enemies of insect crop pests that only target the selected pest host and not the other beneficial insects associated with agricultural cropping systems (Webster, 1973). Parasitism of insects by nematodes has long been known since 17th century but it was only in the 1930's that serious consideration was given to using nematodes as biological control agents to control insect pests (Grewal *et al*, 2006).

There are three distinct methods of BC namely inoculative, augmentation and conservation method (Cook, 1993). The interest is in the conservation method which involves the conservation of existing natural enemies in an environment. Natural enemies are already adapted to the habitat and to the target pest, and their conservation can be simple and cost-effective (Smart, 1995). This is because it is best to use indigenous enemies than foreign for population control purposes. BC agents are advantageous over pesticides because they are environmentally friendly and non-polluting. Biological control processes can be properly understood if chemical, physical and biological interactions are understood in the soil, in order to directly understand how entomopathogenic nematodes react when applied on agricultural fields with the aim to attack and kill insect pests, (Georgis *et al*, 2006).

1.1.3 Entomopathogenic nematodes

Nematodes are non-segmented, colourless roundworms. They are characterized by their excretory, secretory, nervous, digestive, reproductive and muscular systems. They neither have a circulatory nor respiratory systems. Many microorganisms form mutualistic relationships with high order organisms such as animals and plants. It has been hypothesized that coevolution has resulted in the development of beneficial microbial mutualistic associations between plants and animals. Coevolution of mutualistic partners leads to speciation or co-speciation (Maneesakom *et al*, 2011). The nematode-endosymbiotic bacterial-insect host association represents an attractive model system for evolutionary studies. In addition nematodes also represent one of the most diverse and specious animals on earth (O'Leary *et al*, 2011). Nematodes that are vectors for bacteria and are able to penetrate and parasitize the insect larvae are referred to as entomopathogenic nematodes (EPNs). They fall in the genera *Steinernema* and *Heterorhabditis* which are associated with bacterial endosymbionts of the genera *Xenorhabdus* and *Photorhabdus*, respectively. (O'Leary *et al*, 2011). The mutualistic relationship which has evolved between EPNs and their bacterial endosymbionts is highly complex and is under multiple gene co-regulations (Georgis *et al*, 2006). EPN and its bacterial

symbiont are thought to have co-evolved from an association that first involved the nematode as a bacteriovor that fed on an enterobacterial species (Maneesakom *et al*, 2011). From this association the nematode evolved into a vector for the bacteria which it fed on and the bacteria evolved into an insect pathogen that could parasitize a wide range of different species of insect larvae (Maneesakom *et al*, 2011). The EPN-bacterial symbiotic relationship is essential for the lethal infection of insects, especially of insects that attack plants. EPNs infect the larval stage of a diverse range of insects and release their bacterial endosymbiont into the larval haemocoel. The bacterial endosymbionts which are insect pathogens multiply and secrete proteins and secondary metabolites that are lethal to the insect larva and suppress the growth of other competing environmental bacteria, fungi, nematodes and protists while providing a suitable environment for EPNs reproduction and growth (Maneesakom *et al*, 2011).

1.1.4 EPN Taxonomy

Nematodes belong to the phylum Nematoda. The families, Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae, Rhabditidae, Steinernematidae and Heterorhabditidae are more popular in most researches carried by various scientists. However, the nematodes from the families Steinernematidae and Heterorhabditidae have become the most important nematode species for the development of biocontrol agents (Perez *et al*, 2003). The Steinernematidae family consists of Sixty-one species of *Steinernema* and the family Heterorhabditidae consists of 24 species of *Heterorhabditis* nematodes which have been identified to date (Thanwisai *et al*, 2012). Nematodes of the genera, *Steinernema* and *Heterorhabditis* are associated symbiotically with the enterobacteria, *Xenorhabdus* spp. and *Photorhabdus* spp, respectively. The symbionts are gram negative bacterial species and belong to the Enterobactericeae family (Thanwisai *et al*, 2012).

1.1.5 Parasitism of insect larvae by entomopathogenic nematode and bacterial symbiont

Free-living and non-feeding infective juveniles (IJs) are metabolically and developmentally arrested and carry symbiotic bacteria (Kaya and Stock, 1997). *Photorhabdus* and *Xenorhabdus* bacterial endosymbionts are carried by *Heterorhabditis* and *Steinernema* infective juveniles, respectively. These bacterial endosymbionts are genus of bioluminescent enterobacteria under the family Enterobacteraceae and normally colonizes the gut of IJs.

IJs actively search for insects in the soil; they then enter the insect host larvae through natural openings such as the anus, mouth or respiratory spiracles by using mechanical and enzymatic means (Poinar, 1975). *Heterorhabditis* IJs does not only depend on natural openings for entry,

they possess small tooth-like appendages which enables them to tear the cuticle of the insect larvae and gain direct access to the haemolymph. The IJs actively penetrate through the tracheae into the insect body cavity (haemocoel) and releases the symbiotic bacteria from its intestine to the insect haemolymph. The signals that stimulate the IJs to regurgitate the bacteria into the haemolymph post entry have not been identified. The bacteria avoid or silence the immune response of the insect larvae. The bacteria start multiplying and grow exponentially in the nutrient-rich haemolymph while secreting toxins and hydrolytic enzymes including proteases and lipases that hydrolyses the cadaver of the insect larvae until the insect succumbs to septicaemia within 48-72 hours of infection. The IJs recover from their arrested state (dauer stage) and start feeding on multiplying bacteria and disintegrated host tissues (Ciche *et al*, 2006). Toxins produced by the multiplying bacteria kill the insect host. These bacteria also produce a plethora of metabolites, toxins and antibiotics with bactericidal, fungicidal and nematicidal properties, which ensures monoxetic conditions for nematode development and reproduction in insect cadaver (Ciche *et al*, 2006). Heterorhabditid and steinernematid nematodes differ in their mode of reproduction. In heterorhabditid nematodes, the first generation individuals are produced by self-fertile hermaphrodites (hermaphroditic) but subsequent generation individuals are produced by cross fertilization involving males and females (amphimictic) (Pirez-daSilva, 2007). In steinernematid nematodes with an exception of one species, all generations are produced by cross fertilization involving males and females (amphimictic). Prior leaving the cadaver of the insect, the bacteria must colonize the IJ and the transmission of the bacteria to the nematode is a complex process which is not fully understood. IJs emerge from infected larvae in search for new hosts.

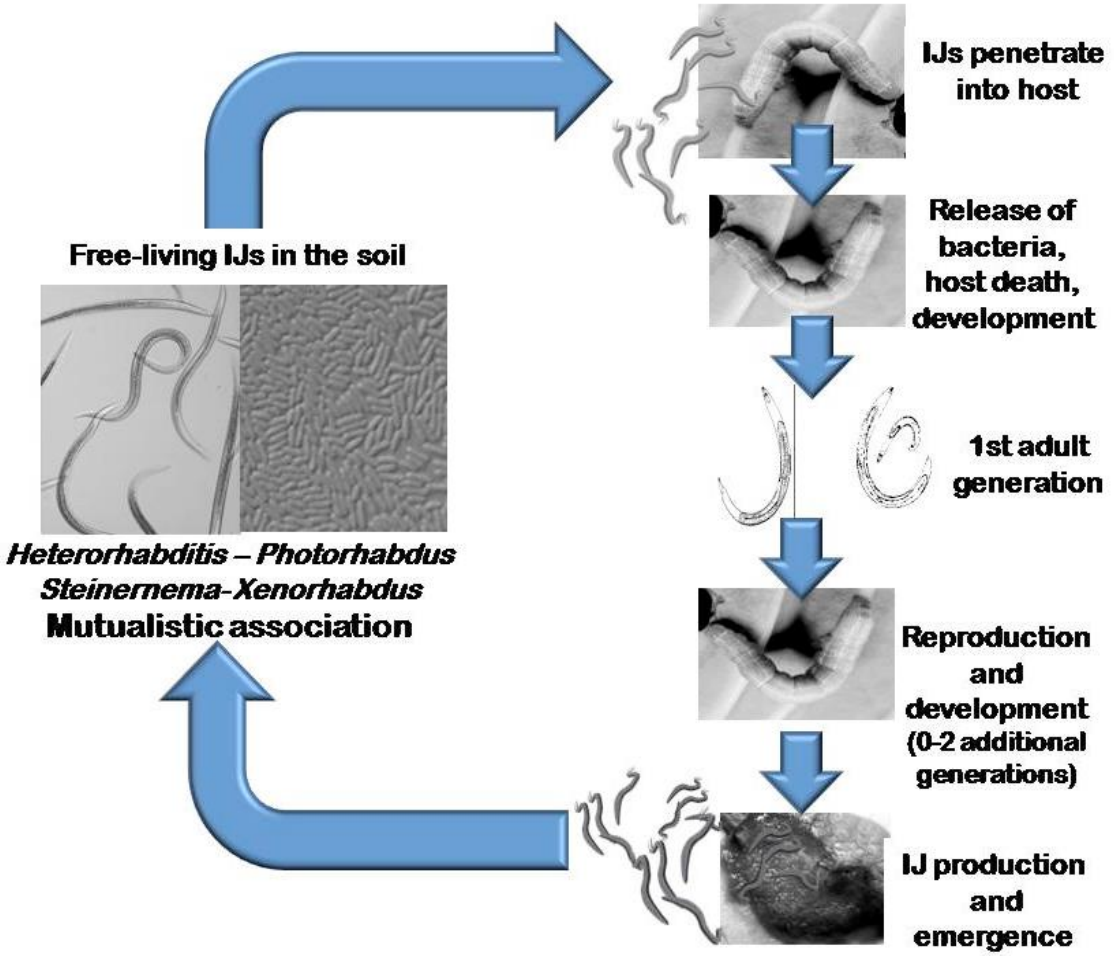


Figure 1.1: The life cycle of entomopathogenic nematodes
(Adapted from Stock and Goodrich-Blair, 2008)

1.1.6 The biology of bacterial symbionts: *Photorhabdus* and *Xenorhabdus*

Xenorhabdus and *Photorhabdus* are members of the family Enterobacteriaceae and phylum Proteobacteria. They are facultative anaerobic gram negative bacteria and are rod shaped and non sporulating. They are lethal to insect pests with the ability to depress their innate immune system. Insects have both cellular and humoral based immune response mechanisms triggered by recognition of foreign particles (Goodrich-Blair and Clarke, 2007). The insect's eicosanoid pathway is induced by phospholipase A2 enzyme and in turn, haemocyte aggregation and nodulation (*Kim et al*, 2005). Cell based immunity involves the encapsulation of invading organisms by circulating haemocytes and subsequent menalization of the capsule by the

enzyme phenoloxidase (Goodrich-Blair and Clarke, 2007). Humoral response involves the production of cationic antimicrobial peptides (CAMPs) that target bacterial membranes.

Photorhabdus and *Xenorhabdus* are not inherently resistant to insect immune system, however both species have developed mechanisms to counteract the immune response of the insect larvae and induce mortality. *Photorhabdus* counteract the immune response of the larval host by the production of a signalling molecule AI-2 that incur resistance to reactive oxygen species (ROS) (Krin *et al,* 2006). ROS is a component of an early immune response in insect larvae. *Photorhabdus* also relies on the modification of the lipopolysaccharide to counteract humoral CAMP response. *Xenorhabdus* species avoid the humoral response through the suppression of CAMPs expression (Park *et al*, 2007).

The bacterial symbionts in the genera *Photorhabdus* and *Xenorhabdus* represent divergent evolution but portray convergent lifestyles. They are both lethal to insect pests and have a specific mutualistic relationship with nematodes in the genera *Heterorhabditis* and *Steinernema,* respectively. This is an obligate EPN-bacterium interaction and there has been no cases where *Photorhabdus* was associated with *Steinernema* or *Xenorhabdus* with *Heterorhabditis*. How can closely related bacterial symbionts which are both pathogenic to insects be specific with their choice of nematode for colonization?

They colonize different sites in their respective nematode associates. The bacterial symbionts are released into the haemolymph of the host and induce mortality. Upon emergence of EPNs from the cadaver of larval host, a key stage involves re-colonization of the nematodes by the bacteria. Events occurring at molecular and cellular interface between the bacteria and nematode are thought to regulate the colonization process, however little is known at genetic level. *Photorhabdus* colonize a substantial fraction of the alimentary lumen of the nematode gut and *Xenorhabdus* are harboured in specialized vesicles known as receptacles.

Photorhabdus colonizing bacteria are reported to be maternally transmitted to infective juveniles during endotika matricida (EM) (Gaudriault *et al,* 2006). The rectal glands of the adult female EPN becomes infected with *Photorhabdus* cells and serves as the source of inoculum for the IJs. Stages involved in nematode colonization are 1. Adult female EPN colonization by *Photorhabdus* cells in the rectal glands, 2. IJ colonization and 3. Outgrowth (Easom *et al,* 2010).

The molecular biology of *Steinernema* colonization by *X. nematophila* is better understood, however the source of colonization have not yet been elucidated. *Steinernema* IJs are colonized in specialized pockets with intravesicular structures (IVS) which have the mucus like substance within the interstitial space which is reported to represent specific adhesion site for colonizing bacteria. The genes that have been predicted to encode membrane proteins for initiation of colonization in *X. nematophila* are Nil A, B and C (nematode intestinal colonization) and these proteins are thought to interact directly with the nematode milieu to facilitate colonization (Goodrich-Blair, 2007).

The genome sequence of *P. luminescens* subsp. *luminescens* strain TT01 revealed a high number of genes encoding proteins potentially pivotal or involved in host-bacterium interaction. A study by Gaudrault *et al* (2006) aimed at identifying bacterial genomic regions that are possibly involved in nematode specificity by comparing two strains of bacteria (*P. luminescens* subsp. *laumondi* TT01 and *P. temperata* subsp. *temperata* XINach) harbored by two nematode species *H. bacteriophora* and *H. megidis*, respectively. Their work showed that DNA microarrays procedure is a powerful tool for selecting some genes or genomic regions potentially involved in bacterium-EPN interaction. In their findings, locus 6 was similar to *Salmonella enterica, Serovar typhimurium* and *Escherichia coli* lsr region which encodes an inner ABC transporter and a cytoplasmic phosphorylation processing system of the autoinducer A1-2 involved in quorum sensing (Gaudrault et al, 2006). The *lsr* locus was similar in 3 bacterial strains carried by *H. bacteriophora* including *P. luminescens* TT01. In *Xenorhabdus* strains and XINach, the *lsr* A, C and D were missing, however various *lsr* A, B and R remnants were observed, showing that the *lsr* locus underwent independent deletions in these latter strains and suggesting that the *lsr* locus is an ancestral locus in the *Photorhabdus* and *Xenorhabdus* strains. Bacterial association with EPNs is suggested to possibly be a selective pressure for the conservation of the *lsr* locus, whereas association with other nematode hosts leads to *lsr* locus loss by genomic decay. These data suggest that the *lsr* locus is possibly involved in the specific interaction with EPNs. In *S. enterica, Serovar typhimurium* and *E. coli*, it was suggested that the *lsr* transporter has a role in removing the AI-2 signal from the external environment in order to terminate cell-cell signalling (Xavier and Bassler, 2005). In nematode interaction, the termination of cell-cell signalling could be an important signal that allows a bacterial physiological shift, for example, in the insect cadaver, when bacteria recolonize the nematode intestinal tract.

1.1.7 Nematode host range and behaviour

EPNs and their bacterial symbionts rapidly kill the insects and do not represent a closely and highly adapted host-parasite relationship characteristics and this allows EPNs to parasitize a wide variety of insect pests. The insects are highly susceptible in a controlled environment such as the laboratory but are seldom impacted in the fields as nematodes tend to be affected by environmental factors such as desiccation, ultraviolet (UV) radiation and temperature fluctuations. Extensive studies have been conducted using *S. carpocapsae* and the results revealed acceptable control of field populations of strawberry root weevils, citrus root weevil and cutworms (Kaya, 1993). Other studies have been conducted using *S. glaseri* and *Heterorhabditis* spp. and positive results have been obtained for black weevil larvae and white grubs using the former species and with the latter species, positive encouraging results have also been obtained in soil treatment against black vine weevil, mole crickets (Cobb and Geogis, 1987), wireworms (Kovacs *et al*, 1980), colorado potato beetles (Wright *et al*, 1987), root maggots (Van Sloun and Sikora, 1986) and cutworms (Lossbroek and Theuiseen, 1985). It is highly crucial to use native EPNs for application as exotic nematode species might not be well adapted to the environment and the target pest and may lead to exclusion of natural biodiversity.

EPNs respond to both physical and chemical stimuli. The foraging ability of the nematode to locate the host is of fundamental importance for biocontrol efficacy (Shapiro-Ilan *et al*, 2006). Species which are ideal for biocontrol application are cruisers (e.g *H. bacteriophora* and *S. glaseri*) as they respond strongly to chemo attractants and are able to search deeper in the soil, whereas ambushers search for host on the soil surface (e.g *S. carpocapsae*). Carbon dioxide (CO2) have been reported to elicit host seeking behaviour in EPNs, however, this cannot explain host adaptation as CO2 is a non-specific, volatile metabolite produced by roots, soil microbes and insects (Stock 1993). Host associated materials such as faeces and cuticle play a role in host recognition (Stock, 1993).

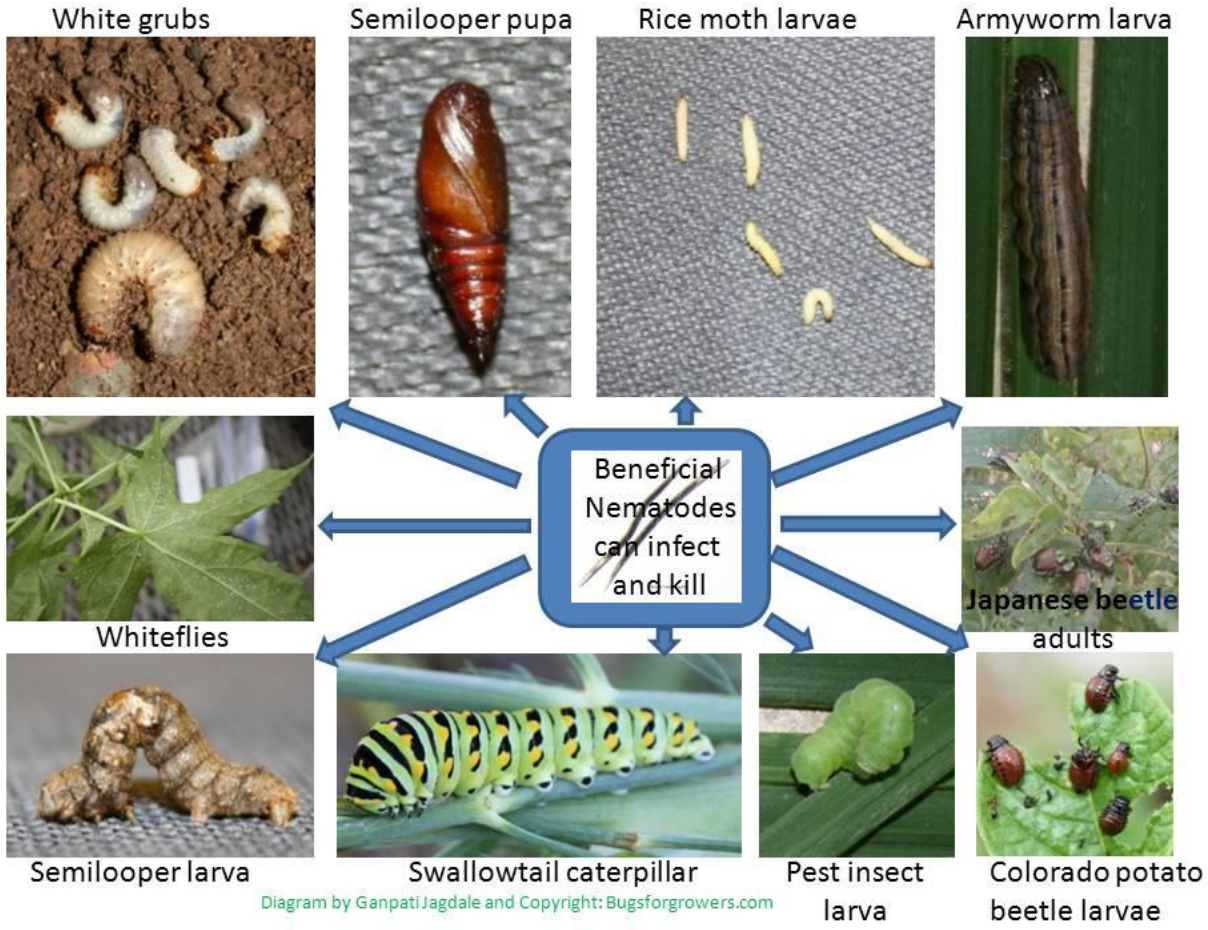


Figure 1.2: Variety of insect pests susceptible to entomopathogenic nematodes.

1.1.8 EPNs as biological control agents

EPNs occur naturally in the soil and are distributed worldwide, with distinctive species and clusters in different geographical regions, and this indicates their genetic ability to adapt and survive environmental stresses such as high temperature and desiccation (Grewal, 2000). Limited shelf-life is a major obstacle to large-scale use of entomopathogenic nematodes in biological control. Anhydrobiosis is induced by dehydration and is considered a vital means of achieving storage stability and increases shelf life of entomopathogenic nematodes as it reduces oxygen and lipid reserve utilization by infective juveniles (Grewal *et al*, 2006). Anhydrobiosis occurs naturally in nematodes and other invertebrates and is believed to be a good survival strategy during drought conditions. True anhydrobiotes can lose up to 95 ± 98% of their body water and, as desiccation persists, they lower their metabolism to below detectable levels, entering a state of cryptobiosis. However a study by Grewal and colleagues (2006) indicated

that entomopathogenic nematodes are partial anhydrobiotes and are referred to as quiescent anyhydrobiotes.

It is important to study the effect of desiccation on EPNs and to identify the genes which are upregulated during desiccation conditions, as environmental stress might have a negative impact on motility, infectivity and longevity of EPNs. Also, controlled desiccation and the induction of dehydration tolerance may be important processes for the formulation of EPNs as biocontrol agents. Soil is an important habitat for nematodes and the EPNs importance as biocontrol agents in agriculture depends on the the IJs ability to survive, disperse and persist in soil that undergo repeated cycles of dehydration and rehydration (Ngoma, 2009). In order for the nematodes to be effective the conditions for growth and amplification are required. These include optimum temperature, moisture content and the type of soil texture (Kopenhofer and Fuzy, 2006). Understanding the natural predator-prey behaviour that is essential for the successful infection of target hosts is vital for developing procedures for the application on EPNs as biocontrol agents, hence the need for behavioural studies to be taken into consideration. For infectivity, nematodes are required to be motile, and be able to search for and infect the host and infectivity is used as an indicator of biological control potential (O'Leary *et al*, 2001). Foraging strategies are used by the nematodes to search for prey in the soil and they vary from species to species (O'Leary *et al,* 2001). Screening for nematodes that have good foraging strategies and tolerance to environmental extremes such as desiccation could lead to application of genetics as a powerful means to enhance the desired traits in nematodes and application of competent and effective strains (Segal and Glazer, 2000). Previous studies on behaviour have shown EPNs to be potential biocontrol agents and might be of use and eliminate the use of chemical pesticides completely.

1.1.9 Gene induction by desiccation stress in EPNs

Whether quiescent or motile, IJs of a parasite are usually adapted for resistance to unfavourable environmental conditions. The dauer juvenile (DJ) stage of the free-living *C. elegans* has been extensively studied. It is a modified third stage juvenile (J3) formed under unfavourable conditions including overcrowding, limited food and high temperatures. *C. elegans* DJs are developmentally arrested with a reduced metabolic rate and can survive for up to 8 times normal life span of approximately 16 days (Klass, 1976).

The infective J3 (DJ) of *Heterorhabditis* and *Steinernema* has received much attention regarding their environmental tolerance and host range. When exposed to moderate levels of

desiccation, EPN DJs respond by aggregating into large clumps and by forming tight individual coils which reduce surface area and slow down the rate of desiccation (O leary *et al*, 2001).

The fully anhydrobiotic *Aphelenchus avenae* have been reported to synthesize large amounts of trehalose in response to desiccation (Madin and Crowe, 1975). DJs of both *Heterorhabditis* and *Steinernema* are not full anhydriobites but quiescent anhydrobites, and have been reported to synthesise moderate levels of trehalose that replaces water in the cell membranes of desiccated cells, maintaining spaces between the phospholipids and retaining the phospholipid bilayer in the liquid crystalline state (Crowe *et al*, 1984).

Recent gene and protein expression studies have identified some of the molecular mechanisms which EPNs use to tolerate desiccation stress. Gal *et al* (2003) used cDNA subtraction to identify expressed sequence tags (ESTs) up-regulated in response to desiccation in DJs of *S. feltiae* 156 strain which are capable of surviving exposure to 75% RH. Among the up-regulated gene classes identified by Gal and his colleagues were transcriptional regulators, molecular chaperones, antioxidants and hydrophilic proteins. This correlates to the findings of the study conducted by Chen *et al* (2005, 2006) where the 2D gels and peptide mass mapping were used to identify proteins whose synthesis was increased in response to desiccation and the proteins included transcriptional regulators, molecular chaperones, antioxidants, proteins involved in cell cycle regulation and actin (a component of cytoskeleton) .

Tyson *et al* (2007) investigated the molecular basis of anhydrobiosis and desiccation tolerance by constructing and analyzing a panel of ESTs that are upregulated in response to desiccation in DJs of the *S. carpocapsae*. *S. capocapsae* is produced commercially and is widely used for biocontrol of insect pests and have been found to have high levels of desiccation tolerance when compared with other species of *Steinernema* (Patel *et al*, 1997). The study showed that the molecular response to desiccation in EPN DJs is complex and parallels many of the adaptive changes which occur in draught tolerant plants during exposure to desiccation (Tyson *et al*, 2007). The study of the complex molecular response of EPNs to different environmental stress conditions continues to be of interest to identify all the genes which are up-regulated during stress and enables the nematodes to overcome or tolerate the stress.

1.1.10 Application technology of nematodes

The application of a strain that is well suited to agricultural fields is of importance following good storage condition. Efficient and effective delivery of EPNs is a necessity. Nematodes can be applied with commercially available ground or aerial spray equipment including pressurized

sprayers, mist blowers and electrostatic sprayers (Georgis, 1990). The equipment for application is highly dependent on cropping system with careful considerations including pressure, spray distribution pattern and environmental conditions (Shapiro-Ilan *et al*, 2006).

High pressure sprayer equipment is reported to result in reduced nematode viability due to mechanical stress from the pump and temperature effects in the liquid after passes through the pump. Recommended sprayers are nozzle type sprayers with openings larger than 50µl and pressure less than 2000KPa (290psi) (Geogis, 1990). However, these recommendations are supported with information obtained on the most well studied EPN, *S. carpocapsae* and may differ from species to species.

EPNs may experience physical stress during flow through the spray system and it is highly crucial to comprehend effects of different stress inducing compartments or factors within the spray system in order to identify the best possible equipment that is least detrimental to EPNs viability. Pressure and temperature are the well-known physical stresses that affect EPN viability during application. Soil is a natural habitat of EPNs and several biotic and abiotic factors must be taken into consideration for successful application (Shapiro-Ilan *et al*, 2006). EPNs are highly effective in sandy soil with pH of 4-8. Other environmental factors that may affect persistence and viability of applied EPNs are UV radiation and desiccation. No matter how well suited a nematode is to the target pest, if it is not delivered in a manner that ensures viability and that ensures access to and infection to the host, application will fail (Shapiro-Ilan *et al*, 2006)

1.1.11 Next generation sequencing

Next generation sequencing which is also referred to as high-throughput sequencing, is used to describe various modern sequencing technologies including Illumina, Rosche 454, Ion torrent: Proton/PGM and SOLiD sequencing. Whole genome sequencing (WGS) advances knowledge at genetic level in the physiological, morphological and metabolic characteristics of organisms (Dillman *et al*, 2012). Next generation sequencing, assembly and annotation of both the bacterial symbionts and the nematode will advance knowledge to comprehend the pathogenicity and symbiosis of nematode-bacterium species, as well to understand the divergent evolution represented by *Xenorhabdus* and *Photorhabdus* species (Gaudriault *et al*, 2006 and Hao *et al*, 2010).

Illumina whole genome sequencing involves isolation of the whole genomic DNA, shearing of genomic DNA, preparation of the library and sequencing using Illumina Miseq for prokaryotes and Illumina Hi-seq for eukaryotes. The genomic library is sequenced on Illumina genome analyzer sequencer in either single or paired-end mode. These reads are then assembled into hundreds up to thousands of larger contigs (contiguous sequence) using assembler programs.

Short read sequencing technology is quick and cheap compared to Sanger sequencing used for whole genome sequencing of *C. elegans* (Sandhu *et al*, 2006). Read assembly is facilitated by knowing the approximate distance between the paired end reads, helping to overcome issues of repeats and homopolymeric regions and the accuracy of next generation sequencing and assembly is improved by high coverage and mate pair data.

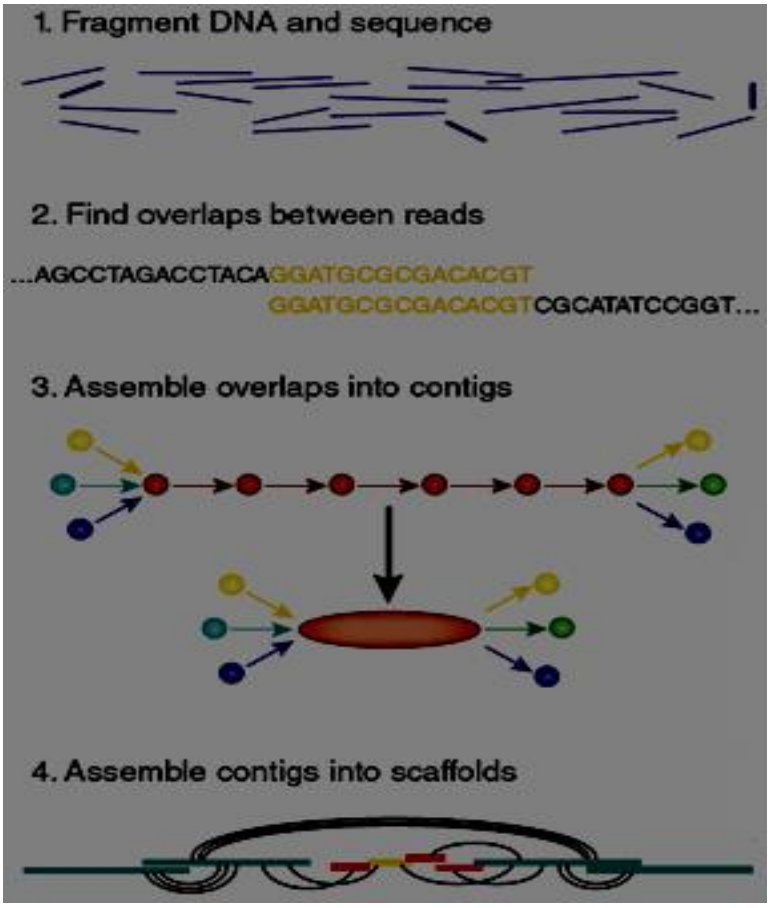


Figure 1.3: Overview of whole genome sequencing and assembly

(http://www.nature.com/nmeth/journal/v9/n4/images/nmeth.1935-I2.jpg)

1.1.12 Research motivation

- The application of EPNs as biocontrol agents requires knowledge of the occurrence, species diversity, biology, ecology, distribution and insect host range of native EPN species. Application of native EPNs as insect biological control agents would be preferable over exotic EPNs which may not be suitably adapted to the South African environment. Non-native EPNs may also have negative impacts on native populations of EPNs. In addition, non-native EPNs may not be able to target local insect pests as they may not be adapted to local environmental conditions.

- Identification by establishing taxonomic and phylogenetic affinities through the application of molecular based techniques based on the PCR amplification and sequencing of the ribosomal DNA has proven to be an efficient and rapid method for identifying EPNS and their bacterial symbionts. This molecular approach to EPN characterization was adopted in this investigation.

- Nematodes that are adapted to the environment with good foraging behavioural characteristics and which are tolerant to environmental stress conditions such as desiccation are ideal for application for effective control of insect pests. Understanding desiccation tolerance attributes of indigenous EPNs will aid in the understanding of the nature of requirements necessary for ensuring good storage conditions and long shelf life of EPNs prior application.

- Genome sequencing and annotation gives insight into the behavioural and physiological attributes of bacterial symbionts and thus their study will contribute to the understanding of pathogenicity, evolution and the specific colonization of bacterial symbionts with respect to their association with selected nematode species.

Aims

- To isolate, identify and assess desiccation tolerance of native South African entomopathogenic nematode species.
- The second aim was to isolate, identify, and do whole genome sequencing and annotation of the associated entomopathogenic bacterial endosymbiont.

