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Characterization of EPNs and their bacterial symbionts

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Characterization of EPNs and their bacterial symbionts

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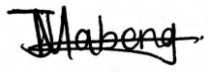
A thesis submitted to the faculty of science under the school of molecular and cell biology in fulfilment of the requirements for the degree of Master of Science.

June 2023

Declaration

I, Nokuthula Busisiwe Mabena, declare that this dissertation is my own work. It is submitted in fulfilment of the Master of Science degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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1645254

June 2023

Abstract

Many synthetic pesticides have been associated with health and environmental issues. Not only are they toxic to non-target organisms but can also pollute the soil, water and vegetation. Hence organic farming has been suggested as an alternative approach of biological pest management. Entomopathogenic nematodes (EPNs), which are obligate parasites to insects and carry pathogenic bacterial symbionts and are effective in managing and controlling populations of a variety of economically important insect pests. These insect pests pose a threat to food security as crop yield losses are primarily caused by them. Identifying natural enemies to insects is crucial as they have no harmful consequences to non-target organism and the environment and can be exploited as biological control agents. The first focus of this research was to isolate, identify and characterize a new nematode species. The nematodes were isolated from soil samples originally collected in Mahobong Leribe, Lesotho. To isolate the nematodes, the soil samples were baited with *Tenebrio molitor* larvae. The methods used for the identification and phylogenetic analysis of the isolated nematodes involved genomic DNA extraction, PCR amplification and Sanger sequencing of the 18S rDNA gene. The nematode that was isolated was a new, previously uncharacterized *Cruznema* species. The phylogenetic analysis and comparative morphometric characterization confirmed it was a new nematode. The results showed that the average body length of the female and male were 1006.45 µm and 868.31 µm, with a standard deviation of 210.95 µm and 162.75 µm. The species was also closely related to *Oscheius* spp., which have been reported to be entomopathogenic. Furthermore, the isolated nematode was able to cause mortality to *T. molitor* larvae within 3 to 6 days. Another focus of this research was to isolate and identify the bacterial symbionts of the isolated nematode. The methods that were used for the isolation involved the homogenization of sterile nematodes. The methods used for the identification and phylogenetic analysis involved genomic DNA extraction, PCR and Sanger sequencing of the 16S rDNA gene as well as culturing on selective and differential media. The isolated bacteria were identified as *Alcaligenes* spp., *Elizabethkingia* spp. and *Enterobacter* spp. They were also tested using lipid agar media to confirm if they were associated with the isolated nematode species. The results showed they were able to feed on and reproduce on the media.

The overall results of this study show that the newly identified *Cruznema* species has the potential of being used as an EPNs.

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Research Outputs

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Venue:

Nelson Mandela Bay, South Africa (Virtual meeting)

Oral presentation:

Characterization of a new insect-killing nematode isolated from a Grassland in Lesotho

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Chapter one: Literature review

1. Literature review

1.1. What are pesticides?

Pesticides are agents formulated to prevent, eliminate, repel, or mitigate any pest, where a pest is defined as any undesired form of life (NSW EPA, 2013). In agriculture, pesticides are used to safeguard crops from pests, weeds, and diseases, as well as to protect humans from vector-borne illnesses including malaria and dengue fever (Nicolopoulou-Stamati *et al.*, 2016). These pesticides function by disrupting the metabolism or behavior of the pest through physical, chemical, and biological means. Their mechanisms of action range from deterring to attracting to sterilizing, producing leaf drops without harming the plant or boosting the activity of another pesticide. They can be natural or synthetic. The different types of pesticides and how they control pests are outlined below (Table 1.1).

Table 1.1. Types of pesticides and how they control pests (NSW EPA, 2013)

Pesticide	**How they control pests**
Bactericide	Destroy, suppress or prevent the spread of bacteria
Fungicide	Control or destroy fungi
Herbicide	Kill, control, or stop the growth of a weeds or other undesired vegetation
Insecticide	Control many insect pests
Rodenticide	Control rodents such as mice and rats
Bait	Can be made up of one or more insecticide active ingredients combined with an alluring food matrix that varies depending on the target pest type and even the species of that pest type
Genetically modified organisms (GMOs)	Have undergone genetic modification to increase their resistance to pests and illnesses
Repellent	Repel a pest instead of killing it

1.2. Impacts of pesticides

Many pesticides have been associated with health and environmental issues (Sanborn *et al.*, 2007). They can pollute the soil, water, and vegetation in the environment and can have an impact on vertebrates like birds, fish, and beneficial invertebrates that take part in controlling insect pests through predation (Kelby, 2021). Therefore, they disrupt the biotic balance of the environment. They can be toxic to non-target organisms, including humans, and most of them are non-selective. Continuous application of pesticides has been associated with human ailments including cancer, liver diseases, and hypertension (Nicolopoulou-Stamati *et al.*, 2016).

Pesticide residues can be detected in a wide range of common food stuff and beverages, including prepared dishes, water, and animal feeds (Mcgill and Robinson, 1968; Cabras and Angioni, 2000; Lorenzin, 2007; Nag and Raikwar, 2011). It should be noted that these residues cannot be entirely eliminated by washing and peeling. The concentrations are often below the legally mandated safe levels (Lorenzin, 2007; Nougadère *et al.*, 2012). However, these "safe levels" may underestimate the synergistic effects on health risks as in the event of continuous exposure to some chemicals, which happens in real time (World health organization, 1990; Kortenkamp, 2007). Pesticide residues have also been detected in human breast milk tests, raising questions about prenatal exposure and the potential effects on children's health (Pirsaheb *et al.*, 2015; Nicolopoulou-Stamati *et al.*, 2016).

Pesticide exposure can occur through ingestion, inhalation, or skin contact. The type of pesticide, the duration of exposure, the route of exposure, and the individual's health status (such as nutritional deficiencies or the condition of one's skin) all have an impact on the potential health consequences (Nicolopoulou-Stamati *et al.*, 2016). Pesticides may be metabolized, eliminated, stored, or bioaccumulated in body fat within a human or animal body (Wesseling *et al.*, 1997). Chemical pesticides have been linked to numerous detrimental health effects, including effects on the skin, gastrointestinal system, nervous system, respiratory system, reproductive system, and endocrine system (Jaeger *et al*., 1999). Furthermore, high levels of pesticide exposure can result in hospitalization and even fatalities (NSW EPA, 2013; Nicolopoulou-Stamati *et al*., 2016).

1.3. Biological pest management

Organic farming has been suggested as an alternative approach of biological pest management in agriculture. This type of farming operates on the principle of non-synthetic use. It utilizes intercropping, organic manure and biological botanical control of pest infestation. Bacteria, plants, and other natural products are used (NSW EPA, 2013). This type of pest management has the advantage of being target specific, whereby it only affects the target pest and other related organisms. Due to its minimal dosage requirements and quick decomposition, it solves the pollution issues caused by conventional pesticides. It is also more environmentally friendly and sustainable than chemicals due to its slow rate of resistance development (Fukruksa *et al.*, 2017). It can also significantly reduce the need for conventional pesticides while still ensuring high crop yields in agricultural fields.

1.4. Impacts of pest infestations

Crop yield losses are primarily caused by insect pests (Price, 2021). They pose a threat to food security, so billions of dollars must be invested in research to find effective control measures (Khan *et al.*, 2014). According to the Centre for Agriculture and Biosciences International, 49% of crop losses in African countries are caused by insect pests. In a region where food security and poverty are major concerns, crop yield loss threatens the source of livelihood for many resource-poor farmers. As a result, there is a significant impact on the developing countries' economies and foreign exchange earnings (Daramola *et al.,* 2021).

The most important food crops in Africa are cereals like maize, rice, wheat, and sorghum, with maize being the most widely grown. More than 300 million people out of a total population of 1 billion in sub-Saharan Africa depend on maize as their primary food source (Khan *et al.*, 2014). Insects such as locusts (*Locustana pardalina*), fall army worms (*Spodoptera frugiperda*), Tomato leaf miners (*Tuta absoluta*) and Oriental fruit flies (*Bactrocera dorsalis*) are among some of the serious pests in South Africa (Price, 2021). They eat leaves, seeds and fruits of crops. Depending on the threshold of infestation, some pests cause crop yields to decline, leading to food shortages and possibly starvation. Lepidoptera Chilo larvae, for instance, have the ability to bore holes in sugarcane stems and harm the crop by sucking the juice. Other pests, like Pyrausta larvae, eat the stems and fruits of maize by boring holes in them. Weevils feed on plant sap, which has an

impact on the plants' growth and development and increases their susceptibility to disease (Kelby, 2021).

Natural enemies of insects come in many different forms. Given that the soil is a natural home and reservoir of many kinds of insect pathogens (including bacteria, nematodes, viruses, protozoans and fungi), these natural enemies can be taken advantage of to help manage insect pests. The identification of naturally occurring enemies is therefore important and needed to explore unexplored geographic areas with the goal of exploiting additional biological control species and evaluating their eligibility as successful biological control agents against pests. This current study, therefore, aims to isolate and identify new nematode species.

1.5. Nematodes

Nematodes are microscopic, colourless, unsegmented worms that are usually 50 µm in diameter and 1 mm in length (Vashisth *et al*., 2013). They have a tube within tube structure: the gut and gonad are enveloped by the body wall that is made up of dorsal and ventral longitudinal muscles, epidermis and cuticle. A pressurized, fluid-filled cavity serves as a hydrostatic skeleton between the inner and outer tubes (Kiontke and Fitch, 2013). This arrangement allows nematodes to only move in the dorsal ventral direction or snake-like movement (Lambert & Bekal, 2002). They can be found in soil with water-filled pore spaces and water films, low oxygen environments, acidic environments, deep sea trenches, polar regions, decaying organic debris, and plant roots, stems, flowers, and seeds (Vashisth *et al*., 2013; Shah & Mahamood, 2017). Their vast distribution is attributed to their ubiquitousness. They are most abundant in organic matter, plant roots, and other places where there are sufficient resources. They are involved in multiple functions within soil systems; they can increase nutrient mineralisation in agricultural systems, contribute to the preservation of adequate plant-available nutrient levels and act as biological control agents (Koppenhöfer *et al*., 2020). They can be free-living, parasitic or predatory (Saari *et al*., 2019); (Shah and Mahamood, 2017). There are trophic groups in which nematodes are categorised based on their feeding habits, these include: (1) plant feeders, which are nematodes that feed on plant tissue; (2) bacterial feeders, which are nematodes that feed on bacteria; (3) omnivore-predators, which are nematodes that feed on a variety of organism and; (4) predatory nematodes, which feed on soil microarthropods and nematodes (Shah and Mahamood, 2017).

Additionally, these nematodes are also categorized based on their associations. (1) Free-living nematodes are not associated with any insects but feed on bacteria and other microorganisms (Dillman *et al.*, 2012). (2) Phoresitic nematodes use insects for dispersal or shelter, with there being no direct nutritional relationship with the host. (3) Necromenic nematodes use dead insects for nutrition (Dillman *et al.*, 2012). (4) Parasitic nematodes may cause important diseases in human, plants and animals (Raja *et al.*, 2021). (5) Entomopathogenic nematodes invade and rapidly kill insects using bacteria (Dillman *et al.*, 2012).

1.6. Entomopathogenic nematodes

1.6.1. Entomopathogenic nematodes as biological pest control agents

Entomopathogenic nematodes (EPNs) are effective in managing and controlling populations of a variety of economically important insect pests through the integrated pest management (IPM) system (Ma *et al.*, 2013; Erdoğan *et al*., 2021). They are safe to all vertebrates, most non-target invertebrates and the food supply, hence they are largely used as biocontrol agents. EPNs and their bacterial symbiont kill their insect host within 24 to 48 hours, whereas other insect-killing biocontrol agents can take up days or weeks to kill their insect host (Vashisth *et al*., 2013). The use of EPNs as a biocontrol agent mostly depends on the environmental state under which the agents are applied. Abiotic factors such as UV radiation, changing soil temperatures, and moisture content could enable EPNs from working successfully as bio-insecticides (Ma *et al.*, 2013). The key to success in biocontrol is matching the correct species of the nematode with the correct pest species, applying them during suitable environmental conditions, and using them with compatible organic biological control (Nikdel and Niknam, 2015). EPNs are lethal obligate parasites to insects and form symbiotic relationships with specific species of pathogenic bacteria (Lephoto and Gray, 2015). The bacteria are dependent on the EPNs for protection against the external environment (Vashisth *et al.,* 2013). They work together to infect and kill various types of commercial crop insects that are soil-dwelling including larval forms of lepidopteran (moths and butterflies), dipteran (flies), and coleopteran (beetles) as well as grasshoppers and crickets in their adult stages (Nikdel and Niknam, 2015). EPNs are living organisms and therefore need to be handled and stored with extreme caution. Besides being safe to humans and other non-target animals, EPNs have received huge attention as biocontrol agents because of the traits they

possess such as (1) seeking for insect pests in cryptic habitats, (2) high reproduction rate, (3) easy mass-production in the laboratory, and (4) they are relatively less expensive than chemical insecticides (Al-zaidawi and Karimi, 2019; Koppenhöfer *et al*., 2020).

1.6.2. Entomopathogenic nematode life cycle

Infective juveniles (IJ) are the free-living and non-feeding forms of EPNs that function as vectors for the insect pathogenic symbiotic bacteria. They are the specialised third stage of the nematode's development adapted to live outside their insect host (Erdoğan *et al.,* 2021; Vashisth *et al*., 2013). They seek for insect hosts, when found, they penetrate the body and eventually release the bacteria into the insect host hemocoel to induce lethal septicaemia (San-blas *et al.*, 2012). The bacteria multiply and release various virulence factors which overwhelm the host's immune system causing the host to ultimately die (Ma *et al.*, 2013). The host tissue is transformed into food resources and the bacteria then produces antibiotics (xenocoumarins, xenorhabdins, and bacteriocins) and metabolites to prevent and inhibit the development of other microorganisms that could be pathogenic to the plant or that would use the resources (Razia *et al.*, 2011). Upon infection, the IJs develop, and mature nematodes reproduce within the insect host for 2-3 generations or until all the resources run out (Erdoğan *et al*., 2021). The IJs then escape and disperse into the soil to seek out other insect hosts.

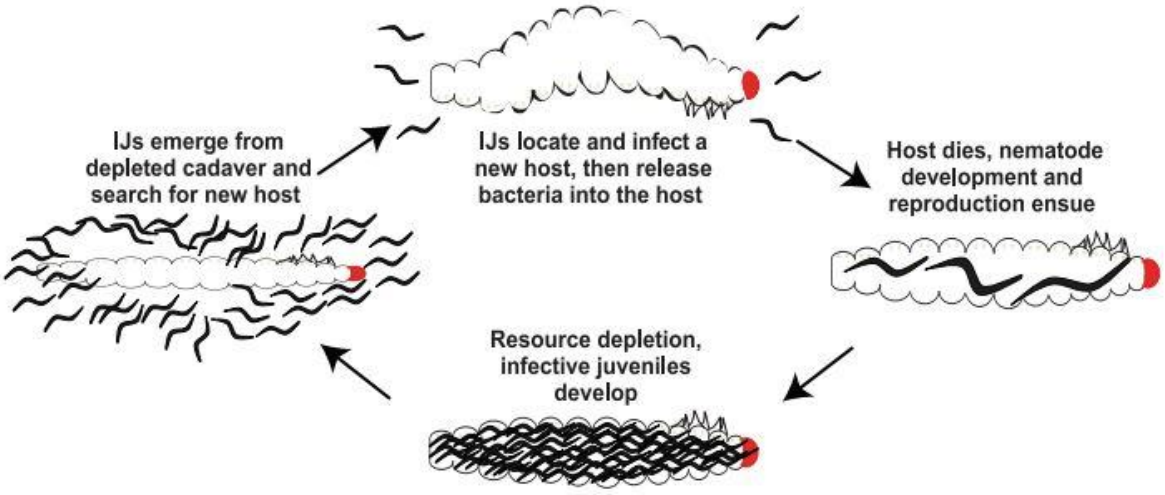

Figure 1.1. EPNs life cycle (Dillman *et al.*, 2012).

1.6.3. EPNs species

Nematodes belonging to the *Steinernema* and *Heterorhabditis* genera are known to exhibit entomopathogenic properties and form mutualistic relationships with bacteria from the *Xenorhabdus* and *Photorhabdus* genera, respectively (Abebe *et al*., 2010; Boemare *et al.,* 1993). As such, they have been used as biocontrol agents to supress pest populations (Vashisth *et al*., 2013). Some nematodes in the *Oscheius* genus have also been identified as EPNs due to their role as vectors for insect pathogenic bacteria in the *Serratia* genus (El-Barbary and Hal, 2017). It is also worth noting that not all *Serratia* species are pathogenic. Species from the *Oscheius* genus are classified as scavengers or bacteriophagous free-living nematodes, while others have been demonstrated to kill insects (Loulou *et al.*, 2022). These include *O. amsactae* (Ali *et al.*, 2011), *O.carolinensis* (Ye, Torres-Barragan and Cardoza, 2010), *O. chongminensis* (Zhang *et al.*, 2008), *O. microvilli* (Zhou *et al.*, 2017), *O. rugaoensis* (Zhang *et al.*, 2012), *O. onirici* (Torrini *et al.*, 2015), *O. safricana* (Serepa-Dlamini & Gray, 2018) and *O. basothovii* (Lephoto & Gray, 2019). The ability to be an EPNs extends beyond the species mentioned above, more research regarding biocontrol capabilities of various species within this genus is on-going. However, there is variability within species in terms of their ability to kill insects- some species can kill specific insect species and others cannot (Loulou *et al.*, 2022).

