

https://dspace.org/

Characterization, host bioassay, and in vitro culture of indigenous entompathogenic nematodes and their bacterial symbionts

Ngoma, Lubanza

2009-04-09T07:22:23Z
http://hdl.handle.net/10539/6880

Downloaded from a DSpace repository. https://dspace.org/

Chapter 1
Literature Review

1.1 Introduction.

1.1.1 Insect pests

The development of agriculture over the last 10 000 years has had a great impact on both man and his environment. Every year a number of insect pests cause enormous damage to vegetables and fruits grown as cash-crops all over the world (Wyniger, 1962). Pests have been defined as living organisms which cause damage or illness to man or his possessions (Matthews, 1984). Pest organisms include insects, mites, ticks, nematodes, fungi, bacteria, weeds, rodents, birds, molluscs, crustaceans and viruses. Whether an organism is a pest or not in its natural habitat, its status can be changed when it comes into conflict with man (Gullan & Cranston, 1994).

Agriculture, throughout its history, has been beset by severe pest outbreaks. Rodents have caused post-harvest losses of disturbing proportions. Certain pest infestations have lead to crop-threatening diseases, while weeds deprive crops of their much needed nutrients (Kadir & Barlow, 1992). The greatest crop loss of corn in history, approximately 1 billion dollars worth, was caused by southern corn leaf blight, which occurred in the U.S 1970 and in 1845. In another example, Ireland lost one third of its population either by death, starvation, or emigration because of the potato blight epidemic (Pimentel & Hanson, 2000a).

It is clearly evident that various pests pose a serious economic burden on agriculture. Most lands that are suitable for agricultural development are already in use. Future improvements in agricultural production will depend on better genetic crop cultivars

with attention being focused on crop nutritional needs, crop interactions with the environment, crop protection from pathogens, and competition with weeds and herbivores (Pimentel & Hanson, 2000a).

To attain the maximal potential yield of new cultivars, man has used pesticides extensively to protect crops from pests. However, the world is faced with new problems of insect resistance to insecticides. The modernization and globalization of the agricultural industry has resulted in an increase in international exchange of infected material (seed, plant and soil), and this also enhances crop susceptibility to new pests. Further factors that have enhanced agro-ecosystem vulnerability to pests include greater reliance on monocultures of major agricultural crops, greater use of fertilizers, improved water management to increase crop yield, multiple cropping, and other agronomic practices which greatly influence pest incidence (Dent, 1997). In addition, the pressure on agro-ecosystems toward greater intensity of production over the years has created new environments for pests. The food and agriculture organization (FAO) panel of experts on integrated pest control has defined integrated control as 'a pest management system that in the context of the associated environment and the population dynamic of the pest species, utilizes all suitable techniques and methods in as compatible manner as possible and maintains the pest populations at levels below those causing injury' (Pimentel & Hanson, 2000b).

An integrated pest management (IPM) strategy aims at manipulating the agro-ecosystem in such a way that pest populations are maintained below the population levels that cause sub-economic crop damage (Pimentel & Hanson, 2000b). Farmers will naturally take action if pests reduce their revenue. Ideally, treatment should be based on estimated losses in revenue in relation to estimated pest populations. The effect of one particular pest may be of crucial importance in a crop or ecological area. More frequently damage is the cumulative results of several pests. Thus lower grades of produce or simply a delay in harvesting can have a considerable impact on the

price paid, particularly with many vegetable crops that undergo price fluctuations (Matthews, 1984).

A crop pest management system can be developed to meet immediate requirements for a practical control program without detailed ecological studies. But, to improve control strategies a thorough knowledge of pest population levels and population dynamics is needed (Boucias & Pendland, 1998; Ruberson *et al.*, 1999).

1.1.2 Chemical control of insect

It is clear that chemical pesticides have helped to boost agricultural output (Hall & Menn, 1999) and synthetic chemical pesticides have played important and beneficial roles in the control of agricultural pests, and also in the reduction of insect born diseases for nearly 50 years. However, chemical pesticides also pose real costs to public health and to the environment (Weinzierl & Henn, 1991). The amount of pesticides released into the environment has risen about 1900% in the period between 1930 and 1980. The improved efficacy of the more recent pesticides has allowed application rates to be reduced in some instances to a few grams per hectare (Pimentel & Hanson, 2000b). However, the capacity to deliver these reduced amounts of agrochemicals remains suspect, and numerous researchers have estimated that only "1 to 2 arrive at the target ". Thus, it is clear that under-efficiency of utilization and application technology has been unduly neglected and suggests that any further improvement in intrinsic activity may in fact be offset by inefficiencies in delivery mechanisms (Pimentel & Hanson, 2000a).