Objectives:

- Isolation, molecular and morphological characterization of native South African EPNs
- Isolation and molecular identification of bacterial symbionts
- Desiccation tolerance of EPNs
- Whole genomic DNA sequencing, assembly and annotation of bacterial symbiont

Research experimental design

1. Soil samples were collected from Brits, North West province, South Africa.
2. Nematodes were isolated from soil samples using the *Galleria mellonella* insect baiting technique
3. Isolation of EPNs from infected larvae was achieved by White trap method
4. Reinfections for confirmation of Koch's postulates and for *in vivo* culturing of EPNs based on consistency of induced symptoms
5. Molecular identification of EPNs through the amplification and sequencing of the 18S and 28S rDNA amplification
6. Morphological characterization of EPNs
7. Isolation of bacterial symbionts from the haemolymph of larvae infected with EPNs using NBTA plates
8. Molecular identification of bacterial symbionts through amplification and sequencing of the 16S rDNA
9. Confirmation of the dependency of EPNs growth and development on bacterial symbionts: culturing of EPNs on bacterial lawns on lipid agar (*in vitro* culturing)
10. Desiccation tolerance of EPNs
11. Whole genomic DNA sequencing of bacterial symbiont
12. *De novo* genome assembly using CLC Bio and SPAdes
13. Genome annotation: National Center for Biotechnology Information (NCBI) prokaryotic genome automatic annotation pipeline (PGAAP) and rapid annotation using systemic technology (RAST)

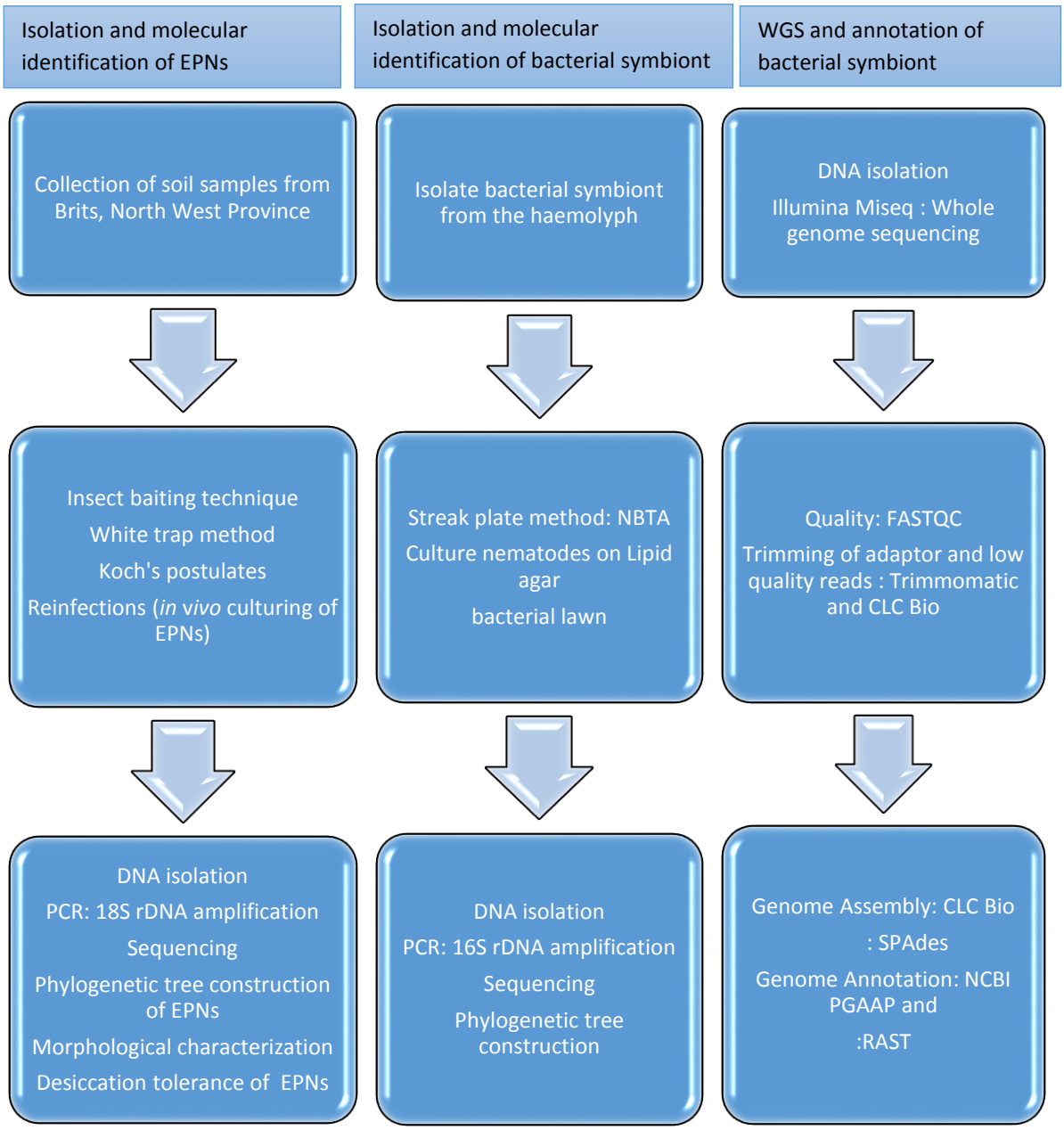


Figure 1.4: Outline of the methodology used in this study.

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CHAPTER 2: Isolation of entomopathogenic nematodes

2.1 INTRODUCTION

Nematodes are inhabitants of virtually every environment and are adapted to both living and non-living substrates (Wright and Perry, 2002). They are the most ubiquitous organisms on earth and most of them are free living and occur in non-living substrates. Free living nematodes inhabit soil, marine, fresh water and estuarine environments whereas parasitic nematodes inhabit plants, vertebrates or invertebrates. Extraordinary and extensive fundamental advances and discoveries on the most well studied nematode *Caenorhabditis elegans,* have made it easier to comprehend the physiology, ecological and behavioural adaptations of nematodes.

Nematodes perceive and respond to signals from the environment and from each other which enables them to locate a host or a mate, undergo development and survive stress (Pionar *et al*, 1990; Gaugler, 2002). A group of specialised nematodes that have received the considerable attention due to their potential as biocontrol agents are entomopathogenic nematodes (EPNs) (Kaya and Gaugler, 1993). These are non-segmented roundworms that harbour bacterial symbionts which are insect pathovars.

EPNs are parasitic to insect pests and kill their hosts with the aid of the bacterial symbiont carried in their alimentary canal (Burnell and Stock, 2000). EPN species belonging to the two genera *Steinernema* and *Heterorhabditis* have emerged as excellent biological control agents and have a specific obligatory association with bacterial symbionts in the genera *Xenorabdus* and *Photorhabdus* (Adams and Nguyen, 2002; Boemare, 2002; Emeliahoff *et al*, 2008). They gained status in the late 1970s as one of the best non-chemical alternatives for control of insect pest population due to 1. Their ability to reach insect pests 2. High reproductive ability 3. Ease of mass production and 4. Harmless to vertebrates and plants (Bemare, 2002). A new genera, *Oscheius* have recently been discovered to be parasitic to insect pests hence EPN, and have been reported to be associated with bacterial symbionts in the genus *Serratia*.

2.1.1 The life cycle of EPNs

The third stage infective juvenile (IJ) is the only free living non-feeding stage that is able to persist in the soils for lengthy periods. When conditions are favourable, IJs are able to search the soil environment for susceptible arthropod hosts. Generally, IJs enter the host larval digestive tract or haemocoel through natural openings such as the mouth, anus and respiratory spiracles. Some species are able to gain direct ingress by penetrating through the insects' outer

integument. Upon entry, the nematodes penetrate into the haemocoel of the larval host and subsequently regurgitate their associated bacterial symbiont. The bacterial endosymbionts produces secondary metabolites that depress the immune system of the insect larvae. The metabolites are also lethal to the larvae which succumb to septicaemia within 48 hours post infection. The bacterial endosymbionts also produces antimicrobial products that elicit toxicity towards other microorganisms thereby resulting in monoxetic conditions within the larval cadaver.

Bacterial enzymatic digested cadaver tissues and the actual bacterial symbiont serve as sources of nutrients for nematode growth, development and reproduction. After two to three reproduction cycles and when the nutrient supply within the cadaver becomes limiting, the juvenile nematode within the martenal body re-associate with bacterial symbionts and develop into its non-feeding infective juvenile and emerge from the insect carcass in search for new susceptible larval hosts.

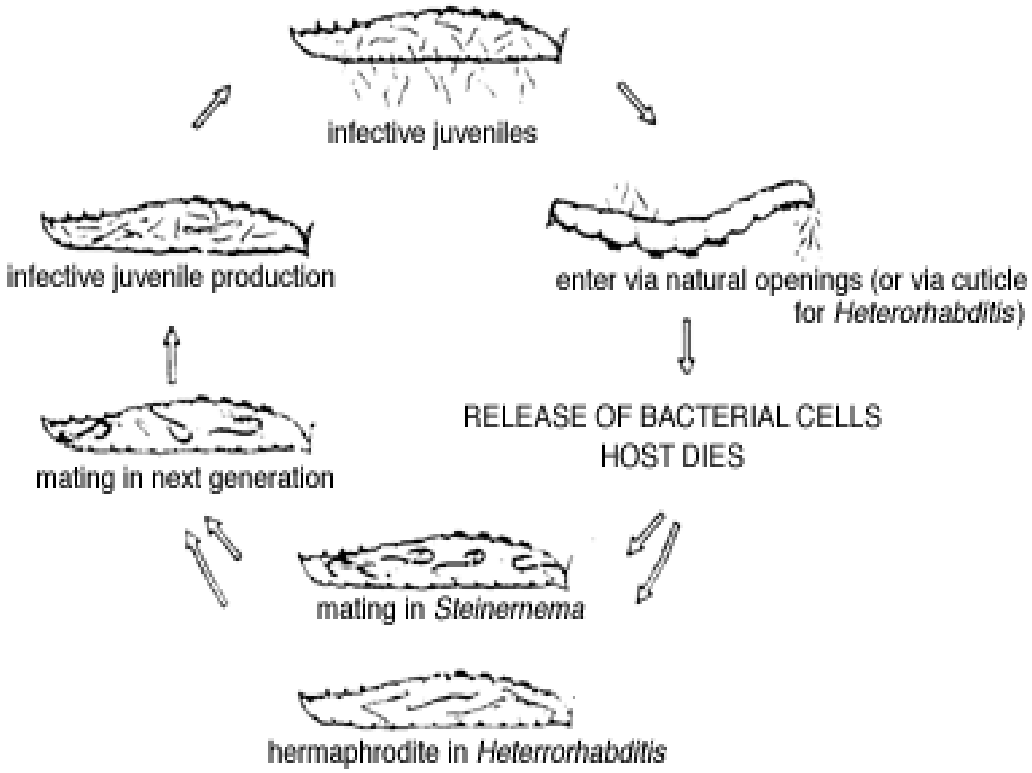


Figure 2.1: The general life cycle of EPNs

2.1.2 Distribution, biological control and host range

Soil is a more suitable habitat for targeting or isolating EPNs. The undisturbed soil profile is the natural reservoir for both steinernematids and heterorhabditids. EPNs naturally inhabit soil and factors that allow for their adaptation to and long term persistence in the soil environment includes soil texture, pH and temperature. They are widely distributed in soils throughout the world and this indicate their genetic ability to adapt to various environmental stresses that they experience in the soil environment (Hominick *et al,* 1996; Adams *et al,* 2006).

Steinernema glaseri is considered to be sub-tropical or possibly tropical in origin and *Steinernema carpocapsae* is widely distributed in temperate areas of the world and have extensively been tested for their potency against soil pests (Pionar, 1986). *S. glaseri* was first used against the Japanese beetle grubs, *Popillia japonica* in the 1930s, however tests gave encouraging results initially and were ultimately unsuccessful, which was followed by a suspension of further on this nematode for 20 years (Gaugler, 1988; Klein and Geogis, 1992). The unsuccessful results was presumably attributed to the researchers being unaware of the nematode's symbiotic bacterial partner with the bacteria being eliminated through the incorporation of antimicrobials into the rearing media. The efficacy of this nematode species have been re-evaluated in recent years with positive results obtained for the black vine weevil larvae and white grubs (Villani and Wright, 1988).

Favourable results have been obtained against soil inhabiting insects where *Heterorhabditis* spp regularly reduced black vine weevil population densities by 90% (Bedding and Miller, 1981 and Stimman *et al,* 1985). Positive encouraging results have also been obtained in soil treatment against mole crickets (Cobb and Geogis, 1987), wireworms (Kovacs *et al,* 1980), colorado potato beetles (Wright *et al,* 1987), root maggots (Van Sloun and Sikora, 1986) and cutworms (Lossbroek and Theuiseen, 1985). The nematodes have not had to adapt to specific host life cycle stages and were able to parasitize hundreds of insect pests. They have demonstrated to have a wide insect host range; while showing high virulence characteristics towards their arthropod hosts and no mammalian pathogenicity have been observed. Numerous surveys have provided evidence of the omnipresence of these nematodes in both natural and agricultural soils (Hominick, 2002). The application of EPNs as biocontrol agents requires knowledge of the occurrence and presence of native EPN species as introduction of exotic species may induce exclusion of natural species and may not be able to target local insect pest

as they may not be adapted to local environmental conditions (Miller and Barbercheck, 2001). Surveys have been conducted in South Africa and throughout African countries for EPN recovery (Malan *et al*, 2006). Steinernematids including *S. karii*, *S. taysaerae*, and *S. yirgalemense* have been isolated from Kenya, Egypt from Ethiopia, respectively (Shamseldean *et al*,1996 and Nguyen *et al*, 2004) .

In South Africa steinernematids including *S. khoisanae*, *S. citrae*, *S. yirgalemense, S. sacchari* and heterorhabditids including *H. safricana, H. bacteriophora, H zealandica* and *H. noeniputensis* have been discovered and described (pionar, 1986; Stokwe *et al*, 2011; Malan *et al*, 2008; Hatting *et al, 2009*). Generally, South Afican climate conditions range from subtropical in the North East, temperate in the interior plateau and Mediterranean in the South Western areas. The North West province is characterized by hot and dry conditions, have an average rainfall of 300 to 700 mm annualy and contribute about 13% to the agricultural sector of the country's provincial GDP. The primary objective of the current study was to isolate South African native EPNs through the insect baiting technique using the model organism *Galleria mellonella* from soil samples collected from the Brits area, in the North West province. The secondary objective was to recover infective juvenile nematodes through White trap method from infected dead larvae.

2.2 MATERIALS AND METHODS

2.2.1 *In vivo* rearing of *Galleria mellonella* larvae

Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Lepidoptera, Family: Pyralidae, Subfamily: Galleriinae, Genus: *Galleria*, Species: *G. mellonella*



Figure 2.2 A) *G. mellonella* insect moths and B) *G. mellonella* larvae.

Aseptic rearing of *G. mellonella* was conducted by placing adult female and male moths in 3L glass bottles (11cm diameter and 23cm height) and allowed to mate. The strips of wax paper on which the eggs can stick were inserted into the glass jars to facilitate the recovery of the eggs (oviposition). Larval growth and development was maintained by continuous supply of *Galleria* medium (wheat, honey and glycerol, see appendix I) in 7 days interval. The metal Consol lids were modified by incorporating stainless meshes into the lid so as to facilitate air exchange while preventing larvae from escaping from jar. The jars were kept in the incubator at 25 C.



Figure 2.3: In *vivo* rearing of *G. mellonella larvae*

2.2.2 Isolation of EPNs from collected soil samples

2.2.2.1 Soil sampling

A total of 50 soil samples (10 -20cm deep) were collected in 2L plastic tubs from Brits, North West province, South Africa. The soil samples were kept at 22-25ºC during transport to the laboratory. Prior to insect baiting technique, the soil samples were sieved to remove the grass tufts. Tap water was added to give moisture content of 8%. The plastic tubs were stored at 25 ºC for 24 hours.

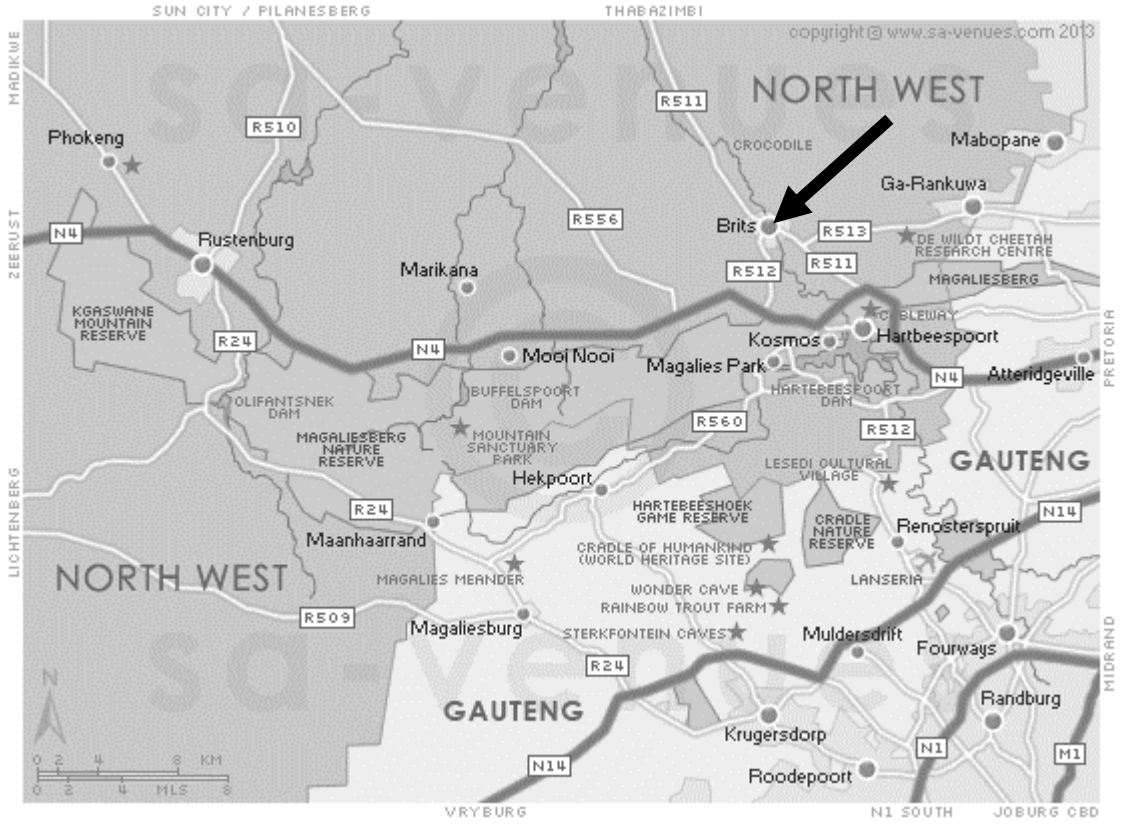


Figure 2.4: The South African Map showing North West Province, Brits – town where the soil samples were collected

(http://www.johannesburg-venues.co.za/magaliesberg-map.htm)

2.2.2.2 Insect baiting technique

Insect-baiting technique of soil samples is a widely used technique for the isolation of EPNs from soil samples. Nematodes were recovered from collected soil samples by baiting with 10 insect larvae of *G. mellonella.* To maximize recovery, second and third baitings were conducted with fresh *G. mellonella* larvae in the same soil. The boxes were inverted and kept at 25 ºC to facilitate or favour infection by EPNs. The boxes were monitored and checked for the presence of dead larvae periodically 48 hours post baiting. Parasitized cadavers were

recognized by change in colour (usually red/purple for heterorhabditids and ocher/brown/black for steinernematids) (Kaya and Stock, 1997). Dead larvae were collected and the symptoms were analysed and used for provisional identification.



Figure 2.5: Insect baiting technique used for isolation of nematodes from soil samples using *G. mellonella* larvae

2.2.2.3 Nematode recovery from infected larvae: White trap method

Infected larvae were surface sterilized by spraying with 70% ethanol. Nematodes were isolated from infected dead larvae by modified White trap method (Kaya and Stock, 1997).

White Trap Method

A lid of a small Petri dish (50 mm) was placed in a large Petri dish plate (90 mm). The former was covered with a 54 mm Whatman No1 filter paper disc and the surface sterilized dead larvae was placed on the filter paper. Water was added into the latter dish until it reaches the edge of the filter paper which remains moist by absorbing the water. The lid of the larger Petri dish was replaced and the white traps were incubated at 25 ºC to facilitate emergence of nematodes.



Figure 2.6: White trap method for isolation of nematodes from infected cadaver

2.2.2.4 Koch's Postulates and maintenance of EPN culture

Nematodes were collected by sedimentation in a 50mL Falcon tube and were surface sterilized with 0.1% sodium hypochlorite. Autoclaved river sand in 90 mm Petri dishes with 8% moisture content were inoculated with sterilized nematodes. Ten *G. mellonella* larvae were placed on top of the sand to confirm pathogenicity based on consistent symptomatic analysis (Koch's postulates).

Koch's postulates

Four criteria established by Robert Koch to identify a pathogen of a particular disease:

1. The microorganism or other pathogen must be present in all cases of the disease

2. The pathogen can be isolated from the diseased host and grown in pure culture

3. The pathogen from the pure culture must cause the disease when inoculated into a healthy, susceptible laboratory animal

4. The pathogen must be re-isolated from the new host and shown to be the same as the originally inoculated pathogen

Nematodes were maintained both *in vitro and in vivo. In vivo* maintenance of nematodes entailed using the insect baiting technique involving the Petri dish river sand soil procedure

that was applied in Koch's Postulates test. EPNs were maintained *in vitro* by culturing on lipid agar bacterial lawn plates.

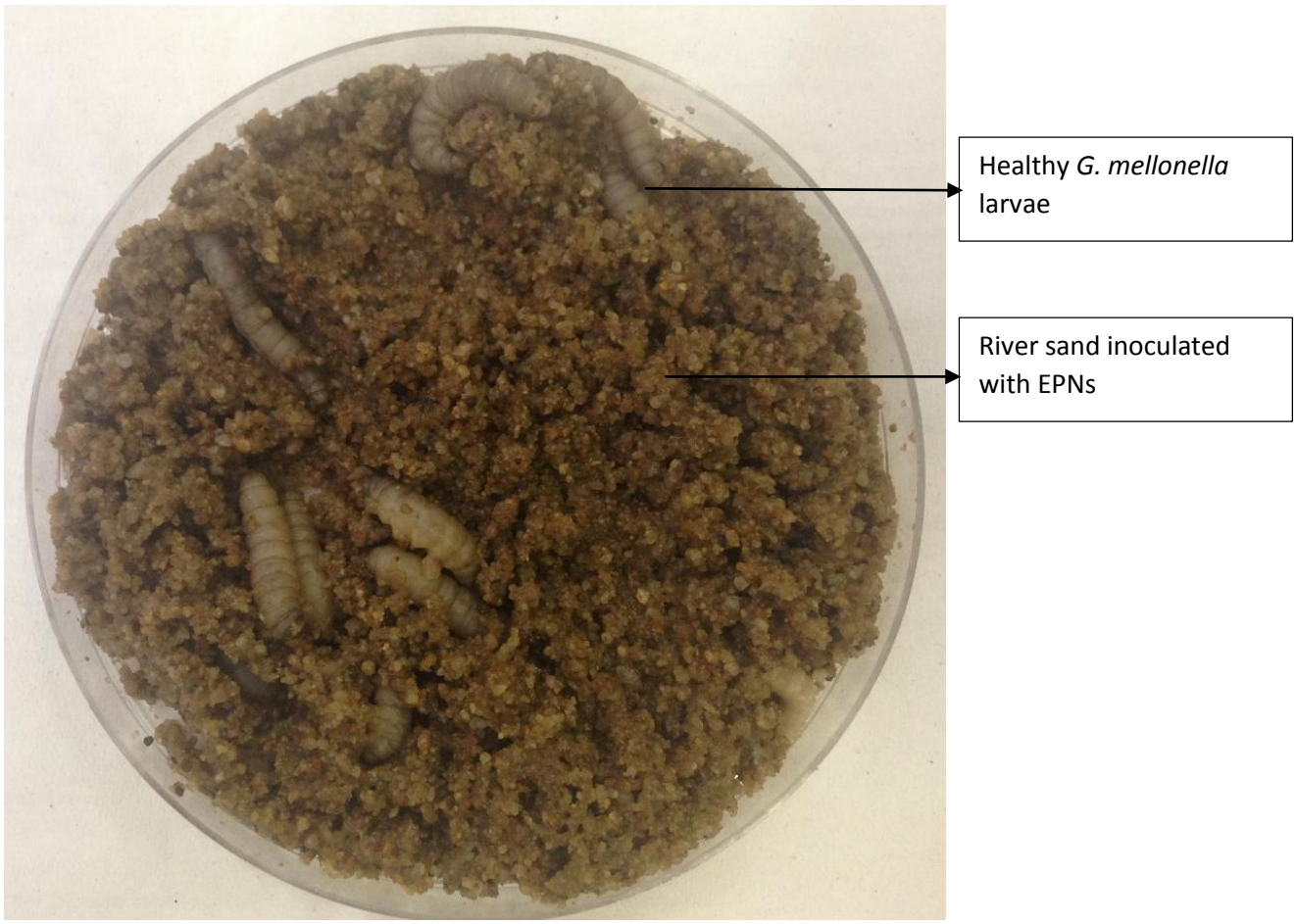


Figure 2.7: *In vivo* maintenance of EPNs.

2.3. RESULTS

2.3.1 Symptom variation and emergence of nematodes on white trap

Entomopathogenic nematodes were successfully isolated from the collected soil samples. Symptoms induced by heterorhabitids varied from those induced by steinernematids. Insect larvae infected by the former were associated with the development of green pigmented larvae change and those infected by the latter turned dark brown. Symptom colour or pigment variations following larval infection and mortality were used as preliminary markers for EPN identification. The nematodes were successfully isolated from infected cadavers by white trap method and successful reinfections favoured Koch's postulates. The steinernematids made full use of the host's tissues and upon IJ emergence the cadaver collapsed and appeared flat. It took 4-5 days for steinernematids to emerge from the cadaver and 7-14 days for heterorhabditids.



Figure 2.8: Symptom variation of insect larvae infected by A) Heterorhabditids and B) Steinernematids

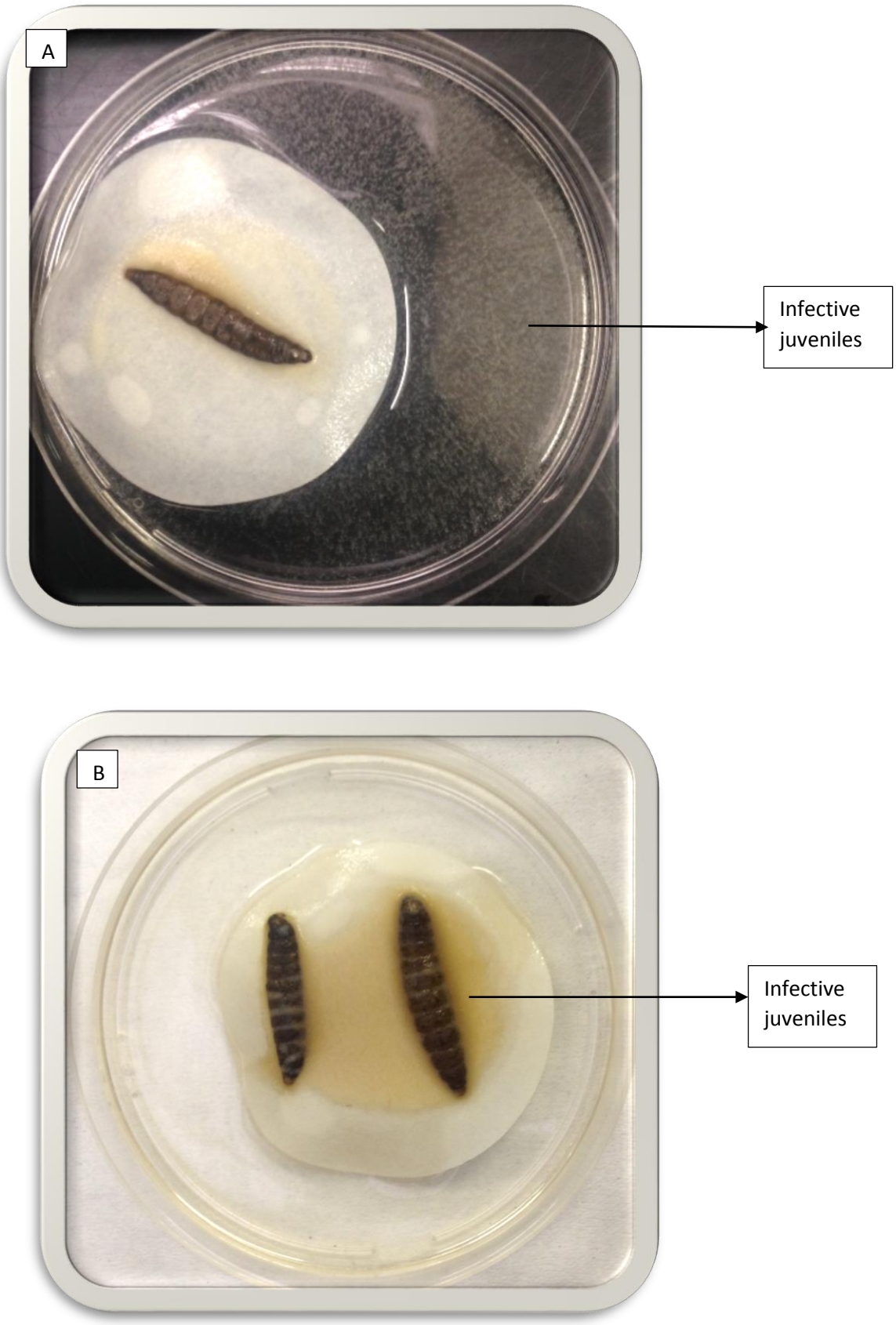


Figure 2.9: White trap method used to isolate EPNs from infected cadavers. A) Nematodes are visibly clear in the white trap water. B) Emergence of nematodes from larvae infected by *Steinernema* species.

Table 2.1: Recovery of nematodes from soil samples collected at different regions at Brits, North West province, South Africa.

Region	Isolate #	Soil type	Larval symptoms	Preliminary species identification
Wild fig tree (WFT)	10	Sandy loam	Dark brown	*Steinernema* spp
Emoe 1	35	Sandy loam	Green	*Heterorhabditis* spp
Emoe 2	42	Sandy loam	Green	*Heterorhabditis* spp
Red Ivory, Tuscaloosa, Boundry road	No recovery	Clay loam, Sandy loam and Clay loam	No recovery	None

2.4 DISCUSSION

Soil is an important habitat for nematodes and various soil properties appear to play a critical role in contributing to the survival and longevity of infective juveniles within the soil profile. The moisture content of soil sample is an important factor for recovery of nematodes and 8% is reported to be sufficient for isolation of heterorhabditids and steinernematids from dry soil samples collected from the field. Of the 50 soil samples collected, nematode isolates were only recovered from 3 regions namely Wild fig tree (isolate #10), Emoe 1 (isolate #35) and Emoe2 (Isolate #42) (table 2.1). Preliminary identification was based on symptom variation post confirmation of Koch's postulates. Steinenematids infection was associated with black, brown and beige larval colour change while infection by heterorhabditids was associated with marron, brick red and green larval colour change. Insect larvae infected by isolate 10 were dark brown in colour and those infected by isolate 35 and 42 were green suggesting that isolate 10 was a steinernematid nematode and both isolate 35 and 42 were heterorhabditids.

G. mellonella larvae used as a model organism for isolation of nematodes is a widely used organism as it is highly susceptible to most steinernematids and heterorhabditids, however species including *S. scapterisci* are incapable of reproducing within *G. mellonella* and isolation of such nematodes may have been missed in this study.

Greater sample size increases chances of positive sites for isolation. An extensive study which was conducted in Turkey for isolation of nematodes involved a sample size of 1080 and of those, 22 were positive for nematode isolation. In South Africa the most abundant species isolated from soil samples has been reported to be those belonging to the genus *Heterorhabditis*, with only few *Steinernema* species isolated and described. Malan *et al*, 2011 collected 202 soil samples (129 samples from the Western Cape with 20 positive sites, 52 samples with 8 testing positive, and 21 from Mpumalanga with 7 testing positive) of which 35 tested positive for nematode isolation and of those 89% were heterorhabditids. A study conducted by Hatting *et al* (2009) recovered nematodes in soil samples collected from different habbitats across seven regions of South Africa. 1506 samples were collected and nematodes were recovered from only 5% of total sample size. Four steinernematids (*S. khoisanae, S.* sp1, 2 and 3 were recovered from humid subtropical and semi-arid regions, with 80% of all steinerenematid isolates recovered from semi-arid climate zones which characterized by sandy and acidic soils (Hatting *et al*, 2009). Another study which was conducted by Stock and Gress (2006) from soil samples collected from oak woodlands in mountain ranges of Coronado national forest in Southern Arizona involved 120 total sample size and recovered nematodes from 28 samples of which 78.5% were steinernematids and only 21.5% were heterorhabditids, suggesting the greater abundance of steinernematids in this instance. In this study, about 50 samples were collected and only 3 tested positive with 2 heterorhabitids and only 1 steinernematid isolated from Brits, North West province in South Africa.

Steinernematids are reported to be found in temperate regions and heterorhabditids throughout much of the tropics and subtropics. Brits is a large district situated in a fertile citrus producing area that is irrigated by the water of the Hartbeespoort Dam in the North West province. EPNs have been isolated from soil samples from different parts of the world. Persistence and occurrence has been reported to be in areas infested with insect pests including agricultural and forested vegetation as infective juveniles are dependent on larval hosts for reproduction. In South Africa, diversity of EPNs have been established in Citrus orchards and two new species have been identified (Malan *et al*, 2011). Previous studies have also reported presence of EPNs in oak woodlands and also organic cultured vines suggesting native biodiversity of EPNs which should be taken into consideration for EPN-based biocontrol programs.

EPNs recovered in this study were recovered from sandy loam samples with no recovery from clay loam samples. This suggest that soil texture plays a pivotal role in persistence of EPNs as pore size and space influence the movement of EPNs to search for insect larval hosts. Clay soil interfere with movement and parasitism of nematodes hence no recovery in clay soil samples. Possibly the effects of high percentages of clay on soil texture also interferes with the IJs capacity to adapt to soil dehydration.

Insect baiting technique and White trap method proved to be effective methods in isolation of nematodes from soil samples. Preliminary identification based on symptoms is limited to genus level and requires validation through molecular based and microscopy techniques for identification to species level.

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Chapter3: Molecular Characterization of Entomopathogenic Nematodes

3.1 INTRODUCTION

Entomopathogenic nematodes (EPNs) belong to the genera *Steinernema*, *Heterorhabditis* and the newly discovered *Oscheius* and hold great potential as biological control agents of economically important insect pests (Gaugler, 1988; Kaya, 1985; Bedding and Akhurst, 1975). They have a mutualistic relationship with bacterial symbionts in the genera *Xenorhabdus*, *Photorhabdus* and *Serratia*, respectively. The bacteria produces toxins that are lethal to insect host larvae and the nematodes serves as vectors for protecting the bacterial symbiont from harsh environmental conditions and for transportation to the target host larvae (Klein *et al*, 1990).

The EPN-bacteria symbiont complex holds great potential as a biological control agent of soil-borne insect pests (Ehlers, 1996; Kaya and Gaugler, 1993; Shapiro-Ilan, 2005). New species and strains are constantly being isolated and discovered, however, their identification is not always straight forward as taxonomic relationships are usually based on morphological identification. Identification that is solely based on morphological characterization is not necessarily sufficient for identification of nematodes.

There has been a considerable debate about the unambiguous reliability of identification methods of EPNs (Curran and Webster, 1989; Gaugler and Kaya, 1990; Pionar, 1990). Prior to our current knowledge on the molecular genetic diversity of nematodes within species and populations, the taxonomic relationships of nematodes have usually been based on morphological characteristics for heterorhabditids as they are hermaphrodites and for steinernematids, morphological characters were combined with cross-breeding data as they are amphimictic (Akhurst and Bedding, 1978; Pionar, 1986, 1990).

Due to morphological similarities amongst numerous strains it has become difficult to accurately identify species based purely on morphology. Researchers have now adopted more reliable methods which include the use of molecular techniques in identifying nematodes, which are not only able to distinguish between the two genera, but also between species within

each genus where phenotypic variation can be low (Omar *et al*, 2014; Stock *et al*, 2008). The benefits from the application of molecular approaches to study the phylogenetic relationships and taxonomic affinities amongst EPNs have been greatly appreciated (Curran, 1990; Emeliahoff *et al*, 2008; Liu *et al*, 1999). These techniques include the polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD (Liu and Berry, 1995; Liu *et al*, 1997; Hashmi *et al*, 1996). PCR and RFLP have been used extensively for the genetic characterization of *Heterorhabditis* and *Steinernema* species (Smith *et al*, 1991, Reid and Hominik, 1992; Joyce *et al*, 1994). The isoenzyme banding patterns have also been used to detect variability amongst species of both genera. (Akhurst, 1987; Curran, 1990). RADP detects polymorphisms and has been used to study genetic diversity and genetic relationships in EPNs (Williams *et al* 1990; Welsh and McClellan, 1990).

The ribosomal DNA (rDNA) has been the targeted region used in identification of many organisms including EPNs, as it is present in high copy numbers in the form of multi-tandem repeat (Susurluk *et al*, 2007; Hasmi *et al*, 1995). The repeat contains both highly conserved regions and potentially highly variable region. The conserved regions allows for amplification using universal primers and the highly variable region referred to as the internal transcribed spaces (ITS) allows for identification of new species.