1.6.4. EPNs associations

Nematode-bacterial associations include: (1) trophism, in which the nematodes consume bacteria. (2) Parasitism, in which bacteria causes disease in the nematodes if not restricted; and (3) mutualism, in which the nematodes and bacteria work collaboratively. The relationship between EPNs and their symbiotic bacteria is mutual (Dillman *et al.*, 2012). This is a species-specific mutual relationship where an individual nematode is associated with only one species of symbiotic bacteria. However, a specific bacterial species might be associated with multiple nematode species from the same genus, this is a horizontal bacterial transfer between nematode species (Goodrich-Blair and Clarke, 2007). It is assumed that each *Steinernema* species forms a symbiotic relationship with only one *Xenorhabdus* species; in turn, many *Xenorhabdus* species can be associated with various nematode species (Boemare and Akhurst, 1988). The symbiotic *Heterorhabditis-Photorhabdus* relationships, however, are more adaptable because

numerous species of bacteria and nematodes can form symbiotic associations with various symbiotic partners (Koppenhöfer, 2007; Koppenhöfer and Gaugler, 2009). Although there is a lot of evidence for this specificity pattern, its underlying mechanisms have not yet been fully understood (Herbert and Goodrich-Blair, 2007; Hillman and Goodrich-Blair, 2016).

There have been other mutualistic interactions between nematodes and bacteria reported. A few examples are: *Serratia sp*., an entomopathogenic bacteria that has been discovered to associate with *Caenorhabditis* nematodes in order to succeed as an insect pathogen, and *Moraxella osloensis*, a member of the *Pseudomonadaceae* family that can form a symbiotic relationship with the slug-parasitizing nematode *Phasmarhabditis hermaphrodita* (Tan and Grewal, 2001). These associations don't appear to be obligate, indicating that nematode symbiosis may develop over time as a result of evolution (Dillman *et al.*, 2012).

Abebe *et al*., 2010, hypothesized that *Caenorhabditis briggsae* was an EPNs as a pathogenic strain of *Serratia* (*S. marcescens*) was isolated from it and was able to cause insect mortality. However, according to Dillman *et al*., 2012, *C. briggsae* cannot be an EPNs as the IJs recovered from the cadavers *were* able to reinfect a new insect host (*Galleria mellonella*) but with less virulent as the bacteria-nematode complex than injection of the bacteria alone. This suggests the *C. briggsae* ranges between a necromenic and entomopathogenic.

1.6.5. What makes an EPNs?

Nematodes are considered entomopathogenic when they have a nematode-bacteria association that is a true obligate mutualism. Hence, they should carry a pathogenic bacterium and be able to actively seek and penetrate the insect host. They should release the bacteria in the insect's haemolymph and cause rapid death (<120 hours) of the insect. There should be nematode and bacterial reproduction, as well as reassociation of the pathogenic bacteria in new IJ generations and emergence of the IJs from the cadaver (Dillman *et al.*, 2012).

1.7. Entomopathogenic bacteria

There are many different types of entomopathogenic bacteria in nature, but the most common ones are from the genera *Bacillus*, *Peanicillus*, *Brevibacillus*, *Serratia*, *Pseudomonas*, *Xenorhabdus*, and *Photorhabdus*. As obligate or facultative insect pathogens, these entomopathogenic bacteria exhibit a variety of host ranges and infection mechanisms (Sajnaga and Kazimierczak, 2020). However, they all share the same capacity to produce a vast array of virulence factors to disrupt the host microbiota and insect immune responses (Sajnaga *et al.*, 2018).

Bacillus thuringiensis (Bt) and *Bacillus sphaericus* are two other important bacteria that are used as biological control agents in addition to *Xenorhabdus* and *Photorhabdus* (Sajnaga and Kazimierczak, 2020). They have been used to manage insect pests from the Coleoptera, Lepidoptera, and Diptera orders (Ruiu *et al*., 2013). However, these bacteria only work once an insect that is susceptible has consumed their toxins (Pigott and Ellar, 2007). This kind of "passive infection" reportedly reduces the extent to which these bacteria are effective at controlling the pest. The symbiotic bacteria of EPNs can, in turn, cause "active infection" when they are introduced into an insect host by their EPNs symbiont and have a broad host range. Their ability to produce toxic compounds, reproduce within the hemocoel, and cause septicemia is what makes them pathogenic (Pervez *et al*., 2020).

1.8. Potential uses of entomopathogenic bacteria

There are ongoing studies on the potential application of EPNs symbiotic bacteria and other entomopathogenic bacteria to control medically important insects including mosquitoes (*Aedes aegypti* and *Aedes albopictus*), which transmit diseases such as dengue, chikungunya, and Zika (Sajnaga and Kazimierczak, 2020). These insects are currently controlled with chemical products that have low specificity to non-target organisms. There has been resistance reported against commonly used insecticides including organophosphate and pyrethroids (Ruiu *et al*., 2013). Studies have shown that *Bacillus thuringiensis israelensis* or *B. sphericus* and their toxins, as well as bioactive compounds of *Xenorhabdus* and *Photorhabdus* have larvicidal action against these mosquitoes (Kovendan *et al.*, 2011, 2012).

Xenorhabdus and *Photorhabdus* bacteria produce variety of bioactive substances that have insecticidal, cytotoxic, and antimicrobial properties (Bode, 2009). As a result, they have been successfully applied in lab settings (Ruiu *et al*., 2013). It was reported that the use of *Photorhabdus luminescens* and Bt *kurstaki* together prevents the *Spodoptera littoralis*, an African cotton leaf worm, from growing (Benfarhat-Touzri *et al.*, 2014). Additionally, Ahantarig *et al*., 2009 reported that as few as 20 *Xenorhabdus nematophilus* cells were able to kill up to 90% of *Manduca sexta* larvae. Even in the absence of EPNs, an oral dose of the cell suspension of *X. nematophila* and *P. luminescens* is fatal to Aedes larvae (da Silva *et al.*, 2013). Therefore, even without the nematodes, some bacteria from the genera *Xenorhabdus* and *Photorhabdus* are entomopathogenic (da Silva *et al.*, 2020). They produce antimicrobial substances that are effective not only against other microorganisms and insects but also against nematodes, protozoa, and cancer cells (Dreyer *et al*., 2018).

There have been few papers published on the regulation and other potential uses of bioactive compounds of these EPNs symbiotic bacteria (da Silva *et al.*, 2020). Most of the studies available investigate the use of these compounds in the agriculture industry. Further studies are required to understand how these bacteria can be used in different industries, including food and healthcare because they are an excellent source of antimicrobial metabolites (Dreyer *et al*., 2018). Identification and characterization of these bioactive compounds are also important for understanding the mechanisms of action so it is known how they can be applied in the different industries.

1.9. Insect-parasitic nematodes

There are approximately 30 different nematode families associated with invertebrates, and their relationships can be anything from casual to parasitic and pathogenic (Camino, María and Achinelly, 2011). In a relationship between an insect and a parasitic nematode, the nematodes have evolved to consume the living insect directly, resulting in some harm and eventual host death (Dillman *et al.*, 2012). In turn, an insect-EPNs relationship occurs when the nematode uses its pathogenic bacteria to cause disease and rapid death of the insect before feeding and growing on the cadavers and bacterial resources (figure 1.2). Therefore, the use of pathogenic bacteria

and the direction of selection (against virulence or avirulence) distinguish these relationships, which either make the nematodes more or less immediately harmful to their host (Dillman *et al.*, 2012). In Argentina, a study conducted by Camino *et al*., 2011 characterizing the populations of nematode parasites infecting soil pest insects discovered 24 nematode species belonging to 6 families in 7 locations. For all the nematodes, the levels of parasitism ranged from 16% to 80%. Hence parasites and pathogens can be used as pest management.

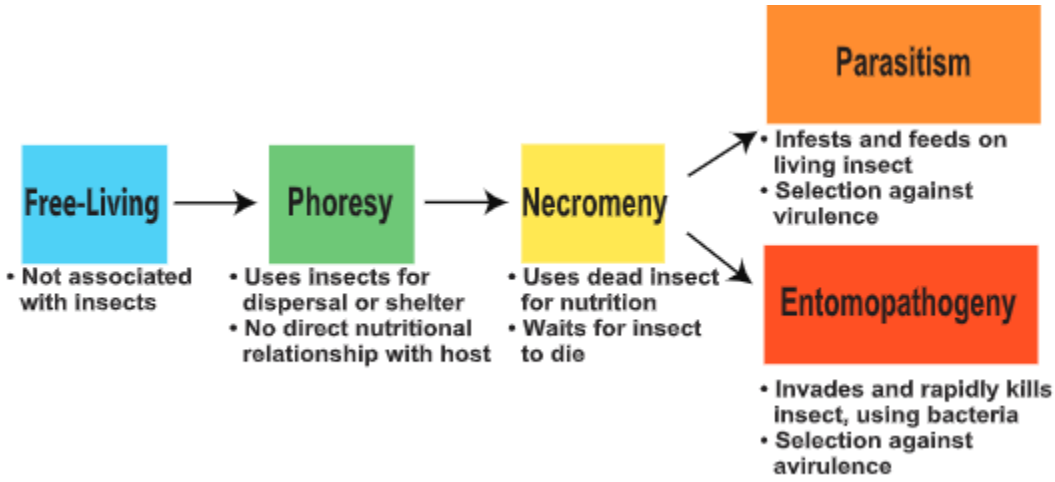

Figure 1.2. The evolution of nematode-insect association (Dillman *et al*., 2012).

1.10. Research motivation

In South Africa, the agricultural sector makes up around 3% of the country's gross domestic product (GDP) (O'Neill, 2022). Annually, the agricultural sector suffers significant declines in the overall yield and quality of agricultural produce and insect pests have been identified as major contributors to the crop damage, resulting in substantial financial losses globally (Price, 2021). Food insecurity also remains high, and farmers have resorted to the extensive use of chemical pesticides and fertilizers. This has led to the need for safer and effective approaches to pest management.

It is necessary to be aware of the occurrence of new EPNS and to identify native species before introducing them as biological control agents at a specific site. Exotic EPNs may cause local populations and species to become extinct, thus reducing the natural diversity. Additionally, they may not be effective against local insect pests because they are not adapted to the conditions of

the area (Emelianoff *et al.*, 2008). Hence, more research is needed to explore unexplored areas with the goal of identifying new EPNs species and evaluating their eligibility as successful biological control against pests. New EPNs will provide alternative ways of controlling and reducing more insect pests with no synthetic chemicals used, hence there will be no harmful consequences to non-target organisms, and the ecosystem, as well as the biodiversity, will not be destroyed. It will also give a better indication of the distribution patterns of EPNs in South Africa and assist in choosing beneficial species for commercial bioprospecting.

1.11. Aims and Objectives

1.11.1. Aims

To isolate, identify and characterize a new nematode species collected from a grassland area and evaluate its eligibility as a biological control agent for insect pests.

1.11.2. Objectives

This study focused on the following objectives:

(1) To isolate nematodes from soil samples collected from a grassland using soil-larval baiting and white trap methods.

(2) To identify successfully isolated nematodes by extracting the DNA, performing PCR, sanger sequencing and phylogenetic analysis based on the 18S rDNA sequence.

(3) To isolate and identify bacteria from the isolated nematodes by extracting the DNA, performing PCR, sequencing and phylogenetic analysis based on the 16S rRNA gene.

(4) To apply Koch's postulate of infectivity to confirm whether the nematodes recovered from the white traps were able to kill insects.

(5) To characterize and morphometrically describe the nematodes using light microscopy.

(6) To test the pathogenicity of the nematodes against *Tenebrio molitor* larvae.

(7) To test the pathogenicity of the bacteria isolated from the nematodes.

1.12. References

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Chapter two: Isolation and Identification of a new nematode

2.1. Introduction

Nematodes are among the most diverse and least researched group of organisms with less than 3 % of approximately one million species being described to date. Among the 3 % (26 000), about 4100 are plant parasitic, which cause drastic economic losses to all crops; and at least 125 are entomopathogenic and can be utilized in crop cultivation as advantageous organisms for biological plant protection against insect infestations (Bogale *et al*., 2020; Raja *et al.*, 2021). Furthermore, 22% of the human population are parasitized by an intestinal nematode parasite, *Ascaris lumbricoides*. Therefore, these organisms play a very significant role in fields of agriculture, medicine and veterinary sciences. Additionally, nematodes are utilized as models in a variety of studies, such as disease detection, ageing, and other significant physiological and developmental investigations (de Oliveira *et al*., 2022).

Nematodes from the genera *Steinernema*, *Heterorhabditis,* and some from *Oscheius* have been described to be entomopathogenic and have mutualistic associations with bacteria from the genus *Xenorhabdus*, *Photorhabdus,* and *Serratia*, respectively (Abebe *et al*., 2010; Boemare *et al*., 1993). There are at least 134 entomopathogenic nematodes (EPNs) reported so far with 100 species described in *Steinernema*, 26 in *Heterorhabditis,* and 8 in *Oscheius* (Yooyangket *et al*., 2018; Raja *et al*., 2021; Ye *et al*., 2018). Not all species in the *Oscheius* genus are entomopathogenic but there are a total of 43 species described so far (Lephoto *et al*., 2015; Ye *et al*., 2018).

In order to comprehend nematode biodiversity, biology, and ecology, as well as to design efficient control and create crucial management techniques, precise detection is essential (Bogale *et al*., 2020; de Oliveira *et al.,* 2022). Different types of techniques have been used for the identification, characterization and classification of nematodes for the past years. The morphological-based method was the only method used for years, it includes identifying the nematodes based on their morphological and anatomical differences using microscopy. The features that are usually observed are the external body structure, total body length, differences in sex organs and stoma, shape of the lips, mouth and tail, and other visible characteristics (figure 2.1) (Bhat *et al.*, 2022). However, using the morphological-based method is challenging as

nematodes have morphological characteristics that are relatively conservative. This results in the lack of clear variation among closely related taxa and leads to the absence of precise categorisation. It also requires a highly skilled taxonomist, whose number is on the decline (de Oliveira *et al.,* 2011). Although morphological-based methods help relate morphology with possible function, it remains inadequate alone, hence other forms of characterisation and identification such as molecular and protein-based methods are required (Lulamba & Serepa-Dlamini, 2020; Bogale, 2020). This increases the resolution and reliability of nematode phylogenetic and diagnostic research (de Oliveira *et al*., 2011). Molecular-based methods include the use of PCR to classify the nematodes and is based on nuclear genes sequence similarity. In addition, the biochemical-based methods are based on the molecular weight of abundant proteins, for instance ribosomal and other proteins used to identify species (Bhat *et al.*, 2022).

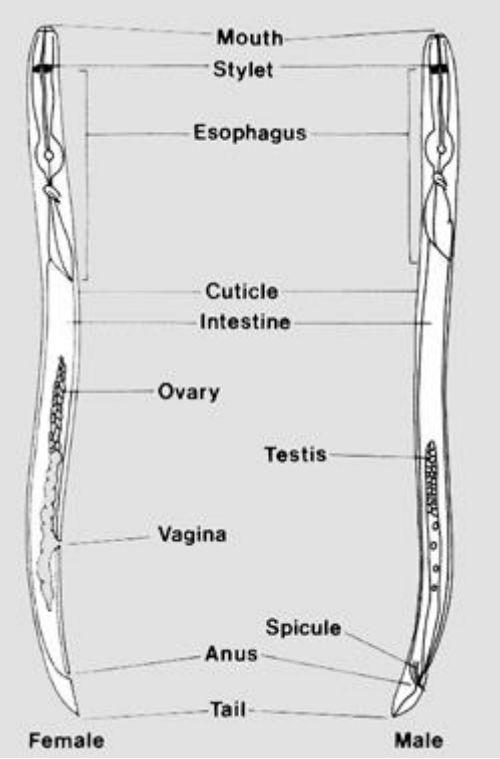


Figure 2.1. The basic body plan of a nematode (Lambert & Bekal, 2002).

Nematode studies make use of sequence data from whole genomes, nuclear and mitochondrial genetic loci to understand phylogenetic relationships. Nuclear genes include an external non-

transcribed spacer (NTS), 18S (small subunit), 28S (large subunit), and the internal transcribed spacer (ITS) which is the non-functional DNA within the 18S and 28S ribosomal DNA (rDNA) genes. They are largely used because they have viable and conserved regions that are can be used to identify different species and differentiate taxonomic levels (Susurluk *et al.*, 2007). Ribosomal RNA (rRNA) and partial messenger RNA copies are also used to predict phylogenetic relationships (Lulamba and Serepa-dlamini, 2020).