Disadvantages of pesticide chemicals

Pest resistance to pesticides

The official definition of insecticide resistance is “an ability in a strain of some insect to tolerate doses of chemical pesticide which would prove lethal to the majority of individuals in normal population of the same species” (Robert & William, 1982). Therefore, resistance occurs simply by selection of populations for rare individuals that can survive chemical pesticides. Resistance of insects to chemical pesticides is not new. For example, in 1914, Melander described resistance of San Jose scale to lime sulfur and in 1916; Quayle reported resistance of California red scale to cyanide (O’brien, 1967). Prior to 1945, 13 insects or tick species had been reported as resistant to arsenical, selenium, rotenone, cyanide and other compounds. However, the problem now concerns the newer synthetic insecticides (O’brien, 1967). In 1945, DDT was patented, and in 1947, first reports came in from Italy and Sweden, suggesting that DDT was becoming less effective for housefly control. Since then the list of insects and pesticides involved in resistance has steadily lengthened. In 1960, the number of species involved was 137; however, when chemical pesticides declined, parasitoids were able to act and reduce insect pests to low levels (Matthews, 1984).

Chemical pesticides are fairly well recognized as an economic approach to control pests, but such chemicals are highly toxic to other beneficial species in the environment. Therefore, there is now a growing concern worldwide over the indiscriminate use of such chemicals. Many arguments have been presented suggesting that over-dependence on chemical control is not in the long-term interest of mankind. Many instances have been cited which show the negative repercussions of indiscriminate use of chemical pesticides (Pimentel & Hanson, 2000b).

It is also obvious that pesticides require proper management and administration. Indiscriminate use can precipitate undesirable consequences such as environmental contamination, especially of surface waters, which is one serious consequence of pesticide run-off, resulting eventually in the contamination of underground aquifers, and other undesirable impacts include toxic pesticides residues that remain on fruits and vegetables (Webster, 1982). An equally serious issue concerns agricultural workers who are in regular contact with pesticides and have suffered ill-health as a result. Pesticides have also been used in suicide attempts. Some chemical pesticides are suspected to be carcinogens. In some regions, pesticide misuse has evolved into a worrying social problem. The World Health Organization estimated (WHO) that there are 25 million of cases of acute occupational pesticide poisoning in developing countries and 20,000 deaths world-wide.

There have been massive upsurges in pesticide use in recent years. Increasing use, and often misuse, has led to increased problems of resistance to insecticides. In addition, there are problems of destruction of beneficial insects and other non target-organisms. Also the accumulation of large stocks of obsolete pesticides gives rise to serious disposal problems and stock piling of obsolete pesticides can lead to spillage or the release of toxic residues which can result in human poisoning and environmental contamination. New chemicals with improved properties are available but they are beyond the financial means of many farmers in developing countries (Harris & Dent, 2000).

1.1.3 Biological control

The development of insect resistance to chemical insecticides and pesticides has been a real driving force for changes in insect pest management. Ehlers (1990) defined the term biological control as "the action of natural enemies (arthropod predators, insect parasitoids and microbial pathogens) that maintains a host population at levels lower than would occur in the absence of these enemies".

Biological control methods based on organisms which are highly specific to the target pest, have gained momentum as a major component of integrated pest management systems. Reduction in the use of synthetic chemical pesticides in cropping systems depends on the development of alternative environmentally friendly crop pest control, such as biological control. Biological control is based on biological agents such as beneficial insects, entomogenous/entomopathogenic nematodes, fungi, bacteria, viruses, and protozoa. Application of biological agents to cropping systems can provide ecological and effective methods for the control of insect pests, especially for pests that have developed resistance to traditional chemical pesticides.

Biological control leaves no toxic residues and is generally harmless to other beneficial insects and other non-target organisms (Harris & Dent, 2000). A major benefit is that they are safe for use by humans and they represent a reduced environmental hazard in terms of disposal and reuse of packaging compared to chemical pesticides. Since they are already in use to control insect pests in high value crops, they have the potential to be used on a large scale in integrated pest management, organic farming and sustainable agriculture systems.