EPNs have 18S and 28S rDNA regions which are highly conserved and the more polymorphic ITS regions (ITS1 and ITS2) which are found in between the 18S and 28S. The two spacers flank a highly conserved region, 5.8S, which is the ribosomal RNA gene having low levels of variation and thus represents a region with slow evolutionary rates. Identification of new species and determination of the variability amongst species and strains requires the amplification and sequencing of the 18S, ITS and 28S rDNA regions. Sequence analysis of these regions have proved to be accurate in the assessment of phylogenetic relationships at taxonomic and species level (Adams *et al*, 1998; Darissa and Iraki, 2014). The ITS is an ideal region for molecular taxonomic studies and due to the conserved genes flanking this region, universal primers are generated to allow for amplification. (Reid *et al*, 1997). This study aimed at applying molecular techniques for identification of EPN isolates.

3.2 MATERIALS AND METHODS

3.2.1 Molecular Identification of Nematodes

3.2.1.1 Genomic DNA extraction

Infective Juveniles (IJs) collected from White traps were allowed to settle under gravity in 50mL Falcon tubes. The IJs were sterilized by incubating for 3 hours in 0.1% sodium hypochlorite. The IJs were rinsed 3 times with 4mL of sterile distilled water post surface sterilization in a laminar flow. The nematodes were pelleted in microfuge tubes by spinning at 13 400rpm for 10 minutes. The tubes were placed on ice for 30 seconds and excess water was discarded. The nematodes were re-suspended in 1mL distilled water and centrifuged at 13 400 rpm for 3 minutes. After removing the supernatant, 600 µL of cell lysis solution and 3ul of proteinase k solution were added into the microfuge tube with EPNs and the tube was inverted 25 times. The samples were incubated at 55ºC for 24 hours to allow for nematode cell lysis and degradation of cytoplasmic proteins.

About 3µl of RNAse was added to the cell lysate and incubated at 37ºC for 30 minutes for catalysis of RNA degradation. Post incubation, 200µl of protein precipitation solution was added to the proteinase-k and RNAse treated cell lysate followed by centrifugation at 13 400 rpm to precipitate degraded proteins. The supernatant containing the DNA was transferred into a clean centrifuge tube containing 600µl of 100% isopropanol and centrifuged to pellet the genomic DNA and the supernatant was discarded. The DNA pellet was subsequently washed with 70% ethanol via centrifugation and the supernatant was discarded. About 100ul of DNA was added into the tube followed by incubation at 65ºC. The genomic DNA was stored at 4ºC.

3.2.1.2 PCR: amplification of the 18S and 28S rDNA

Polymerase chain reaction for the amplification of the 18S rDNA was conducted for identification of the nematode species. The universal primers (TW81-forward primer and AB28-reverse primer) were used for the amplification of the 18S and 28S rDNA. The reaction mixture was prepared by adding the reagents Master Mix, genomic DNA, forward primer, reverse primer and nuclease free water, with the exception of addition of genomic DNA in the control tube as illustrated in table3.2.

Table 3.1: The 2 universal forward and reverse oligonucleotide primers used to amplify the ITS regions found between the 18S and 28S rDNA region of the nematode genomic DNA (Joyce *et al.* 1994).

Oligonucleotide primer	**Sequence**	**Tm (°C)**	**Ta (°C)**
TW81 Forward Primer	5'-GCGGATCCGTTTCCGTAGGTGAACCTGC -3'	71.94	66.4
AB28 Reverse Primer	5'-GCGGATCCATATGCTTAAGTTCAGCGGGT -3'	68.87	63.87

Table 3.2 PCR reaction mixture for amplification of the 18S and 28S rDNA.

Reagent/sample	Volume (µl) Experiment	Volume (µl) Control
Master Mix	25	25
Nematode genomic DNA	3	0
Forward primer	3	3
Reverse primer	3	3
Nuclease free water	16	19
TOTAL	**50**	**50**

Amplification cycle

25 cycle amplification series:

Denaturation at 95°C for 60 seconds

Annealing at 64°C for 60 seconds

Extension at 72°C for 120 seconds

Final extension after cycling: 72°C for 10 minutes

3.2.1.3 Sequencing of the 18S and 28S rDNA

The PCR products of the 18S rDNA amplification were purified and sequenced using Sanger sequencing technology at Inqaba Biotechnical Industries. The rDNA sequences were edited and error corrected using FinchTV.

3.2.1.4 Identification: NCBI BLASTn

The National Centre for Biotechnology Information (NCBI) database, basic local alignment search (BLASTn) algorithm was used for identifying the nematode species through local alignment by finding species that have high similarity percentage to the query sequence.

3.2.1.5 Multiple alignment: MEGA 6.1 CLUSTAL W

The query sequence and other sequences for the partial 18S, ITS1, 5.8S, ITS2 and partial 28S rDNA of the already existing, identified and known species from the NCBI-BLASTn search results were loaded onto MEGA 6.1 (molecular evolutionary genetics analysis, version 6.1) program and were multi-aligned with clustalW using the default parameters. This was conducted for unidentified species of *Heterorhabditis* and *Steinernema* species that had been isolated, respectively. *Caenorhabditis elegans* was used as the outgroup. The evolutionary divergence between aligned sequences was analysed by MEGA 6.1 pairwise distance.

3.2.1.6 Phylogenetic analysis: MEGA 6.1

Phylogenetic relationships between the aligned isolates were established using MEGA 6.1 following the Maximum Likelihood method. Evolutionary distances between the unknown and known isolates were computed using the Kimura-2 parameter which is favourable for phylogenetic analysis of sequences with different base composition such as for the ITS regions. Phylogenetic trees were constructed by clustering of associated taxa based on 1000 replicates in the bootstrap statistical test.

The following taxa were used for phylogenetic tree construction of *Heterorhabditis:*

Caenorhabditis elegans isolate X5005 (FJ589008.1), *Heterorhabditis zealandica* strain Bartow (GU174009.1), *Heterorhabditis zealandica* strain NZH3 (EF530041.1), *Heterorhabditis marelatus* (AY321479.1), *Heterorhabditis megidis* (AY321480.1), *Heterorhabditis bacteriophora* (AY321477.1), *Heterorhabditis amazonensis* (DQ665222.1) and *Heterorhabditis bacteriophora* isolate IRA24 (EU598232.1), *Heterorhabditis safricana* (EF488006.1)**, *Heterorhabditis noenieputensi*s strain WS17 (KP335198.1)**,

Heterorhabditis noenieputensis strain SF669 (JN620538.1)**, *Heterorhabditis bacteriophora* strain J172 (EU716335.2)**, *Heterorhabditis zealandica* strain SF41 (EU699436.1)** and Caenorhabditis elegans isolate X5005 (FJ589008.1)

The following taxa were used for phylogenetic tree construction of *Steinernema :*

Steinernema nyetense (JX985266.1), *Steinernema cameroonense* (JX985267.1), *Steinernema carpocapsae* (LN624759.1), *Steinernema carpocapsae* strain Dok83 (KJ950293.1), *Steinernema carpocapsae* strain NCR (KJ950292.1), *Steinernema glaseri* (AF122015.1), *Steinernema cubanum* (AY230166.1), *Steinernema thermophilum* (EF431958.1), *Steinernema yirgalemense* (AY748450.1), *Steinernema oregonense* (GU569055.1), *Steinernema oregonense* strain OS-10 (AF331891.1), *Steinernema poinari* strain 1160 (KF241749.1), *Steinernema poinari* strain 1093 (KF241751.1), *Steinernema poinari* strain tomsk (KF241750.1), *Steinernema arenarium* (AF331892.1), *Steinernema intermedium* (AF331916.1), *Steinernema everestense* (HM000103.1), *Caenorhabditis elegans isolate* X5005 (FJ589008.1), *Steinernema khoisanae* strain 106-C (EU683802.1)**, *Steinernema yirgalemense* strain 157-C (EU625295.1) **, *Steinernema sacchari* strain SB10 (KC633096.1)**, *Steinernema tophus* isolate ROOI-352 (KJ701241.1)**, *Steinernema innovation*i isolate SGI-60 (KJ578793.1)**.

All species marked with "**" are isolates recovered in South Africa and C elegans was used as an output.

3.3 RESULTS

3.3.1 Sequencing of the 18S and 28S rDNA of the isolates.

Successful amplification of the 18S and 28S rDNA of isolate 10, 35 and 42 was followed by successful sequencing and the sequences obtained from the Inqaba biotechnical laboratories are illustrated in figure 3.1 -3.3.

CGGGGTGTATGGAACATCGATTAAGTTCACAATGCGTCCGCTGATCAACGAAAC
GTGTTAAATCAATGACATGGTTACCGACTCTTCAAGAAACGATTACGCACAAAG
CAGAAGCTTAGTACAAGTCAGCTGGCAAGAGACACGCCAAAACCACTAACGCAC
CGAACCGATATGATAGCAGCGACACAGAGAGCAATCTCGCGAAAGAAACGCTCG
CAAGAATGAAACTGCGCGCCATTAAACTATCGCACAAAAGCGCAATCACACCGA
AAAGGTACTCTTTGCAGAGTAGCTCATCGAAAAAACAGTCCAAGCTGACTGCAA
GTAACTAGTTAATCGACCCTCAACCAAACATACTATCAGATATAAACCTGATAGT
GCCATTTGCGTTCAAAATTTTAGCGTTCAATATGTCTGCAATTCACGCCAAATAA
CGGTTTTTGCCCCGTTTTTCATCGAACTACGAACCGAGTGATCCACCGATAAGAC
TTGATAAAGATTGTTGGTTACGCTACCATACGCATGGAGCATAATCAATGTAAAA
AATGTTAATAGTAGGGTTGGCCATGGGGGGTCCCCCAATGGACTCCTTTATTTCC
ATCCCCCGATGGTCCAGAAACTGGCCGAACCCACCGGAAAAAGAAGGTAAAAGT
CCCTTATAACACCCCCTCTCATTCAGGTGCCCCCTAAGCCTATAAAAACCATTAA
AAGCCGATCCAGACCAAATTCACTTTAAAAACACCACCCGGCCCCGTTCCATCCT
TCAAACCCTTATGGGAATAGCTTCATAATGGTCCTTCCCCAGTCCCCCCCGGGAA
ACAA

Figure 3.1: The sequence of isolate #10. The NCBI BLASTn results revealed high affinity to *Steinernema* species.

GGGAGGTGCCAAGTAAGATAGCATAAGAAGCACCTCCTAGCCATGTATGGGAAG
CATCATATTTCATACGCGACACATCCATAGGTACAGACTTATACTCTCTACGGCG
TCTTCACGAAGAAGACATGTTGCCAATATTCGGAACTAGAACGGGGCCAGTAGT
AACACTAACGCCGCTCGACATTCGGCGTTGCCACTTTCGCAGCAACACCGCAGTA
CTTTAAGCAACCCTGAGCCAGACGTGCCGAAGGGAAAACCCAACGGCGCTGTGC
GTTCAAAACTTCACCACTCTAAGCGTCTGCAATTCGTGGTAAATAACGCAGCTAG
CTGCGTTTTTCATCGATACGCGAATCAACCGATCCATCGCTGAAGCTAATACATT
GTGCCAAAATGTATTAACACGTATCCAAGAGACAAGATAACGATACGTACCCAA
AAATCAAATATCGCAAGGCGGTTGACGGAACAACTCATTGAGGCTCTCCGCATA
GCGGATCTCGATGTGACCGAAGTCACATCACAGCCACCGGTCCACGATAGTTTG
GCTGAGGCAACAGTCCGAGAGTACTGTTCATTAAGTCTATGCGCCACCCATACGG
GCAACACACCCAATCATCACTGCCGTCACTCCAAGCGACTCCAATTGGGAATAG
ACACCTCACCAAGAGCTTAGATGGGGTGAGCTACCGATTGAAATCAAACGTGAG
CCTGATTCCATGAGCACCGATAGCTCGTGACCAAAGCATAACCATTAAGGTTTTC
AGCGATGATCCATCTGCAGGTTCACCATACGGAAAAC

Figure 3.2: The sequence of isolate #35. The NCBI BLASTn results revealed high affinity to *Heterorhabditis species*

CCAGTAAGATAGCATAAGAAGCACCTCCTAGCCATGTATGGGAAGCATCATATT
TCATACGCGACACATCCATAGGTACAGACTTATACTCTCTACGGCGTCTTCACGA
AGAAGACATGTTGCCAATATTCGGAACTAGAACGGGGCCAGTAGTAACACTAAC
GCCGCTCGACATTCGGCGTTGCCACTTTCGCAGCAACACCGCAGTACTTTAAGCA
ACCCTGAGCCAGACGTGCCGAAGGGAAAACCCAACGGCGCTGTGCGTTCAAAAC
TTCACCACTCTAAGCGTCTGCAATTCGTGGTAAATAACGCAGCTAGCTGCGTTTT
TCATCGATACGCGAATCAACCGATCCATCGCTGAAGCTAATACATTGTGCCAAAA
TGTATTAACACGTATCCAAGAGACAAGATAACGATACGTACCCAAAAATCAAAT
ATCGCAAGGCGGTTGACGGAACAACTCATTGAGGCTCTCCGCATAGCGGATCTC
GATGTGACCGAAGTCACATCACAGCCACCGGTCCACGATAGTTTGGCTGAGGCA
ACAGTCCGAGAGTACTGTTCATTAAGTCTATGCGCCACCCATACGGGCAACACAC
CCAATCATCACTGCCGTCACTCCAAGCGACTCCAATTGGGAATAGACACCTCACC
AAGAGCTTAGATGGGGTGAGCTACCGATTGAAATCAAACGTGAGCCTGATTCCA
TGAGCACCGATAGCTCGTGACCAAAGCATAACCATAAAGGTTTTCAGCGATGAT
CCATCTGCAGGTTCACCAAGGGAAAACCA

Figure 3.3: The sequence of isolate #42. The NCBI BLASTn results revealed high affinity to *Heterorhabditis species.*

3.3.2 Evolutionary divergence

Evolutionary divergence based on genetic variation of the ITS region was analysed using MEGA6 pairwise distance using multi-aligned ITS sequences of species obtained from NCBI and the undescribed isolates (table 3.3 and 3.4). The lowest evolutionary divergence was observed between isolate 10 and *Steinernema khoisanae* strain 106-C** (0.187), and between undescribed isolate 35 and 42 with *Heterorhabditis zealandica* strain Bartow (0.00; 0.00), *H. zealandica* strain NZH3 (0.002;0.002) and the South African isolate *H. zealandica* strain SF41 (0.002;0.002) respectively, suggesting close relation. There was no divergence between *Heterorhabditis zealandica* strain Bartow and isolates (35 and 42) suggesting that they are of the same species.

Table 3.3: Estimates of evolutionary divergence between CLUSTALW multi-aligned *Steinernema* species and the undescribed isolate 10 ITS sequences. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model (Tajima and Nei, 1984). The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 527 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*, 2013)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. Undescribed Steinernema species (Isolate 10)		0.032	0.034	0.032	0.138	0.138	0.030	0.030	0.035	0.036	0.224	0.224	0.236	0.236	0.236	0.232	0.034	0.031	0.084	0.019	0.036	0.150	0.019	0.020
2. Steinernema nyetense JX985266.1	0.381		0.008	0.031	0.214	0.214	0.029	0.030	0.035	0.034	0.301	0.301	0.311	0.311	0.311	0.344	0.045	0.027	0.101	0.029	0.035	0.191	0.027	0.027
3. Steinernema cameroonense JX985267.1	0.388	0.037		0.031	0.181	0.181	0.030	0.030	0.035	0.035	0.229	0.229	0.291	0.291	0.291	0.294	0.046	0.027	0.118	0.031	0.036	0.166	0.029	0.029
4. Steinernema carpocapsae LN624759.1	0.380	0.347	0.356		0.166	0.166	0.034	0.033	0.035	0.032	0.194	0.194	0.162	0.162	0.162	0.304	0.042	0.030	0.092	0.030	0.032	0.159	0.029	0.030
5. Steinernema carpocapsae strain Dok83 KJ950293.1	1.246	1.417	1.344	1.335		0.000	0.191	0.166	0.127	0.138	0.017	0.017	0.022	0.022	0.022	0.018	0.220	0.177	0.484	0.172	0.137	0.016	0.151	0.142
6. Steinernema carpocapsae strain NCR KJ950292.1	1.246	1.417	1.344	1.335	0.000		0.191	0.166	0.127	0.138	0.017	0.017	0.022	0.022	0.022	0.018	0.220	0.177	0.484	0.172	0.137	0.016	0.151	0.142
7. Steinernema glaseri AF122015.1	0.339	0.330	0.325	0.413	1.326	1.326		0.008	0.038	0.038	0.316	0.316	0.329	0.329	0.329	0.341	0.042	0.030	0.122	0.025	0.037	0.255	0.024	0.023
8. Steinernema cubanum AY230166.1	0.342	0.336	0.324	0.413	1.276	1.276	0.035		0.039	0.038	0.255	0.255	0.238	0.238	0.238	0.298	0.042	0.030	0.115	0.026	0.038	0.214	0.024	0.023
9. Steinernema thermophilum EF431958.1	0.430	0.389	0.382	0.368	1.200	1.200	0.445	0.460		0.020	0.158	0.158	0.173	0.173	0.173	0.171	0.041	0.032	0.090	0.033	0.020	0.151	0.032	0.032
10. Steinernema yirgalemense AY748450.1	0.435	0.381	0.378	0.346	1.222	1.222	0.432	0.442	0.183		0.165	0.165	0.255	0.255	0.255	0.176	0.040	0.033	0.093	0.035	0.003	0.148	0.033	0.034
11. Steinernema oregonense GU569055.1	1.424	1.522	1.436	1.424	0.142	0.142	1.495	1.447	1.292	1.286		0.000	0.023	0.023	0.023	0.015	0.255	0.267	0.293	0.212	0.161	0.012	0.232	0.185
12. Steinernema oregonense strain OS-10 AF331891.1	1.424	1.522	1.436	1.424	0.142	0.142	1.495	1.447	1.292	1.286	0.000		0.023	0.023	0.023	0.015	0.255	0.267	0.293	0.212	0.161	0.012	0.232	0.185
13. Steinernema poinari strain 1160 KF241749.1	1.475	1.543	1.521	1.372	0.202	0.202	1.510	1.423	1.329	1.455	0.206	0.206		0.000	0.000	0.024	0.413	0.277	0.855	0.326	0.248	0.022	0.397	0.354
14. Steinernema poinari strain 1093 KF241751.1	1.475	1.543	1.521	1.372	0.202	0.202	1.510	1.423	1.329	1.455	0.206	0.206	0.000		0.000	0.024	0.413	0.277	0.855	0.326	0.248	0.022	0.397	0.354
15. Steinernema poinari strain tomsk KF241750.1	1.475	1.543	1.521	1.372	0.202	0.202	1.510	1.423	1.329	1.455	0.206	0.206	0.000	0.000		0.024	0.413	0.277	0.855	0.326	0.248	0.022	0.397	0.354
16. Steinernema arenarium AF331892.1	1.430	1.546	1.490	1.530	0.167	0.167	1.503	1.481	1.300	1.295	0.096	0.096	0.225	0.225	0.225		0.315	0.274	0.386	0.187	0.172	0.015	0.247	0.189
17. Steinernema intermedium AF331916.1	0.437	0.520	0.527	0.495	1.481	1.481	0.483	0.489	0.491	0.475	1.549	1.549	1.654	1.654	1.654	1.572		0.033	0.079	0.032	0.040	0.214	0.034	0.035
18. Steinernema everestense HM000103.1	0.366	0.301	0.300	0.324	1.338	1.338	0.328	0.331	0.373	0.384	1.490	1.490	1.494	1.494	1.494	1.476	0.421		0.123	0.026	0.033	0.227	0.026	0.027
19. Caenorhabditis elegans isolate X5005 FJ589008.1	1.007	1.057	1.119	1.010	1.824	1.824	1.129	1.128	0.996	1.005	1.662	1.662	2.080	2.080	2.080	1.759	0.942	1.161		0.091	0.096	0.506	0.080	0.079
20. Steinernema khoisanae strain 106-C EU683802.1 **	0.187	0.353	0.357	0.343	1.377	1.377	0.255	0.272	0.386	0.401	1.444	1.444	1.593	1.593	1.593	1.391	0.391	0.290	1.015		0.035	0.171	0.015	0.016
21. Steinernema yirgalemense strain 157-C EU625295.1 **	0.442	0.385	0.382	0.352	1.213	1.213	0.427	0.438	0.183	0.004	1.276	1.276	1.442	1.442	1.442	1.285	0.472	0.381	1.017	0.404		0.145	0.034	0.034
22. Steinernema sacchari strain SB10 KC633096.1 **	1.298	1.396	1.327	1.355	0.141	0.141	1.418	1.377	1.290	1.235	0.085	0.085	0.202	0.202	0.202	0.103	1.488	1.436	1.873	1.368	1.226		0.200	0.154
23. Steinernema tophus isolate ROOI-352 KJ701241.1 **	0.187	0.335	0.339	0.323	1.301	1.301	0.241	0.240	0.385	0.394	1.461	1.461	1.626	1.626	1.626	1.461	0.430	0.289	0.916	0.122	0.398	1.414		0.009
24. Steinernema innovationi isolate SGI-60 KJ578793.1 **	0.197	0.336	0.344	0.334	1.268	1.268	0.239	0.238	0.384	0.402	1.384	1.384	1.594	1.594	1.594	1.366	0.435	0.298	0.903	0.133	0.406	1.315	0.049	

Table 3.4: Estimates of evolutionary divergence between CLUSTALW multi-aligned *Heterorhabditis* species and the undescribed isolates (35 and 42) ITS sequences. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Tajima-Nei model (Tajima and Nei, 1984). The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 643 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*,

2013)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Heterorhabditis zealandica strain Bartow (GU174009.1)		0.000	0.000	0.002	0.013	0.014	0.019	0.019	0.023	0.013	0.022	0.022	0.019	0.002	0.035
2. Undescribed Heterorhabditis species (isolate 35)	0.000		0.000	0.002	0.013	0.014	0.019	0.019	0.023	0.013	0.022	0.022	0.019	0.002	0.035
3. Undescribed Heterorhabditis species (isolate 42)	0.000	0.000		0.002	0.013	0.014	0.019	0.019	0.023	0.013	0.022	0.022	0.019	0.002	0.035
4. Heterorhabditis zealandica strain NZH3 (EF530041.1)	0.002	0.002	0.002		0.013	0.014	0.019	0.019	0.023	0.013	0.023	0.023	0.019	0.002	0.036
5. Heterorhabditis marelatus (AY321479.1)	0.095	0.095	0.095	0.096		0.011	0.016	0.016	0.020	0.008	0.021	0.021	0.016	0.013	0.037
6. Heterorhabditis megidis (AY321480.1)	0.114	0.114	0.114	0.116	0.076		0.018	0.018	0.021	0.010	0.021	0.020	0.018	0.014	0.039
7. Heterorhabditis bacteriophora (AY321477.1)	0.196	0.196	0.196	0.198	0.151	0.177		0.002	0.020	0.016	0.020	0.020	0.000	0.019	0.043
8. Heterorhabditis bacteriophora isolate IRA24 (EU598232.1)	0.198	0.198	0.198	0.200	0.153	0.175	0.005		0.020	0.017	0.021	0.020	0.002	0.019	0.044
9. Heterorhabditis amazonensis (DQ665222.1)	0.257	0.257	0.257	0.260	0.220	0.238	0.218	0.223		0.020	0.016	0.015	0.020	0.023	0.048
10. Heterorhabditis safricana (EF488006.1)**	0.098	0.098	0.098	0.100	0.037	0.065	0.151	0.153	0.222		0.020	0.020	0.016	0.013	0.039
11. Heterorhabditis noenieputensis strain WS17 (KP335198.1)**	0.261	0.261	0.261	0.263	0.236	0.233	0.222	0.224	0.144	0.231		0.003	0.020	0.023	0.048
12. Heterorhabditis noenieputensis strain SF669 (JN620538.1)**	0.258	0.258	0.258	0.261	0.231	0.228	0.222	0.224	0.144	0.226	0.005		0.020	0.023	0.048
13. Heterorhabditis bacteriophora strain J172 (EU716335.2)**	0.196	0.196	0.196	0.198	0.151	0.177	0.000	0.005	0.218	0.151	0.222	0.222		0.019	0.043
14. Heterorhabditis zealandica strain SF41 (EU699436.1)**	0.002	0.002	0.002	0.003	0.096	0.116	0.198	0.200	0.260	0.100	0.263	0.261	0.198		0.035
15. Caenorhabditis elegans isolate X5005 (FJ589008.1)	0.480	0.480	0.480	0.483	0.504	0.528	0.601	0.606	0.648	0.520	0.641	0.637	0.601	0.480	

3.3.3 Phylogenetic relationships

Phylogenetic relationships analysis was conducted by MEGA6 and Maximum Likelihood method was used to identify the isolates to species level. Isolate 10 clustered with *Steinernema khoisanae* strain 106-C ** on the same clade (figure 3.3.1d) which was isolated by Mallan, 2006 in South Africa, however the branch length indicates evolutionary distance between the two species, suggesting that isolate 10 diverged from *S. khoisanae* and is a different species. Close relation was observed with other two South African species *S. tophus* ** and *S innovationi* **. A great evolutionary divergence is observed between Isolate 10 and *S. sacchari* **. Both isolates 35 and 42 clustered with *Heterorhabditis zealandica* strain Bartow, NZH3 and the SA heterorhabditid *H. zealandica* strain SF41** on the same clade and were identified to be *Heterorhabditis zealndica* strain 35 and 42.

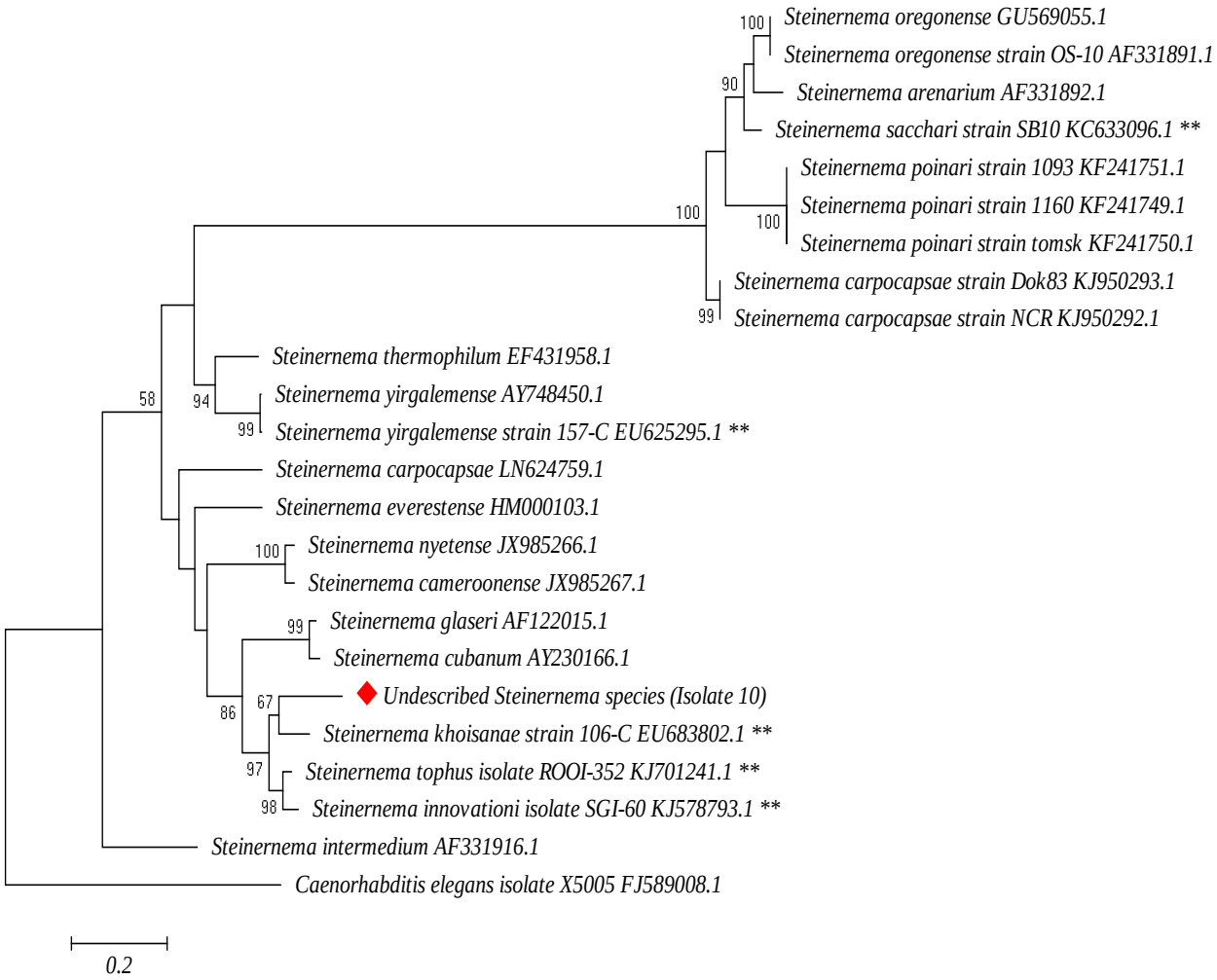


Figure 3.4: Phylogenetic relationships between *Steinernema* species based on 18S rDNA sequences. *C. elegans* isolate *X5005* was used as an out-group and the NCBI accession numbers of the species used to generate the tree are given next to species name. The numbers shown next to the tree branches are bootstrap percentages. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-6408.5986) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 524 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*, 2013).

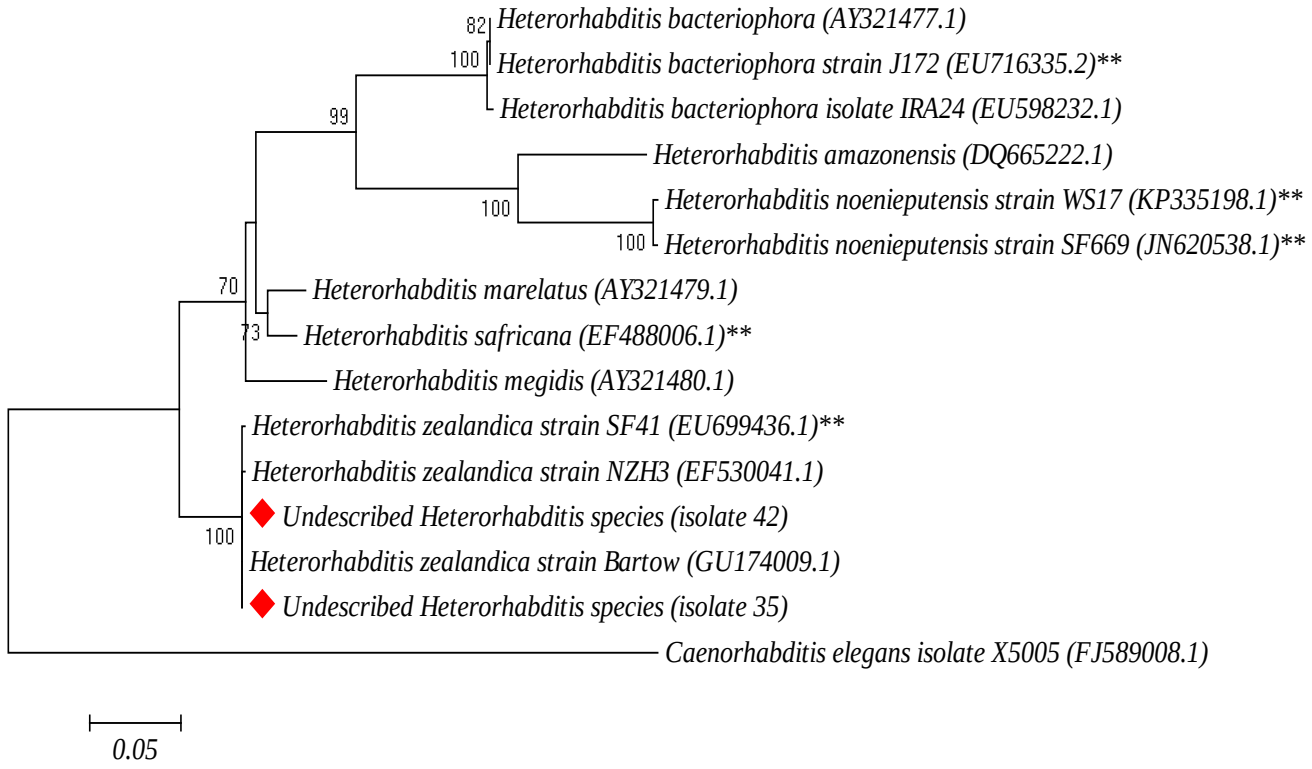


Figure 3.5: Phylogenetic relationship analysis between *Heterorhabditis* species obtained from NCBI and isolate 35 and 45 based on 18S rDNA sequences. *C. elegans X5005* was used as an out-group and the NCBI accession numbers of the species used to generate the tree are given next to species name. The numbers shown next to the tree branches are bootstrap percentages. Isolate 35 and 45 were both identified to be *Heterorhabditis zealandica* strain 35 and 42. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [Kimura, 1980). The tree with the highest log likelihood (-3065.2315) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 643 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*, 2013).

3.4 DISCUSSION

Accurate identification of nematode isolates is crucial for understanding geographical distribution, habitat preference and occurrence of EPNs. It also has vital implications for population genetics and is important for selection of species for use in biological control (Valadas *et al*, 2011). Molecular identification allows researchers to identify organisms to genus and species level and is also important in identification of new species. In this study, molecular based techniques were employed for the identification of isolates 10, 22 and 35 isolated from Brits in the North West province.

The 18S rDNA sequences of isolates obtained from Inqaba Biotechnical laboratories were subjected to BLASTn tool on NCBI and results revealed 83% similarity percentage between isolate 10 and *Steinernema khoisanae* 106-C** (accession number EU683802), and isolate 35 and 42 both revealed a 98% similarity to *Heterorhabditis zealandica* strain Bartow (accession number GU174009.1) and NZH3 (accession number EF530041.1).

Pairwise distances revealed that isolate 10 differs from its closest relatives with evolutionary divergence of 0.187 observed between isolate 10 and *Steinernema khoisanae* 106-C suggesting divergent evolution hence isolate 10 was identified to be an unknown *Steinernema* species (table 3.3). Evolutionary divergence estimates between isolate 35 and 42 with *Heterorhabditis zealandica* strain Bartow were revealed to be 0.000; 0.000, strain NZH3 0.002;0.002 and the South African heterorhabditid *H. zealandica* strain SF41 0.002;0.002, respectively, suggesting close relation. There was no divergence (0.000) between *Heterorhabditis zealandica* strain Bartow and both isolates suggesting that they are of the same species. These results were futher confirmed by phylogenetic tree construction to analyse phylogenetic relationships. Phylogenetic tree construction is a method that is reliable for assessing phylogenetic relationships between undescribed isolates and described isolates. Phylogenetic tree was constructed using multi-aligned ITS regions of the related *Steinernema* species obtained from NCBI and the isolates. *Steinernema khoisanae* 106-C and isolate 10 clustered on the same clade with bootstrap percentage support of 67%. The branch length between the two species further confirms genetic variation. Isolate 35 and 42 clustered on the same clade with *Heterorhabditis zealandica* strain Bartow, NZH3 and SF41 with clade bootstrap percentage of 100%. These results validated preliminary identification of nematodes based on symptom variation. Steinernematids including *S. khoisanae* strain 106-C (EU683802.1), *S. yirgalemense* strain 157-C (EU625295.1) , *S. sacchari* strain SB10 (KC633096.1), *S. tophus* isolate ROOI-352 (KJ701241.1), *S. innovation*i isolate SGI-60 (KJ578793.1) have been recovered in different

habitats of South Africa and have shown a great level of adaptation (Hatting *et al*, 2009; Malan *et al*, 2011).