The use of all these approaches has been proven sufficient and has an important role in improving nematode taxonomy, which has further contributed to a greater understanding of the nematode biodiversity, geographic distributions, ecology, behaviours, and co-evolution (Lulamba and Serepa-dlamini, 2020). Identifying new nematode species and EPNs populations is an essential step towards achieving effective biological control against pests because of their better adaptation to local environments (Nikdel and Niknam, 2015). Characterising EPNs accurately is critical and necessary to match appropriate EPNs species with compatible target insect pests for effective biocontrol potential and to prevent species from out-competing native species and jeopardizing the local biodiversity from being imported (Peat *et al.*, 2010).

2.2. Methods and Materials

2.2.1. Rearing of insects

Tenebrio molitor commonly known as yellow wheat mealworms were used to procure the nematodes. The mealworms were grown in the lab, in a 25°C incubator, in 2 litre plastic containers and were fed oat bran and apples, which served as a sugar and water source. The apples were added once a week and left in the containers for a day, to prevent the growth of mould.

2.2.2. Isolation of nematodes

2.2.2.1. Collection of soil samples

The soil samples were collected from different places; however, we were able to get potential insect killing nematodes from the samples collected from Mahobong Leribe, Lesotho

(28°59'17.411" S, 28°8'25.027" E). The area they were collected in used to grow vegetables which are affected by different insect pests. The soil was collected from a depth of 20 cm using a hand shovel sterilised with 70% alcohol and placed in clean 2 litre ice cream plastic containers stored at 22- 25°C, which is the optimum temperature for nematodes. Water was added to the samples to keep them moistened with a moisture content of no more than 8% and kept at room temperature (Lephoto & Gray, 2019).

2.2.2.2. Insect baiting

Insect larvae (*T. molitor*) were used to capture the nematodes from the collected soil. This was done by placing 10 insects on top of each soil sample, which was then turned over. The soil samples were then stored at 25 ˚C in the dark until there were infections. The insect larvae were observed daily for mortality and symptoms of infections were recorded. The dead larvae were then gathered and left to dry at room temperature before placing them on white traps.

2.2.2.3. Isolation of nematodes from infected larvae by White Trap Method

The white trap method was used to isolate the nematodes from the infected larvae. Two petri dishes of different sizes were used, the smaller petri dish (60 mm) was inverted and placed inside the bigger petri dish (90 mm). A filter paper (55 mm) was then placed on top of the small petri dish and water was added to the bigger petri dish and filled to about 5 mm. The dead larvae removed from the soil were placed on the filter paper and kept at room temperature to isolate emerging nematodes. Precautions were taken to not allow the filter paper to get too dry or too wet while the dead larvae are still on the trap because the nematodes could die before they emerge. The emerged nematodes were rinsed in sterile autoclaved water and used for morphological and molecular characterisation. The nematodes were kept in 95% alcohol for molecular characterisation to dehydrate the samples and protect the DNA by denaturing proteins that could degrade it. While the ones used for morphological characterization were stored at 4°C and had to be used within 3 days.

2.2.2.4. Infectivity assay

Koch's postulate of pathogenicity was used to confirm that the insect mortality was caused by the nematodes. This was done using the nematodes that emerged from the cadavers in the white traps. The nematodes were used to infect living *T. molitor* in petri dishes with 40 g of sterile sandy loam soil. 5 insect larvae and approximately 100 IJs in 1 ml sterile distilled water were contained in each petri dish. A plate with 5 insect larvae and no IJs was used as a control to make sure there were no contaminations. The larvae and the IJs were incubated at room temperature and observed daily.

2.2.2.5. In vivo maintenance of nematodes

The nematodes were maintained and cultured *in vivo* through their recovery from larval cadavers in the white traps and reinfection of fresh larvae.

2.2.3. Molecular characterisation of nematodes

2.2.3.1. Genomic DNA extraction

Genomic DNA was extracted from the nematodes using the E.Z.N.A. Insect DNA Kit (Omega Bio-tek) as per the protocol (Protocol in appendix).

2.2.3.2. PCR amplification and sequencing of 18S rDNA

The extracted DNA was sent to Inqaba Biotechnological Industries Pty (Ltd) for amplifying and sequencing of the 18S rDNA sequence using forward primer TW81 (5'-GCGGATCCGTTTCCGTAGGTGAACCTGC-3') and reverse primer AB28 (5'-GCGGATCCATATGCTTAAGTTCAGCGGGT-3').

2.2.3.3. Phylogenetic analysis using MEGA 11

The sequences from Inqaba Biotechnological Industries Pty (Ltd) were edited and viewed for errors using Geneious Prime 2022.2.1. The edited 18S rDNA FASTA sequence was uploaded onto the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLASTn) to compare the unknown isolate sequence with the known sequences on the NCBI database.

The phylogenetic tree was constructed using the 18S rDNA sequences from BLASTn that were somewhat similar to the unknown nematode isolate sequence to identify and establish phylogenetic relatedness of the isolates at species level. Molecular Evolutionary Genetics Analysis 11 (MEGA 11) was the program used for the phylogenetic analysis and alignment of both known and unknown sequences. FASTA files of all the selected nematode species' 18S rDNA sequences were uploaded onto MEGA11 and aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool. The aligned sequences were then used to construct the phylogenetic tree. The maximum likelihood method was used to determine the phylogenetic relationships between the aligned sequences. The tree was built using taxon clustering based on 1000 replications in the bootstrap statistical test.

2.2.4. Morphological characterization of nematodes

2.2.4.1. Light microscopy

The nematodes were taken from the white traps and rinsed in sterile distilled water; this was repeated 3 times. A drop (100 µl) with water and nematodes was then placed on a slide, which was then covered with a cover slip and sealed with nail polish to prevent the nematodes from drying out and the cover slip from sliding off.

A light microscope (Olympus) with a camera and different magnifications (x10, x40 and x100) was used for morphological analysis. Observations were made following the taxonomic criteria (table 2.1).

2.2.4.2. Measurements

A total of 32 female, 32 male and 6 IJs were morphologically characterized. The Olympus light microscope using Zeiss software was used to measure the nematodes. The morphometric table was generated based on the mean and standard deviation statistical calculations according to Hominick *et al*. (1997).

Table 2.1. The following abbreviations were used:

BL	Body length
BW	Body width
PL	Pharynx length
TL	Tail length
AB	Anal body width
SL	Spicule length
AV	Anterior end to vulva
V	Vulva width
M	Mouth
C	Cuticle thickness

2.3. Results and discussion

2.3.1. Isolation of nematodes

The larvae infected by the nematodes, which had been isolated from the soil from Lesotho, displayed symptoms of dark brown patches and black-brown colour change, as well as softening of the cadaver (figure 2). The white trap method facilitated the trapping and isolation of the emerging IJs. The testing of Koch's postulate confirmed that the isolated nematodes were responsible for the death of the cadaver. It took 3 to 6 days for the nematodes to emerge from the cadaver. There was no mortality for the control larvae with no nematodes.

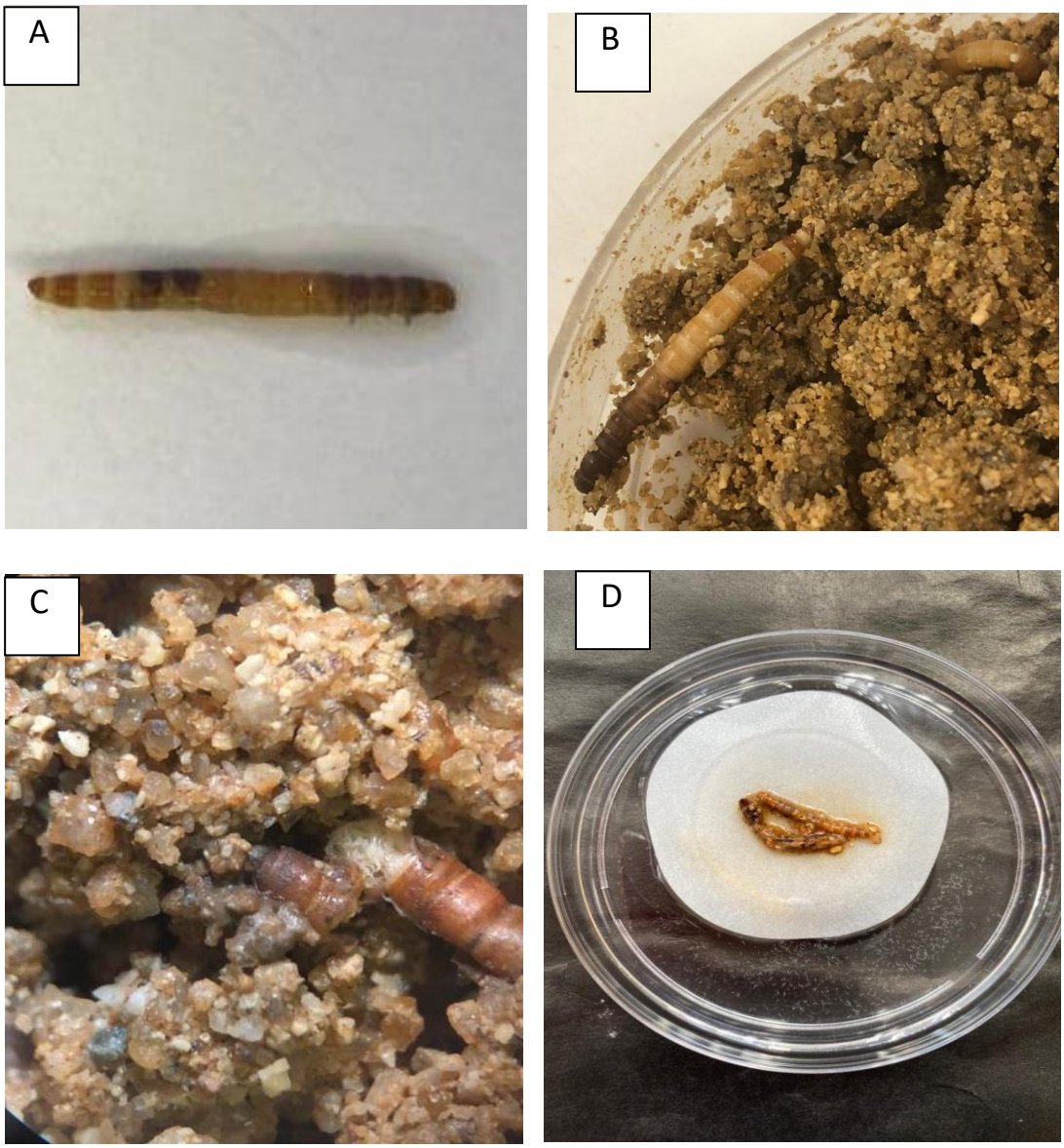


Figure 2.2. Infected *T. molitor* larvae, larval symptoms indicating form of infection A) Larvae with black-brown patches along body. B) Larvae from infectivity assay presenting black-brown patches from top half of the body. C) Disembodied cadaver displaying nematodes reproducing inside. E) Emergence of nematodes from the white traps.

2.3.2. Molecular Characterisation of nematodes

The sequences for the PCR amplicons of the 18S rDNA regions were received from Inqaba Biotechnological Industries Pty (Ltd) in the form of chromatograms. The chromatograms were viewed for errors, edited using Geneious prime version 2020.0.5, and saved as FASTA formatted

files. The 18S rDNA sequence of the unknown nematode isolate is in figure 2.3. The NCBI BLASTn results revealed a high affinity to *Cruznema* species: *Cruznema sp. CS50* (MW228469) and *Cruznema sp. JN2018* (MK156051), suggesting that the unknown species was also a *Cruznema* species. The sequence to be deposited in GenBank and assigned the name *Cruznema sp. NTM-2021* with accession number OQ408141.

GCCGGTGGATCATTGCCGAAACGCAAATATCCGAAAACCTACCTAACTATCTCTTCACGAGGTGTAGTCG
TGTCGATGAGCTGTCTCCGGGAGAGGATGGCGATGAATCGATGCACGTTGCTTTTGCGACGATACTTAA
TCGCTCTGCCTGAGAGTGGCCTCGGATTGGTTTGGTGGCTTGGAAGCCTTGTGCTTCTAGTCTTAATGAG
ATAGCTTGCTATCCGCCATCCAATCACTTCTTTCATAACTGAGTTGGTAATCGAAACGTGACAGGCTCAC
GCTAAAAAGCCTACTAACTAGCATTAGCGATGGATCGGTTGATTTGGTCATCGATGAAAAACGCAGCCA
GCTGCGTTATTTACCACGAATCGCAAATTTGTAGAGTGGTAAAATTTTGAATGCATAGCGCCGAAGGGT
ATCATCCCTTCGGCACGCCTGGTTCAGGGTTGTTTAATCGAATTCCGTCGACAGACAGTTGATGGATAGT
CGAGGGGCTGCGCTTCGGCGTACCCTCTCTGGTTTCGAGAACTATGTTCCTAGTGAACTGTGAATTCGCG
TTTGCGTAGCCTCCATGGCTCTGTGCTTGAATTAGTGCAGTGTCGATGGCGCTTATAAGGCAGCAACGT
GCATTTTGCAATCGCAACCTGAACTCAGACGTGACTACCCG

Figure 2.3. The 18S rDNA sequence of *Cruznema sp. NTM-2021*.

The isolated nematode underwent phylogenetic analysis using MEGA 11, and the Maximum Likelihood Method was employed to identify or determine the phylogenetic relatedness of the isolates at the species level. The phylogenetic tree (figure 2.4) shows that the unknown nematode isolate (*Cruznema sp. NTM-2021*) is clustered on the same clade with *Cruznema sp. CS50* (MW228469) and *Cruznema sp. JN2018* (MK156051), which supports that they are closely related with a bootstrap of 100%. However, *Cruznema sp. CS50* (MW228469) and *Cruznema sp. JN2018* (MK156051) have both not been described yet. The isolate is also sister species with *Oscheius myriophilus* KP792651, *Oscheius sp. TEL-2014* KM492926 (*O. basothovii*) and *Oscheius sp. MCB* KF684370 (*O. safricana*). They have bootstrap of 42% suggesting re-evaluation to confirm if these species are in the same clade. Furthermore, *O. basothovii* and *O. safricana* has been reported to

be infectious to insect and have the potential of being used as EPNs (Serepa-Dlamini and Gray, 2018; Lephoto and Gray, 2019a).

The following taxa were used for the construction of the phylogenetic tree:

Cruznema sp. CS50 (MW228469), *Cruznema sp. JN2018* (MK156051), *Trachypharynx sp. 4-YT-2019* (LC512865), *Ancylostoma braziliense* (MG890386), *Oswaldocruzia filiformis* (KR029784), *Nematodirus oiratianus* (HQ389233), *Poteriostomum imparidentatum* (KY495604), *Cylicodontophorus bicoronatus* (MH636326), *Cvlicostephanus minutus Csm4* (MT382660), *Cyathostomum catinatum* (KY495603), *Cyathostomum tetracanthum* (MF489231), *Cylicocyclus nassatus* (MT581488), *Cylicocyclus nassatus* (KX819274), *Heterorhabditis bacteriophora 56-C* (FJ217351), *Heterorhabditis argentinensis* (AF029706), *Heterorhabditis safricana* (EF488006), *Heterorhabditis zealandica* (AY321481), *Steinernema kraussei* (AY230174), *Steinernema jeffreyense J194* (KC897093), *Steinernema khoisanae BMMCB* (KT027382), *Steinernema khoisanae LAOS* (KM275351), *Oscheius sp. MCB* (KF684370), *Oscheius sp. TEL-2014* (KM492926*), Oscheius myriophilus* (KP792651), *Caenorhabditis elegans* (NR 000054*).*