When compared with chemical insecticides, biological control has many advantages such as no side effects, no residual pollutants residing in surrounding ecosystems and potentially low production costs, plus long term sustainability in terms of the capacity

of the biocontrol agent to become self-propagating and self perpetuating within the cropping system (Emden & Service, 2004).

Biological control has in recent years received much attention particularly from entomologists since increasing publicity regarding the problems associated with the use of chemical pesticides (Burges & Hussey, 1971). It should be an important part of any integrated pest management program, especially in approaches which combine a variety of pest control methods to reduce pest levels below an economic damage threshold. Virtually all insect and mite pests have some natural enemies. Managing these natural enemies can effectively control many pests. Often the use of insecticides or other practices can injure or kill natural enemies, increasing the survival of the remaining pest insects (Wright, 2006).

There are three basic components of biological control: importation, conservation and augmentation. A great number of predators and parasites prey on insect hosts which are crop pests, thus contributing to their population density in crop systems (Wyniger, 1962).

The reduction in pest population density below the level at which they cause economic damage (economic threshold) by the deliberate introduction of exotic natural enemies has been referred to as classical biological control. The designation ”classical” has been applied in recognition of its relatively early first use in the 1800s. Although the origins of many pests are uncertain the impression inferred from the literature is that success is largely dependant on obtaining the natural enemies of the pest from its area of origin. (Waage, 1999).

Classical biological control, however, has been very cost-effective because once established the system becomes self-sustaining. Pests can also be controlled by the propagation and local release of large numbers of natural enemies, which are often indigenous. Maintaining the facilities to produce large numbers of natural enemies for these inundative or augmentative releases, especially if the pest does not exceed its

economic threshold every year, is likely to be very expensive. In addition, the number of natural enemies can be increased locally by cropping practices that provide alternative prey or hosts for them to attack and propagate on before the target pest exceeds its economic threshold. The expectation is that these natural enemies will switch to attacking the pest as soon its relative density increases and thus prevent it from reaching levels of abundance that could inflict economic damage. Both are included under the heading of augmentative biological control (Hoy & Herzog, 1985)

1.1.4 Entomopathogenic nematodes (EPNs) as biological control agents

Out of the different biological control methods, EPNs of the genera *Steinernema* and *Heterorhabditis* have attracted the attention of nematologists for a number of years for their use in controlling economically important insect pests (Sturhan & Mracek, 2000). With increasing restrictions on the use of chemicals and the mounting problem of resistance, such nematodes have become a valuable addition to the range of biological control agents available for insect pest management since they possess many of the attributes of an effective biocontrol agent. For example, they kill their target hosts within 24-48 hrs following application, their application does not require special equipment, they have no negative effects on ground water contamination, residues, chemical trespass and pollinators, and they pose no health, environmental or ecological risks. (Sturhan & Mracek, 2000).

Nematodes are considered as one of the most abundant groups of living animals, even though they morphologically are very simple compared to the specious Arthropod and Mollusca phyla. They have a worldwide distribution and occur in a variety of soil types and habitats (Travasoss & Sobre, 1927). Most of us are educated to consider nematodes as harmful organisms, attacking plants and parasitizing vertebrates,

including man. It is true that most studies on nematodes are done on species that cause some damage to man and his products, but there are other types of nematodes that are beneficial to man as well (Poinar, 1979). Nematodes can be classified into several groups depending on their nourishment. For example, there are the free-living microphagous forms, invertebrate predatory forms, plant-parasitic forms, vertebrate parasitic forms, and forms that are parasites of invertebrates. It is the latter category that is the focus of this study. Nematodes can parasitize spiders, leeches, annelids, crustaceans, molluscs, and other groups of invertebrates, including insects. When this parasitism results in death of the host, these forms are potential biological control agents. When the host is an insect pest, then the possibility of control becomes more intriguing. To be included in this work, an EPN must meet three criteria. First, it must attack insects that are considered pests of man. Second, it must kill, sterilize or seriously hamper the development and propagation of the insect; and third, the artificial or laboratory propagation of the nematode must be possible. For the latter criteria on propagation must be possible through at least one complete generation, either *in vivo* or *in vitro*, under laboratory conditions, in order to demonstrate the potential for mass production (Gaugler, 1998).