Heterorhbditis zealandica isolated in this study have also been isolated in the Western Cape and Mpumalanga provinces in South Africa. *Heterorhabditis bacteriophora* has been reported to be the most abundant in SA, however was not isolated in the present study (Hatting *et al*, 2009; Malan *et al*, 2011). Heterorhabditids that have been recovered so far include *H. safricana* (EF488006.1), *H. noenieputensi*s strain WS17 (KP335198.1), *H. noenieputensis* strain SF669 (JN620538.1), *H. bacteriophora* strain J172 (EU716335.2), *H. zealandica* strain SF41 (EU699436.1).

Evolutionary relationships amongst *Steinernema* and *Heterorhabditis* species are recently assessed by DNA sequence analysis of the mitochondrial genes including cytochrome oxidase II (COII) (Szlanski *et al*, 2002), the 12S rDNA and cox I genes (Nadler *et al*, 2006) in conjunction with nuclear genes such as ITS, 18S and 28S rRNA genes (Nguyen *et al*, 2001; Stock and Hunt, 2005). In the present study, the ITS region of isolates were compared with those of described nematodes and have proven and confirmed to be an ideal candidate for identification purposes.

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Chapter4: Isolation and Molecular Characterization of Bacterial Endosymbiont

4.1 INTRODUCTION

Xenorhabdus and *Photorhabdus* bacterial species are members of the family Enterobacteriaceae. They are motile, gram-negative gamma proteobacteria and are highly virulent and pathogenic to a wide variety of insect host larvae. (Goodrich-Blair and Clarke, 2007). These bacterial species have an obligate specific mutualistic symbioses with rhabditoid nematodes belonging to the genus *Sternernema* and *Heterorhabditis. Photorhabdus* and *Xenorhabdus* species have a broad host range and a dose of less than 5 colony forming unit (CFU) directly injected into the haemolymph of insect larvae is sufficient to induce mortality within 48 hours post inoculation.

The bacteria is dependent on the nematode as it vectors the symbiont to the new larval host. All of the *Xenorhabdus* and most of the *Photorhabdus* isolates studied so far have been obtained from nematodes recovered from soil samples (Rainey *et al*, 1995). The free living forms of the bacterial endosymbionts have not yet been isolated from soil or water sources which suggest that symbionts are dependent on the nematode for survival in the soil environment (Forst *et al*, 1997).

4.1.1Taxonomy

Xenorhabdus and *Photorhabdus* are members of the family Enterobacteriaceae and phylum Proteobacteria. They are facultative anaerobic gram negative bacteria and are rod shaped and non sporulating. They have been reported to be oxidase negative and are chemoorganotrophic heterotrophs with respiratory and fermentative metabolisms. They belong to group 5 and subgroup1 of the family Enterobacteriaceae (Akhurst and Boemare, 1990; Forst *et al*, 1997)

4.1.2 The life cycle of the bacterial symbiont

Phase I and II bacteria are released into the haemolymph of the host larvae and Phase I bacteria begin to secrete secondary metabolites and toxins which are lethal to the host. Only Phase I bacteria have been reported to produce antimicrobial compounds which are toxic to microorganisms and this ensures monoxetic conditions within the infected larvae (Akhurst, 1980, 1982; Brown et al, 2004). The bacterial symbionts establish and maintain suitable conditions for nematode growth and development (Liu *et al*, 2001). Re-association of the

bacterial symbionts and nematodes occur after two to three reproduction cycle to form infective juveniles which emerge from an insect cadaver in search for new hosts.

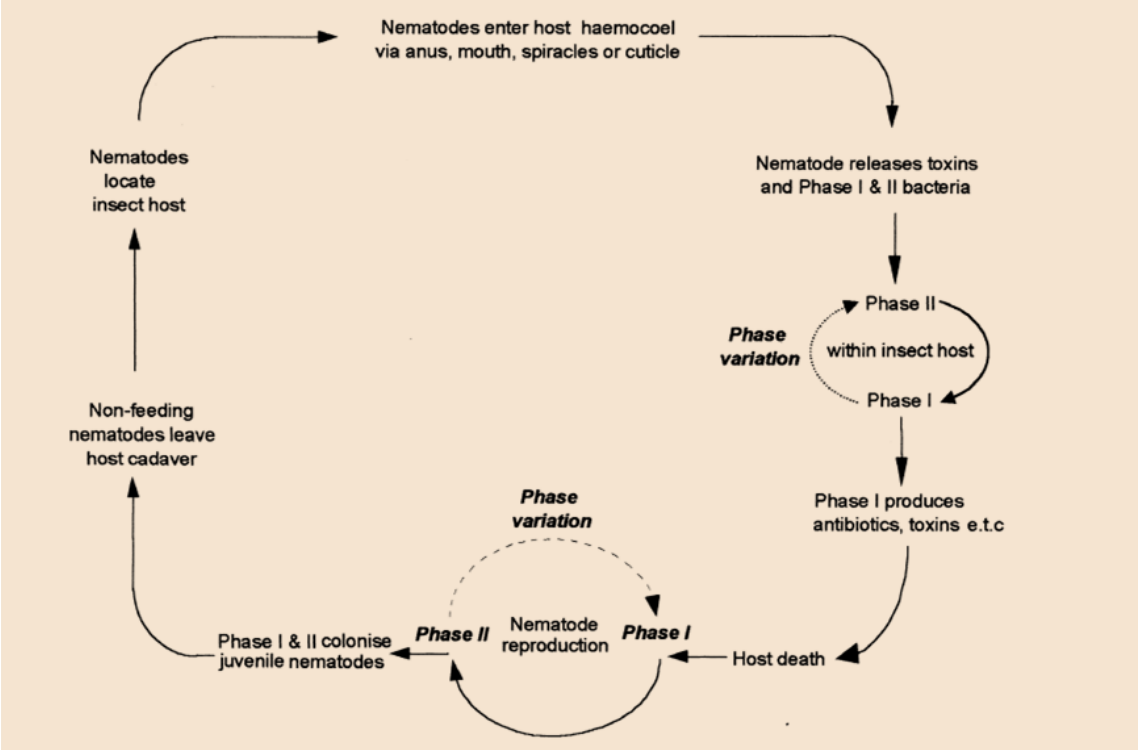


Figure 4.1: General life cycle of entomopathogenic bacterial symbionts associated with nematodes. (Adapted from Owuama, 2001)

4.1.3 Insect larval host immune system depression

The larval innate immune system comprises of both cellular and humoral components that are targeted upon recognition of foreign particles (Leulier *et al*, 2003). These include haemocytes that are able to recognize invading particles and encapsulate them to protect the insect larvae. Humoral response, involves the production of cationic antimicrobial peptides that directly target the bacterial membranes. *Photorhabdus* and *Xenorhabdus* species have developed mechanisms to depress the immune system of the insect larvae (Goodrich-Blair and Clarke, 2007). *Photorhabdus* is reported to produce Al-2, a signalling molecule which results in resistance to the reactive oxygen species (ROS) which is a component of the early insect immune response (Krin *et al*, 2006). *Photorhabdus* also incurs resistance through the modification of the lipopolysaccharides (LPS). *Xenorhabdus* in contrast to *Photorhabdus*, suppresses the expression of CAMPs (Park *et al*, 2007; Ji and Kim, 2004).

4.1.4 Divergent evolution and convergent lifestyles

The bacterial endosymbionts, *Photorhabdus* and *Xenorhabdus* species have divergenced evolutionarily wise but evolved convergently similar lifestyles. They are both associated with nematodes and are insect pathogens but their mode of pathogenicity is different. They colonize different sites in the alimentary canal of their associated nematodes. Both bacterial species are located just posterior to the pharynx in both nematodes, however, *Photorhabdus* colonizes a substantial fraction of the lumen of the nematode gut whereas *Xenorhabdus* appears to be located within vesicles or pockets (Bird and Akhurst, 1983)

4.1.5 Molecular identification of bacterial symbionts

The ribosomal DNA (rDNA) has been employed in molecular characterization of taxonomic relationships and identification of new bacterial species and strains (Tailliez *et al*, 2006). Amplification of the 16S and 23S ribosomal genes which are highly conserved have proved extremely useful for species identification and comparison of phylogenetic relationships of closely related species (Reney *et al*, 1995; Suzuki *et al*, 1996; Brunel *et al*, 1997). This study focused on isolation and molecular characterization of the entomopathogenic bacterial symbiont associated with a novel *Steinernema* species (isolate #10).

4.2 MATERIALS AND METHODS

Two techniques were carried out for the isolation of bacterial endosymbiont associated with a novel nematode species.

4.2.1a isolation from the haemolymph of infected larvae

About 5 instar *G. mellonella* larvae were placed in a Petri dish plate with river sand inoculated with EPN infective juveniles (IJs). This was to allow for infection of larvae with EPNS. The bacterial endosymbiont is released into the haemolymph and initiate infection post EPN entry. At 48 hours post infection, infected and dead larvae were collected and prepared for dissection. Infected *G. mellonella* larvae were primarily surface sterilized by spraying with 70% ethanol. Secondary surface sterilization was conducted prior dissecting by dipping the larvae in 70% ethanol followed by slight heating of the larval surface for 2-3 seconds to avoid heat-killing the bacteria.

Sterilized larvae were cut open or dissected using sterile scissors and scalpel, working aseptically. The syringe was used to draw the sticky fluid or haemolymph from the cadaver into an Eppendorf tube containing Nutrient Broth or sterile distilled water. The haemolymph containing the bacterial endosymbiont was streaked on NBTA (nutrient bromothymol triphenyltetrazolium agar) plates for the isolation of bacteria.

4.2.1b Isolation from infective juveniles

Infective juveniles (IJs) were collected from white traps and allowed to settle under gravity in a 50ml Falcon tube. The IJs were surface sterilized with 0.1% sodium hypochlorite for 3 hours to get rid of any possible bacterial and fungal contaminants on the surface of the nematodes. The sterilized nematodes were rinsed with Ringer's solution (pH 7.3) under the laminar flow to avoid contamination and were allowed to settle and were transferred into Eppendorf tubes.

Using a sterile plastic pestle, the nematodes were crushed and homogenized. The homogenate was then transferred into sterile 1.0ml of Nutrient Broth and was incubated at 25ºC for 24 hours. 24 hours post incubation, the broth containing the bacterial endosymbiont culture was streaked onto NBTA plates.

The NBTA plates for both protocols were incubated at 25ºC for 2-3 days. The blue-green Phase I colonies were screened for and sub-cultured 6-7 times to obtain a pure culture. The plates were stored at 4ºC until required for analysis. Nutrient Broth supplemented with 10% glycerine was inoculated with bacterial colonies and stored at -70ºC to preserve the culture.

4.2.2 Confirmation of the dependency of nematode growth and development on lipid agar bacterial lawns

The Phase I blue green colonies from NBTA plates were inoculated in 1.0ml sterile Nutrient Broth in 2.0ml Eppendorf tubes and incubated for 24 hours at 25ºC. Spread plates of the bacterial endosymbiont broth culture (0.1ml) were prepared on lipid agar plates and incubated at 25ºC for 48 hours. Nematodes collected by white traps were sterilized for 1 hour in 0.1% hypochlorite and about 200IJs/ml were inoculated onto bacterial lawn lipid agar plates. The plates were incubated at 25ºC and inspected daily for EPN propagation and development.

4.2.3 DNA isolation

Colonies of pure bacterial endosymbiont culture were picked from NBTA plates and re-suspended in 200µl of distilled water. Isolation of genomic DNA was conducted using the ZR fungal/ bacterial DNA kit (catalog #D6005)

4.2.4 Polymerase Chain Reaction: amplification of the 16S rDNA

PCR was conducted to amplify the 16S rDNA using universal primers EUB968 and UNIV1382. The reaction mixture reagents were prepared to make up a total of 50ul of the sample which was allowed to run on the PCR machine (GeneAmp PCR system 2700).

Table 4.1: The forward and reverse universal oligonucleotide primers used to amplify the 16S rDNA region of the bacterial isolate (Brunel *et al*, 1997).

Oligonucleotide primer	**Sequence**	**Tm (°C)**	**Ta (°C)**
EUB968 Forward primer	5'-ACGGGCGGTGTGTRC-3'	62	57
UNIV1382 Reverse primer	5'-AACGCGAAGAACCTTAC-3'	66	61

Table 4.2: PCR reaction mixture for amplification of the 16S rDNA

Reagent/sample	Volume (µl) Experiment	Volume (µl) Control
Master Mix	25	25
Bacterial genomic DNA	2	0
Forward primer	3	3
Reverse primer	3	3
Nuclease free water	17	19
TOTAL	**50**	**50**

16S rDNA amplification cycle

35 cycle amplification series:

Denaturation at 94ºC for 30 seconds

Annealing at 57°C for 45 seconds

Extension at 72ºC for 90 seconds

Final extension after cycling: 72ºC for 7 minutes

4.2.5 Sequencing of the 16S rDNA

The PCR products were sequenced at the Inqaba Biotechnical Industries (pty) (LTD). The generated sequence of the undescribed bacterial endosymbiont species was edited and error corrected using FinchTV. The edited sequence was subjected to NCBI BLASTn algorithm for identification based on similarity percentage with existing species in the database.

4.2.6 Multiple alignment: CLUSTALW

Using the NCBI BLASTn search results, the 16S rDNA sequences with the highest similarity percentage to the query sequence were uploaded on MEGA 6.1 and multi-aligned using clustalW. *Escherichia coli* was used as the out-group.

4.2.7 Phylogenetic analysis

Aligned sequences of closely related *Xenorhabdus* species and the undescribed *Xenorhabdus* species as well as *E. coli* as the out-group were subjected to phylogenetic analysis for establishment of phylogenetic relationships. This was achieved through MEGA 6.1 Maximum Likelihood tree construction tool using the Kimura-2 parameter. Bootstrap analysis was carried out with 1000 datasets.

The following taxa were used for phylogenetic tree construction of bacterial species: *Xenorhabdus griffiniae* (GU480979), *Xenorhabdus* sp. MY8 KsSu155 (AB507812), *Xenorhabdus ishibashii* (AB243427), *Xenorhabdus poinarii* (GU480978), *Xenorhabdus poinarii* strain Iran 2 (EU250472), *Xenorhabdus szentirmaii* (GU480989), *Xenorhabdus magdalenensis* strain IMI (NR 109326), *Xenorhabdus khoisanae* strain SF87(NR_117921.1)**, *Xenorhabdus khoisanae* strain SF362(JX623978.1)**, *Xenorhabdus*

khoisanae strain 106-C (JX623972.1)** and *Escherichia coli* strain EcSC4 (KC504011.1). All species marked with "**" have been isolated in South Africa.

4.3 RESULTS

4.3.1 Isolation of bacterial endosymbiont from the haemolymph of infected larvae

The bacterial endosymbiont was successfully isolated from the cadaver of *G. mellonella* insect host larvae that had been infected with *Steinernema* spp. isolate 10. Phase I colonies were blue green and deep blue on NBTA plates with bromothymol blue concentration of 0.025g/L and 0.06g/L (figure 4.1). Phase II colonies were rust on NBTA plates (plates not shown). The Phase I bacterial colonies were granulated, convex, opaque and circular with irregular margins and a colony diameter of 1-2.5 mm. The Phase II colonies were flat and translucent with irregular margins and a colony diameter of 2-4mm.

Table 4.3: Comparison of Phase I and Phase II bacterial endosymbiont BMMCB colony morphological characteristic with other *Xenorhabdus* species (Kaya and Stock, 1997)

Organism	Phase I colony Morphology	Colony colour on NBTA	Phase II colony Morphology	Colony colour on NBTA
Xenorhabdus isolate	Granulated, convex, opaque and circular with irregular margins Colony diameter = 1-2.5mm Sticky consistency	blue-green	Flat, transluscent with irregular margins Colony diameter = 2-4mm	shaded from red to rust
X. bedingii		Blue		rust
X. pionar		Red		rust
X. nematophilus		Blue to deep purple		rust

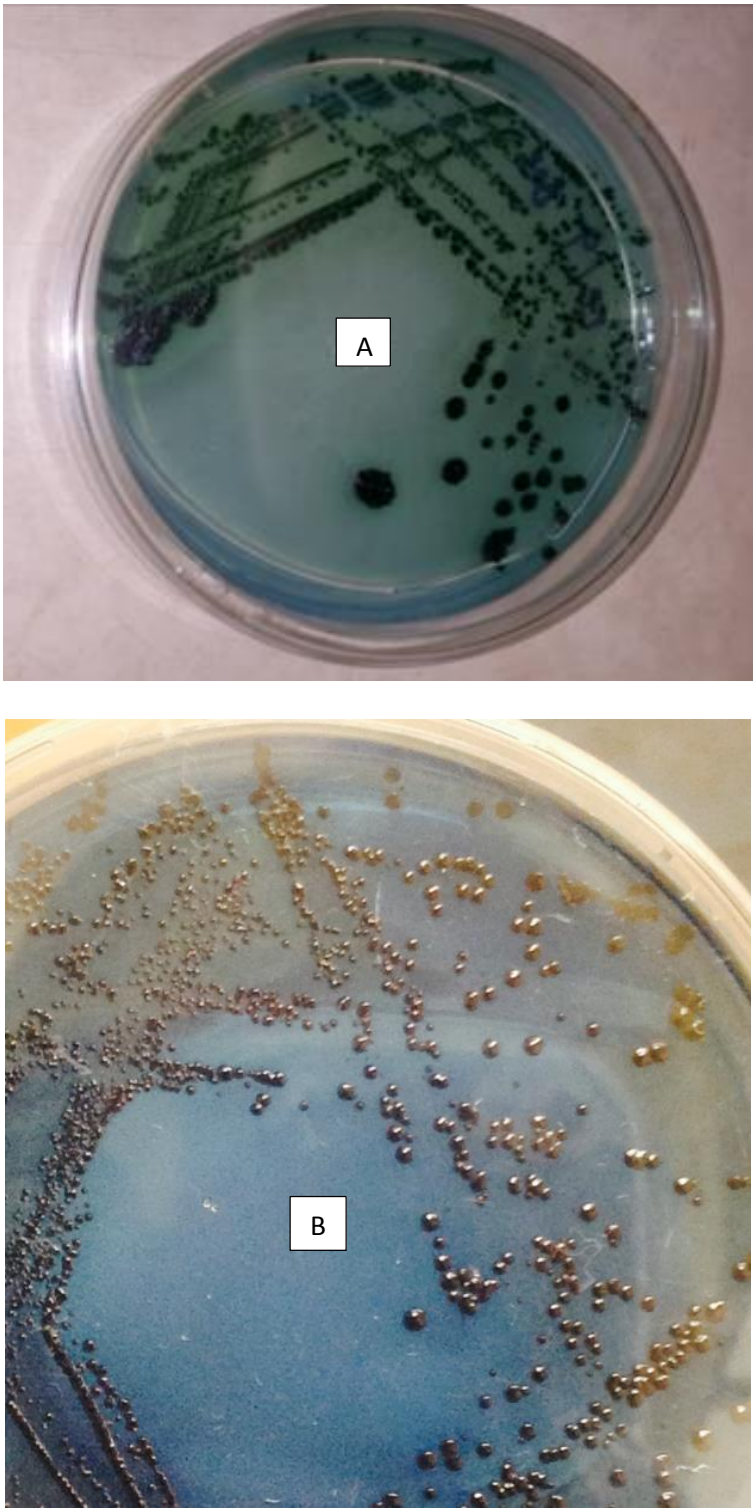
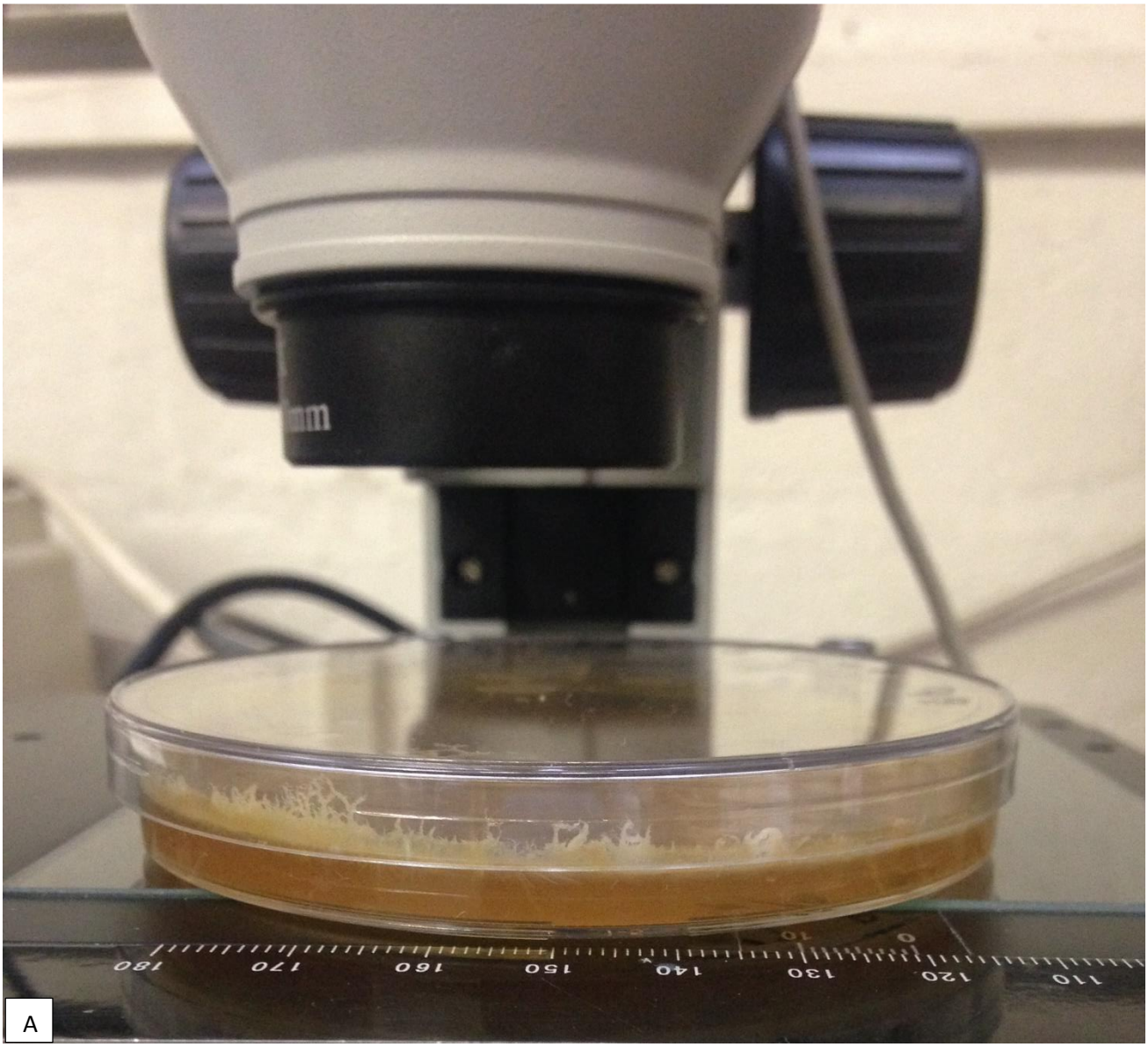


Figure 4.2: Bacterial colonies of bacterial endosymbiont isolated from *Steinernema* spp. isolate 10 A) blue green colonies on NBTA plate with bromothymol blue concentration of 0.025g/L and B) deep blue colonies on NBTA with bromothymol blue concentration of 0.06g/L

4.3.2. Confirmation of the dependency of nematode growth and development on lipid agar bacterial lawn

The bacterial symbiont was spread on lipid agar plates and allowed to grow in a lawn. The sterilized IJs were inoculated onto the bacterial lawn and the nematodes developed into adults suggesting that nematodes depend on the bacterial symbionts for growth and development.



A

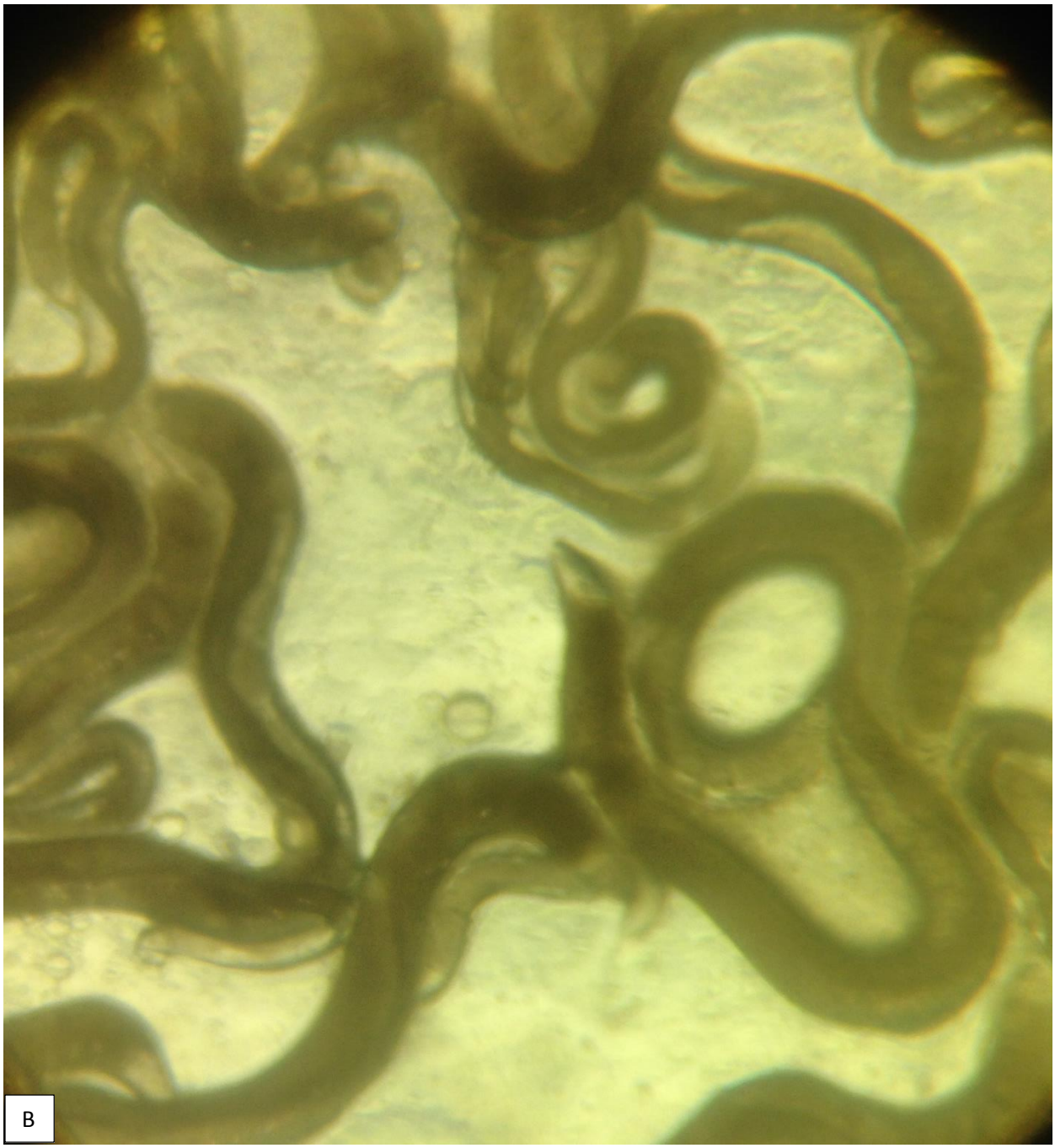


Figure 4.3: Confirmation of the dependency of nematodes on bacterial endosymbiont A) increased nematode reproduction and B) nematodes developed from infective juvenile stage to adult stage.

4.3.3 Molecular identification of isolated bacterial symbiont

Sequencing of the 16S rDNA was subsequently achieved post successful PCR amplification. The sequence obtained from Inqaba Biotechnical Industries is illustrated in figure 4.4. The sequence was subjected to NCBI and revealed high affinity to *Xenorhabdus griffiniae* species. Phylogenetic relationships were assessed and the isolate clustered on the same clade with *Xenorhabdus griffiniae* species and were supported by bootstrap percentage of 98%, however estimate of evolutionary divergence revealed genetic variation.

GAAACATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTC
CAATCCGGACTACGACAGACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTT
GTATCTGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTC
ATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTTGAGTTCCCACCATCACGTGCT
GGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACG
AGCTGACGACAGCCATGCAGCACCTGTCTCACGGGTCCCGAAGGCACTTCCGCATCTCTG
CAGAATTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAATCTTGCGACCGTAC
TCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCACAGCTCAAGGCCACAACCTC
CAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACG
CTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCC
ACATCTCTACGCATTTCACCGCTACACGTGGAATTCTACCCCCCTCTACGAGACTCTAGTC
AACCAGTCTTAGATGCCATTCCCGGGTTAAGCCCGGGATTTCACATCTAACTTAATTGAC
CGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGC
GGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTGGGTAACGTCAATCACAGGGTGTA
TTCAACCCTGTGCCTTCCTCCCCACTGAAAGTACTTTACAACCCGAAAGGCCTTCTTCATA
CACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCCACTGCTGCCTC
CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGCTGGTCATCCTCTCAGACAGCTAG
GATCGTCGACTAAGTGAGCATAACCCCACTACTAGCTATTCCCATCTGGGATTCCTTCCC
GGAATTG

Figure 4.4: 16S rDNA sequence of the undescribed bacterial endosymbiont isolated from larval haemolymph of insect larvae infected with *Steinernema* spp. isolate 10.

Evolutionary divergence was also assessed using MEGA 6.1 pairwise distance and revealed genetic variation between the bacterial isolate and its closest relatives. The bacterial isolate identified belongs to the genera *Xenorhabdus*. The isolate clustered on the same clade with *Xenorhabdus griffiniae* (accession number GU480979) and *Xenorhabdus* sp. MY8 KsSu155 (AB507812), with evolutionary distance of 0.022 and 0.024 suggesting divergent evolution and the isolate was identified to be *Xenorhabdus* bacterial isolate. A great evolutionary divergence (1.019; 1.012 and 1020) was observed between the isolated bacterial symbiont and South African isolates X. khoisanae strain SF87, SF362 and 106-C , respectively.

Table 4.4 Estimates of evolutionary divergence between sequences of undescribed bacterial isolate and its closely related species. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). The analysis involved 12 nucleotide sequences. Codon positions1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1122 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*, 2013).

	1	2	3	4	5	6	7	8	9	10	11	12
1. Undescribed Xenorhabdus species		0.004	0.004	0.068	0.068	0.069	0.068	0.070	0.073	0.066	0.065	0.065
2. Xenorhabdus griffiniae (GU480979)	0.022		0.003	0.071	0.071	0.072	0.071	0.073	0.076	0.069	0.068	0.068
3. Xenorhabdus sp. MY8 KsSu155 (AB507812)	0.024	0.015		0.070	0.071	0.071	0.070	0.072	0.076	0.068	0.067	0.068
4. Xenorhabdus ishibashii (AB243427)	1.041	1.072	1.058		0.004	0.004	0.004	0.004	0.009	0.004	0.004	0.004
5. Xenorhabdus poinarii (GU480978)	1.045	1.076	1.062	0.017		0.001	0.004	0.005	0.009	0.005	0.005	0.005
6. Xenorhabdus poinarii strain Iran2 (EU250472)	1.046	1.078	1.064	0.018	0.001		0.004	0.005	0.009	0.005	0.005	0.005
7. Xenorhabdus szentirmaii (GU480989)	1.030	1.060	1.046	0.019	0.021	0.021		0.004	0.009	0.004	0.004	0.004
8. Xenorhabdus magdalenensis strain IMI (NR 109326)	1.043	1.074	1.060	0.024	0.026	0.025	0.017		0.009	0.005	0.005	0.005
9. Escherichia coli strain EcSC4 (KC504011.1)	1.068	1.101	1.086	0.082	0.082	0.081	0.083	0.078		0.009	0.009	0.009
10. Xenorhabdus khoisanae strain SF87 (NR 117921.1)**	1.019	1.049	1.035	0.026	0.031	0.031	0.022	0.029	0.085		0.002	0.002
11. Xenorhabdus khoisanae strain SF362 (JX623978.1)**	1.012	1.042	1.028	0.025	0.030	0.030	0.022	0.028	0.086	0.003		0.001
12. Xenorhabdus khoisanae strain 106-C (JX623972.1)**	1.020	1.050	1.036	0.027	0.030	0.030	0.024	0.028	0.087	0.004	0.002	

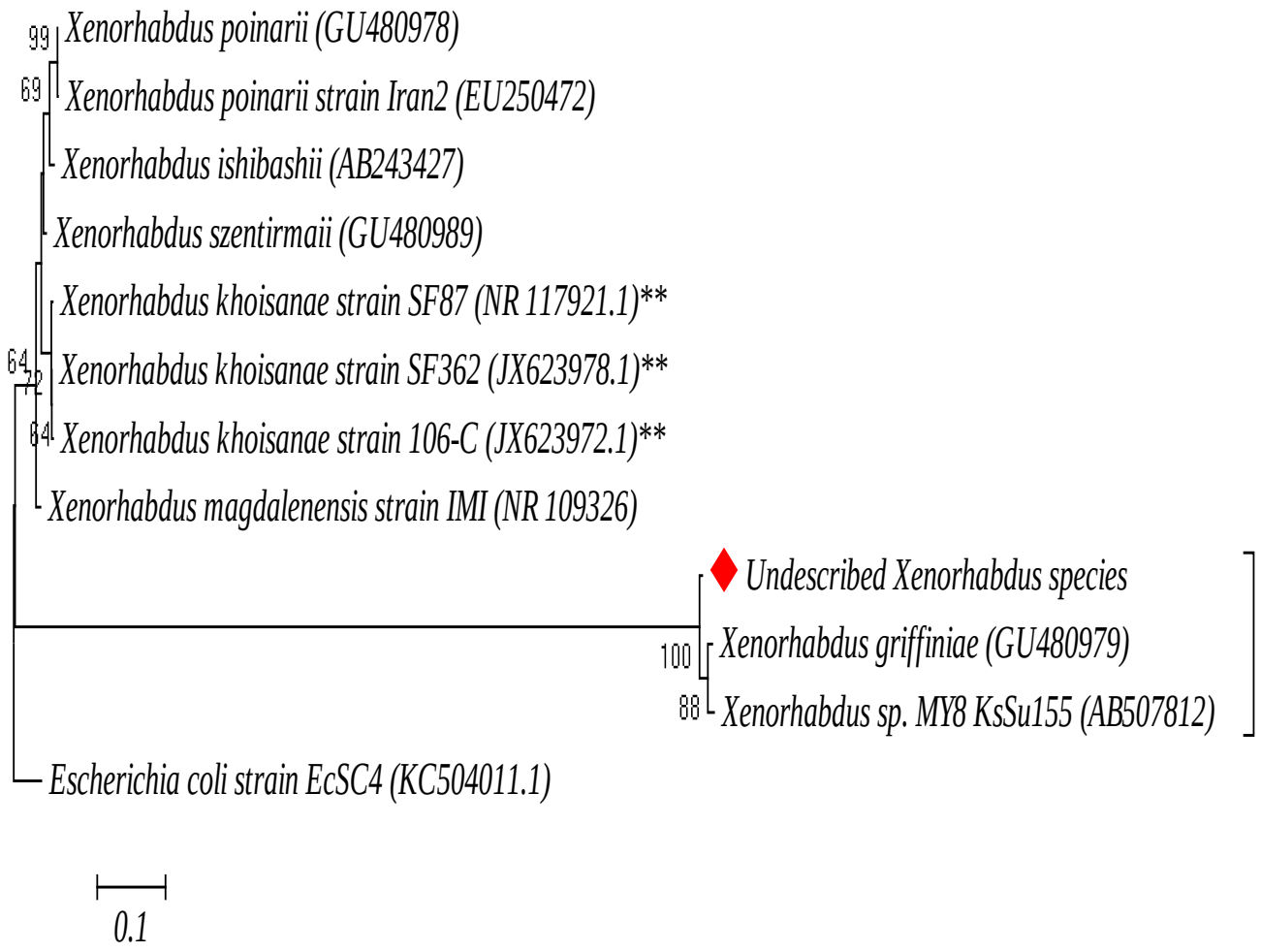


Figure 4.5: Phylogenetic relationship analysis of 16S rDNA sequences of described species obtained from NCBI and the undescribed *Xenorhabdus* isolate using MEGA 6.1. The accession numbers are shown next to species name. The numbers shown next to the tree branches are bootstrap percentages. The tree with the highest log likelihood (-4086.1469) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1122 positions in the final dataset. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980).