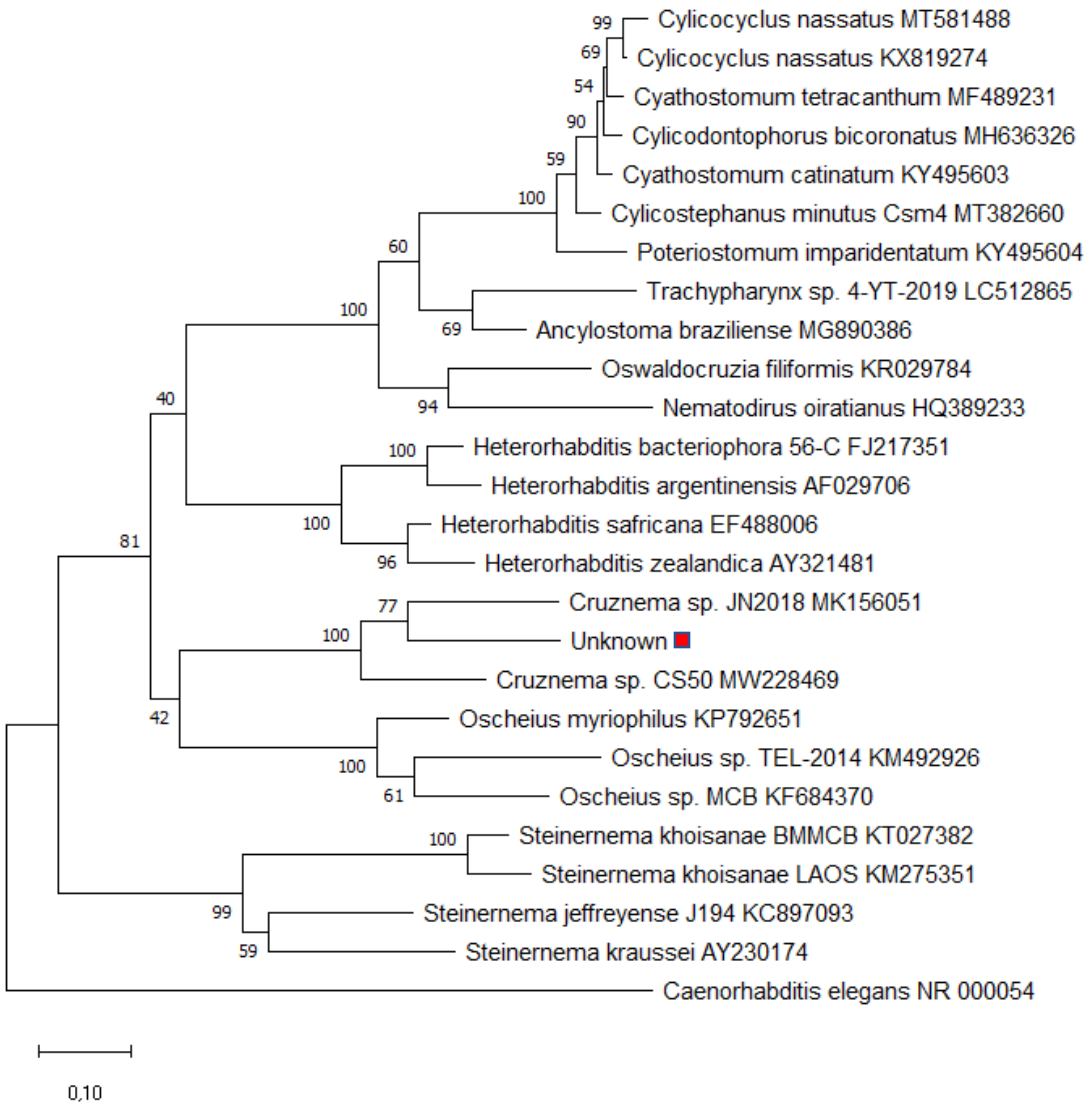


Figure 2.4. Phylogenetic tree showing the phylogeny of the unknown nematode isolate (*Cruznema sp. NTM-2021*) in relation to other *Cruznema* species that are somewhat similar to it, as well as EPNs species. *C. elegans* was used as the outgroup.

2.3.3. Morphological Characterisation

2.3.3.1. Description

Male

The *Cruznema sp. NTM-2021* male body was J shaped and posterior ventrally curved, had a mean body length of 880,183 µm. The head was truncated to slightly round and continuous with the

body, it had a conoid tail, and the spicule was paired, symmetrical, curved with ochre brown coloration.

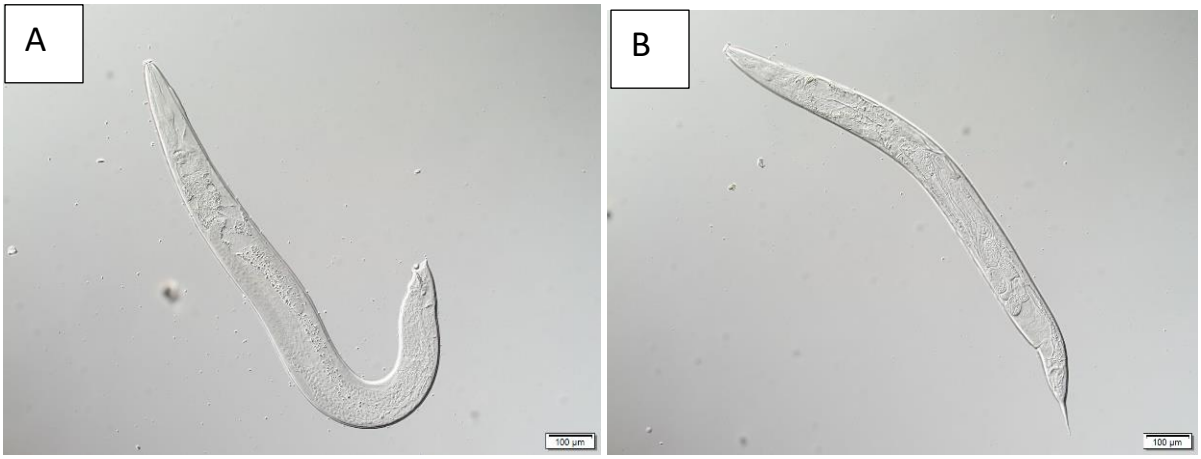
Female

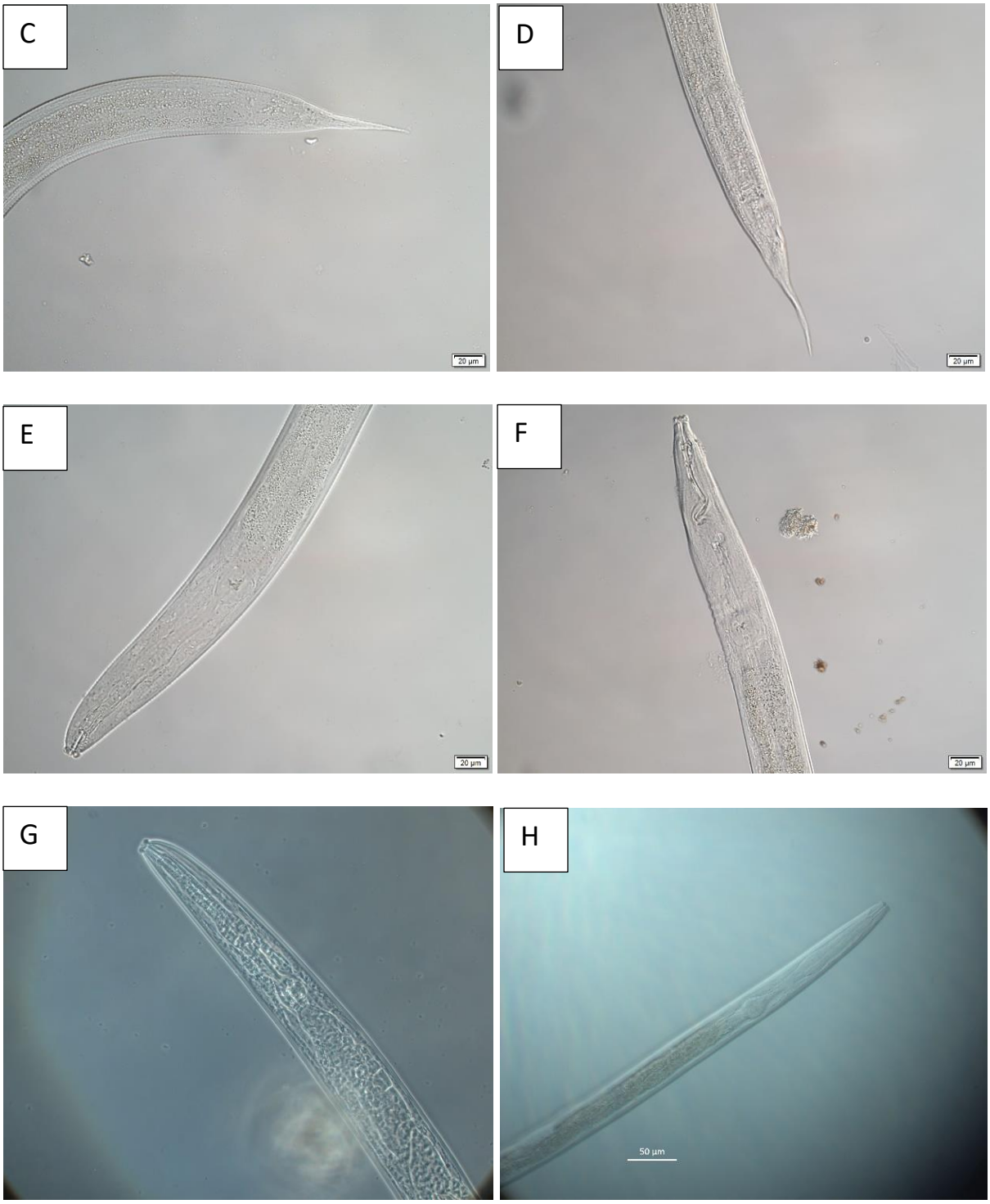
The female was J-shaped, almost straight, body length had a mean of 1144,274 µm which is larger than the male. The head was slightly round and continuous with body. It was gradually tapering posteriorly and had a tail tapering gently to a sharp point. The vulva was located posteriorly with lips slightly protruding and mostly symmetrical.

Infective Juvenile

The infective juveniles had a straight, slender body with a body length of 491,145 µm. It was gradually tapering posteriorly, had a head region continuous with the body and a tail region conoid with a pointed terminus.

2.3.3.2. Morphological analysis by light microscopy




C
D
E
F
G
H
20 µm
20 µm
20 µm
20 µm
50 µm

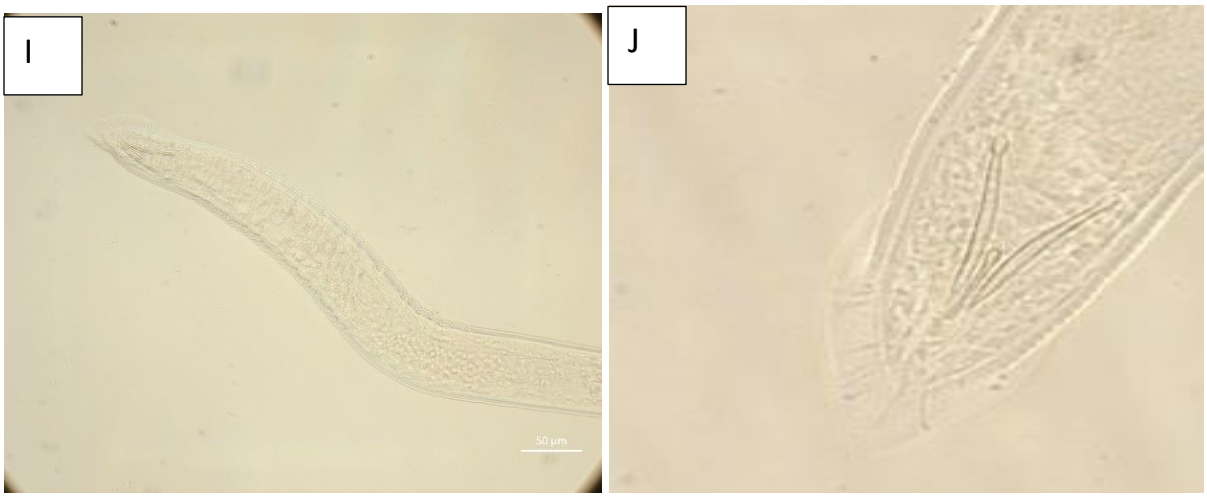


Figure 2.5. Morphology of *Cruznema sp. NTM-2021* under a light microscope. A) J-shaped male body. B) Female body. C-D) Female posterior end and tail. E-H) Anterior end showing mouth and pharynx. I-J) Male posterior end showing tail and spicule. J) Paired, symmetrical and curved male spicule. Scale bar indicated.

2.3.3.3. Measurements

Table 2.2. Morphometrics of Cruznema sp. NTM-2021 presented as mean ± SD (range). All measurements in µm

	Cruznema sp. NTM-2021		
Character	**Female** 32	**Male** 32	**IJs** 6
BL	1006.43 ± 210.95 (651.44-1434.51)	868.31 ± 162.75 (631.09-1282.54)	411.15 ± 188.40 (277.92-544.37)
BW	81.95 ± 20.68 (40.43-121.06)	71.86 ± 13.06 (45.46-104.27)	49.34 ± 16.65 (17.18-66.21)
PL	171.53 ± 27.37 (108.84-221.7)	164.58 ± 28.46 (119.85-225.9)	87.65 ± 26.78 (58.93-138.83)
TL	68.78 ± 18.42 (19.25-101.88)	28.75 ± 12.8 (18.25- 69.36)	59.13 ± 23.29 (14.43-81.71)
S		41.74 ± 7.16 (22.32- 52.7)	
AV	998.67 ± 145.77 (855.62-1205.42)		
M	17.36 ± 2.59 (10.51-21.31)	15.71 ± 1.5 (12.95- 19.03)	10.98 ± 2.83 (5.45- 12.80)
C	3.91 ± 1.6 (1.99- 9.05)	4.00 ± 2.04 (1.95- 10.70)	2.31 ± 0.57 (1.03- 2.57)
V	24.19 ± 6.51 (16.09-39.36)		

Table 2.3. Morphometric comparison between unknown Cruznema isolate and other species of Cruznema genus presented as mean ± SD (range) (all measurements in µm) (Latheef and Seshadri, 1972; Reboredo and Camino, 1998, 2000; Tahseen *et al*., 2012)

	C. sp NTM-2021	***C. tripartitum***	***C. helalii***	***C. brevicaudatum***	***C. campestris***	***C. lincolnensis***
Female						
BL	1006.43 ± 210.95 (651.44- 1434.51)	831 ± 103.2	921 ± 42.8	1130 ± 1270	1100 (950-1210)	1435 (1370-1490)
PL	171.53 ± 27.37 (108.84- 221.7)	165 ± 10.5	174 ± 14.8	285 (275-300)	232 (197.4-253.8)	211.42 (209.15-213.85)
TL	68.78 ± 18.42 (19.25- 101.88)	50.3 ±5.6	61.3 ± 6.6	25 - 40	66.7 (44.6 – 82.8)	87 (84.6- 89.3)
CT	3.91 ± 1.6 (1.99-9.05)			3		
Male						
BL	868.31 ± 162.75 (631.09- 1282.54)	656 ± 48.9	825 ± 66.2	1060 - 1080	812 (730 – 890)	972 (870-1090)
PL	164.58 ± 28.46 (119.85- 225.9)	178 ± 10.9	162 ± 8.7	250 (235 – 265)	199.9 (173.9 – 213.8)	173.83 (169.2-181.7)
TL	28.75 ± 12.8 (18.25- 69.36)	28.3 ± 7.8	29.1 ± 2.9	74		
S	41.74 ± 7.16 (22.32- 52.7)	44.1 ± 4.5	51.6 ± 1.8	75 - 95	42.4 (35.5 – 45.8)	40.40 (37.6-44.65)
CT	4.00 ± 2.04 (1.95-10.70)					

Table 2.4. Morphometric comparison between unknown Cruznema isolate and entomopathogenic nematode species presented as mean ± SD (range)(all measurements in µm) (Sagun *et al*., 2015; Lephoto and Gray, 2019b; Didiza, Lephoto and Gray, 2021)

	Cruznema sp. NTM-2021	***Oscheius basothovii***	***Stienernema batswanae***	***Heterorhabitis bacteriophora***
Female				
BL	1006.43 ± 210.95 (651.44- 1434.51)	1125.18 ± 201.35	7969 ± 1012	3180 - 3850
PL	171.53 ± 27.37 (108.84- 221.7)	169.45 ± 0.79	154 ± 15	155-0183
TL	68.78 ± 18.42 (19.25- 101.88)	87.67 ± 1.22	40 ± 12	71 - 93
CT	3.91 ± 1.6 (1.99- 9.05)	7.35 ± 0.68		
Male				
BL	868.31 ± 162.75 (631.09- 1282.54)	1125.18 ± 160.61	1953 ± 157	3630 - 4390
PL	164.58 ± 28.46 (119.85- 225.9)	155.44 ± 1.43	123 ± 9	99 - 105
TL	28.75 ± 12.8 (18.25- 69.36)	60.55 ± 2.04	33 ± 5	22 - 36
S	41.74 ± 7.16 (22.32- 52.7)	48.55 ± 2.04	86 ± 4	36 - 44
CT	4.00 ± 2.04 (1.95- 10.70)	8.32 ± 0.43		
IJs				
BL	411.15 ± 188.40 (277.92- 544.37)	510.71 ± 19.17	975 ± 72	520 - 600
PL	87.65 ± 26.78 (58.93- 138.83)	164.91 ± 2.74	108 ± 12	119 - 130
TL	59.13 ± 23.29 (14.43- 81.71)	21.96 ± 1.26	19 ± 8	83 - 99
CT	2.31 ± 0.57	3.57 ± 1.75		

	(1.03- 2.57)			

The morphometrics traits data of *Cruznema sp. NTM-2021* (Table 2.2) were compared with five described *Cruznema* species (table 2.3). They had minimal similarities with each other, however, *Cruznema sp. NTM-2021* and *Cruznema helalii* were within the same range with a body length of both female and male (1006.45 µm vs 921 µm and 868.31 µm vs 825 µm), tail length of female and male (68.78 µm vs 61.3 µm and 28.75 µm vs 29.1 µm) and spicule (41.74 µm vs 51.6 µm). Additionally, a morphometrics table to compare *Cruznema sp. NTM-2021* with EPNs species from each genus (*Oscheius*, *Steinernema* and *Heterorhabditis*) was tabulated (Table 2.4). According to this, *Cruznema sp. NTM-2021* was more similar to the *Oscheius basothovii* with regards to size. *Cruznema sp. NTM-2021* and *Oscheius basothovii* are both small nematodes compared with the other EPNs species. This suggests that they could be closely related as seen on the phylogenetic tree as well. This source of similarity and variation could be due to genetics or the environmental conditions (Bolnick *et al.*, 2011). Furthermore, the differences in the morphometric data might result from variations in their habitat or host-seeking techniques (Burnell and Stock, 2000).