4.4 DISCUSSION

The bacterial endosymbiont isolation was achieved by streaking the haemolymph of dead larvae infected by *Steinernema* spp. isolate 10, on NBTA plates. Pure culture of the bacterial symbiont was obtained by multiple streak sub-culturing and was used for molecular based techniques for identification.

Bacterial symbiont appears in two phases, Phase I and Phase II. No major DNA rearrangements between the two Phases have been reported, however phase II colonies are reported to lack morphological traits and have reduced levels of numerous biochemical and physiological characteristics (Brunel *et al*, 1997). Phase II appears during the stationary phase of *in vitro* culture or during nematode rearing on artificial diet (Brunel *et al*, 1997).

Xenorhabdus species colony colour on NBTA range from blue, blue-green and deep purple depending on the species and the concentration of BTB on the media. All *Xenorhabdus* species are known for their ability to adsorb bromothymol blue except for *Xenorhabdus pionar* which exhibit red colonies on NBTA for both Phase I and II (Kaya and Stock, 1997; Hurlbert *et al*, 1989; Nealson et al, 1990). *Xenorhabdus* bacterial isolate Phase I colonies were blue-green for normal BTB concentration (0.025g/1L) and were deep blue when the concentration was higher (0.06g/1L) on NBTA plates (Figure 4.1). The colonies were surrounded by clearing around the colonies due to adsorption of BTB. The colony morphology was convex, opaque and circular with irregular margins. Phase I colonies were small to middle sized (1-2.5mm colony diameter) and displayed a sticky consistency. Colonies were sometimes reddish on NBTA which is a result of absorption and reduction of triphenyltetrazolium chloride (TTC) in the media (Kaya and Stock, 1997). Phase II colonies were flat, translucent with irregular margins and a greater diameter (2-5mm). Colonies were shaded from red to rust from adsorption and reduction of TTC. There were no clear zones around phase II colonies as they are incapable of adsorbing BTB.

The 16S rDNA sequence obtained from Inqaba Biotechnical Industries were subjected to BLASTn tool on NCBI to compare with organisms already described and stored on the database. NCBI BLASTn results revealed high affinity to *Xenorhabdus griffiniae* (accession number GU480979) and *Xenorhabdus* sp. MY8 KsSu155 (accession number AB507812), with evolutionary distance of 0.022 and 0.024 suggesting divergent evolution and the isolate was identified to be *Xenorhabdus* bacterial isolate. Phylogenetic relationships were assessed using Maximum likelihood method in MEGA 6 and the isolate clustered with *X. griffinae and X.* sp.

MY8 KsSu155 strains with bootstrap percentage of 100%. Despite clustering on the same clade, results revealed high evolutionary distance or variation between the *Xenorhabdus* isolate, *Xenorhabdus griffiniae and X*. sp. MY8 KsSu155 suggesting evolutionary divergence and the isolate was identified to be *Xenorhabdus* bacterial isolate. Despite the close relation between *Steinernema* isolate 10 and *S. khoisanae* 106-C, their bacterial symbionts reveal distant relation and great level of evolutionary divergence.

Nematodes are known to be dependent on Phase I bacterial symbiont for growth and development. To confirm this dependency, spread plates of *Xenorhabdus* bacterial isolate Phase I colonies on lipid agar were prepared and sterilized IJs (200IJs/ml) were cultured on the bacterial lawn. Nematodes were observed to develop from IJs to adults and this confirms their dependency on bacterial symbiont for development.

This study revealed that PCR based techniques for amplification of the 16S rDNA and bacterial colony morphological characterization are accurate and reliable methods for identification of *Xenorhabdus* bacterial symbionts.

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Chapter5: Morphological Observations of Entomopathogenic Nematodes

5.1 INTRODUCTION

Nematodes in the genera *Steinernema* and *Heterorhabditis* have physiologically and behaviourally adapted to different habitats for survival and longevity. Some species are specific with regard habitat preference due to prevalence of suitable host. Morphological characteristics of EPNs play a pivotal role in environmental adaptation (Hominick 2002; Smits and Ehlers, 1991; Dolinski *et al*, 2008). Different species amongst both genera lack morphological variation and some valid characters such as the spicule and gubernaculum requires proper processing of nematodes samples and high definition observation methods. Morphometric measurements of special characters are currently used by researchers to differentiate between shape and size of species and are taken into consideration for identification of new species (Stock and Kaya, 1996; Phan *et al*, 2005) in addition to molecular identification.

In Africa, the amount of information available for EPN taxonomy is currently limited (Malan *et al*, 2006; Nthenga *et al*, 2013). Thus, there is a need for isolation and accurate identification of indigenous EPN strains for application as biocontrol agents (Kanga *et al*, 2012). The first EPN to be isolated in South Africa, was by Hamington in Grahamstown, Eastern Cape. Three *Steinernema* and only one *Heterorhabditis* species were identified in 1988 in Kwazulu Natal (Spaull, 1988, 1990). In 1991, 15 steinernematids and 7 heterorhabditids were isolated however were not identified to species level. Heterorhabditids have been identified to be the most abundant species in South Africa (Malan *et al*, 2011; Hatting *et al*, 2009). Morphological identification using light microscopy and scanning electron microscope have proven to be the best and reliable methods for observation of both internal and external features (Kaya and Stock, 1997).

Accurate identification requires morphological characterization and morphometric measurements of adult males, females and IJs. Currently, a total of four heterorhabditids (two of which are new descriptions for South Africa) and eight steinernematids (of which 7 are new descriptions for SA) have been reported (malan *et al*, 2011). This study aimed at characterizing the morphological features of a novel *Steinernema* species using light microscopy (different observation methods) and scanning electron microscopy.

5.2 MATERIALS AND METHODS

Morphological observations of infective juveniles was conducted using light microscopy and scanning electron microscopy. (modification of protocol by Courtney *et al*, 1995 and Steinhorst, 1959)

5.2.1 Light microscopy

5.2.1.1 Heat-killing and fixing nematodes

Infective juveniles were collected from white traps, sterilized for 1 hour using 0.1 sodium hypochlorite and were rinsed with distilled water 3 times. The nematodes were transferred into a small Petri dish plate and were heat killed by suspension in 80 ºC distilled water. The water was discarded after 5 minutes and replaced with 85 ºC TAF. The TAF solution was replaced with double strength TAF and the samples were stored at 4 ºC to relax nematodes for 1 hour. The double strength TAF was replaced with 65ºC TAF and allowed to infiltrate for 24 hours to achieve fixation.

5.2.1.2 Processing nematodes to pure glycerine

Fixed nematodes were transferred into a clean small Petri Dish plate containing 0.5ml of solution I (20ml 95% ethanol, 1ml glycerine and 79ml distilled water). The plate was placed in a desiccator and was incubated in an oven at 35ºC for 24 hours. The nematodes were re-suspended in solution II (15ml glycerine, 95ml 95% ethanol) and were incubated at 40ºC for 3 hours and mount on microscopic slides. Images were captured using the Olympus BX63 fluorescence microscope using different observations (BF – bright field, DF – dark field and DIC – differential interference contrast).

5.2.2 Scanning electron microscopy

5.2.2.1 Heat-killing, fixing and processing

Sterilized nematodes were heat killed by suspending them in 65 ºC distilled water for 3 minutes. Heat killed nematodes were rinsed 3 times in Ringer's solution (pH 7.3) and were pre-fixed in TAF solution for 24 hours at 25 ºC. The nematodes were rinsed with distilled water and dehydrated with ethanol gradient of varying concentration (30, 50, 70, 90, 95 and 100% V/V) at 10 minutes interval. The nematode samples were left in 100% ethanol overnight prior freeze drying.

5.2.2.2 Freeze-drying

Ethanol was allowed to evaporate for 48 hours and the nematodes were spread evenly using a needle in an Eppendorf tube. A hole was created on the lid using a dissecting needle to allow for drying. The samples were placed in a freeze drying machine and were allowed to freeze at -85.4 ºC. The frozen samples were allowed to dry at a pressure of 101mT.

5.2.2.3 SEM: Mounting and observation of samples

The nematodes were picked from the tube using a needle and were mount on SEM stubs. The samples were coated with carbon and gold Palladium. The samples were viewed and images were captured using the FEI Quanta 200 scanning electron microscope.

5.3 RESULTS

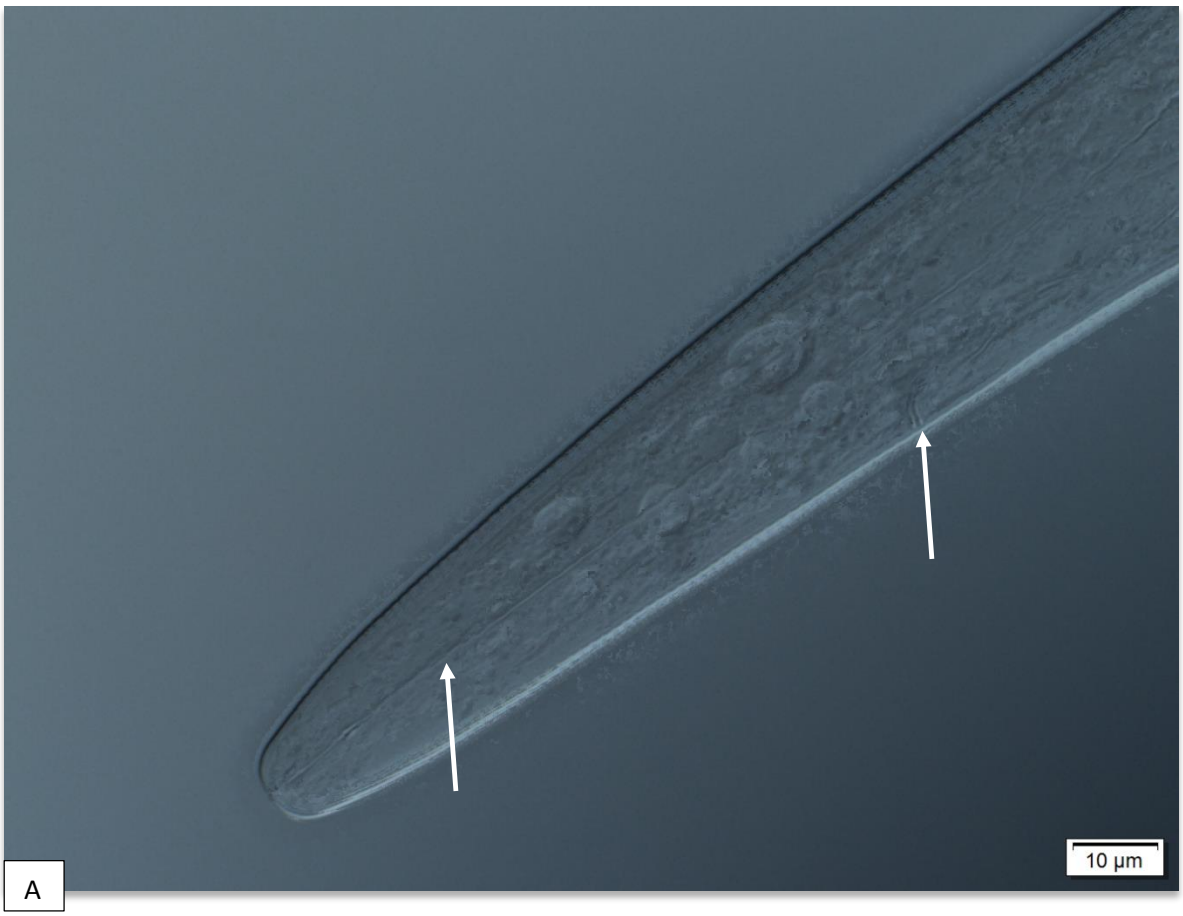
Description:

Infective juveniles: Heat killing of the IJs resulted in straight body shaped nematodes. The nematodes retained the second stage cuticle. The stoma was closed and the oesophageal tract was observed with minimal characters of the pharynx. Excretory pore (EP) at mid pharynx-level. The tail region showing the anal tract on the posterior region. The bacterial pouch showing the rod shaped bacterial symbionts and the thick cuticle. The scanning electron microscopy results of infective juveniles' lateral view revealed 8 equally spaced ridges through the mid body region.

First generation female adult: Remains curved after heat killing. Head region is rounded and continuous with body. Funnel shaped stoma with six labial papillae more prominent under light microscopy and only 4 visible under scanning electron microscopy. Pharynx with distinct isthmus, basal bulb enlarged and valvated. The nerve ring surrounds the isthmus just anterior to basal bulb. The pharingo-intestinal tract is prominent. The Excretory pore is positioned at mid basal bulb and the excretory tract is elongated from mid basal-bulb to close to the gut. Vulva protruding from the body with double flapped epiptygma.

Second generation female adult: Body remains curved after heat killing. Vulva slightly protruded from body with double flapped epiptygma. Tail dome shaped with peg-like mucron present.

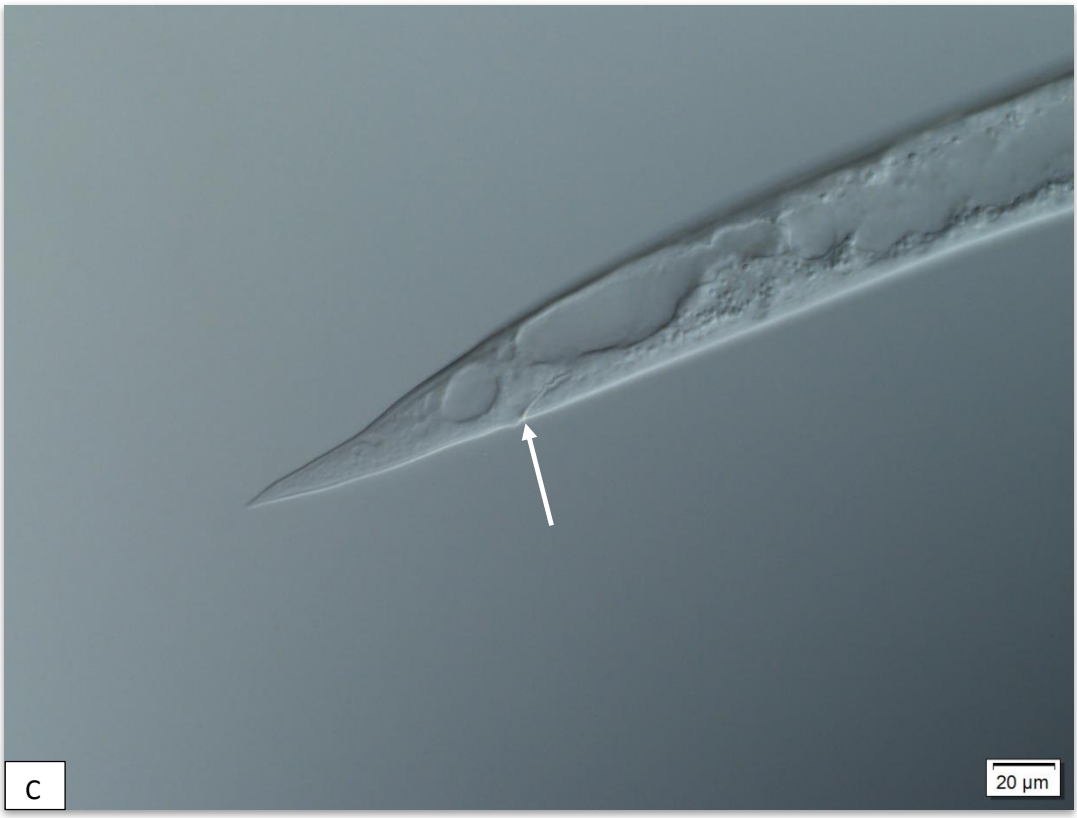
First generation male: The funnel shaped stoma is observed at the anterior region. Spicules paired, light brown in colour. Head (manubrum) of spicules oblongate, each spicule with two internal ribs. Gubernaculum boat-shaped in lateral view, anterior end curved. The tail of male adult is mucronless.

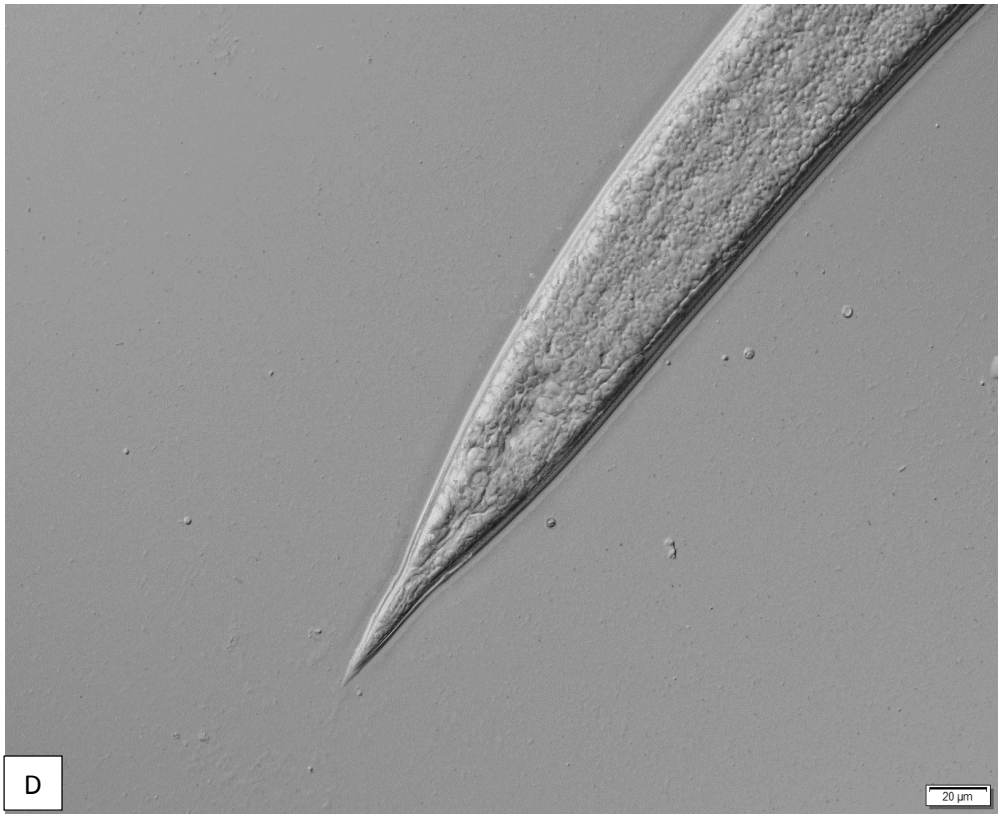


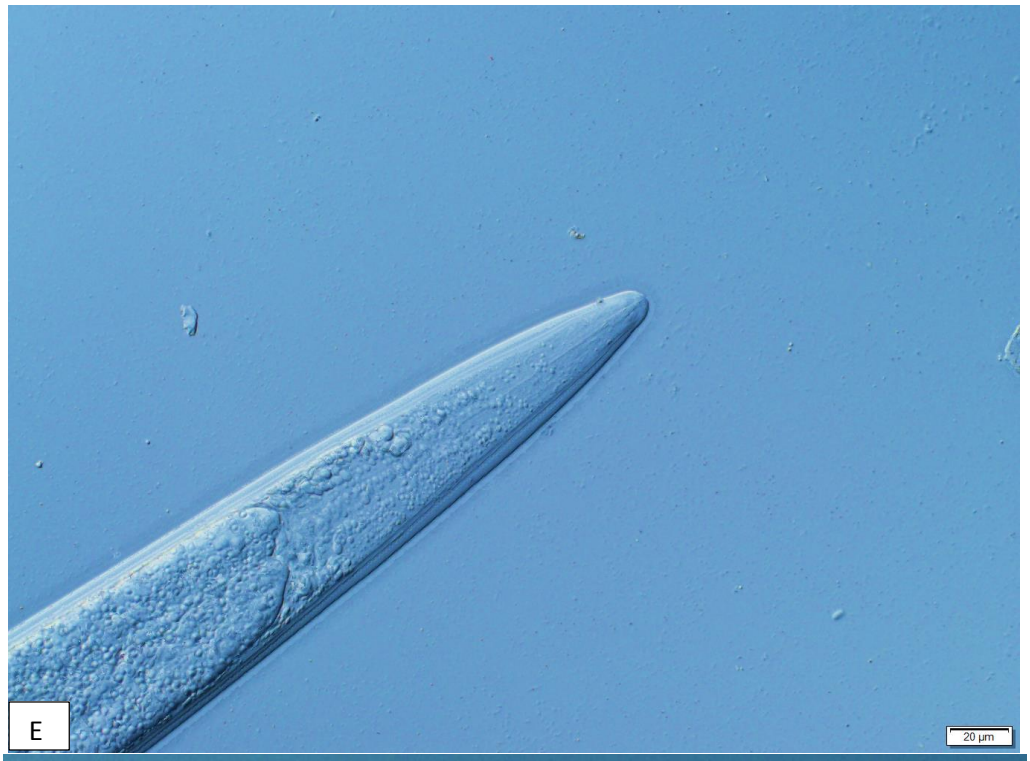
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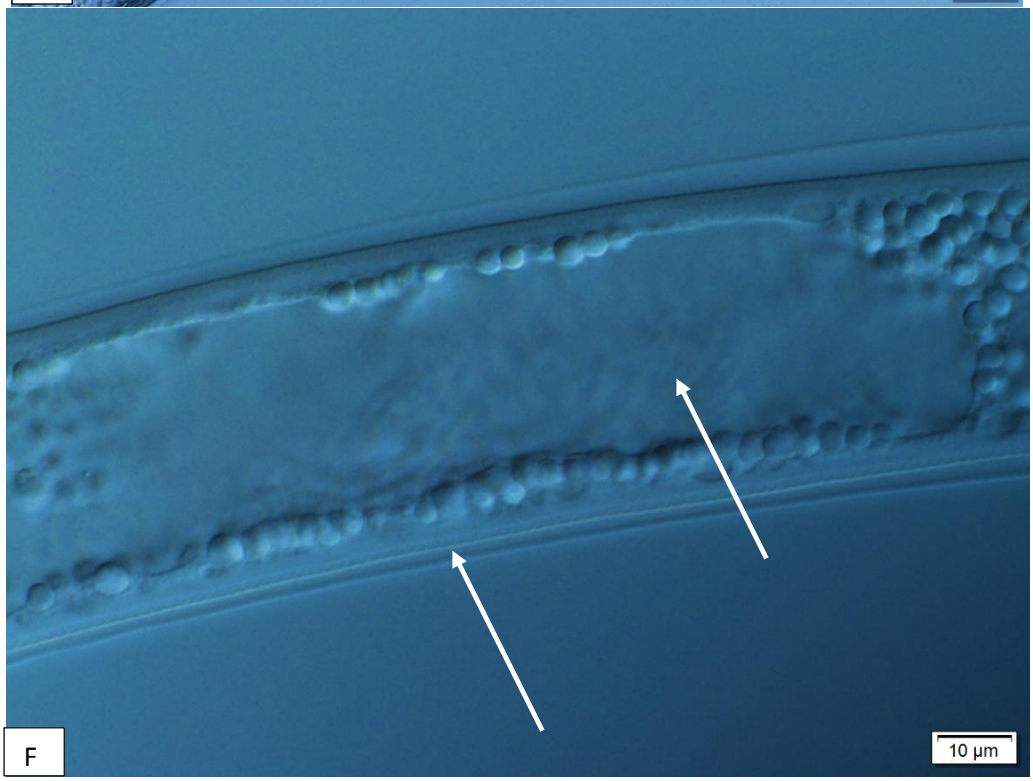


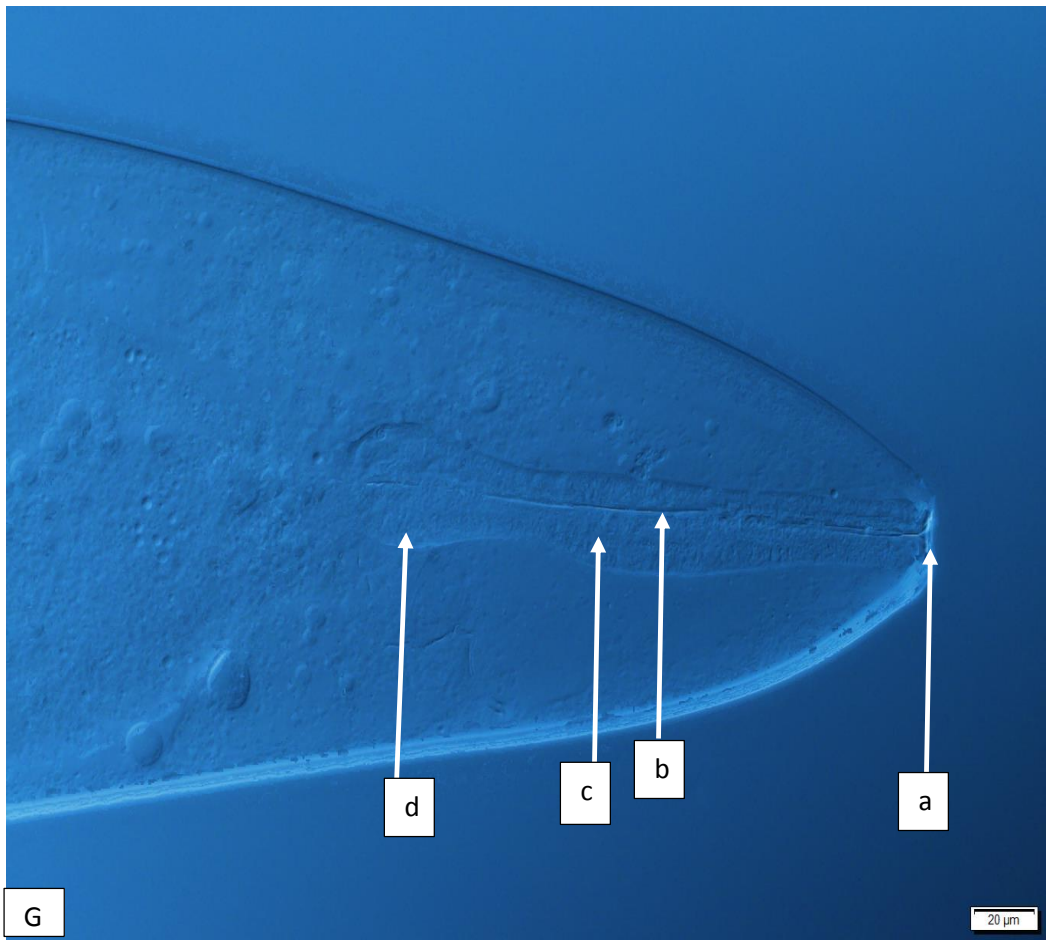
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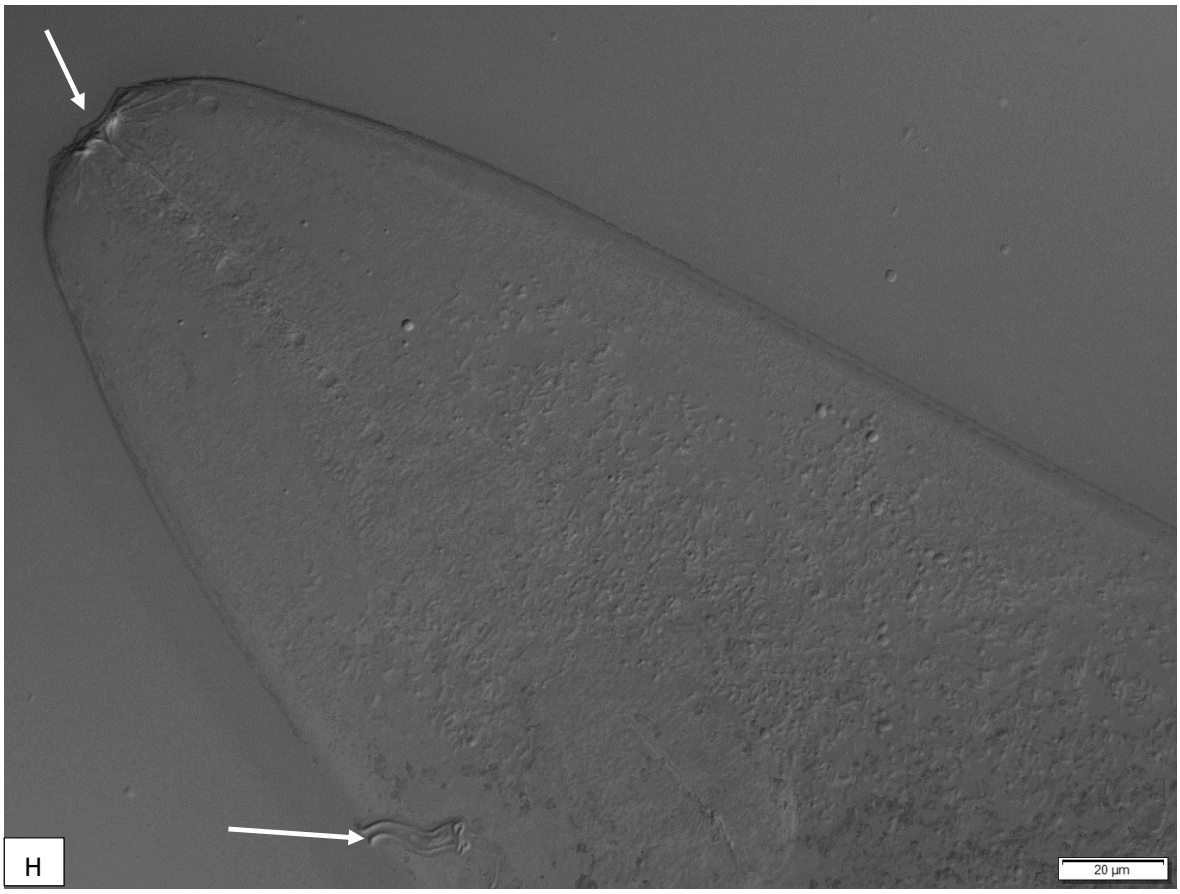

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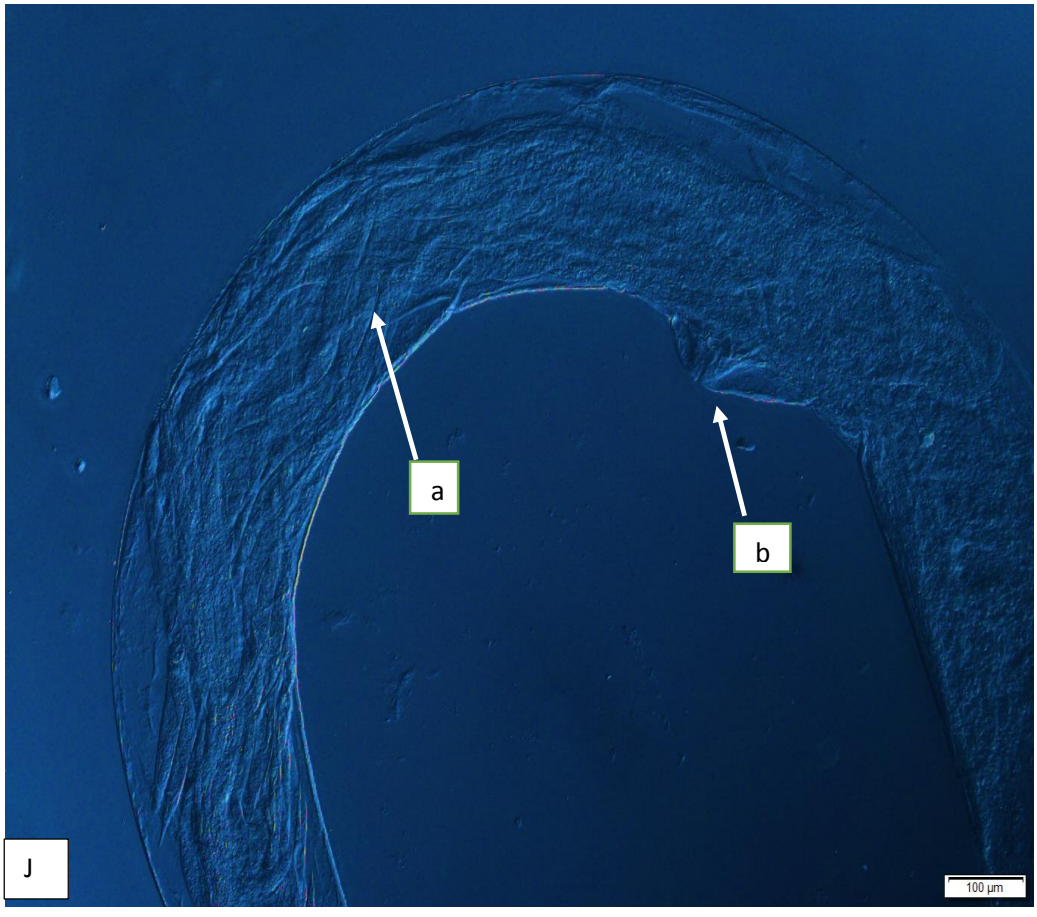

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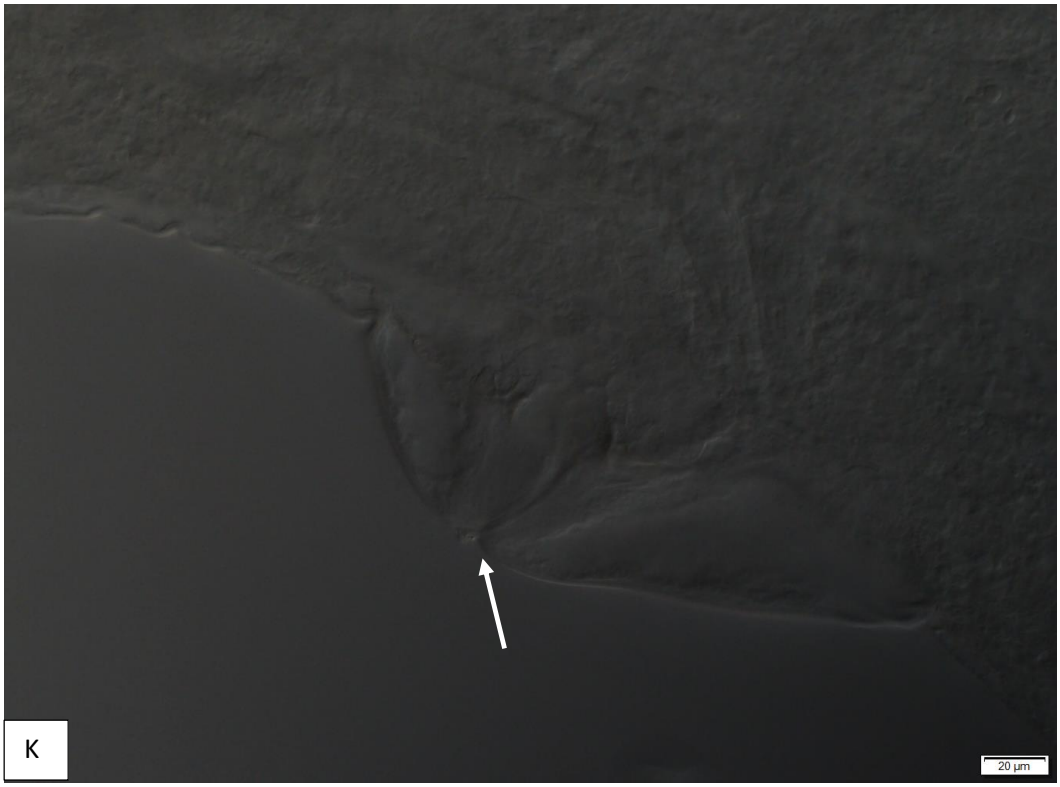

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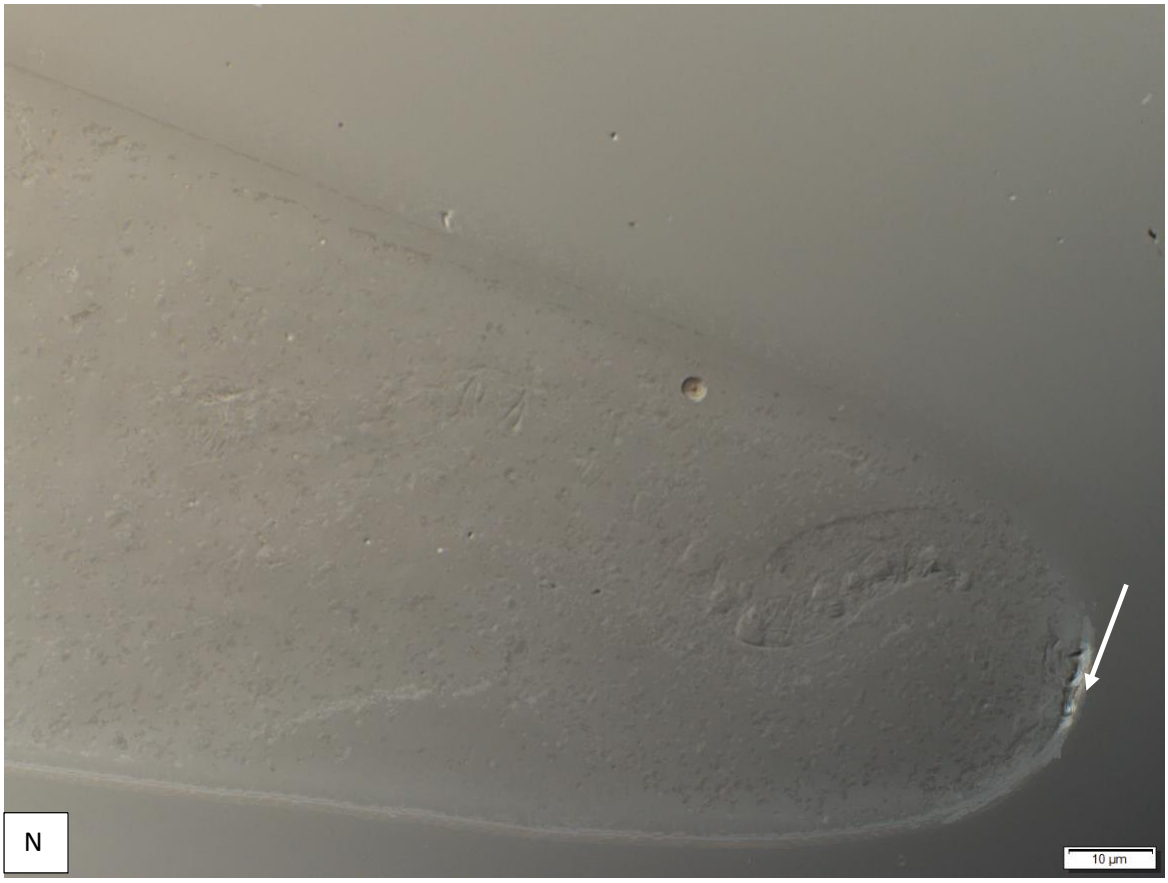

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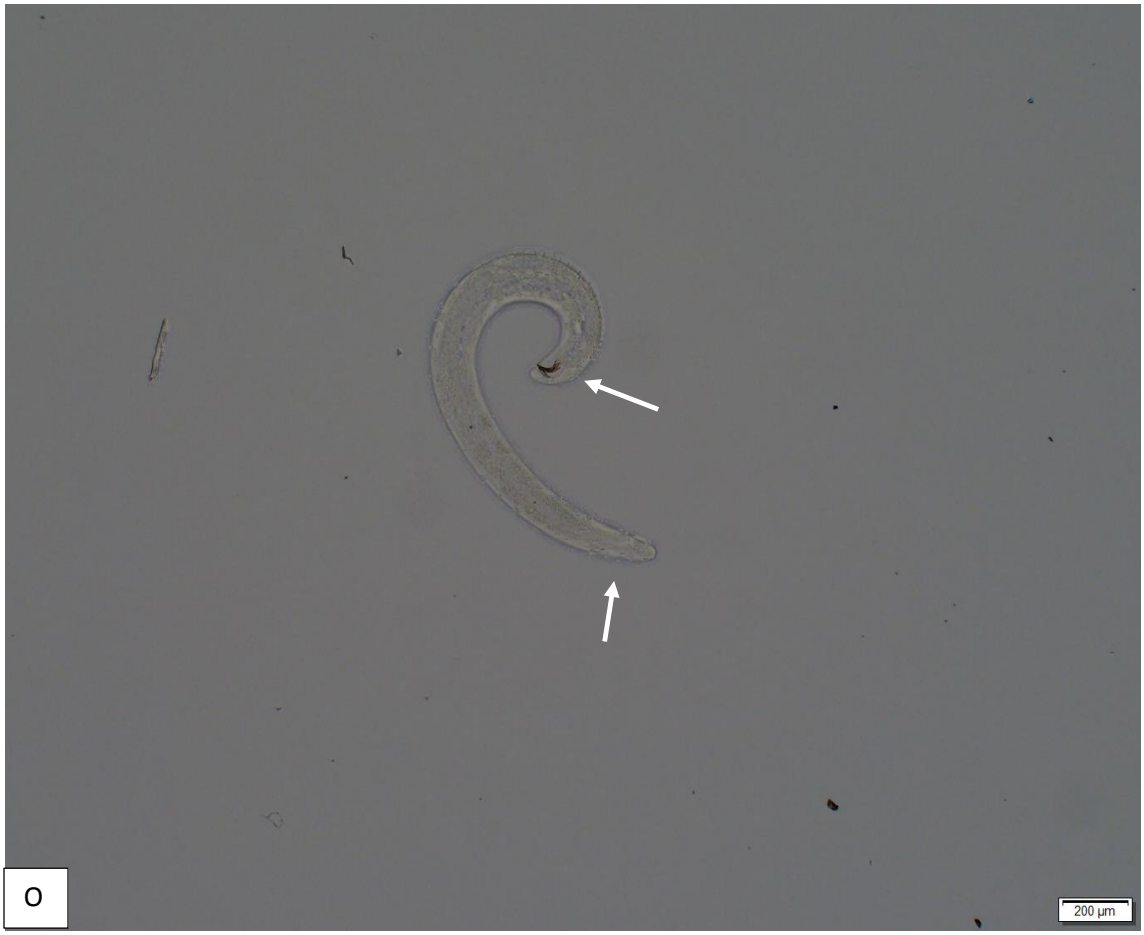

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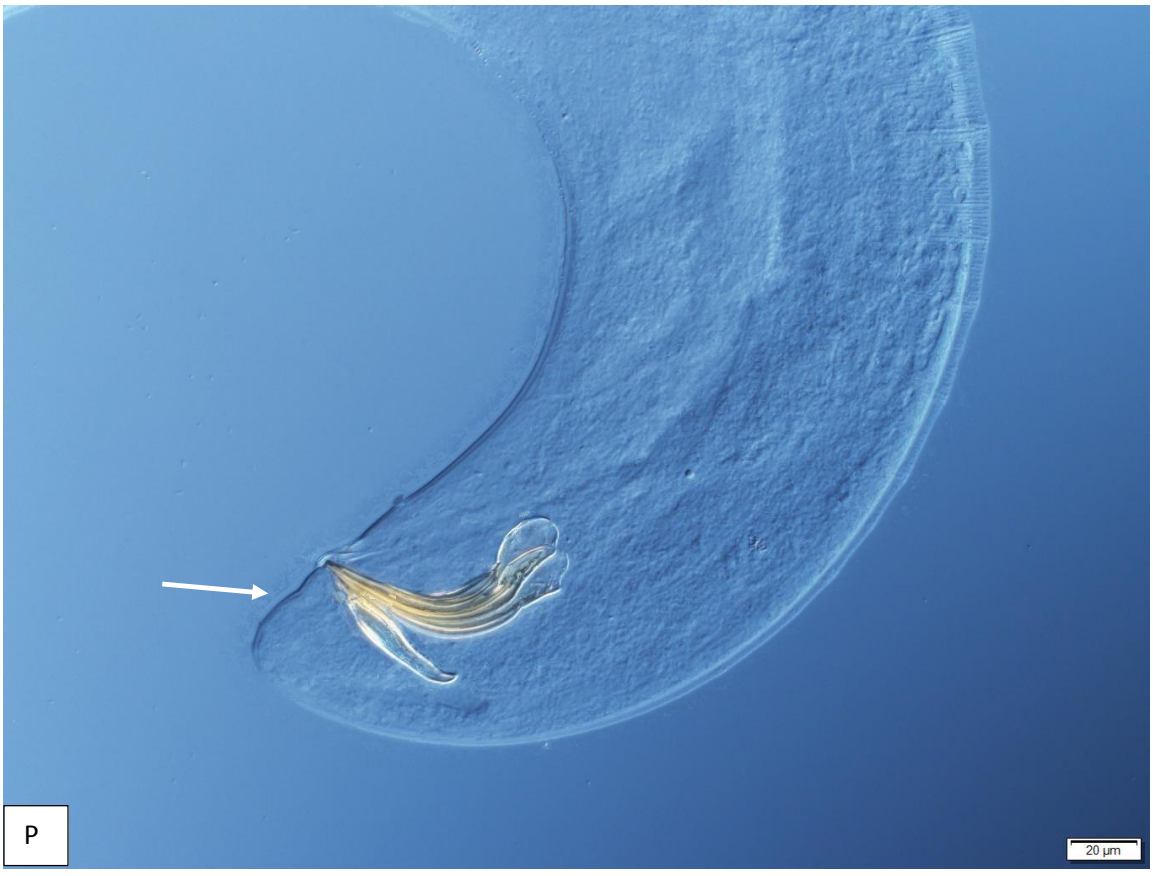

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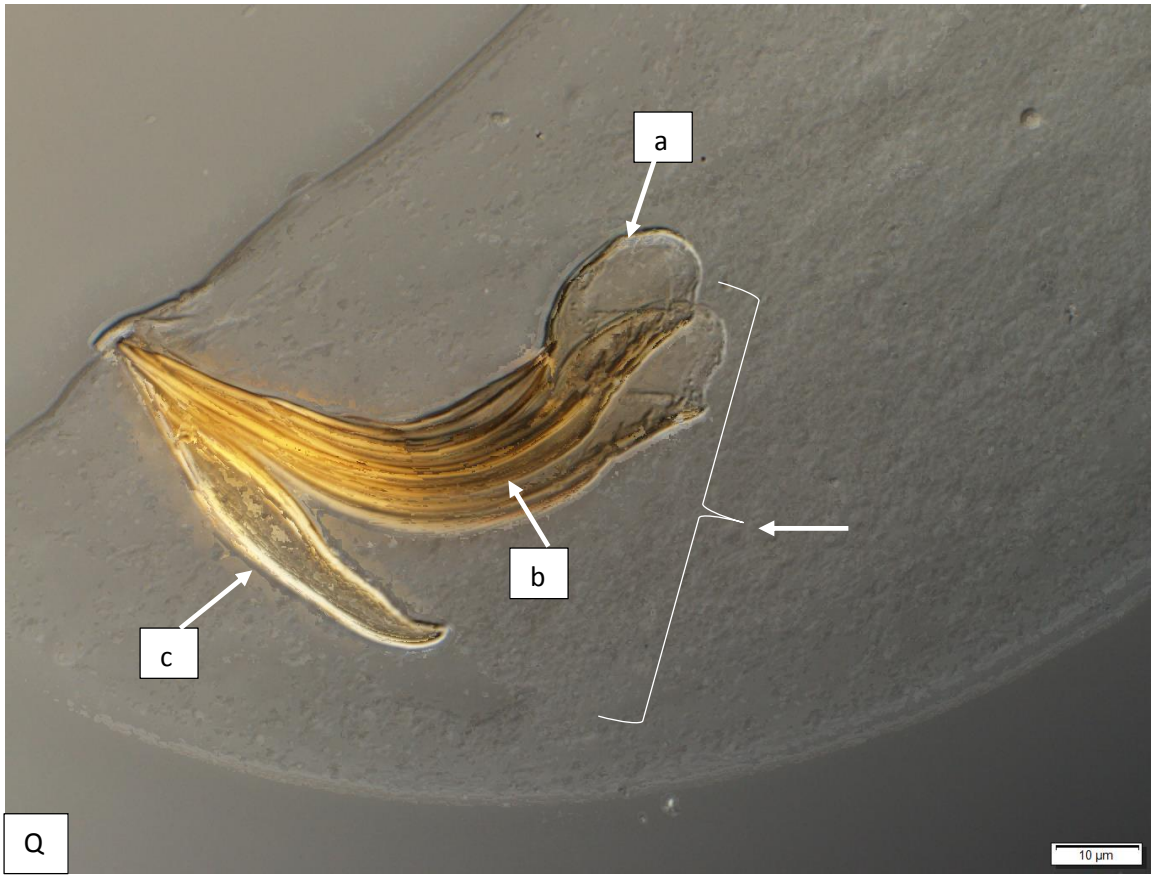
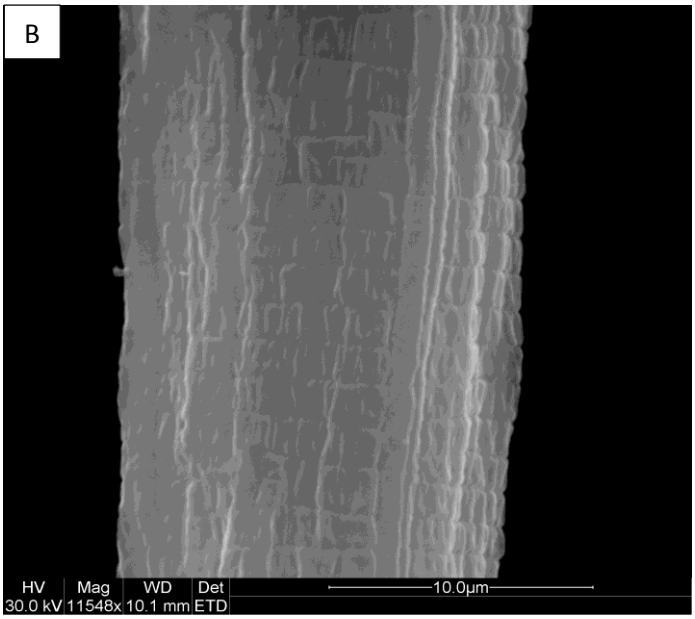
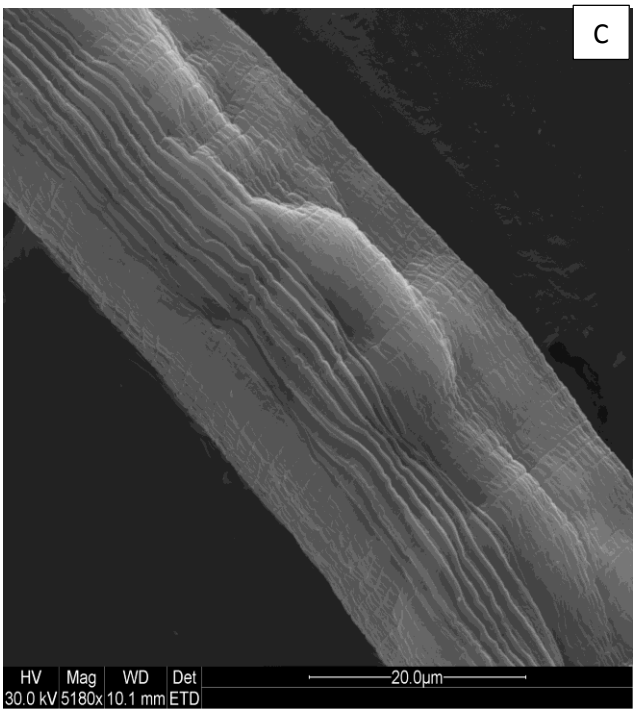
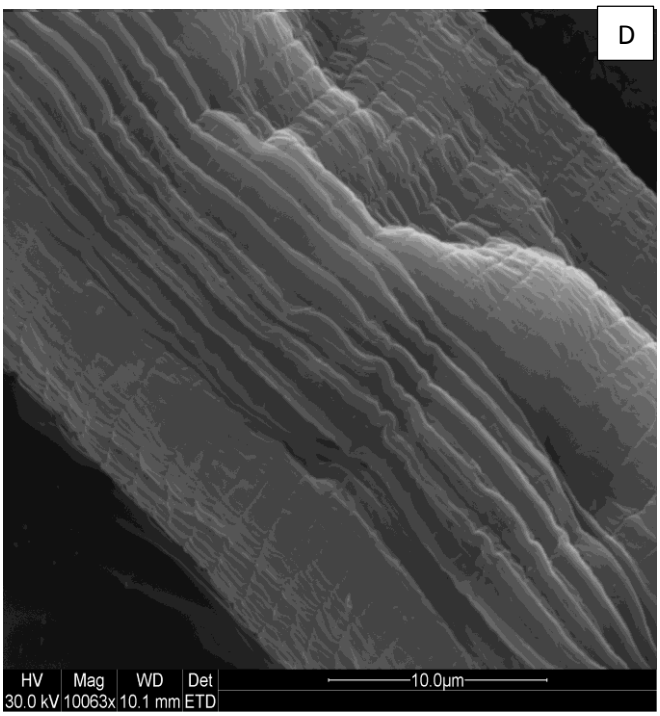


Figure 5.1: Morphological observations of *Steinernema* sp. isolate 10 using the light microscope: *1. Infective juveniles* (A-F)- A) Anterior region showing the excretory pore (EP) and Oesophageal tract, B) Infective juvenile, C) Posterior region showing the anus position, D) tail region, E) Head region, F) rod shaped bacterial endosymbiont enclosed in a vesicle. 2. *First generation female adult* (G-K)- G) Posterior region showing the position of the a) stoma b) oesophageal tract, c) pharynx and d)basal bulb, H) Head region showing 6 labial papillae And the EP, I) Excretory duct (ED) postion, J) Endotika matricida a) infective juveniles developing inside the adult female EPN and b) protruded vulva lips (VL), K) VL with double flapped epiptygma. *3. Second generation female adult* (L-M) - L) VL showing the epiptygma, (M) tail region showing the anus. *4. Male adult* EPN (N-Q)- N) Head region showing the stoma, O) male adult head, body and tail, P) tail region Q) Posterior region showing the position of the spicules (b) with manubrum (a) and the gubernaculum (c).


A
HV
30.0 kV
Mag
9966x
WD
10.1 mm
Det
ETD
20.0µm


B
HV
30.0 kV
Mag
11548x
WD
10.1 mm
Det
ETD
10.0µm


C
HV
30.0 kV
Mag
5180x
WD
10.1 mm
Det
ETD
20.0µm


D
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30.0 kV
Mag
10063x
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10.1 mm
Det
ETD
10.0µm

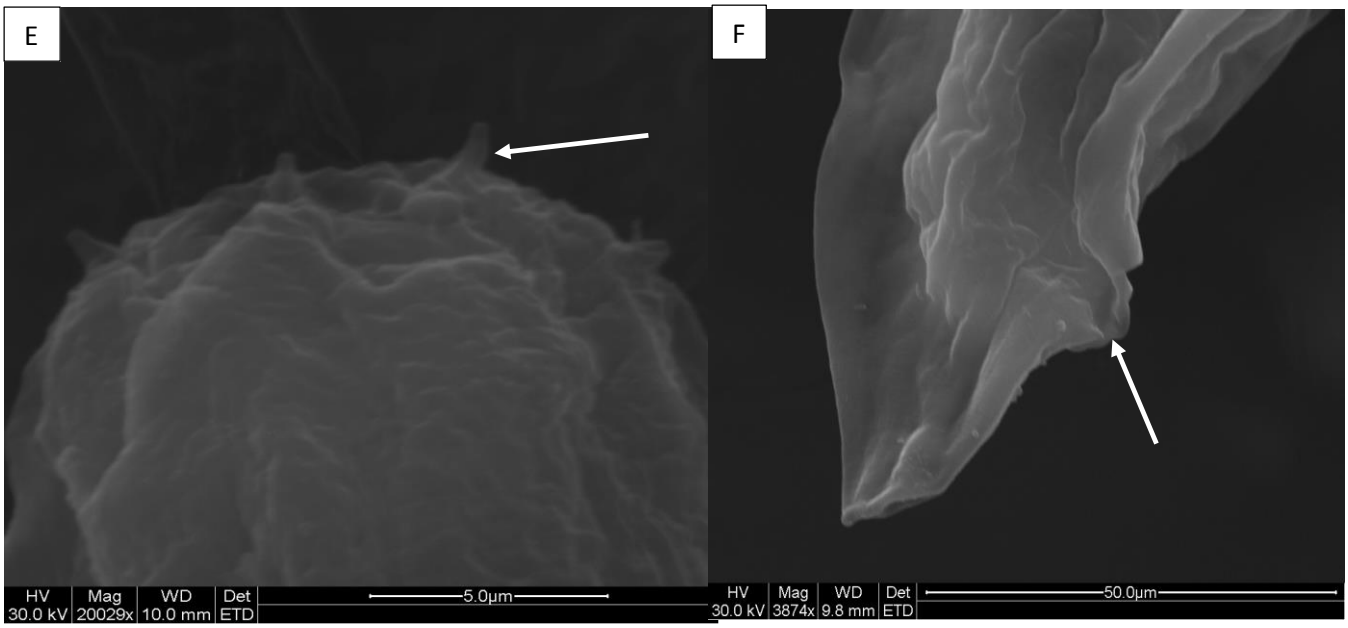


Figure 5.2: Morphological observations of *Steinernema* spp. isolate 10 using the scanning electron microscope. 1. *Infective juveniles* (A-D) - A) and B) Head showing the stoma and mid body region showing the longitudinal striations and tessellate patterns. C) and D) 8 equally spaced ridges on mid body region. 2. *Female adult* (E-F) - E) 3 papillae on the anterior region and F)Tail region showing the anus.

5.4 DISCUSSION

Morphological chracacterization of *Steinernema* spp. isolate 10 resembled those of *Steinernema khoisanae* 106-C and the original description of *Steinernema khoisanae* (Nguyen, *et al*, 2006). The head region of Infective Juveniles (IJs) was truncated and slightly rounded and were located at the anterior region. The excretory pore was prominent at mid pharynx-level. The rod shaped bacterial endosymbiont were observed in a pocket within the gut of IJs which further confirms the association of entomopathogenic nematodes with bacterial symbionts. The tail was pointed and differed with those of the adult female and males. The male tail was robust and curved with prominent spicules and gubernaculum present and the tail of the adult females were curved with a peg like mucron and anus located quite close to the end of the tail. The shape of the spicules and gubernaculum resembled those of *Steinemema sacchari*, a recently described entomopathogenic nematode from South Africa (Nthenga *et al*, 2014).

The body length of the 1st generation adult females was twice the size of the 1st generation male and about four times the size of IJs. The vulva lips of the 1st generation females were protruded and that of 2nd generation females were somewhat slightly protruded and were both characterized by a double flapped epiptygma. Endotika matricida (EM) is a process whereby infective juveniles develop within the adult female EPN and upon emergence, the mother bursts and dies. EM was observed in both generations with IJs visible within adult EPNs. Six papillae were evident on the head stoma region of the adult female opening to the oesophageal tract which opens to the intestines just after the basal bulb of the pharynx. Both the excretory pore and the excretory tract aiding in the emission of waste products to the exterior were evident at the anterior region.

Scanning electron microscopy aided in observation of surface characteristics of the IJs and females. Longitudinal striations and tessellate patterns were evident on both IJs and females on the head region and body. The anus and shape of the tails were observed. The 8 equally spaced and developed ridges on the surface of Ijs have been reported and is consistent with the *Steinernematid* spp "*glaseri* group" infective juveniles identified in Portugal (Nguyen and Smart, 1995; Valadas *et al*, 2011)

Light and scanning electron microscopy revealed detailed important characteristics of *Steinernema* spp isolate 10. Special morphometric measurements such as body length (L), tail length (T) and the size of the spicule and gubernaculum should be considered for detailed morphometric identification (Nguyen *et al*, 1995).

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CHAPTER6: Desiccation Tolerance of Entomopathogenic Nematodes

6.1 INTRODUCTION

Control of insect pests remains problematic to agricultural industries as they usually evolve resistance to chemical pesticides with time. This inevitable result necessitates an alternative approach to crop pest management involving the use of biocontrol agents in place of synthetic chemical pesticides. Entomopathogenic nematodes represent a possible alternative crop pest management biocontrol agent. The economic importance of EPNs in the genera *Steinernema* and *Heterorhabditis* is growing exponentially and generates a demand for application of suitable strains with high reproductive potential *in vivo*, longer shelf life or storage ability, and field efficacy against target pests (Tamalak, 1997).

The third stage infective juvenile (IJ) is the only stage in the life cycle of EPNs that can survive outside the insect larval host. IJs are free living nematodes ensheathed by the second stage stage cuticle (Pionar, 1970). Nematodes have developed strategies to resist and tolerate adverse environmental conditions (Serwe-Rodriguez *et al*, 2004). They can tolerate both abiotic and biotic factors in the soil. Adaptation to permit survival include morphological specialization, behavioural and biochemical mechanisms.

EPNs have chemoreceptors and are motile, which enables them to be able to locate susceptible hosts in the soil. They respond to both chemical and physical stimuli emitted by the potential prey, however some nematode species are able to search for hosts either at or near soil surface and are referred to as ambushers, whereas others are adapted to search deeper in the soil surface and we refer to them as cruisers (Koppenhofer *et al*, 1995). The most important traits for a suitable strain include best behavioural adaptation as well as the ability to tolerate environmental factors such as desiccation (Rodriguez *et al*, 2004).

Desiccation tolerance is a highly vital factor affecting the commercial use of nematodes from mass production to application and it has been stipulated to be important for storage and shelf life of nematodes prior application. Extensive research have been conducted on EPNs behavioural, biochemical and molecular mechanisms and has increased knowledge of their stress responses (Glazer, 2002; Gal *et al*, 2001 and Chen *et al*, 2006). Molecular studies have identified several genes that may be potential markers for desiccation tolerance in *Steinernema*

feltiae. Of the identified genes, some were known to be related to stress-response and some were homologues to *C. elegans* hypothetical proteins (Somvanshi *et al*, 2008).

Survival under low moisture conditions is highly crucial for the persistence of EPNs in the soil. Soil is characterized by rapid desiccation on the surface and gradual desiccation within the soil hence it is highly important to commercialize strains that will be able to tolerate desiccation once applied to agricultural fields. The study aimed at investigating desiccation tolerance of both *Steinernema* and *Heterorhabditis* species.

6.2 MATERIALS AND METHODS

6.2.1 Desiccation studies

The ability of *Steinernema* spp. isolate 10 and *Heterorhabditis* species to tolerate desiccation was assessed. The effect of soil texture on infectivity was also assessed. River sand and loamy sand were autoclaved at 121°C to get rid of contaminants and about 35g of soil samples were weighed and put in a Petri dish and rehydrated to 8% moisture. The IJs were collected from White traps and allowed to settle and were sterilized for 1 hour with 0.1% sodium hypochlorite. The IJs were rinsed with water and the soil samples were inoculated with 200IJs/ml. The control plates were kept at 8% moisture and experimental plates were allowed to undergo desiccation for different days. During the day of dehydration, the respective experimental plates were rehydrated to 8% moisture, 5^{th} instar *G. mellonella* larvae were added and percent mortality was recorded daily for 96 hours. Infected larvae were put on White traps to confirm EPN induced mortality and Two way ANOVA-with replication was used to analyse the results.

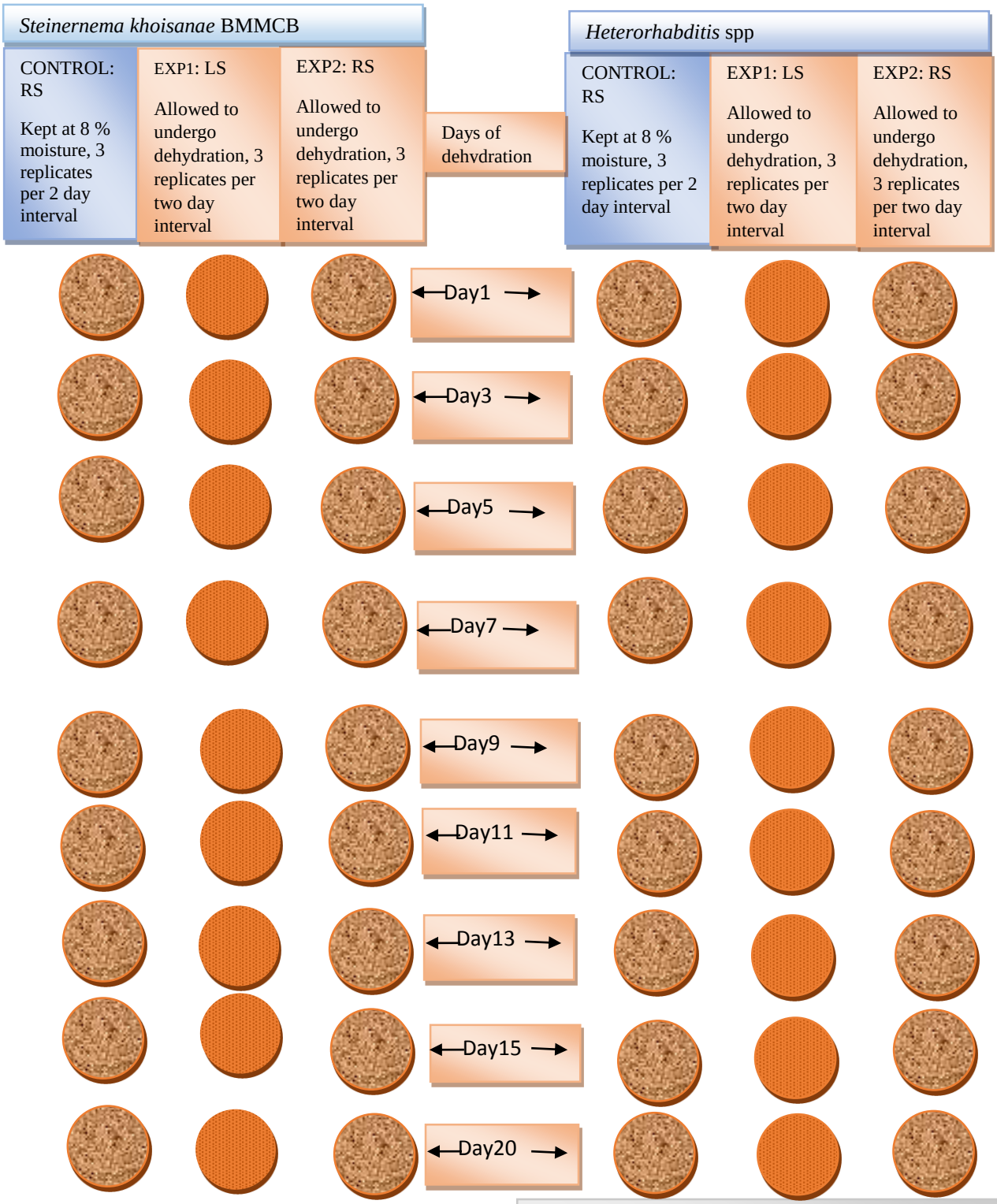


Figure 6.1: Experimental design for dehydration studies

6.3 RESULTS

Both *Steinernema* spp. isolate 10 and the *Heterorhabditis* species could tolerate desiccation. *Steinernema* spp. isolate 10 was tolerant up to 11 days of desiccation exposure in loamy sand and up to 9 days of exposure in river sand, causing 26, 6% and 13,4% cumulative larval mortality after 96 hours, post resuscitation by rehydration, respectively. *Heterorhabditis* spp. could tolerate desiccation up to 13 days of exposure and induced 26.6% cumulative larval mortality on both loamy and river sand after 96 hours post resuscitation. Both *Steinernema* spp. isolate 10 and *Heterorhabditis* spp were highly effective on loamy soil samples from which they were isolated from. The Two way ANOVA statistical analysis revealed no significant difference between the two species on infectivity as 0, 15211 = F < Fcritical= 3,125945 and and the p value = 0, 85945 < 0, 05.