2.4. Conclusion

Molecular methods were used to successfully identify the unknown nematode as a new species. Morphological and phylogenetic analysis were then used to confirm the identification and characterize. The new nematode species from Mahobong Leribe, Lesotho belonging to the *Cruznema* genera was identified using the 18S rDNA sequence and given the name '*Cruznema sp. NTM-2021'*. This nematode isolate caused insect mortality within 3 to 6 days hence it has the potential of being used as a biological control agent in the agricultural sector. However, further studies are required to determine its host range, temperature range, desiccation capabilities and foraging strategies.

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Chapter three: Bacterial isolation and identification

3.1. Introduction

Bacteria frequently form symbiotic relationships with mammals, insects, plants and nematodes. These relationships may have pathogenic or mutually beneficial effects. Bacteria from the genus *Xenorhabdus, Photorhabdus* and *Serratia* are symbiotically associated with entomopathogenic nematodes (EPNs) from the genera *Stienernema, Heterorhabidis* and *Oshcieus* respectively. They belong to the family Enterobacteriaceae within the gammaproteobacteria (Koppenhöfer, 2007). They are defined as gram negative, facultatively anaerobic and non-spore forming rod bacteria (Sajnaga and Kazimierczak, 2020). The bacteria work in concert with the EPNs to infect and kill insects. Therefore, they have been largely used as biological control agents (Erdoğan *et al*., 2021). These symbiotic bacteria are carried in the gut of the infective juvenile stages of EPNs, as they are provided with protection and transportation (Stock and Goodrich-Blair, 2012). The symbiotic bacteria in turn kills insect hosts; develop and maintain favorable conditions for nematode reproduction, providing nutrients and anti-microbial substances that that prevent the growth of a wide variety of microorganisms (Yooyangket *et al.*, 2018).

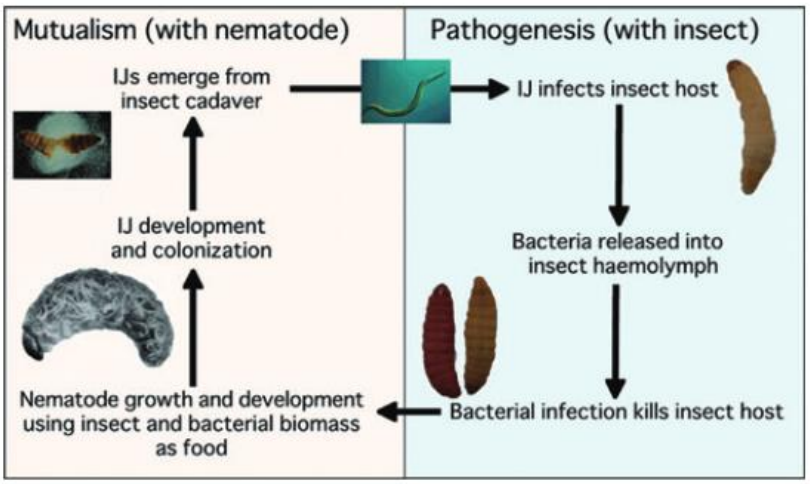


Figure 3.1. The lifecycle of all microsymbionts in EPNs follows a consistent pattern that includes three phases: phoresis within the nematode host, pathogenicity during an insect infection and finally saprophytism once inside a deceased insect's carcass (Sajnaga and Kazimierczak, 2020). EPNs bacterial symbionts must successfully complete three different roles throughout the life cycle: (1) rapid insect killing; (2) production of nutrients from the insect cadaver to aid nematode

growth and development. (3) Nematode colonization and growth during the IJ stage (Goodrich-blair and Clarke, 2007).

Xenorhabdus, *Photorhabdus* and *Serratia* bacteria stand out for their entomopathogenic potential. They release several toxins or secondary metabolites that cause immunosuppression, hemolymph overabundance and killing of the insect (Yooyangket *et al.*, 2018). There have been reports of several secondary metabolites produced by symbiotic bacteria that have bioactive properties (Bode, 2009). These properties include cytotoxic, antimicrobial, antiparasitic, and insecticidal cues. Therefore, these bacteria have been the focus of numerous research organizations who hope to use them in pharmaceutical, industrial, and agricultural products (Bode, 2009). The nematode-bacteria association is critical for nematode infection, mass reproduction, and registration as biological control agents. It is essential to identify which bacterial species are found in nature in association with different EPNs species (Liu *et al*., 2011). Hence, more studies are needed due to their possible use as new biological control agents of agricultural pests and vector insects such as mosquitoes (da Silva *et al.*, 2020).

The main criteria for identifying and distinguishing bacteria were symbiotic properties and some morphological characteristics (Liu *et al*., 2011). Morphological characterisation of bacteria yields a great deal of information, such as gram reactions, motility, arrangement, presence of spores, capsules, inclusion bodies, and its shape. However, this technique only allows for identification of the bacteria at the genus level hence molecular characterisation is required (Janda and Abbott, 2007). Bacterial characterisation studies now make use of rRNA gene sequences, namely: the 16S rRNA gene, which has proved effective in establishing broader phylogenetic relationships and identification. It comprises of variable and conserved regions and is used for comparative taxonomy (Liu *et al*., 2011). 16S rRNA gene sequence analysis has now become 'the gold standard' in the taxonomy of bacteria (Sajnaga and Kazimierczak, 2020). This information is used to understand the past evolutionary path of the organism to see how related they are to other organisms, and it provides the best assumption.

3.2. Methods and materials

3.2.1. Isolation of bacteria from nematodes

The bacteria were isolated from *Cruznema sp. NTM-2021* (OQ408141), which was obtained from Mahobong Leribe, Lesotho. The nematodes were taken from white traps (Refer to 2.2.2.3), placed in 50 ml falcon tubes and surface sterilized with 0.1% sodium hypochlorite for 1 hour. This was done to kill any viable bacteria adhering to the surface of the nematodes that would result in contamination and interfere with the isolation of pure bacterial cultures residing within the nematode. The nematodes were then rinsed with Ringer's solution and homogenized with sterile plastic pestles in sterile Eppendorf tubes. The homogenate was then transferred into a sterile Eppendorf tube with 0.1 ml of sterilized nutrient broth, and it was incubated there for 24 hours at 25 °C.

The homogenate was steaked onto nutrient agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue (NBTA) and MacConkey agar; and kept at 25 ˚C for 24 hours. The different colour colonies of bacteria were separated and sub-cultured several times to ensure pure cultures. Each colony colour had three plates dedicated to it, bringing the total to 6 plates for each bacteria. The plates were then stored at 4 °C until required for further use.

3.2.2. Molecular characterization

3.2.2.1. Bacterial DNA extraction

Bacterial DNA of the pure colonies from the NBTA was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek) (See protocol in appendix).

3.2.2.2. PCR amplification and sequencing of 16S rRNA gene

The extracted DNA was sent to Inqaba Biotechnological Industries Pty (Ltd) for amplifying and sequencing of the 16S rRNA gene. The following bacterial universal primers were used: 16S-27F (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 16S-1492R (5'-CGGTTACCTTGTTACGACTT-3'). The PCR reactions were performed using NEB OneTaq 2X MasterMix with Standard Buffer. Genomic DNA samples at a concentration of 10-30 ng/µl were used as templates. The forward and reverse primers were used at a concentration of 10 µM each.

Nuclease-free water was added to achieve the desired reaction volume. The PCR cycling conditions are as follows: an initial denaturation step at 94°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 68°C for 1 minute. These steps were repeated for a total of 35 cycles. A final extension step was performed at 68°C for 10 minutes, followed by holding the reaction at 4°C.

Agarose Gel Analysis was performed to visualize the integrity of PCR amplicons on a 1% agarose gel stained with EZ-vision Bluelight DNA Dye. The NEB Fast Ladder served as a size standard. Following this, the PCR amplicons were enzymatically purified using the ExoSAP procedure for subsequent applications. For Sanger sequencing, the purified amplicons were processed with the Zymo Research ZR-96 DNA Sequencing Clean-up Kit and sequenced in both forward and reverse directions using the Nimagen BrilliantDy Terminator Cycle Sequencing Kit on the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

3.2.2.3. Phylogenetic analysis

The sequences from Inqaba Biotechnological Industries Pty (Ltd) were edited and viewed for errors using Geneious Prime 2022.2.1. The edited 16S rRNA FASTA sequences were uploaded onto the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLASTn) to compare the unknown isolate sequence with the known sequences on the NCBI database.

The phylogenetic tree was constructed using the 16S rRNA sequences from BLASTn that were similar to the unknown bacterial isolate sequences to identify the isolates at species level. Molecular Evolutionary Genetics Analysis 11 (MEGA 11) was the software tool used for the phylogenetic analysis and alignment of both known and unknown sequences. The alignment was done using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool; and the phylogenetic tree was constructed using the neighbor-joining method and taxon clustering based on 1000 replications in the bootstrap statistical test.

The following taxa were used for the construction of the phylogenetic tree for unknown 1:

Alcaligenes faecalis 3e (LC376950), *Alcaligenes sp.* strain WWTP-0321-S-MHB100-D7 (OQ160690), *Alcaligenes sp*. strain SNH-K76 (MN493925), *Alcaligenes faecalis* strain YZ19 (MT579857), *Alcaligenes aquatilis* strain RC43 (MT572474), *Alcaligenes sp.* strain SNH-K70 (MN493920), *Alcaligenes sp*. strain A-TC2 OP804216), *Alcaligenes faecalis* strain BJD1 (MT378145) and *Alcaligenes aquatilis* strain NVS12 (OP810742)

The following taxa were used for the construction of the phylogenetic tree for unknown 2:

Elizabethkingia sp. NB0903 (MN706626), *Elizabethkingia miricola* CW2012072510 (OP750227), *Elizabethkingia miricola* Adu42 (MN709334), *Elizabethkingia miricola* 2022EM07 (OP778141), *bacterium* FY70815-3 (MF800860), *Elizabethkingia sp*. B916 (KM678253), *Elizabethkingia sp.* CV2012072501 (OP221516), *Elizabethkingia occulta* G4070 (MH789418), *Elizabethkingia ursingii* G4122 (MH789420), *Elizabethkingia ursingii* EM/514-03 1 (ON714894)

The following taxa were used for the construction of the phylogenetic tree for unknown 3:

Enterobacter cloacae AFS055079 (OP986832), *Enterobacter ludwigi* NFB1 (OP941461), *Pantoea agglomerans* 600 (MT585395), *Enterobacter ludwigii* AFS065241 (OP986255), *Enterobacter cloacae* AFS066524 (OP986227), *Enterobacter ludwigi* D8 (MT374261), *Pantoea sp.* BT3 (MT355737), *Enterobacter sp.* AN3 (MT557019), *Enterobacter huaxiensis* AY5 (MT557013), *Enterobacter sp.* EC18 (OP412787), *Enterobacter sp.* BT22 (MT355740).

3.2.3. Morphological characterization

3.2.3.1. Gram stain

On microscope slides, NBTA agar-grown colonies were smeared and heat-fixed. Crystal violet was applied to the slides for 60 seconds. The slide was gently washed with running water after the excess dye was poured out. The Gram's Iodine was then applied to the smears for 60 seconds, poured off, and then washed with running water. 90% alcohol was poured on the slide for 7 seconds and washed off with running tap water to stop the decolorization. The smear was then counterstained for 30 seconds with safranin, washed off and dried with a paper towel. The slides were then viewed with oil immersion under a light microscope at a 100x magnification.

3.2.4. Lipid agar test

To understand the mutualistic relationship between the nematodes and the isolated, potentially associated bacteria, a lipid agar test was performed. Lipid agar is ideal for growing lawns of bacteria which can be used to support the growth and reproduction of associated nematodes under suitable conditions (McMullen and Stock, 2014). The method on how the lipid agar media was made is outlined in the appendices. Pure colonies of isolated bacteria (Refer to 3.2.1) were grown in 1 ml nutrient broth in a sterile Eppendorf tube for 4 hours at 25 °C. The lipid agar plates were inoculated with the 1 ml nutrient broth culture and incubated for 24 hours at 25 °C. IJs taken from the white traps (Refer to 2.2.2.3) were counted and 4 IJs (2 male and 2 female) in 4 µl were added to the lipid agar bacterial lawns. Observations for growth and reproduction of the IJs were done daily. Confirmation of the unknown bacteria's ability to support nematode growth would be obtained if the infective juveniles (IJs) thrived and multiplied on the bacterial lawn in the lipid agar medium.

3.2.5. Bacterial pathogenicity test

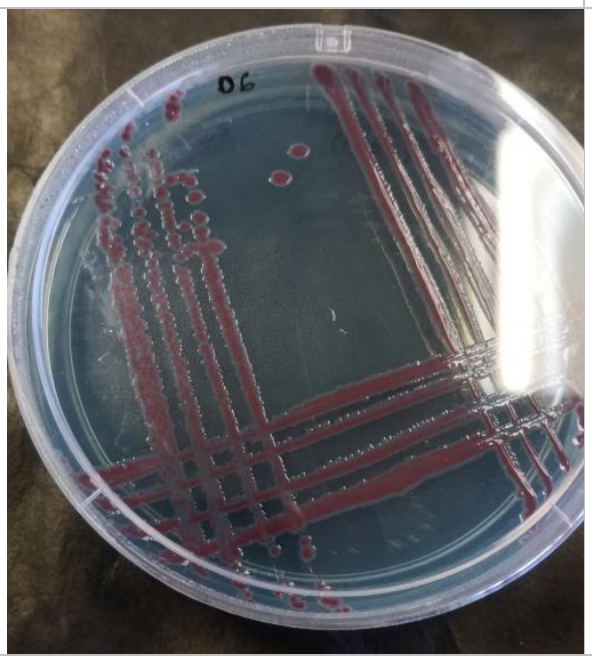
Pure colonies of the isolated bacteria were spread on nutrient agar and incubated for 24 hours at 30 °C. When the bacterial lawns were established, 4 surface sterilized *Tenebrio molitor* larvae were added on each plate and observations of mortality were done daily. *E. coli* was used as a positive control and uninoculated nutrient agar was used as a negative control.