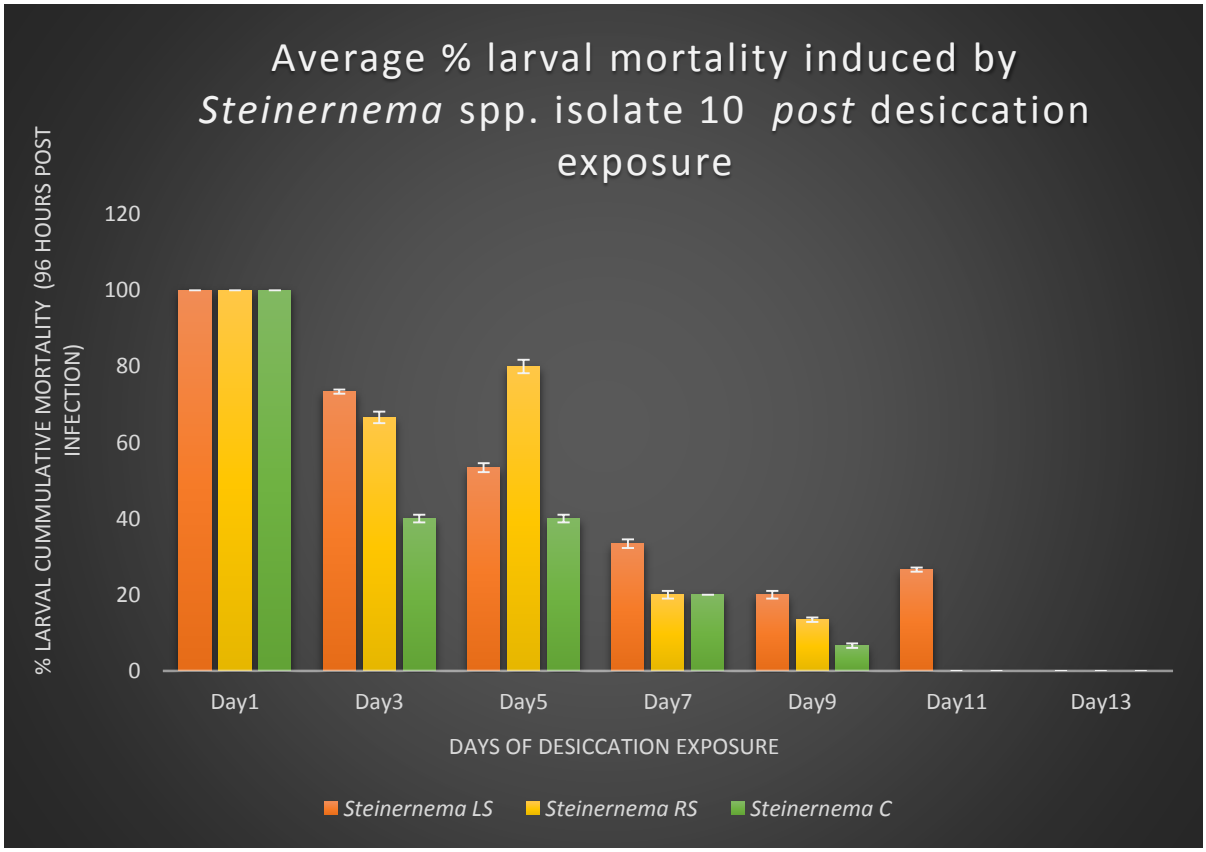


Figure 6.2: Average cumulative larval mortality percentage induced by *Steinernema* spp. isolate 10 post different days of desiccation exposure (LS = loamy sand, RS = river sand and C= control).

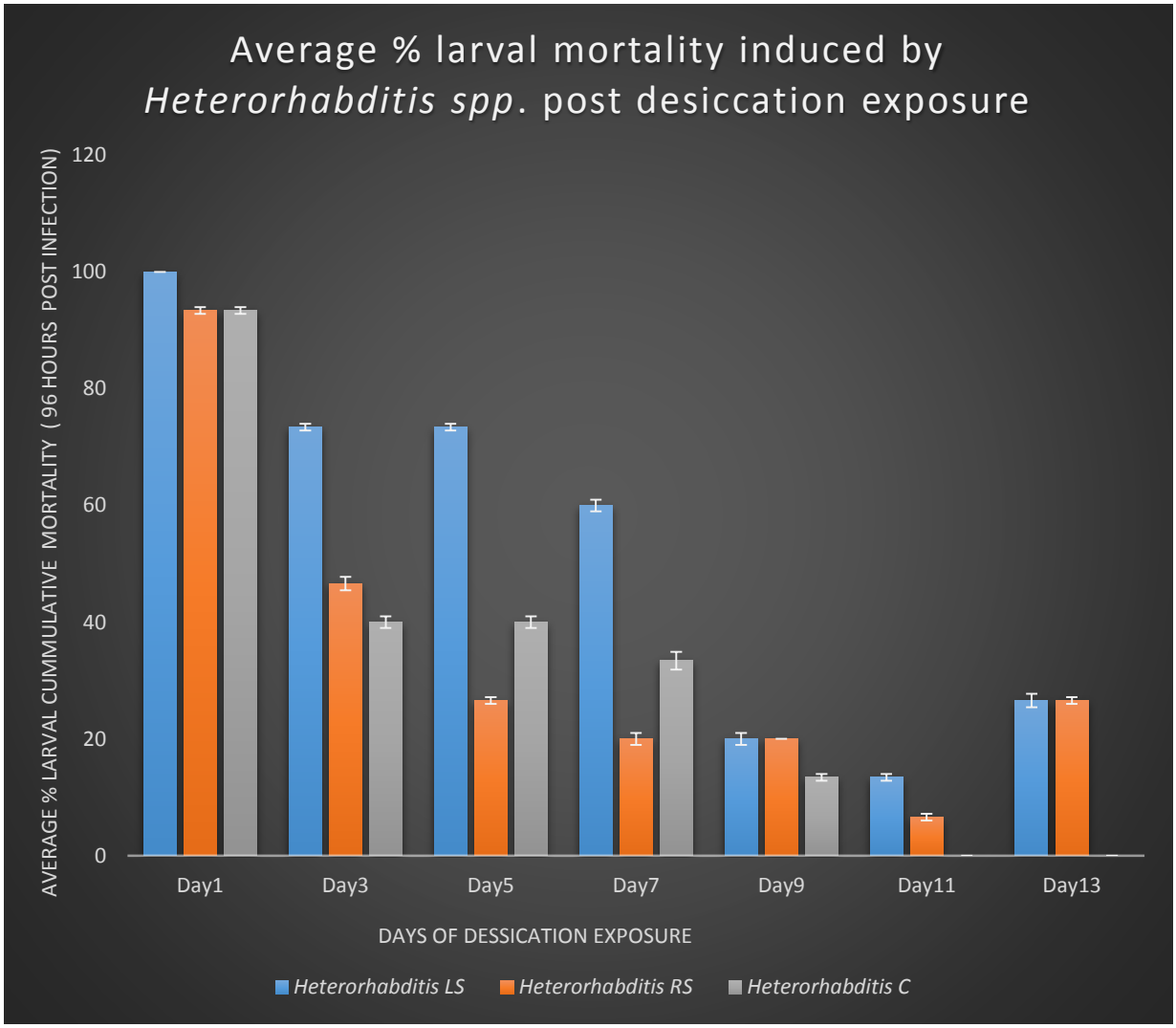


Figure 6.3: average cumulative larval mortality percentage induced by *Heterorhabditis* spp. post different days of desiccation exposure (LS = loamy sand, RS = river sand and C= control)

Swarming, aggregation, coiling and clumping behavioural characteristics were observed when *Steinernema* spp. isolate 10 was exposed to desiccation (figure 6.4). *Heterorhabditis* species displayed no equivalent or similar behavioural characteristics associated with desiccation tolerance. The thick cuticle and sheath which are both known to lower the rate of water loss were observed using the light microscopy on infective juveniles of *Steinernema* spp. isolate 10 (figure 6.5).

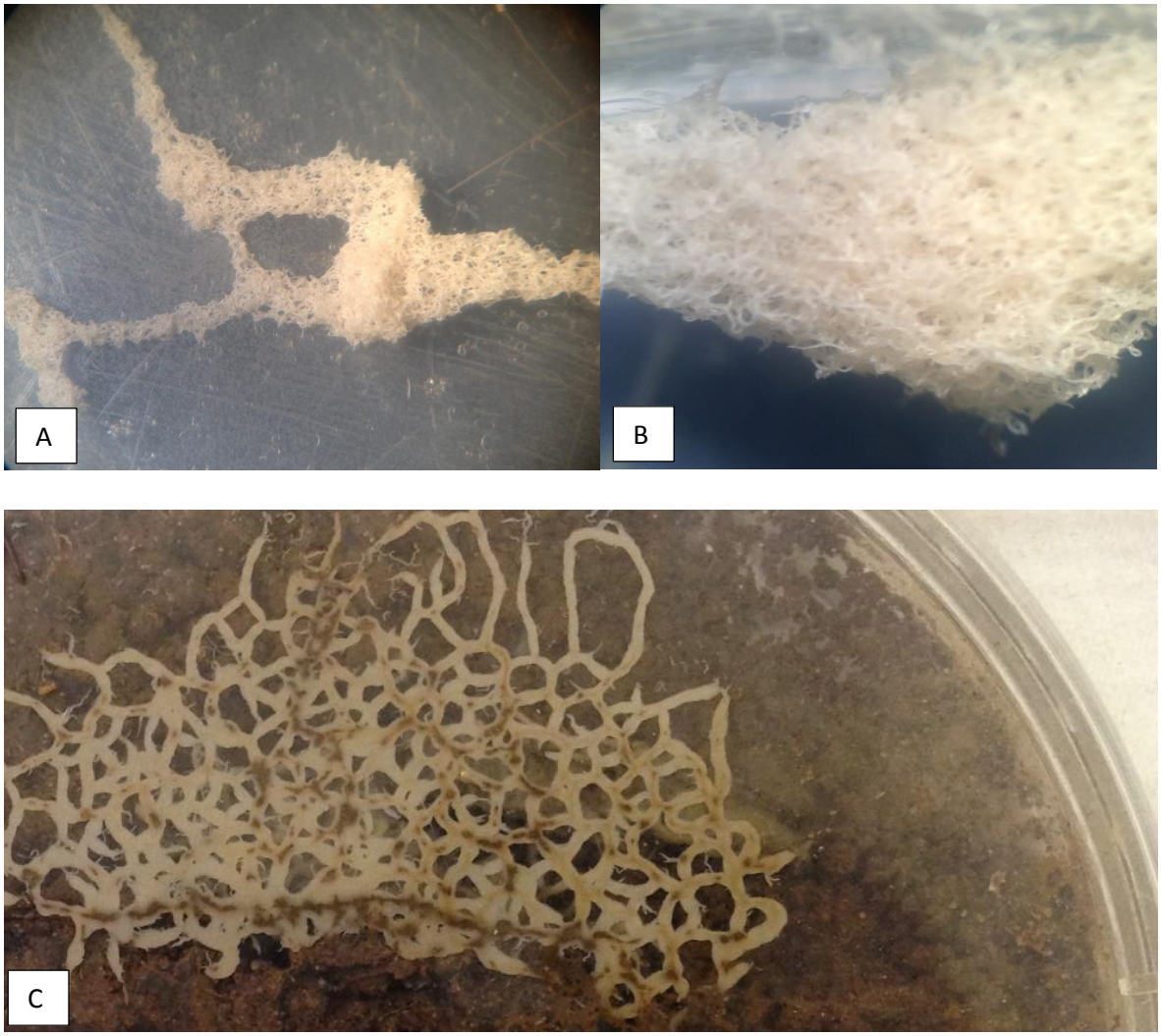


Figure 6.4: Behavioural characteristics of *Steinernema* spp. isolate 10 when exposed to desiccation conditions: A and B) coiling, clumping and aggregation C) swarming.

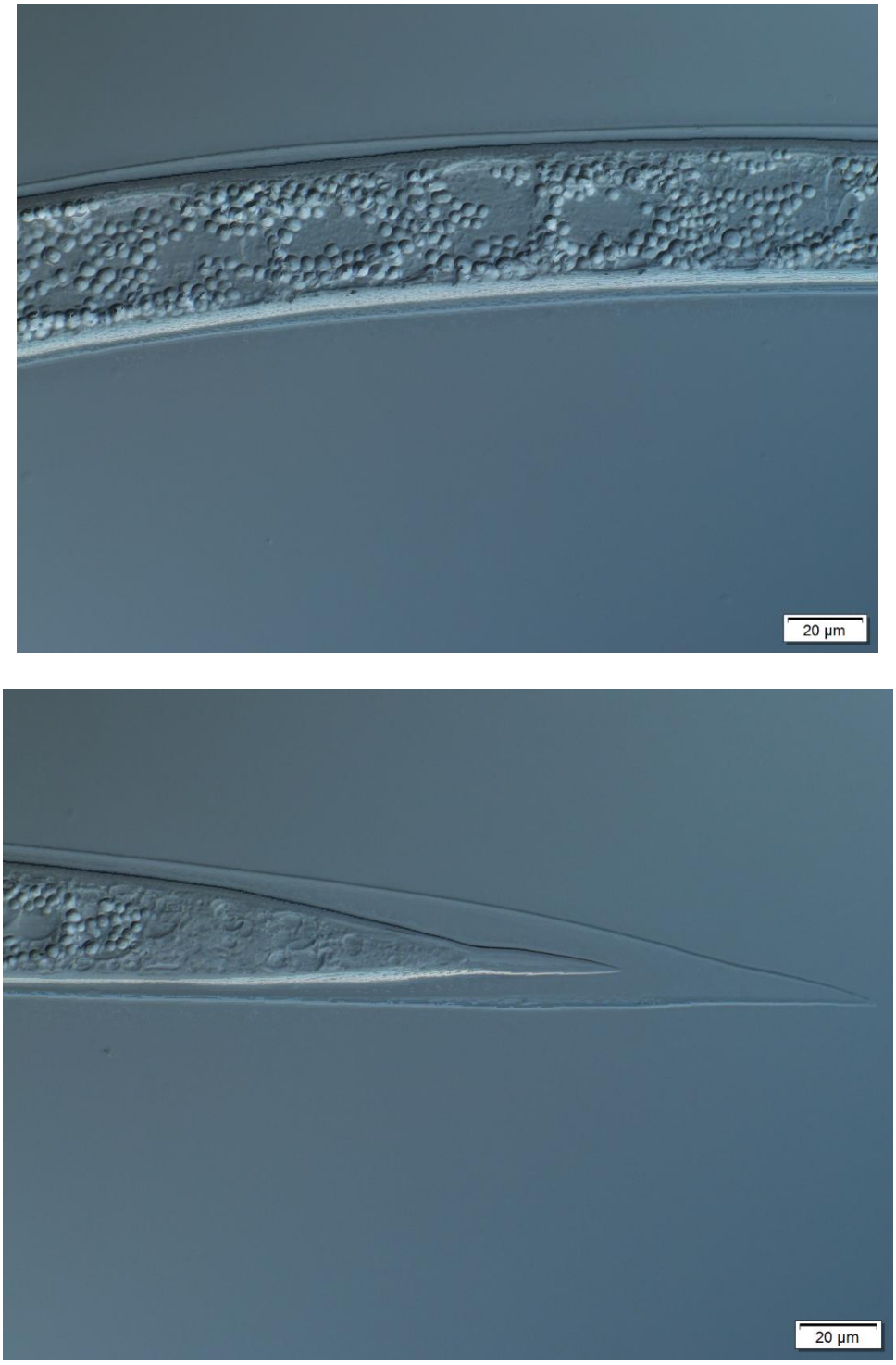


Figure 6.5: Thick cuticle and the retained sheath believed to aid in desiccation tolerance of *Steinernema* spp. isolate 10.

Table 6.1: Average percent cumulative larval mortality after 48, 72 and 96 hours post infection by *Heterorhbditis* and *Steinernema* species exposed to desiccation conditions for different days of dehydration. The control plates were kept at hydrated (8% moisture) conditions at all times. (LS= loamy sand, RS= river sand and C= control)

	Average percent cumulative larval mortality								
Heterorhabditis	LS			RS			Control		
Days of dehydration	48 hours	72 hours	96 hours	48 hours	72 hours	96 hours	48 hours	72 hours	96 hours
1	40	86,6	100	60	80	93,4	80	86,6	93,4
3	53,4	60	73,4	40	40	46,6	33,4	33,4	40
5	53,4	66,6	73,4	13,4	26,6	26,6	20	26,6	40
7	20	26,6	60	0	20	20	6,6	20	33,4
9	13,4	20	20	0	20	20	13,4	13,4	13,4
11	6,6	13,4	13,4	6,6	6,6	6,6	0	0	0
13	6,6	26,6	26,6	6,6	20	26,6	0	0	0
Steinernema	LS			RS			Control		
Days of dehydration	48 hours	72 hours	96 hours	48 hours	72 hours	96 hours	48 hours	72 hours	96 hours
1	46,6	93,4	100	53,4	100	100	53,4	93,4	100
3	33,4	66,6	73,4	13,4	33,4	66,6	13,4	40	40
5	26,6	40	53,4	33,4	33,4	80	20	33,4	40
7	13,4	33,4	33,4	6,6	6,6	20	13,4	20	20
9	0	13,4	20	6,6	13,4	13,4	0	6,6	6,6
11	13,4	13,4	26,6	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0

6.4 DISCUSSION

Steinernematids and heterorhabditids have developed mechanisms to tolerate environmental stress conditions including desiccation. Desiccation tolerance of *Steinernema* spp. isolate 10 and *Heterorhabditis* species was investigated in the current study and both species were tolerant to desiccation stress. *Steinernema* spp. isolate 10 was tolerant up to 11 days of desiccation exposure in loamy sand and up to 9 days of exposure in river sand, causing 26, 6% and 13, 4% cumulative larval mortality after 96 hours, post resuscitation by rehydration, respectively. *Heterorhabditis* spp. could tolerate desiccation up to 13 days of exposure and induced 26.6% cumulative larval mortality on both loamy and river sand after 96 hours.

Both species were highly effective on loamy sand for all days of desiccation exposure and this shows that habitat preference plays a pivotal role in infectivity and longevity. The control soil samples were maintained at 8% moisture at all times and were never exposed to desiccation, hence 100% infectivity was expected, however nematodes infectivity was drastically affected suggesting that desiccation conditions could be one of the most important factors for storage

conditions. Both species could not infect post 13 days of exposure and this suggest that high levels of desiccation exposure affect infectivity and longevity of nematodes.

Nematodes have been reported to tolerate desiccation through the process called anhydrobiosis. This is a reversible, physiologically and metabolically arrested state of dormancy that results due to water loss (Liu and Glazer, 2000). EPNs are referred to as quiescent anhydrobites as they are only capable of low level of dormancy. A study conducted by Liu and Glazer (2000) reported *Heterorhabditis* nematodes to be poor anhydrobiotes. *Steinernema carpocapsae* have been stipulated to survive low relative humidities from gradual desiccation (Simons and Pionar, 1973; Campbell and Gaugler, 1991).

The infective juveniles (IJs) of EPNs are ensheathed by the second stage cuticle (Pionar, 1979) of which is believed to protect them against both biotic (Timper and Kaya, 1989; Grewal *et al*, 1994)) and abiotic factors (Womersley, 1990). The sheath is reported to act as a water loss barrier resulting in a very slow rate of water loss during desiccation. The nematode sheath has a restricted permeability, hence slow rate of water loss which enables the enclosed infective juveniles to survive anhydrobiotically (Wharton, 1980). High survival rate of nematodes exposed to desiccation is attributed to slow water removal from nematodes body and this is required to enable metabolic changes necessary to enter anhydrobiotic state (Crowe *et al*, 1992)

Steinernema spp. isolate 10 IJs are ensheathed and the sheath enables the nematodes to be able to tolerate desiccation conditions. Aggregation, coiling and clumping behavioural characteristics have been observed for *Steinernema* spp. isolate 10 and no similar behaviour was observed for *Heterhabditis* species when exposed to desiccation conditions. Aggregation and swarming behaviour are reported to be associated with response to environmental stress, including desiccation (Womersly *et al*, 1990). Least tolerant strains of nematodes does not show clumping behaviour hence *Steinernema* spp. isolate 10 is reported to be the most tolerant and *Heterorhabditis* spp least tolerant to desiccation stress (Solomon *et al*, 1999). A recent study conducted by Shapiro-Ilan and colleagues on desiccation tolerance of *Steinernema* and *Heterorhabditis* species revealed *S. carpocapsae* to have the highest level of desiccation tolerance among species followed by *S. feltiae* and *S. rarum*; the *Heterorhabditid* species exhibited the least desiccation tolerance (Shapiro-Ilan *et al*, 2014). This results are further supported by coiling behaviour that have been observed and reported for EPN species including *S glaseri, S carpocapsae and S feltiae* (Womersly *et al*, 1990; Partel *et al*, 1997).

The two way Anova-with replication statistical analysis was conducted to assess infectivity variation between *Steinernema* spp. isolate 10 and *Heterorhabditis* spp groups based on cumulative larval mortality for different days of desiccation exposure and the results revealed no significant difference as 0,15211 = F < Fcritical= 3,125945 and and the p value = 0,85945 > 0,05.

There are also biochemical and physiological responses attributed to desiccation stress tolerance. In their anhydrobiotic state, nematodes are reported to synthesize elevated levels of disaccharide trehalose which is believed to play a pivotal role in stress tolerance (Pellerone *et al*, 2003). Trehalose is thought to stabilize membranes by attaching to the head groups of the phospholipids and preventing phase changes that may cause the membrane to become leaky and this is referred to as the "water replacement hypothesis" (Behm, 1997). By means of this association, trehalose protects biological membranes during desiccation by replacing the water that normally associates with the phospholipid bilayer (Hoekstra and Crowe, 1992). Other functions of trehalose associated with desiccation tolerance include prevention of protein denaturation and oxidative damage and provision of inert energy (Higa and Womersly, 1993). *Steinernema* spp. isolate 10 and *Heterorhabditis* species are both tolerant to desiccation stress.

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CHAPTER7: Genome Sequencing and Annotation of Entomopathogenic Bacterial Symbiont

7.1 INTRODUCTION

Next generation sequencing which is also referred to as high-throughput sequencing, is used to describe various modern sequencing technologies including Illumina, Rosche 454, Ion torrent: Proton/PGM and SOLiD sequencing. These sequencing technologies enable researchers to sequence DNA and RNA quickly and cheaply than the previously used Sanger sequencing (Tatusova *et al*, 2013). NGS have radically transformed the study of genomics and molecular biology.

Microbial whole genome sequencing (WGS) advances knowledge at genetic level in the physiological, morphological and metabolic characteristics of organisms. The ability to sequence and analyse whole genomes has begun to provide insight into evolutionary patterns amongst organisms (Chaston *et al*, 2011). WGS allows for mapping genomes of novel organisms, finishing the genomes of known organisms as well as comparative genomics. Microbial divergent and convergent evolutionary relationships can be better understood through WGS by identification of shared protein families (Pfams) and evolutionary patterns generated from the 16S rDNA. Through sequencing, a number of approaches have been used to study how bacterial genomes reflect evolutionary divergence and convergence and these include phylogenetic relationships based on average amino acid identity and shared gene orthology. Sequencing of genomes is highly important for generation of reference genomes which can be used for reference guided sequencing and assembly.

De novo WGS involves sequencing and assembling a genome without a guide of a genomic reference and is frequently used to sequence novel microbial genomes. The steps involved in genome sequencing are genomic DNA extraction, Library preparation, sequencing, assembly and interpretation.

Bacterial genomes are susceptible to many alterations through genome reduction, gene duplication, divergence and horizontal gene transfer which are incurred by pressures such as envrionmental stress, mutation and competition. It is possible to detect results of these mechanisms, however it is difficult to characterize the evolutionary path. One example is that of endosymbionts in the genera *Photorhabdus* and *Xenorhabdus*, which have undergone divergent evolution but have convergent lifestyles. They are both associated with nematodes

in the genera *Heterorhabditis* and *Steinernema,* respectively. They are both insect pathogens and physiological and genetic studies have revealed that their colonization within nematodes varies, and that they use functionally different approaches for colonization and pathogenicity. Both *Xenorhabdus* and *Photorbdus* phase I variants have been tested against fungal species and revealed to produce antimicrobial compounds with antifungal activity (Chen *et al*, 1994). Several compounds with antibiotic activity secreted by *Xenorhabdus spp.* include benzylineacetone (Ji *et al*, 2004), xenorhabdins and xenocoumacin (McInerney *et al*, 1991), phenethylamides (Li *et al*, 1995), and cyclolipopeptide (Gualtien *et al*, 2009), xenortides and xenematide, as well as nematophin (Chen *et al*, 1994; Wang *et al*, 2014). Insecticidal proteins have also been identified and could be of important use in agriculture (Duchaud *et al*, 2003; Ffrench-Constant *et al*, 2007). The tripartite nematode-bacteria-insect larvae system serves as the best model system to better understand evolutionary relationships, mutualism and pathogenic processes.

In this study, Illumina Miseq whole genomic sequencing, assembly and annotation of *Xenorhabdus* bacterial endosymbiont is discussed. The assembly was done using CLC Genomics Workbench version 6.5.0 and SPAdes version 3.5.0. The best assembly based on the total length, N50 and number of contigs was submitted to National Centre for Biotechnology Information (NCBI) prokaryotic genome automatic annotation pipeline (PGAAP) and rapid annotation using systemic technology (RAST) for annotation.

7.2 MATERIALS AND METHODS

7.2.1 Genomic Sequencing

Total genomic DNA was extracted from colony bacterial cultures of the *Xenorhabdus* bacterial endosymbiont using the ZR fungal/bacterial DNA kit (catalog #D6007). Purification of isolated genomic DNA was conducted using the DNA clean and concentrator-5 kit. Illumina libraries were generated using the Illumina Nextera DNA sample preparation kit (FC-121-1031) and paired-end sequencing was performed using Illumina Miseq instrument version 3, chemistry 300 x 300 bp.

7.2.2 Quality

The quality of generated reads was assessed using CLC Genomics Workbench 8.0.2 (CLC Bio) and FASTQC version 0.11.3.

7.2.3 Genome Assembly: CLC Bio

7.2.3.1 Trimming of low quality bases and adaptors, and assembly

CLC Bio was used to trim the Illumina nextera transposase adaptor sequences, as well as the low quality reads at default parameters. The reads were merged and both the merged and not merged reads were used for assembly. The minimum contig length parameter was set to >/= 400bp.

7.2.4 Genome assembly: SPAdes

7.2.4.1Trimming of low quality bases and adaptor sequences, and assembly

The low quality reads (Phred score < 20, base calling duplicates and adaptors were trimmed using Trimmomatic version 0.32 with the parameters: ILLUMINACLIP: NexteraPE-PE, MAXINFO: 50:0.8, MINLEN: 50 and LEADING: 20. The quality of the trimmed reads was assessed using FASTQC. The trimmed reads of high quality (Phred score> 20) were assembled into contigs using SPADES version 3.5.0. The assembly was assessed using QUAST version 2.3.

7.2.5 Genome annotation

The assembled reads (contigs) were submitted to national centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), prokaryotic genome automatic annotation pipeline for annotation. Subsystem and functional annotation was conducted through rapid annotation using systemic technologies (RAST) (http://rast.nmpdr.org) web based pipeline. Genome comparison was conducted using *E. coli* as a reference and two *Photorhabdus* species.

7.3 RESULTS

7.3.1 Sequencing and quality analysis

The Miseq-sequencing machine generated 6, 525, 888 sequences (in pairs) of 301 bases long. The total number of nucleotide bases generated 1, 964, 292, 288 bp. Distribution revealed all sequences of the same length represented by a single peak for 100% of sequences. The fragment length generated from the insert DNA fragment was specified and the generated sequences revealed expected fragment length for all the reads suggesting that the reads were successfully

generated. The GC content is a very important factor in genome analysis. It represents the sum of guanine and cytosine nucleotide bases compared to all bases including ambiguous bases in all the generated reads. The relative GC content of sequences in percentages ranged from 24% to 69%. About 506 sequences had the least GC content (24%) and about 430 sequences had the highest GC content (69%). Of all 6, 525, 288 sequences, 86% of those had a GC content that is </= 50% and which would result in average GC content being less than 50% which is favourable for generation of best assemblies. Ambiguity of base content was analysed and only 0.07% (normalized to the total number of sequences) of sequences featured N-percentages. The ambiguous base percentages observed were 1%, 2%, 3%, 4% and 5% in 1, 702; 772; 650; 496; and 420 sequences, respectively.

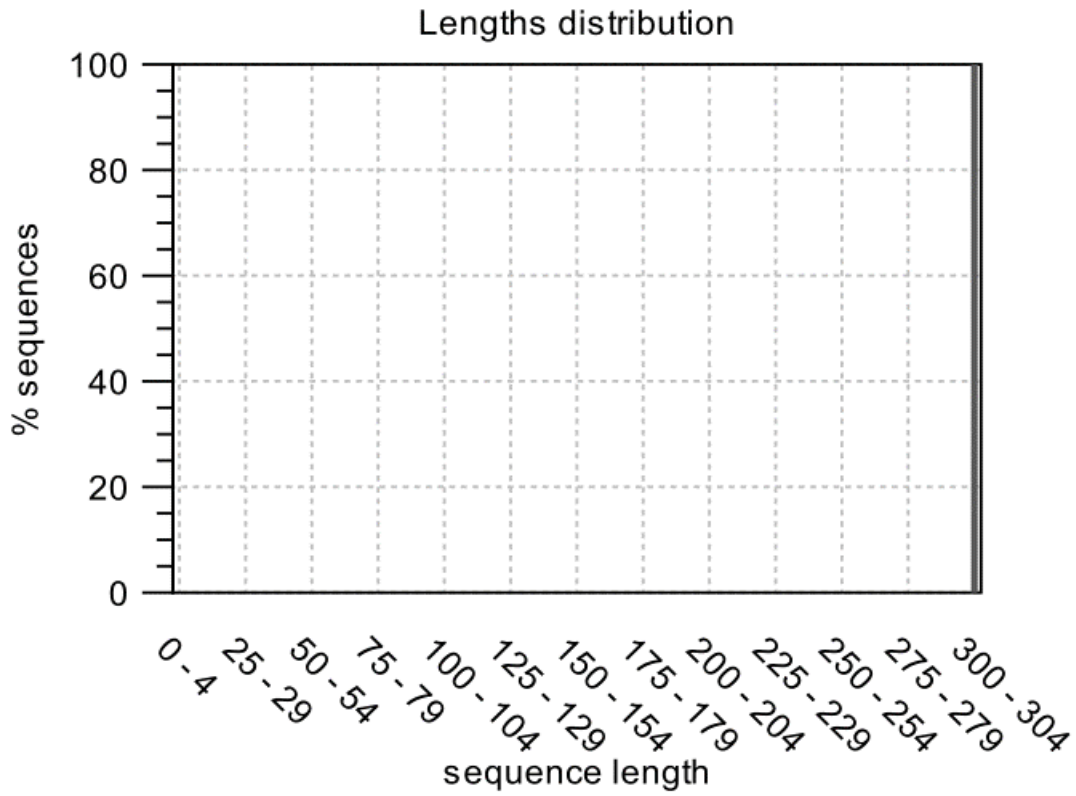


Figure 7.1: Length distribution of all sequences after sequencing

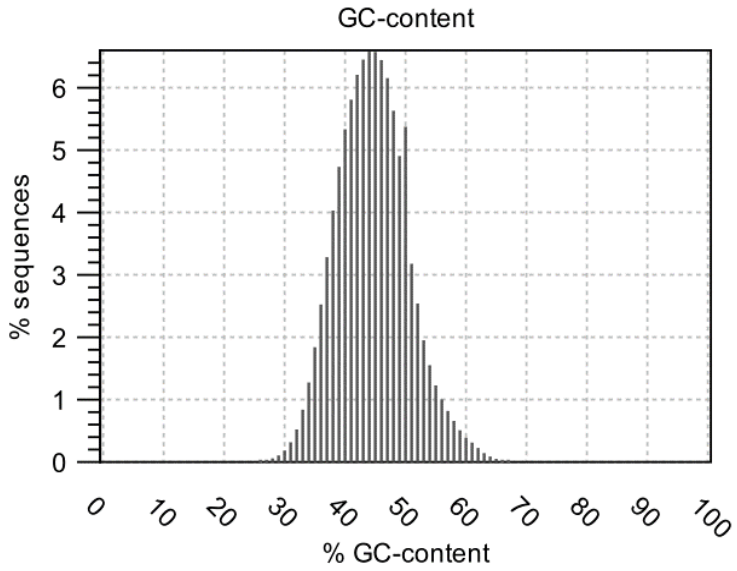


Figure 7.2: Distribution of GC content percentage across all sequences

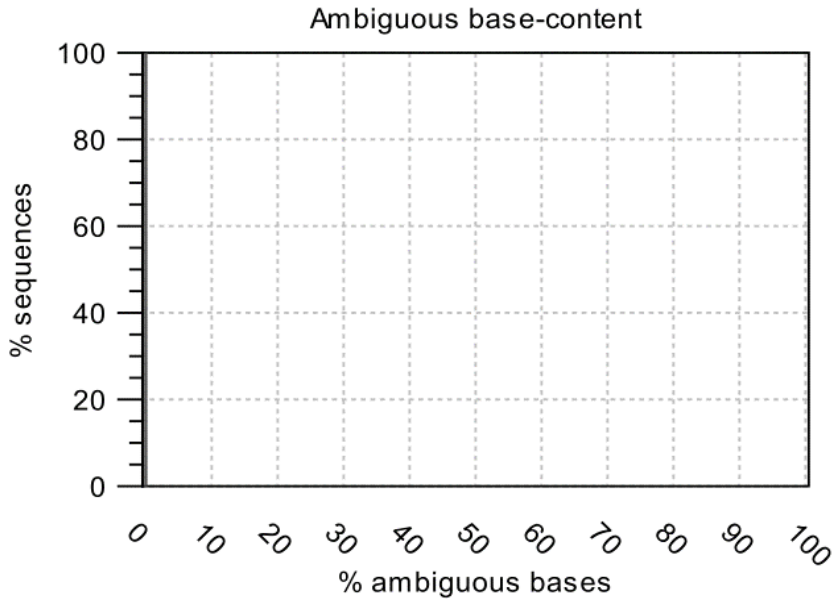


Figure 7.3: Distribution of nucleotide bases other than ATGC (N-bases) across all sequences. The number of sequences featuring a particular N-bases percentage is normalised to the total number of sequences.

7.3.1.1 Quality distribution

The average PHRED score quality distribution of nucleotide bases across the generated reads ranged between 9 and 38. The sequences were of good quality with 99,96% of all bases lying between an average Phred score of 15 and 38 and only 0.014% had a quality score of </=14.

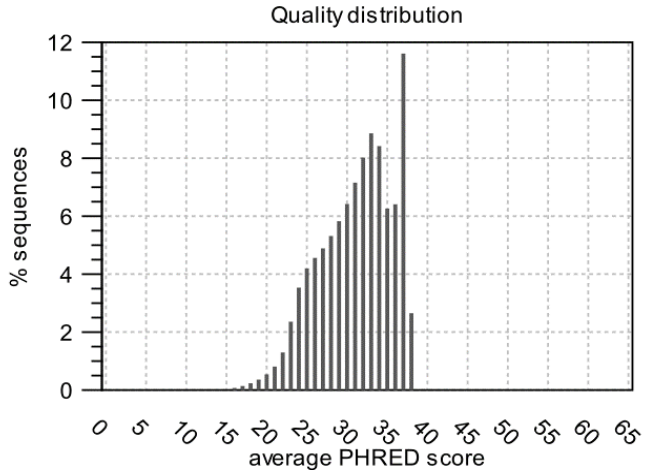


Figure 7.4: Average PHRED quality score distribution across all sequences. This illustrate the arithmetic mean of base qualities per sequence.

7.3.1.2 Coverage

The number of sequences that support (cover) the individual base positions was 100% for all sequences i.e. all individual bases were covered 100 times during sequencing to support the probability of that base being at the specified position.