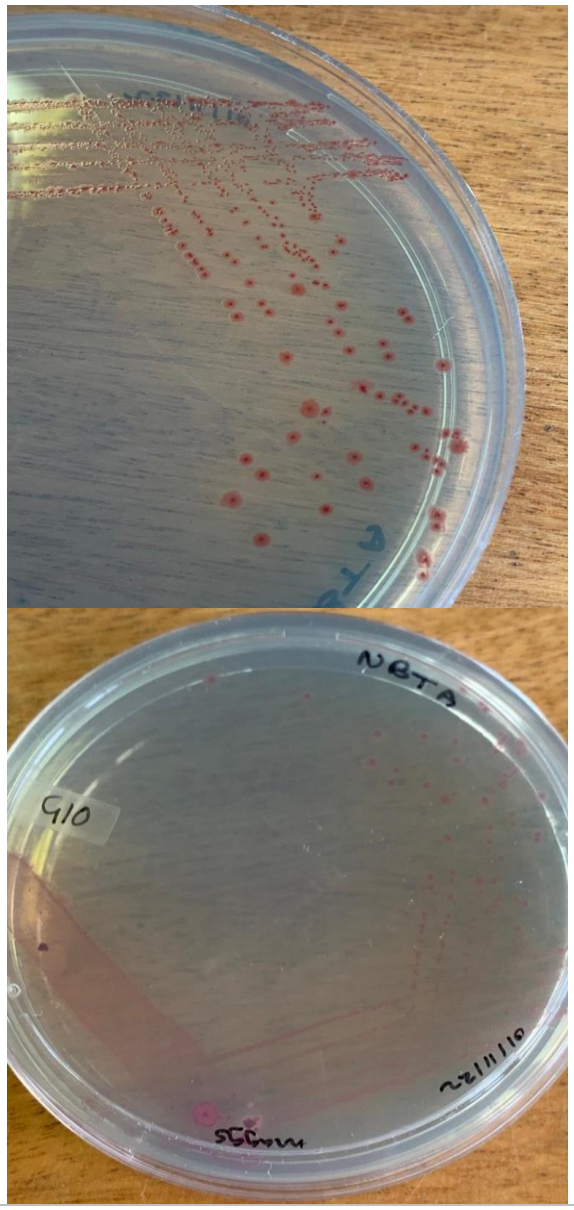
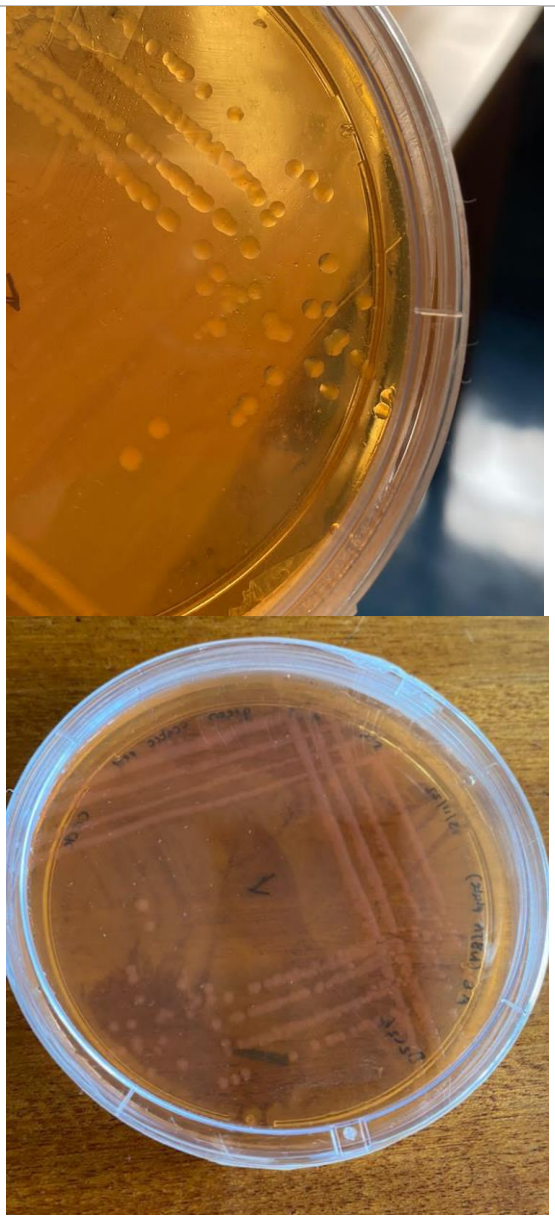
3.3. Results

3.3.1. Isolation of bacteria

The bacteria were isolated from the nematodes and cultured on NBTA and MacConkey (Table 3.1). Colony and cell morphology was determined (Table 3.2).

Table 3.1. Unknown isolate 1, 2 and 3 colonies cultured on NBTA and MacConkey

	NBTA	MacConkey
Unknown 1		

Unknown 2		

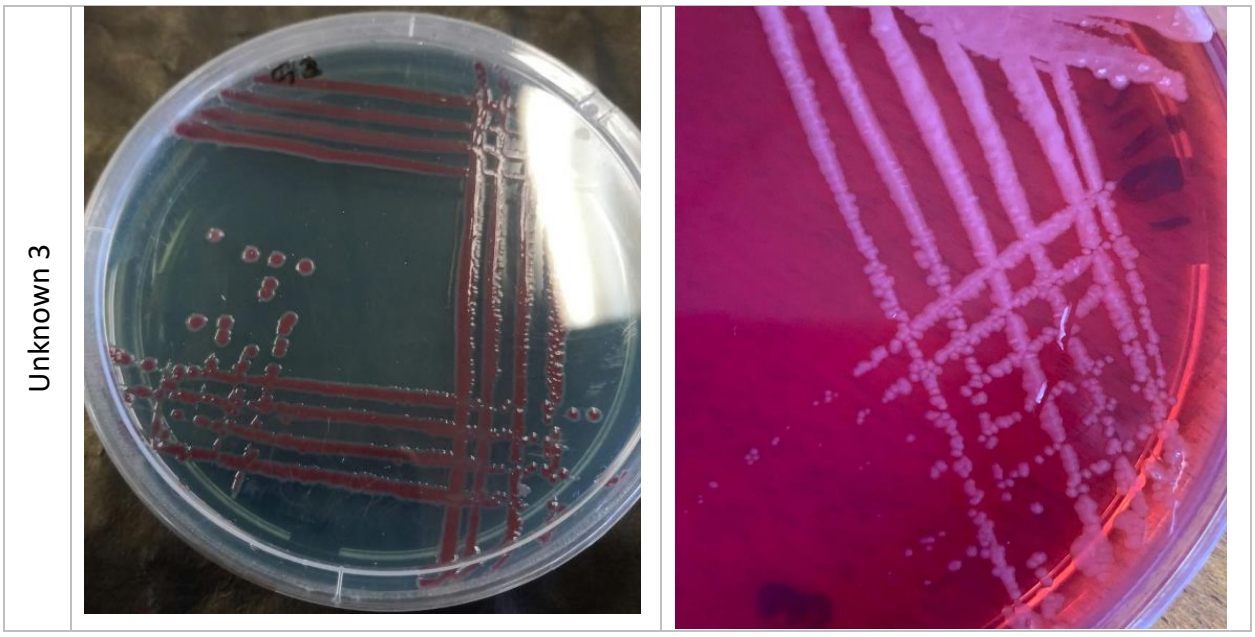


Figure 3.2. Isolated bacteria cultured on NTBA and MacConkey plates. D6, G10 and G2 represent unknown 1,2 and 3, respectively.

Table 3.2. Morphological characteristics of bacterial colonies on NBTA plates and MacConkey for unknown bacterial isolate 1, 2 and 3

Isolate	Morphological characteristics on NBTA				Lactose fermentation	Cell shape	Gram reaction
	Colony color	Surface texture	Colony elevation	Shape			
Unknown 1	Dark red	Smooth	Raised	Circular	-	Rod	-
Unknown 2	Red	Rough	Flat	Irregular	-	Rod	-
Unknown 3	Dark red	Smooth	Raised	Circular	+	Rod	-

3.3.2. Molecular characterization

The sequences for the PCR amplicons of the 16S rRNA genes were received from Inqaba Biotechnological Industries Pty (Ltd) in the form of chromatograms. They were viewed for errors, edited using Geneious prime version 2020.0.5, and saved as FASTA formatted files. The 16S rRNA gene sequences for the unknown bacteria isolate 1,2 and 3 are indicated below (Figure 3.3, 3.4 and 3.5).

>GGTGAGCCCTGGGATTTCACATCTTTCTTTCCGAACCGCCTACACACGCTTTACGCCCAGTAATTCCGATTAACGC
TTGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTGSAGATACCGTCAGCAGTATCC
CGTATTAGGGGATACCTTTTCTTCTCTGCCAAAAGTACTTTACAACCCGAAGGCCTTCATCATACACGCGGGATGGC
TGGATCAGGGTTTCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCTGGTCGTCCTCTCAAACCAGCTACGGATCGTTGCCTTGGTGAGCCTTTACCCCACCAACTAGCTAATCCG
ATATCGGCCGCTCCAATAGTGAGAGGTCTTGCGATCCCCCCCTTTCCCCCGTAGGGCGTATGCGGTATTAGCCACT
CTTTCGAG

Figure 3.3. The bacterial sequence of unknown isolate 1 (468 bp). The NCBI BLASTn results revealed a high affinity to *Alcaligenes* spp.

>TACCGGCCATTGTAGCACGTGTGTGGCCCAAGACGTAAGGGCCGTGATGATTTGACGTCATCCCCACCTTCCTCT
CTACTTGCGTAGGSAGTCTCACTAGAGTCCTCAACTTAATGTTAGCAACTAGTGACAGGGGTTGCGCTCGTTGCAG
GACTTAACCTAACACCTCACGGCACGAGCTGACGACAACCATGCAGCACCTTGAAAAATGTCCGAAGAAGGATCT
ATTTCTAAATCTGTSATTTCCCATTTAAGTCTTGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCACC
GCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGATTACTTATCACTTTC
GCTTAGTCTCTGAATCCTAAAACCCAAAAACGAGTAATCATCGTTACGGCGTGGACTACCAGGGTATCTAATCCTG
GTTCGCTCCCCACGCTTTCGTCCATCAGCGTCAG

Figure 3.4. The bacterial sequence of unknown isolate 2 (492 bp). The NCBI BLASTn results revealed a high affinity to *Elizabethkingia* spp.

>TACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCAT
TCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTA
TGAGGTCCGCTTGCTCTCGCGAGTTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGG
GCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCG
CTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCC
ATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAGTTCTCTGGATGTCAAGAGTAGGTAAG
GTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGC

Figure 3.5. The bacterial sequence of unknown isolate 3 (504 bp). The NCBI BLASTn results revealed a high affinity to *Enterobacter* spp.

The BLASTn search for unknown 1 revealed that the bacterial isolate belonged to the *Alcaligenes* genus, with a high sequence similarity of 99.79% to *Alcaligenes aquatilis* RC43 (MT572474), *Alcaligenes faecalis* strain YZ19 (MT579857) and *Alcaligenes* 3e (LC376950), among others. The phylogenetic tree revealed that unknown isolate 1 was indeed closely related to *Alcaligenes faecalis* 3e (LC376950) with a bootstrap of 83 but had *Alcaligenes faecalis* strain YZ19 (MT579857) as a distant ancestor (Figure 3.6).

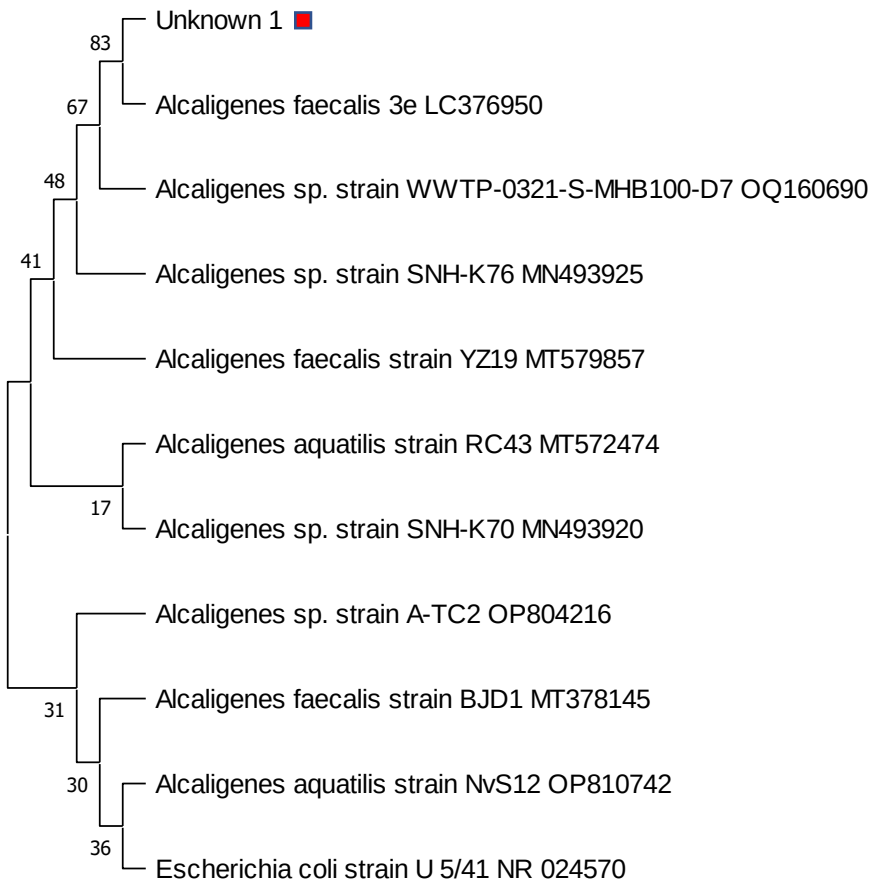


Figure 3.6. Neighbor-joining tree of unknown 1 and closely related bacteria inferred by homologous sequences based on the 16S rRNA gene sequence data. The numbers on the tree are bootstrap values from 1000 replicates.

The BLASTn results for unknown 2 revealed this bacterial isolate had high percentage similarities with *Elizabethkingia* species including *Elizabethkingia sp*. strain NB0903 (MN706626) and *Elizabethkingia ursingii* strain G4122 (MH789420) among others, with a percentage similarity of 99.19%. However, the phylogenetic tree shows that unknown 2 is more closely related to *E. coli* (NR024570) with a bootstrap of 53 (Figure 3.7).

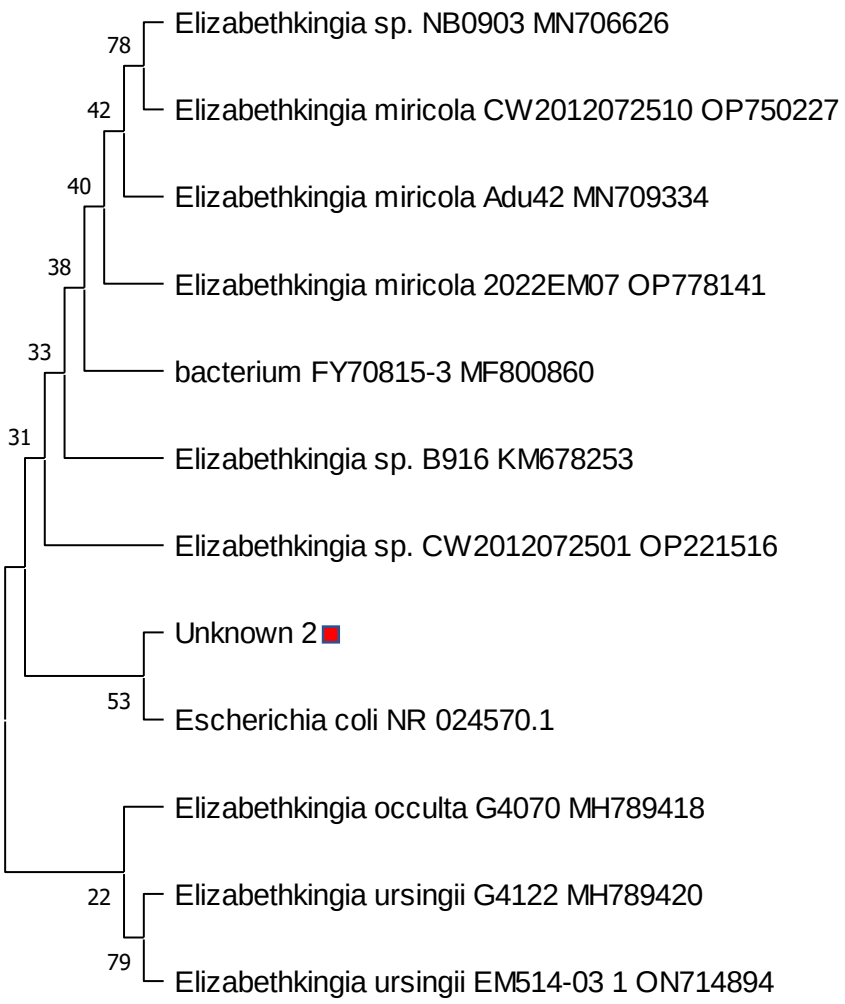


Figure 3.7. Neighbor-joining tree for unknown 2 and closely related bacteria inferred by homologous sequences based on the 16S rRNA gene sequence data. The numbers on the tree are bootstrap values from 1000 replicates.

Bacterial unknown isolate 3's 16S rRNA gene sequence BLASTn results revealed that it had a high percentage similarity of 99.8% with multiple species including: *Pantoea agglomerans* strain 600 (MT585395), *Enterobacter sp.* strain AN3 (MT557019) and *Enterobacter huaxiensis* strain AY5 (MT557013). The phylogenetic tree revealed that unknown isolate 3 is closely related to *Enterobacter sp*. EC18 (OP412787) with a bootstrap of 41 (figure 3.8). It is also in the same clade with more Enterobacter species including *Enterobacter huaxiensis* AY5 (MT557013) and *Enterobacter sp*. AN3 MT557019), with very low bootstrap values of 31 and 20, respectively.

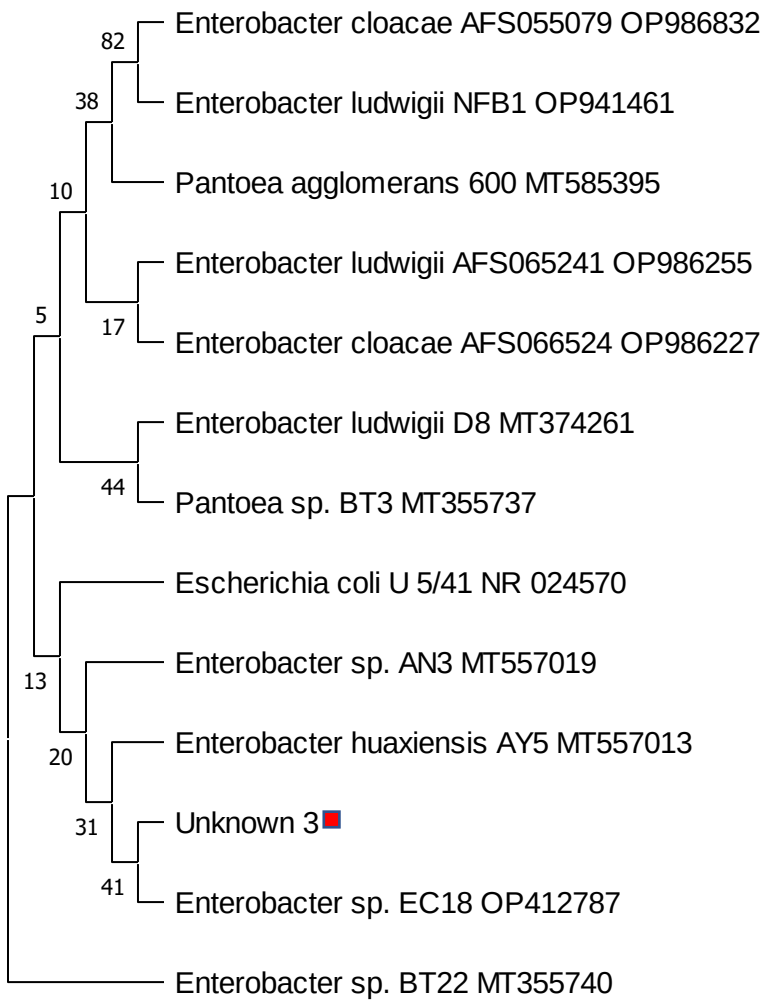


Figure 3.8. Neighbor-joining tree for unknown 3 and closely related bacteria inferred by homologous sequences based on the 16S rRNA gene sequence data. The numbers on the tree are bootstrap values from 1000 replicates.

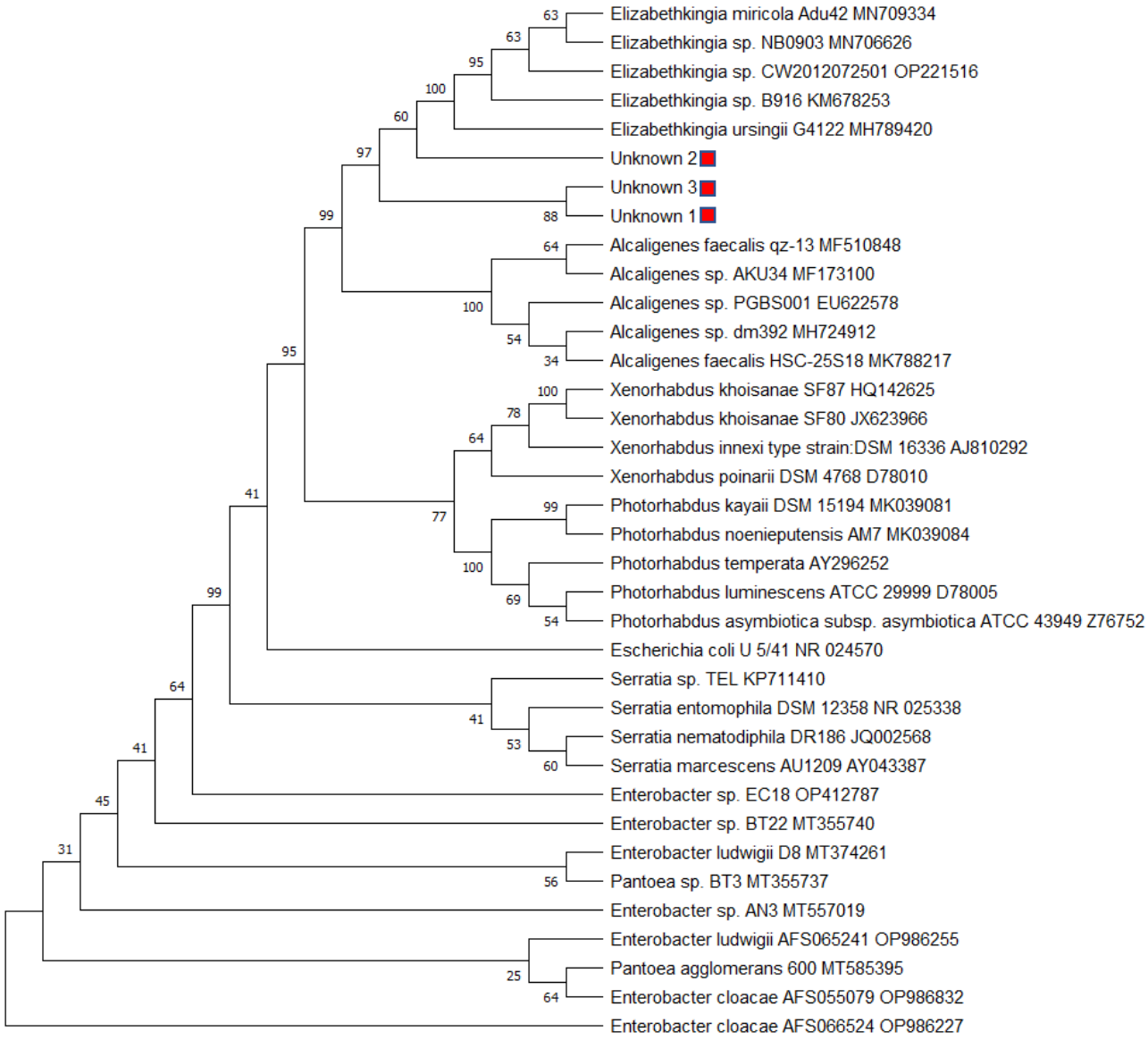


Figure 3.9. Maximum likelihood tree for unknown 1, 2 and 3, as well as their closely related bacteria and bacteria from genera reported to be entomopathogenic (*Xenorhabdus*, *Photorhabdus* and *Serratia*) inferred by homologous sequences based on the 16S rRNA gene sequence data. The numbers on the tree are bootstrap values from 1000 replicates.

3.3.3. Lipid agar test

Cruznema sp. NTM-2021 was able to reproduce on the lipid agar with all the bacterial isolates.

3.3.4. Bacterial entomopathogenicity

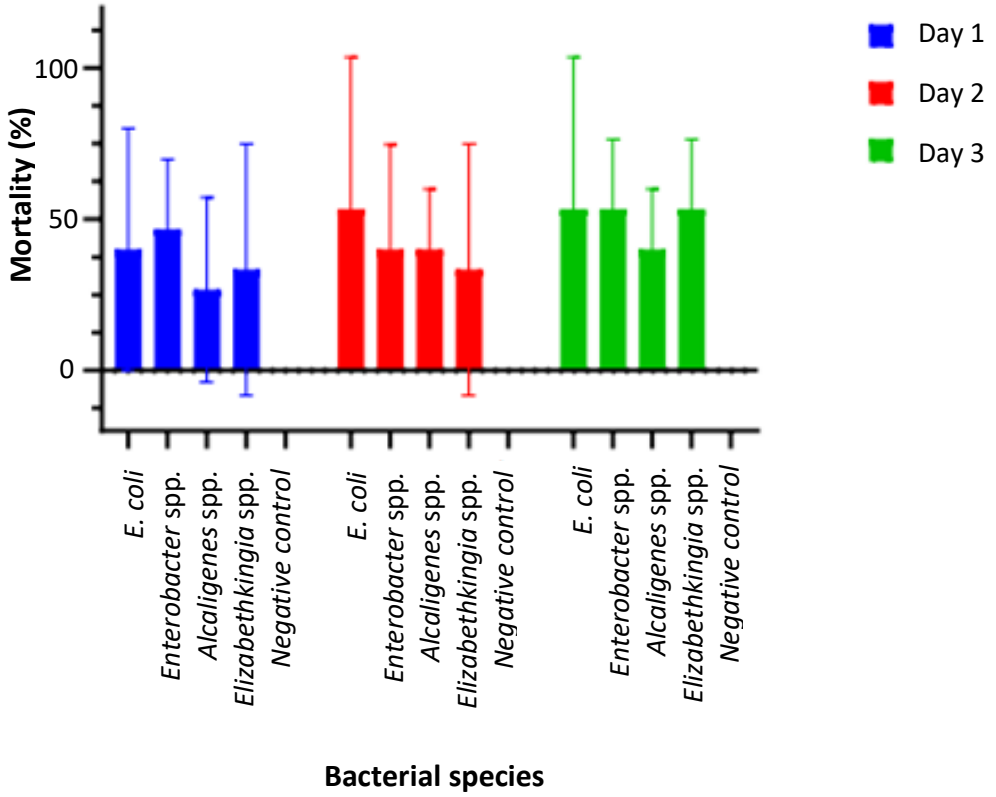


Figure 3.10. Graph showing the percentage of mortality of larvae (n=4) killed by bacteria isolated from the *Cruznema sp. NTM-2021 (Enterobacer* spp.*, Alcaligenes* spp. and *Elizabethkingia* spp.), positive control (*E. coli*) and negative control, over a period of 3 days. The data represents independent observations made at various time points of each replication and the error bars represent the variability of data points.

3.4. Discussion

Three bacterial species were isolated from *Cruznema sp. NTM-2021* (OQ408141) using selective and differential media (NBTA and MacConkey). Selective media promotes the growth of one bacterial species or genus in some cases while inhibiting the growth of others; and differential media distinguishes between bacterial cultures based on their biochemical properties (Al-blooshi *et al.*, 2021). NTBA was used to isolate and select gram-negative bacteria based on their ability to absorb bromothymol blue and reduce triphrnyltetrazolium chloride (TTC), resulting in red colonies. All the isolates were found to be rod shaped, had a negative gram reaction and red or dark red color colonies (Table 3.2). MacConkey agar is also selective in that it inhibits the growth

of many gram-positive bacteria while favoring the growth of gram-negative bacteria, particularly those belonging to the Enterobacteriaceae family. Furthermore, the presence or absence of a pink color distinguishes lactose fermenters from non-lactose fermenters in gram-negative rods (Al-blooshi *et al.*, 2021). The colony morphology on MacConkey agar for unknown 1 and 2 were yellow suggesting they were non-lactose fermenting and unknown 3 was pink suggesting it was a lactose fermenter. According to the CDC Division of laboratory systems (Appendix, Figure F), gram-negative bacterial rods that grow on MacConkey agar and are lactose non-fermenters could be *Pseudomonas* spp., *Alcaligenes* spp., *Vibrio* spp., *Aeromonas* spp., *Burkholderia* spp., *Acinetobacter* spp., *Stenotrophomonas* spp., *Proteus* spp., *Yersinia* spp., *Salmonella* spp., *Shigella* spp. or *B. mallei*, depending on the outcome of the oxidase test. Lactose fermenters, however, could be *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter diversus*, *Edwardsiella tarda*, *Cronobacter* spp., *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae* or *Hafnia alvei* based on the indole test. This morphological identification method narrowed down the possible species for unknown isolate 1, 2 and 3. However, molecular identification was necessary for more further identification.

Molecular identification of the isolated bacterial species was done by the analysis of the 16S rRNA gene. This gene has been most commonly used as it is relatively large (1500 bp long) and has remained conserved over time (Clarridge, 2004). According to the BLASTn results, *Alcaligenes* spp., *Elizabethkingia* spp. and *Enterobacter* spp. were more similar to unknown isolates 1, 2 and 3, respectively. According to the phylogenetic trees, unknown 2 and *E. coli* were in the same clade with a bootstrap value of 53, and unknown 3 and *Enterobacter* spp. were in the same clade with a bootstrap value of 41. These bootstrap values were extremely low, which suggests that the clades might not be reliable. A bootstrap higher than 65% is considered robust (Dorris, de Ley and Blaxter, 1999). There is more confidence that *Alcaligenes faecalis* and unknown isolate 1 are closely related as they have a bootstrap value of 83. Figure 3.9 shows that unknown 1 and 3 are clustered together, with unknown 2 clustering with the *Elizabethkingia* spp. Re-evaluation is required to confirm if these species do form part of the same clade.

EPNs like *Oscheius* species have been found to be associated with bacteria strains from three classes: (1) beta-proteobacteria (*Alcaligenes* spp.), (2) gamma-proteobacteria (*Enterobacter*,

Proteus, *Providera*, *Pseudomonas*, *Stenotrophomonas*), and (3) *Bacilli* (*Bacillus*, *Enterococcus*, *Lynsinibacillus)* (Sangeetha *et al.*, 2016; Shan *et al.*, 2019). Additionally*, Steinernema feltiae, S. carpocapsae,* and *Heterorhabditis bacteriophora*'s oesophagus and intestine contained *Alcaligenes faecalis strain MORO2.* This strain was later confirmed to be an entomopathogenic bacterium. According to Quiroz-Castañeda *et al*., 2015, *G. mellonella* larvae suffered more than 97% mortality by this bacterial strain after 24 hours. A study by Legrifi *et al*., 2022 further observed that *Alcaligenes faecalis* and *Pantoea agglomerans* were highly effective in the management of pathogens causing soil-borne diseases.

The isolated bacteria have not been reported to be symbiotic with EPNs. However, they have all been isolated from them. *Elizabethkingia* species have been found in microbial communities and through life cycles of *Aedes Aegypti* larvae (Onyango *et al*., 2021). Thus, it has been suggested that it is very likely that they are symbionts of mosquitoes (Boissière *et al.*, 2012). Nonetheless, a study by Ávila-López *et al*., 2021 isolated *Elizabethkingia sp*. from mosquito larvae infected by *Heterorhabditis indica*. However, the uninfected larvae had no *Elizabethkingia* species, suggesting it could have been from the EPNs.

The *Cruznema sp. NTM-2021*-isolated bacteria were able to cause infection and kill approximately 50% of *T. molitor* larvae within 24 hours (Figure 3.10). The bacteria might also be potential symbionts of the *Cruznema sp. NTM-2021* nematode, as the nematode was able to feed and reproduce on all the bacteria. More nematode-bacteria associations are being reported, which can be beneficial or harmful, as well as temporary, facultative, or stably maintained (Hernández-Mendoza *et al.*, 2014). Additionally, there are non-symbiotic bacteria that can coexist with or colonize nematodes and insect cadavers (Quiroz-Castañeda *et al.*, 2015).

3.5 Conclusion

Morphological and molecular techniques were used to identify the unknown bacteria. *Alcaligenes* spp., *Enterobacter* spp. and *Elizabethkingia* spp. were isolated from the *Cruznema sp. NTM-2021* species isolated in Mahobong Leribe, Lesotho. The bacteria were entomopathogenic to *T. molitor* and are potential symbionts of the *Cruznema sp. NTM-2021* nematode, as the nematode was able to feed and reproduce on all the bacteria.

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Chapter four: Conclusions

A new nematode species was isolated from Mahobong Leribe, Lesotho. According to the BLASTn results and phylogenetic analysis of the 18S rDNA gene, the species was from the *Cruznema* genus, and was closely related with *Cruznema sp. CS50* (MW228469) and *Cruznema sp. JN2018* (MK156051) which have both not yet been described. The *Cruznema* genus is a nematode genus that belongs to the family Rhabditidae under the order Rhabditida (Du *et al.*, 2022). The majority of soil Rhabditidae are considered to feed on bacteria, be invertebrate parasites or have commensal relationships with insects (entomophilic); and some species have necromenic techniques (Tahseen et al., 2012; Akhurst and Brook, 1984). *Cruznema* species have been identified as free-living bacterivores or parasites of crickets and mollusks.

Accurately identifying and inferring phylogenies can reveal important information about evolutionary patterns and mechanisms (Du *et al.*, 2022). Molecular and morphological techniques were used to identify and characterize the new nematode species. The 18S rDNA gene sequence was uploaded onto NCBI GenBank and given the name *Cruznema sp. NTM-2021* with accession number OQ408141. *C. sp. NTM-2021* was characterized as having a female and male body length of 1006.45 µm and 868.31 µm, a tail length of 68.78 µm and 28.15 µm, respectively and a spicule of 41.74 µm in males.

Cruznema sp. NTM-2021 was able to cause mortality within 3 to 6 days in *Tenebrio molitor* larvae. The cause of this mortality was distinguishable from being necromenic as the larvae with no IJs did not die which suggests that there were no contaminations. The likely cause of mortality could have been linked to some form of parasitism as necromenic nematodes wait for the demise of their insect hosts before continuing to develop on the microorganisms of the decomposing cadaver (Koneru *et al.*, 2016). *Cruznema brevicaudatum* n sp. was found in the intestine of the mole cricket *Gryllotalpa Africana Beauvois* (Latheef and Seshadri, 1972). Adults of *Gryllodes laplatae* were found parasitized by *C. lincolnensis n. sp*. (Reboredo and Camino, 1998), and the larvae of *Cyclocephala signaticollis* was parasitized by *C. campestris n. sp.* These parasitic association have the potential of regulating insect populations and communities (Camino *et al*., 2011).