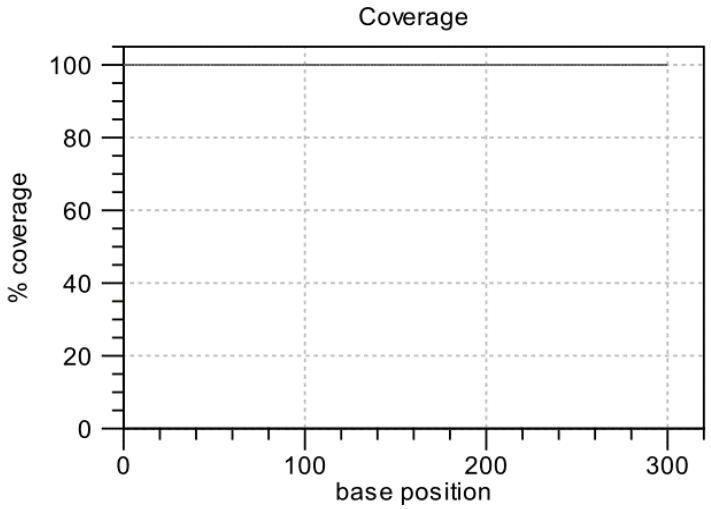


Figure 7.5: Coverage of all bases across all sequences. All bases were covered 100X.

7.3.1.3 Duplication levels

There were duplicated sequences observed in the generated sequences. About 43,84% of the sequences appeared only once and were not duplicated, with about 56,16% of the genome sequences duplicated with variation in duplicate count and of those, 34,15% had a duplicate count of less than 5.

7.3.2 FASTQC Quality distribution before and after trimming

FastQC is similar to CLC in terms of quality analysis of sequences, however FASTQC analyse the reads and identify the adapter contamination.

7.3.2.1 Adaptor contamination

There was a contamination of a sequencing universal adaptor nextera transposase sequence in all the generated reads. The adaptor sequence was trimmed off from all the sequences using Trimmomatic version 0.32.

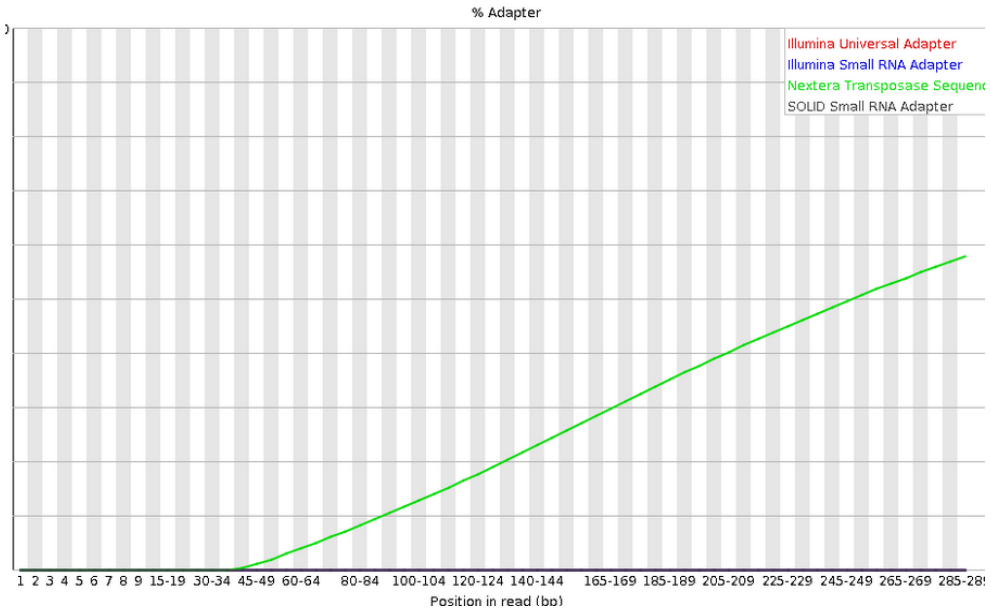


Figure 7.6: Adaptor contamination revealed by FASTQC quality analysis.

7.3.2.2 Trimming of adapters and low quality reads

The reads were trimmed for adapters and low quality reads and FASTQC of trimmed sequences was evaluated. Only bases with a PHRED score >= 20 were kept and used for for genome assembly. FASTQC identified adaptor contamination of nextera transposase sequence which was successfully trimmed using Trimmomatic (figure 7.8B).

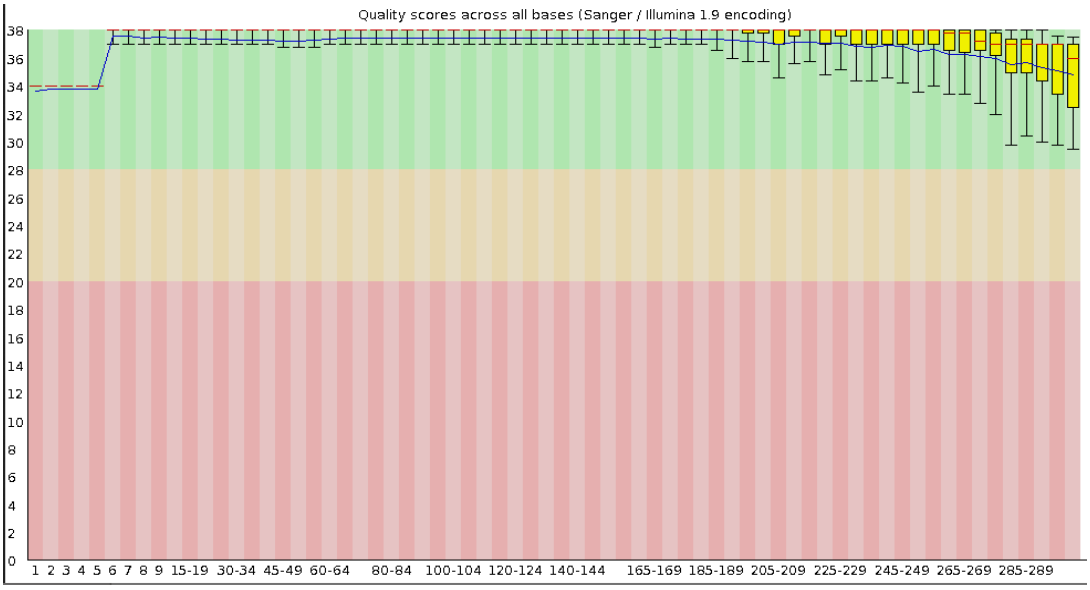


Figure 7.7: Average Phred score quality of the reads after trimming of low (<20 average Phred score) quality bases.

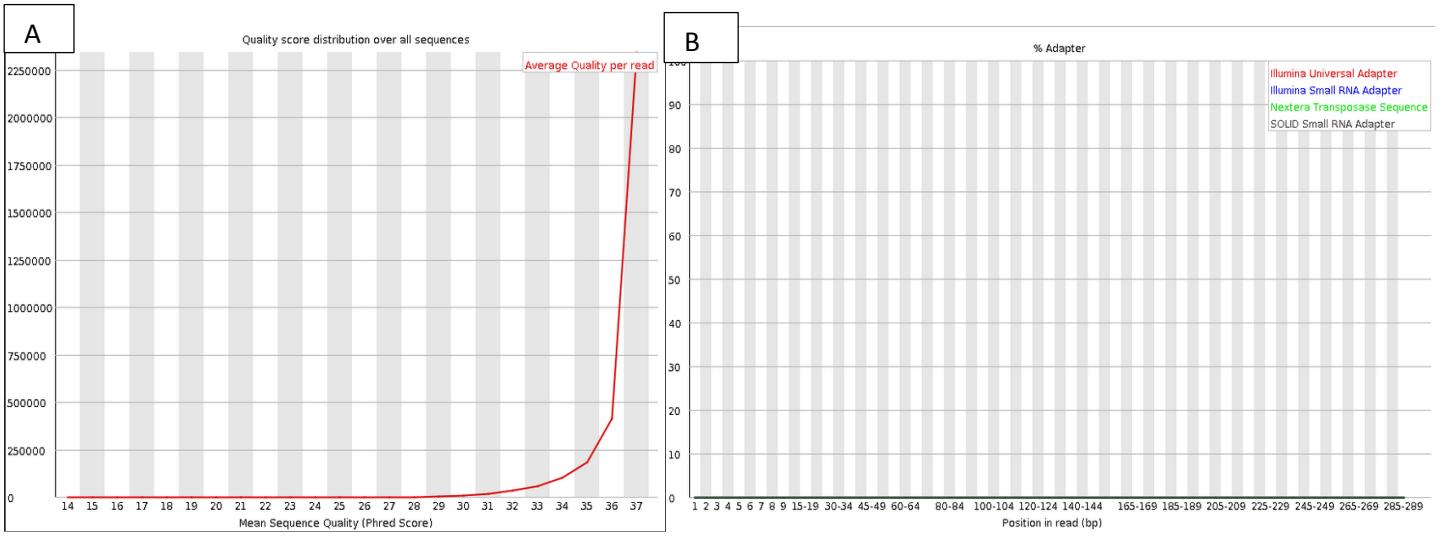


Figure 7.8: A) Quality score distribution over all sequences and B) adaptor contamination sequence removed.

7.3.3 Genome assembly: CLC Bio

The genome assembly of *Xenorhabdus* bacterial isolate using CLC Bio revealed a total length of 4, 183, 779 bp with 231 contigs (>=400bp), GC content of 44.7%, N50 of 57,901.

Table 7.1: Nucleotide distribution revealing the frequency of each nucleotide base in all sequences: GC content of 44.7%

Nucleotide	Count	Frequency
Adenine (A)	1,159,054	27.7%
Cytosine(C)	935,309	22.4%
Guanine (G)	934,031	22.3%
Thymine (T)	1,155,026	27.6%
Any Nucleotide (N)	359	0

Table 7.2: CLC assembly contig measurement of *Xenorhabdus* bacterial isolate

Contig	Count
N75	**31,527**
N50	57,901
N25	108,331
Minimum	402
Maximum	164,495
Average	18,112
Count	231
Total	4,183,779

7.3.4 Genome Assembly: SPAdes

The genome assembly of *Xenorhabdus* bacterial isolate using SPAdes revealed a total length of 4, 165,463 bp with 290 contigs (>=500bp), GC content of 44.62% and N50 of 64,801

Table 7.3: SPAdes assembly contig measurement of *Xenorhabdus* bacterial isolate. Unless stated, all results represent contigs that are >= 500bp.

Contigs	Count
# contigs (>= 0 bp)	419
# contigs (>= 1000 bp)	185
Total length (>= 0 bp)	4,202,961
Total length (>= 1000 bp)	4,093,713
#contigs	290
Largest contig	204,152
Total length	4,165463
GC(%)	44.62
N50	64,801
N75	29,174
L50	18
L75	43
Ns per 100kbp	0.00

7.3.5 Genome annotation:

Contigs were submitted to national centre for biotechnology information (NCBI), prokaryotic genome automatic annotation pipeline (PGAAP) for annotation and the results revealed 3,950 genes (3,601 protein coding sequences (CDS) and 266 pseudogenes), 12 rRNAs and 70 tRNAs.

Table 7.4: Annotation of *Xenorhabdus* bacterial isolate using NCBI PGAAP

Annotation provider	**NCBI**
Annotation pipeline	NCBI Prokaryotic Genome Annotation Pipeline
Annotation method	Best-placed reference protein set; GeneMarkS+
Annotation software revision	3.0
Features annotated	Gene; CDS; rRNA; tRNA; ncRNA; repeat region
Genes	3,950
CDS	3,601
Pseudo Genes	266
CRISPR Arrays	1
RRNAs	4, 4, 4 (5S, 16S, 23S)
Complete rRNAs	4, 1 (5S, 23S)
Partial rRNAs	4, 3 (16S, 23S)
TRNAs	70
NcRNA	1
Frameshifted Genes	62
Frameshifted Genes on Monomer Runs	1
Frameshifted Genes Not on Monomer Runs	1

The contigs were also submitted to rapid annotation using systemic technology (RAST) annotation server for annotation. The results revealed 4,083 coding sequences, 487 subsystems and 80 RNAs. The subsystem features revealed 55 of virulence, disease and defense features which are involved in the pathogenicity of *Xenorhabdus* bacterial isolate.

Table 7.5: Overview of RAST annotation for *Xenorhabdus* bacterial isolate.

Genome	*Xenorhabdus* bacterial isolate
Domain	Bacteria
Taxonomy	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriaceae; *Xenorhabdus*, *Xenorhabdus* bacterial isolate
Size	4,183,779
Number of contigs (with PEGs)	231
Number of subsystems	487
Number of coding sequences	4083
Number of RNAs	80

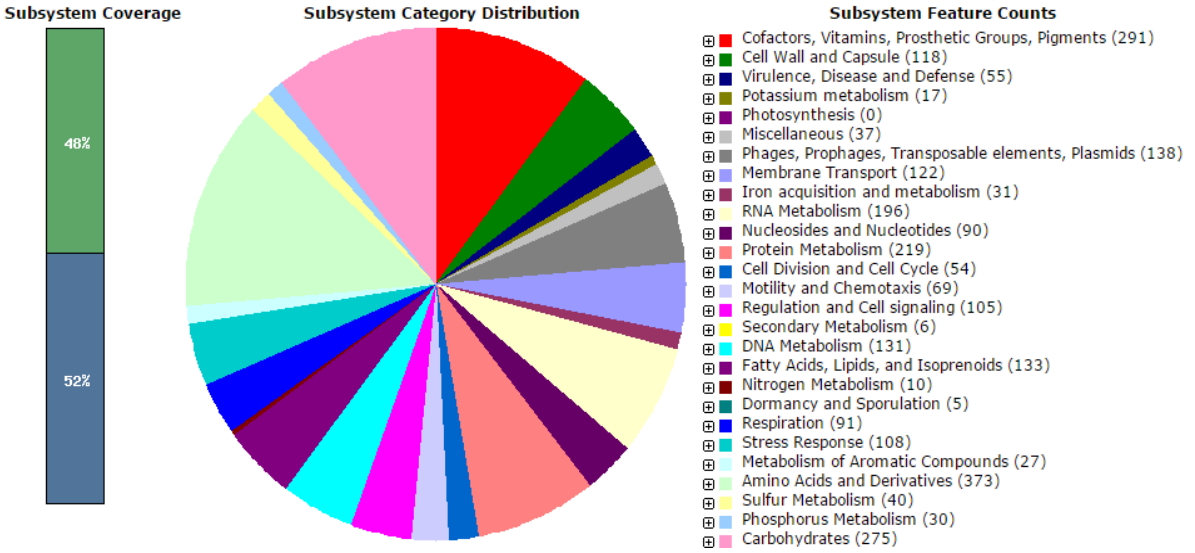


Figure 7.9: Representation of genes encoding for different physiological and metabolic systems

The comparison organisms were aligned to the reference genome. The result list the genes of the reference organism in chromosomal order and display hits on the comparison organisms accordingly

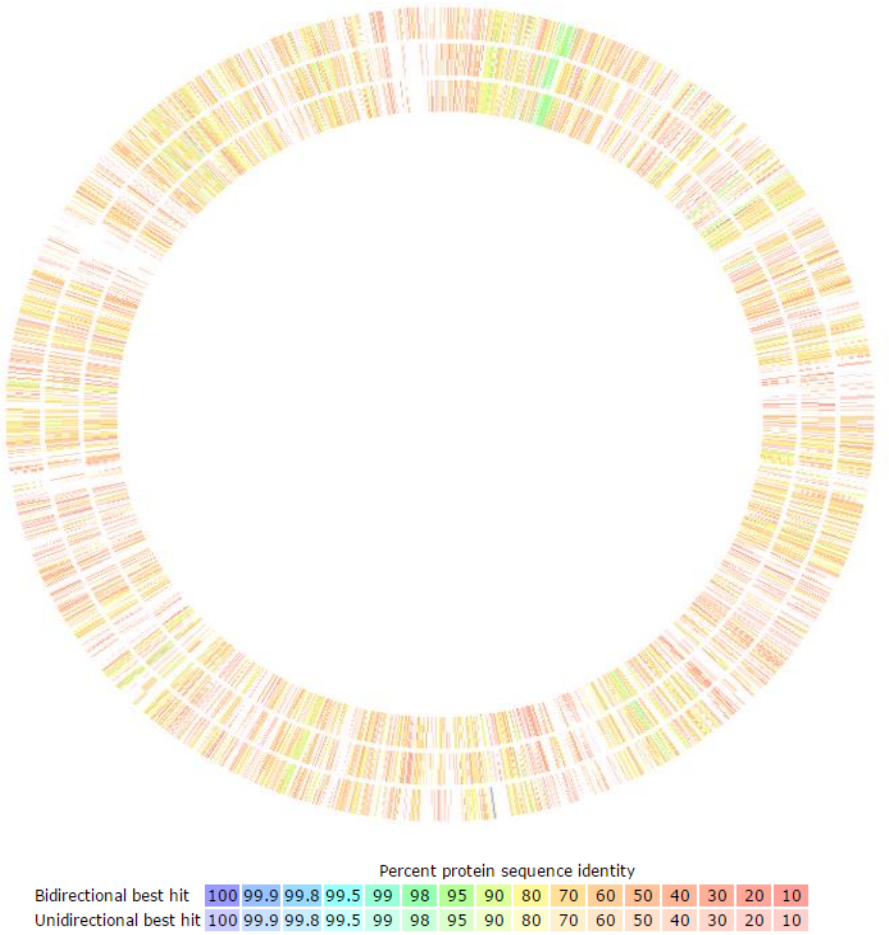


Figure 7.10: Circle plot comparing percent protein sequence similarity of *Xenorhabdus* bacterial isolate (1), *P. asymbiotica* subsp. Asymbiotica(2) and *P. luminescens* subsp. Laumondii TT01(3) to the reference genome *E. coli* strain 0.42. The outer most map shows the comparison between organism 1 and reference and the middle and inner most map compares organism 2 and 3 with the reference, respectively.

7.4 DISCUSSION

The generated reads were successfully trimmed for adaptor sequences and low quality bases. The trimmed reads of 20 and above average Phred score were assembled using CLC genomics workbench (CLC Bio) version 6.5.0 and SPAdes version 3.5.0. The CLC Bio assembly revealed a total length of 4, 183, 779 bp (minimum contig length >/= 400bp), with 231 contigs, an N50 of 57, 901 bp and a GC content (sum of guanine and cytosine nucleotide base pairs) of 44, 7%. The results were compared with those of other *Xenorhabdus* species assemblies. *X nematophila* ATCC and *X bovienii* SS-2004 have been sequenced and their full assembled genome consist of 4, 432, 590 bp and 4, 225, 498bp, with GC content of 44.2% and 45%, respectively, which is quite similar to results of draft whole genome sequence of *Xenorhabdus*

bacterial isolate presented in this study. The results were also compared to the whole genome sequence of *Photorhabdus luminescens* strain TT01 which possess a single circular chromosome of length 5,688, 987 bp and a GC content of 42.8% with a total of 4, 839 protein coding genes, 157 pseudogenes, 7 complete sets of (23S, 5S and 16S) ribosomal RNA operons and 85tRNA genes (Duchaud *et al*, 2003)

SPAdes assembly for *Xenorhabdus* bacterial isolate, revealed a total length of 4, 165, 463 bp with 290 contigs (minimum conting length >/= 500bp), N50 of 64, 801bp and GC content of 44, 62. The best assembly was decided based on the N50 and total length. The CLC assembly comprised of both high total length, N50 and lesser number of contigs, whereas the SPAdes assembly had a high N50 but the total length was less than that of the CLC assembly and number of contigs were higher. This suggested that there might be important sequences missing on the SPAdes assembly which are present on the CLC assembly.

The CLC draft sequence assembly of *Xenorhabdus* bacterial isolate was deposited to NCBI and submitted at prokaryotic genome automatic annotation pipeline (PGAAP (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) for annotation. PGAAP annotation revealed 3,970 genes and of those 3, 614 were protein coding (CDs) and 271 were pseudogenes. The genome has 12 rRNAs (5S, 16S and 23S), 70 tRNAs and 3 non coding RNAs.

The CLC draft sequence assembly was also submitted to a web-based annotation server, rapid annotation using system technologies (RAST) (http://rast.nmpdr.org), for subsystem and functional annotation. RAST revealed 4083 coding sequences and 80 RNAs. The entomopathogenicity of *Xenorhabdus* bacterial isolate is dependent on the ability of the bacterial symbiont to avoid and silence humoral and cellular innate immunity of the insect larvae and to be able to produce toxins that are lethal to the larval host. About 55 coding counts was revealed for virulence, disease and defense. Of all classified systems, 105 counts was revealed for regulation and cell signalling which may be involved in colonization of the associated nematode gut. High subsystem feature count (373) was revealed for amino acids and derivatives, 275 for cofactors, vitamins and pigments, 275 for carbohydrates and 219 for protein metabolism as well as 196 for RNA metabolism.

Using *E. coli* as a reference, genome sequences of *Xenorhabdus* bacterial isolate, *Photorhabdus asymbiotica* and *Photorhabdus luminescens* were compared using RAST compare sequences tool and the results revealed high protein similarity. The reference is not shown, only the map of the compared genomes showing different colour coding for percent similarity of the associated gene on the reference. The colour coding and percentage similar associated with that specific colour is shown in figure 7.10.

Genome sequencing and annotation gives insight to behavioural and physiological attributes of bacterial symbionts and thus this study will contribute to the understanding of pathogenicity, evolution and specific colonization of bacterial symbionts to their associated nematodes.

7.5 REFERENCES

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RESEARCH SUMMARY

Insect pests remains to be problematic in agricultural industries as insects evolve resistance towards the currently employed chemical pesticides, leading to low yield loss. The indigenous entomopathogenic nematodes isolated in this study, *Steinernema* spp. isolate 10 and *Heterorhabditis* species, holds great potential as biological control agents as they are able to tolerate environmental extremes such as desiccation. Soil is an important habitat for EPNs and undergoes gradual desiccation, hence the importance of application of desiccation tolerant EPNs. The EPNs identified in this study have only been tested against the *G. mellonella* larvae and would be interesting to further test their infectivity on South African problematic insect pests and conduct field trials. The limitations include different micro flora in the soil as in the lab it is only the host and the EPNs, which is different in the agricultural fields as all other organisms and competitors are part of the biodiversity and the temperature conditions keeps on changing which might have an effect.

Xenorhabdus bacterial isolate have not yet been described and is a bacterial symbiont associated with *Steinernema* spp. isolate 10 and this bacterial-nematode complex hold great potential as biological control agent. Genomics of the bacterial symbiont will provide insights to pathogenicity of insect pests by *Xenorhabdus* bacterial isolate as annotation revealed the presence of genes involved in virulence, defense and disease. Future studies include the whole genome sequencing of *Steinernema* spp. isolate 10 to identify genes involved in environmental

stress tolerance and to understand the co-evolution between *Xenorhabdus* bacterial isolate and *Steinernema* spp. 10. Behavioural studies will need to be conducted to comprehend the foraging behaviour of *Steinernema* spp. 10 and *Heterorhabditis* species to assess they will be able to locate soil inhabiting insect larval pests and induce mortality. It is highly crucial to understand the behavioural and physiological characteristics of EPNs prior formulation, production and application. *Steinernema* spp. isolate 10 and *Heterorhabditis* species both isolated in this study holds potential as biological control agents of insect pests.

Appendix I

Galleria mellonella media

(Adapted from Woodring and Kaya, 1988)

The following modifications were made:

- Calcium propionate substituted with benzoate
- Multivitamin bran substituted with ProNutro (banana flavour)

Recipe:

500g ProNutro

200ml pure natural honey

200ml glycerol

5 teaspoon yeast extract

200ml boiled distilled water

1 teaspoon benzoate

Protocol:

1. Mix honey, glycerol and ProNutro together.
2. Add yeast extract, boiling water and benzoate to ProNutro mixture.
3. Mix contents thoroughly.
4. Place mixture in tin foil and seal adequately.
5. Autoclave at 121˚C and 15 psi for 25 min.

Appendix II

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 atleast 30 seconds and discard the supernatant.
5) Add 600µl Cell Lysis Solution (from kit) and invert several times.
6) Add 3µl Proteinase K solution (from 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 RNase A 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 RNase A 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 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.

Appendix III

1. Isolation of bacterial symbionts from the haemolymph of larvae infected by EPNs

1) Place 5 instar *G. mellonella* larvae in a Petri dish plate with river sand inoculated with EPN infective juveniles (IJs).
2) At 48 hours post infection, collect infected and dead larvae.
3) Surface sterilize infected *G. mellonella* larvae by spraying with 70% ethanol.
4) Secondary surface sterilization: dip the larvae in 70% ethanol followed by slight heating of the larval surface for 2-3 seconds to avoid heat-killing the bacteria.
5) Cut open or dissect sterilized larvae using sterile scissors and sculpit, working aseptically.
6) Use a syringe to draw the sticky fluid or haemolymph from the cardaver into an Eppendorf tube containing 200 µl of nutrient broth or distilled water.
7) Streak on NBTA plate NBTA (nutrient bromothymol triphenyltetrazolium agar) and incubate for 48-72 hours at 25ºC.
8) Screen for blue green colonies

2. Isolation of bacterial symbionts from from EPNs

1) Sterilize IJs for 3 hours and suspended in 500µl of nutrient broth
2) Grind and crush the nematodes using a sterile pestle to form a homogenate
3) Transfer the homogenate aseptically to sterile tubes containing fresh Nutrient Broth.
4) Incubate at 25 ˚C in the dark for 48 hours
5) Streak the broth culture in NBTA plates and incubate at 25 ˚C for 48-72 hours
6) Screen for blue-green colonies

Appendix IV

DNA isolation of bacterial cells associated with EPNs

Bacterial genomic DNA isolated using DNA extraction kit (ZR Fungal/Bacterial DNA Kit, catalog no: D6005)

1) Pick a colony of isolated bacteria from NBTA plate and suspend in a ZR BashingBeadTM Lysis Tube.

2) Secure in bead beater and process at maximum speed for 5 minutes.

3) Centrifuge the ZR BashingBeadTM 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-SpinTM 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-SpinTM 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-SpinTM II Column and centrifuge at 10000rpm for 1 minute.

10) Transfer the Zymo-SpinTM 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.

Appendix V

Protocol for confirmation of the dependency of EPNs on bacterial symbionts for growth and development

1) Inoculate 1.0ml of nutrient broth with Phase I blue green colonies from NBTA plates in 2.0ml Eppendorf tubes
2) Incubate the tubes for 24 hours at 25ºC.
3) Prepare spread plates using 0.1ml of broth culture on lipid agar plates and incubate for 48 hours at 25ºC
4) Sterilize nematodes for 1 hour in sodium hypochlorite and inoculate 100IJs/ml and inoculate onto bacterial lawn lipid agar plates.
5) Incubate the plates (unsealed) at 25ºC and inspected daily for EPN propagation and development

Appendix VI

Protocol for EPN specimen preparation for morphological identification:

Light Microscopy

Processing Nematode Specimens to Glycerin (Stock & Kaya, 1997)

Killing and fixing nematodes

1. Place nematodes in a Syracuse watch glass in 1ml distilled water.
2. Add 3-4ml 100ºC TAF and leave for 24 hours.
3. Replace TAF with double-strength TAF and store at 4ºC to relax nematodes for up to one hour.
4. Add 65ºC TAF. Allow fixative to infiltrate for at least 24 hours. Remove most of the fixative.

Processing nematodes to pure glycerine

1. Transfer fixed nematodes to a Sycaruse watch glass containing 0.5ml of solution.
2. Add 95% ethanol to a desiccator until the space below the holding shelf is half- full. Place the watch glass containing the nematodes in the desiccator.
3. Place the desiccator in an oven preheated to 35ºC for 12 hours.
4. Remove the watch glass/nematodes from the desiccator.
5. Fill the watch glass with Solution II and place the watch glass in a glass Petri dish.
The Petri dish is left partially open to allow for slow ethanol evaporation.
6. Place the Petri dish containing the watch glass in an oven preheated to 40ºC for 3 hours.

Solutions

TAF

8 ml 35% formaldehyde , 2.28 ml triethanolamine and 104 ml distilled water

Double-strength TAF

8 ml 35% formaldehyde , 2.28 ml triethanolamine and 52 ml distilled water

Solution I

20 ml 95% ethanol, 1 ml glycerine, 79 ml distilled water

Solution II

5 ml glycerine

95 ml 95% ethanol

Appendix VII

Protocol for EPN specimen preparation for morphological identification:

Scanning electron Microscopy

Preparation of Nematodes for Scanning Electron Microscopy (Kaya & Stock, 1997).

1. Kill nematodes by placing them in a water bath at 60ºC for 2 minutes.
2. Rinse three times (5 minutes each) in Ringer‟s solution (pH 7.3).
3. Prefix in 8% gluteraldehyde (25% EM grad gluteraldehyde diluted in Ringer‟s Solution (pH 7.3) overnight.
4. Rinse three times (5 minutes each) in Ringer‟s solution (pH 7.3).
5. Rinse once, for 5 minutes, in sterile double distilled water.
6. Post-fix in 1% osmium tetroxide for 2 hours at 4ºC.
7. Rinse three times (5 minutes each) in sterile double distilled water.
8. Dehydrate with a series of ethanol washes (30%, 50%, 70%, 90%, 95% for 30 minutes each.
9. Finally wash in several changes of 100% ethanol over 30-60 minutes.
10. Freeze dry the nematodes at -85 ºC and 101mT for 48 hours. Alternatively,Dry nematodes to critical point with liquid CO2.
11. Mount specimens immediately onto SEM stubs and coat with carbon and gold Palladium

Solutions:

Ringer‟s solution pH 7.3 (9g NaCl and 0.4g KCl)

Appendix VIII

In Vitro Media and sterilization solution

1. Nutrient Broth

4.0% (W/V) Canola oil

25mg/ml glucose

Protocol:

1. Weigh out nutrient broth powder and suspend in 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.

Autoclave at 121˚C and 15 psi for 15 min

2. NBTA (adapted from Akhurst, 1980)

1 litre nutrient agar

0.04g triphenyltetrazolium chloride (TTC)

0.025g bromothymol blue (BTB)

Protocol:

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.

3. 0.1% jik solution for infective juvenile sterilization

34ml distilled water

1ml 3.5% jik

Protocol:

1. Autoclave distilled water at 121˚C and 15 psi for 15 min..
2. Mix jik and autoclaved distilled water in bottles.

Appendix IX

ANOVA statistical analysis

Anova: Two-Factor With Replication														
SUMMARY	LS	RS	C	Total										
Steinernema														
Count	7	7	7	21										
Sum	15,34	13,99667	10,33333	39,67										
Average	2,191429	1,999524	1,47619	1,889048										
Variance	2,923181	4,257779	3,142857	3,193077										
Heterorhabditis														
Count	7	7	7	21										
Sum	18,34	11,99	11,01	41,34										
Average	2,62	1,712857	1,572857	1,968571										
Variance	2,684133	2,05789	2,62159	2,435303										
Total														
Count	14	14	14											
Sum	33,68	25,98667	21,34333											
Average	2,405714	1,85619	1,524524											
Variance	2,637442	2,937049	2,66303											
ANOVA														
Source of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*								
Sample	0,066402	1	0,066402	0,022525	0,881536	4,113165								
Columns	5,546221	2	2,77311	0,940705	0,399737	3,259446								
Interactio	0,896783	2	0,448391	0,152105	0,859447	3,259446								
Within	106,1246	36	2,947905											
Total	112,634	41												

Anova: Tw	Anova: Two-Factor V	Anova: Tw	Anova: Two-Factor W
SUMMARY	SUMMARY	LS	SUMMARY	SUMMARY
Steinernema	*teinernema*	*Steinernema*	*teinernema*
Count	Count	21	Count	Count
Sum	Sum	37,16667	Sum	Sum
Average	Average	1,531429	Average	Average
Variance	Variance	3,302915	Variance	Variance
Heterorhabditis	*rorhabditis*	*Heterorhabditis*	*rorhabditis*
Count	Count	21	Count	Count
Sum	Sum	37,675	Sum	Sum
Average	Average	1,445	Average	Average
Variance	Variance	2,404031	Variance	Variance
Total	*Total*		*Total*	*Total*
Count	Count	14	Count	Count
Sum	Sum	14,66667	Sum	Sum
Average	Average	1,047619	Average	Average
Variance	Variance	2,771429	Variance	Variance
ANOVA	ANOVA		ANOVA	ANOVA
ce of Varia	*ce of Varia*	*SS*	*ce of Varia*	*ce of Variation*
Sample	Sample	3,00846	Sample	Sample
Columns	Columns	0,67983	Columns	Columns
Interactio	Interactio	2,078899	Interactio	Interaction
Within	Within	-54,8192	Within	Within
Total	Total	-30,634	Total	Total

APPENDIX X:

FASTQC and TRIMMOMATIC Scripts

1. FastQC before trimming

```
#!/bin/bash
#PBS -N FastQCBac
#PBS -q medium
#PBS -l walltime=01:00:00,mem=2gb
#PBS -l nodes=1:ppn=1
#PBS -o /home/mothupib/fastqcbac_TEST/logs/output.log
#PBS -e /home/mothupib/fastqcbac_TEST/logs/error.log
WORK_DIR=/home/mothupib/fastqcbac_TEST
cd $WORK_DIR

fastqc $(ls /home/mothupib/fastqcbac_TEST/*fastq.gz) -o $WORK_DIR --noextract
```

2. FastQC after trimming

```
#!/bin/bash

#PBS -N FastQC_trimmed_bac
#PBS -q WitsLong
#PBS -l walltime=03:00:00,mem=2gb
#PBS -l nodes=1:ppn=1
#PBS -o /home/mothupib/fastqc_trimmed1_bac/logs/output.log
#PBS -e /home/mothupib/fastqc_trimmed1_bac/logs/error.log

WORK_DIR=/home/mothupib/fastqc_trimmed1_bac
cd $WORK_DIR

fastqc $(ls /home/mothupib/fastqc_trimmed1_bac/*fastq.gz) -o $WORK_DIR --noextract
```

3. Trimmomatic

```
#!/bin/bash

#PBS -N Trim_bac
#PBS -q WitsLong
#PBS -l walltime=03:00:00,mem=2gb
#PBS -l nodes=1:ppn=1
#PBS -o /home/mothupib/Trim_bac/logs/output.log
#PBS -e /home/mothupib/Trim_bac/logs/error.log

WORK_DIR=/home/mothupib/Trim_bac
OUT_DIR=$WORK_DIR/trimmed_bac

cd $WORK_DIR

count=0

for file in $(ls *R1*fastq.gz)
do
   (( ++count ))
   base_in=$file
   base_out="Bacteria_trimmed_rep"$count".fastq.gz"

   java -jar /opt/exp_soft/bioinf/trinity/trinity-plugins/Trimmomatic/trimmomatic.jar \
      PE -phred33 \
      -trimlog $OUT_DIR/trimmomatic.log \
      -basein $base_in \
      -baseout $OUT_DIR/$base_out \
      ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:1:true \
      MAXINFO:50:0.7 \
      MINLEN:50
      LEADING:20
```

Appendix XI

Web based annotation server URL

NCBI PGAAP: http://www.ncbi.nlm.nih.gov/genome/annotation_prok/

RAST: http://rast.nmpdr.org