Oscheius species including *Oscheius myriophila* and *O. colombiana* like some Rhabditidae species, live a necromenic lifestyle. However, when associated with *Serratia marcesens,* an entomopathogenic bacterium, *O. carolinensis* is a facultative entomopathogenic nematode (EPNs) that can invade, reproduce, and kill insect hosts. *O. carolinensis* is a free-living nematode and does not require the *Serratia* bacteria to survive. Due to their ability to associate with pathogenic bacteria, entomophilic nematodes have the potential to develop from non-parasitic to parasitic through non-obligate and initially low specific bacterial affiliations (Dillman *et al* 2012).

Entomopathogenicity is a form of insect-parasitism that uses the nematode-bacteria complex. Three bacteria were isolated from *Cruznema sp. NTM-2021*, namely: *Alcaligenes* spp., *Enterobacter* spp. and *Elizabethkingia* spp. These bacteria have been previously isolated from EPNs including the *Oscheius* and *Steinernema* genus. However, they have not been reported as symbionts of EPNs. In our study, they were entomopathogenic to the *T. molitor* larvae, and *Cruznema sp. NTM-2021* was able to reproduce by feeding on them on lipid agar media.

Cruznema sp. NTM-2021 exhibited some parasitic and entomopathogenic traits. Further studies like metagenomics are required to investigate its bacterial association as well as their abundance within the nematode before we can confidently report it as an EPNs. Based on the observations made, it is likely to be insect parasitic. However, like EPNs, insect-parasitic nematodes develop only in the insect and do not attack mammals, birds or plants so they have the ability to act as biological control agents.

Future studies should also include more entomopathogenicity tests for the bacterial isolates and the *Cruznema sp. NTM-2021* nematode against different insects. The determination of host range, temperature range, desiccation tolerance and foraging strategies should also be conducted to evaluate if this new nematode species can be applied in agriculture as biological control agents.

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Appendices

Rearing of insects



Figure A. *Tenebrio molitor* larvae and beetles in oat bran and apples.

Isolation of nematodes



Figure B. Soil samples collected from Mahobong Leribe, Lesotho (28°59'17.411" S, 28°8'25.027" E)



Figure C. Soil baiting method using *Tenebrio molitor* larvae.

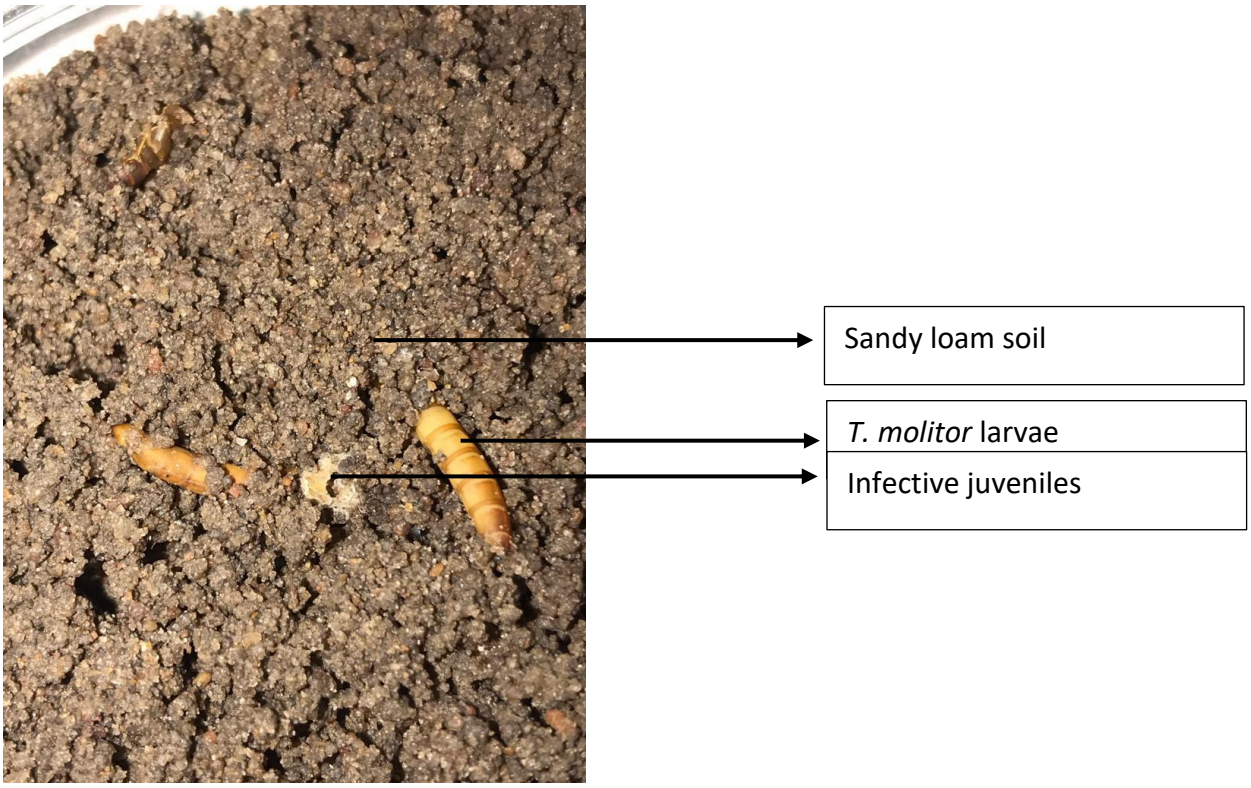

Figure D. Infective juveniles on the soil with *T. molitor* larvae.

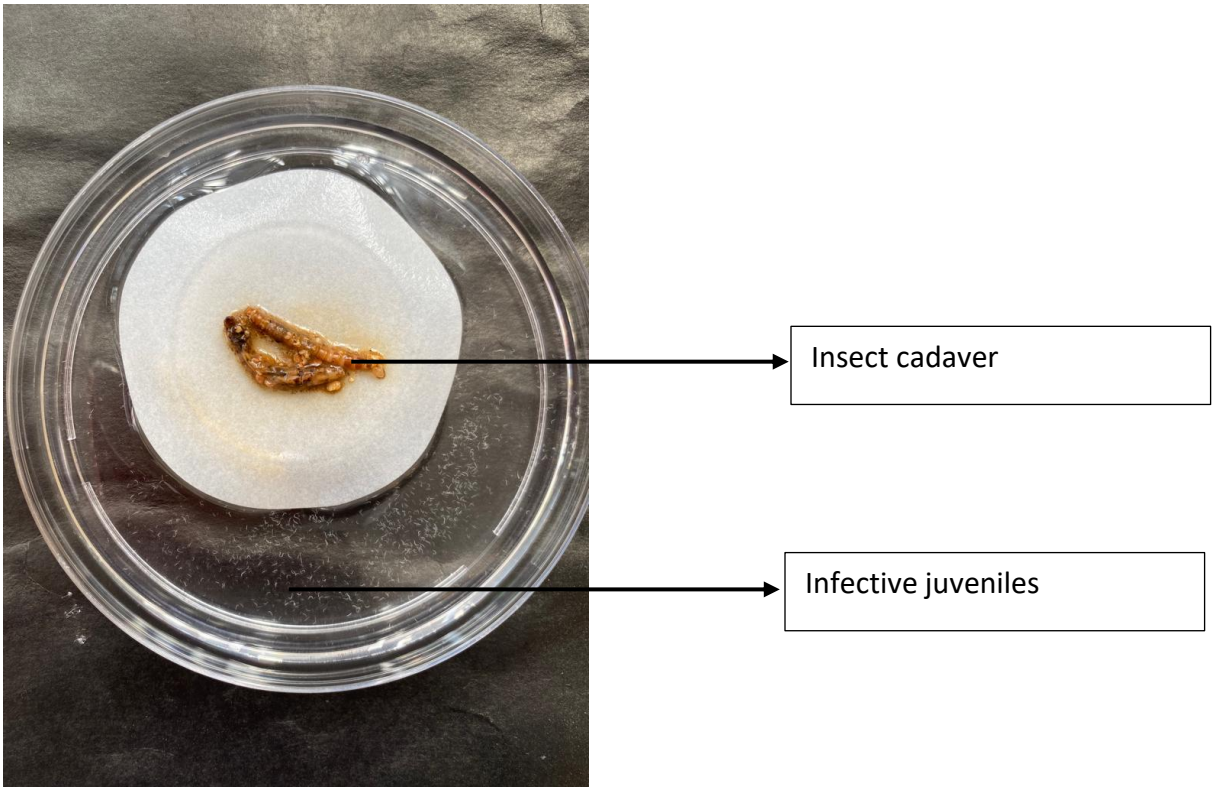

Figure E. Illustration of white trap used to collect infective juveniles from *T. molitor* larvae.

0.1% JIK solution for surface sterilization of IJs

1.4 mL 3.5% JIK

48.6 mL distilled water

Mix JIK and distilled water in bottles and autoclave at 121 °C for 15 minutes.

Lipid agar

10 g honey

5 g yeast extract

2 g nutrient agar

2.5 mL cod liver oil

2 g MgCl2.6H2O

1 L distilled water

Mix ingredients well and autoclave at 121 °C.

NTBA

1 L nutrient agar

0.04 g triphenyltetrazolium chloride (TTC)

0.025 g bromothymol blue (BTB)

Mix nutrient agar and BTB in a 1 L bottle and autoclave at 121 °C for 15 minutes. Wait for it to cool down (<50 °C) then add TTC and swirl to mix. Pour media into petri dishes and allow to solidify.

Nematode DNA extraction protocol (protocol from E.Z.N.A Insect DNA Kit Protocol)(Omega Bio-Tek)

The surface sterilized nematodes were pulverized in an Eppendorf tube using a pestle. 350 µL CTL buffer and 25 µL Proteinase K Solution were added to the pulverized nematodes sample. It was briefly vortexed to mix and incubated at 60 °C for 30 minutes. 350 µL of chloroform:isoamyl alcohol (24:1) was added, the sample was briefly vortexed and centrifuged at 10 000 xg for 2 minutes at room temperature. The upper aqueous phase was then carefully transferred into a clean 1.5 mL microcentrifuge tube. One volume of BL buffer and 2 µL RNase A was added. The sample was vortexed at maximum speed for 15 seconds and incubated at 70 °C for 10 minutes. One volume of 100% ethanol was added and vortexed at maximum speed for 15 seconds. A HiBind DNA Mini Column was inserted into a 2 mL collection tube and 750 µL of the cleared lysate was transferred into the Column. The tube was centrifuged at maximum speed for 1 minute and the filtrate and collection tube were discarded. The Column was placed in a new 2 mL collection tube and 500 µL HBC buffer was added. The sample was then centrifuged at maximum speed for 30 seconds. The filtrate was discarded, and the collection tube reused. 700 µL DNA Wash Buffer was added and the mixture was centrifuged at maximum speed for 1 minute. The filtrate was

discarded, and the collection tube reused. The DNA Wash Buffer step was repeated for the second time. An empty Column was centrifuged for 2 minutes at maximum speed to dry the column matrix. The Column was then transferred in a clean 1.5 mL microcentrifuge tube. 50 µL Elution buffer was added directly to the center of the column membrane. It was let to sit at room temperature for 2 minutes and centrifuged at maximum speed for 1 minute. The DNA was then stored at -20 °C until further use.

DNA extraction of bacterial isolates (Protocol from E.Z.N.A Bacterial DNA Kit)(Omega-Biotek)

The bacteria were cultured in nutrient broth overnight. 3 ml of the culture was centrifuged at 4000 xg for 10 minutes at room temperature and the supernatant was discarded. 100 µL TB buffer was added to the pellet. The mixture was then vortexed to resuspend the pellet. 10 µL of lysozyme resuspended with elution buffer was incubated at 37°C for 10 minutes. 100 µL TL buffer and 20 µL Proteinase K Solution were added. The mixture was then vortexed thoroughly and incubated at 55 °C in a shaking water bath for 20- 30 minutes. 5 µL RNase A was added and the tube was inverted several times to mix the contents, which were then let to sit at room temperature for 5 minutes. The tube was centrifuged at 10 000 xg for 2 minutes to pellet any undigested material. The supernatant was then transferred to a new 1.5 mL microcentrifuge tube. 220 µL BL buffer was added, the tube was vortexed to mix thoroughly then incubated at 65 °C for 10 minutes. 220 µL of 100% ethanol was added and the mixture was vortexed for 20 seconds at maximum speed to mix thoroughly. A HiBind DNA Mini Column was inserted into a 2 mL collection tube and the entire sample was transferred into the column. The sample was then centrifuged at 10 000 xg for 1 minute and the filtrate and the collection tube were discarded. The column was inserted into a new 2 mL collection tube and 500 µL HBC buffer diluted with 100% isopropanol was added, and the sample was centrifuged at 10 000 xg for 1 minute. The filtrate was discarded, and the collection tube was reused. 700 µL DNA Wash Buffer diluted with 100% ethanol was added, and the sample was centrifuged at 10 000 xg for 1 minute. The filtrate was discarded, and the collection tube reused. This was repeated for a second DNA Wash Buffer wash step. An empty HiBind DNA Mini Column was centrifuged at maximum speed for 2 minutes to dry the column. The HiBind DNA Mini Column was inserted into a new nucleases-free 1.5 mL microcentrifuge tube. 50-100 µL Elution Buffer was added and the sample was heated to 65°C

and left to sit at room temperature. The Elution step was repeated for the second time. The eluted DNA was then stored at -20°C for further use.

Identification of isolated bacteria

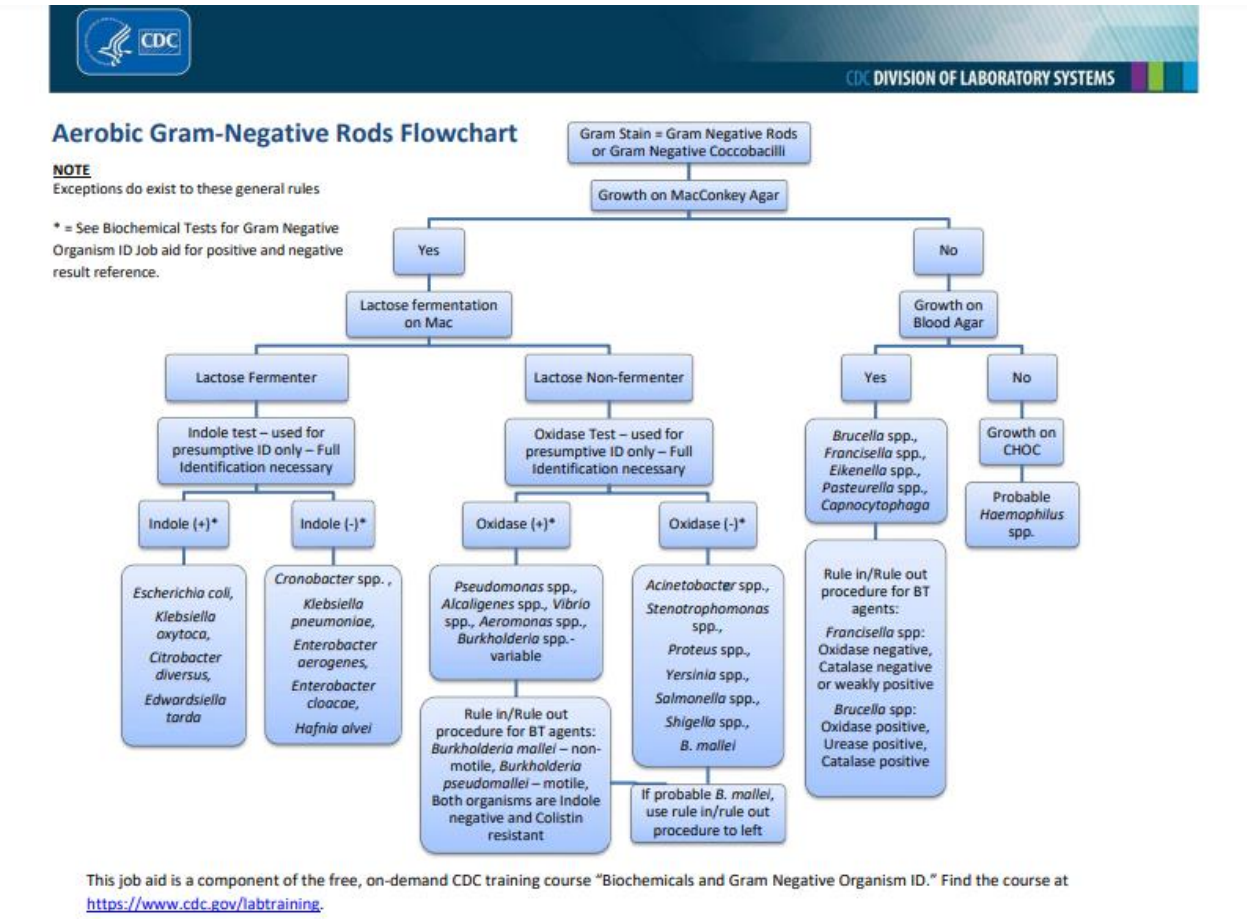

Figure F. Aerobic gram-negative bacteria rods flowchart used for bacterial identification.