EPNs represent one important part of the spectrum of biological control agents that has been shown to be economically feasible and environmentally safe (Bathon, 1996: Ehlers & Hokanen, 1996). Since they have already been used to control insect pests in high value crops, they also have the potential to be further used in large scale integrated pest management in organic farming and in sustainable pest management programmes in various agriculture systems. The success of biological control, however, will also depend on the understanding of the adaptation and establishment of introduced biological control agents in agricultural ecosystems (Gaugler, 1998).
The discovery of EPN species and the rate at which new species have been described has been stimulated by the historical need for developing biological alternatives for the management of insect pest. Nematode parasites of insects have been known since the 17th century, but it was only in the 1930s that serious consideration was given to

using nematodes to control insect pest (Adams & Nguyen, 2002). In 1929, Glasser and Fox found a nematode infecting grubs of the Japanese beetle, *Popillea japonica P. japonica*) (at the Tavistock golf course near Handdonfield, New Jersey. Steiner described the nematode that same year as *Steinernema glaseri* (*.S. glaseri*), we now know that these nematodes carry a symbiotic bacterium which provides essential food for nematodes both *in vivo* and *in vitro* (Smart, 1995).

Research on EPNs remained somewhat dormant as chemical-based pest control measures remained cheap, effective and relatively unregulated. Upon recognition of their negative environmental effects in the 1960s, pesticides gradually became more restricted, less effective, and much more costly. Consequently, the search for biological alternatives to chemical-based pest management programs received renewed attention from scientists (Griffin *et al*., 2001).

1.1.5. Biology and life cycle of EPNs

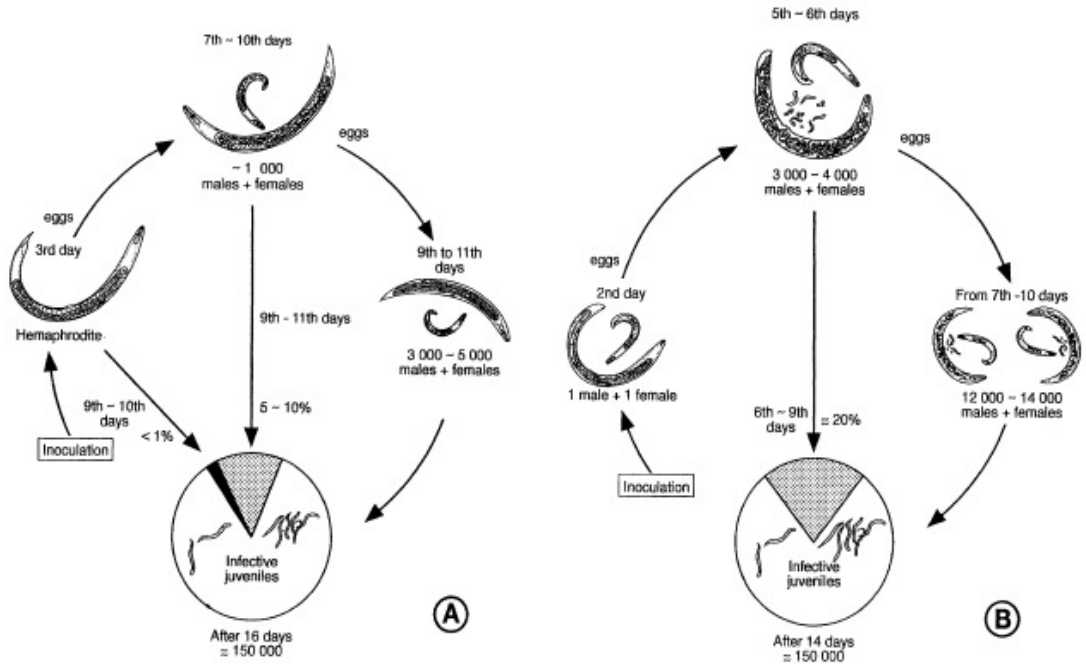


Figure1.1. Life cycle of: A) *Heterorhabditis.bacteriophora* and B) *Steinernema. carpocapsae* (Taken from Wang & Bedding, 1996).

EPNs are ubiquitous, natural microscopic parasites, simple rounds worms, colorless, unsegmented and lacking appendages. They are extraordinarily lethal pathogens to many important soil insect pests, yet they are safe for plants, vertebrate animals and humans (Gaugler, 1998). The parasitic cycle of nematodes is initiated by several developmental stages, starting with eggs, developing through three juvenile stages (Js3) and the adult stage (Fig 1.1). One of the juvenile stages can be become arrested in a free living, non-feeding development state. This free living, non-feeding stage constitutes the infective juvenile stage (IJs) or Dauer stage which is derived from the 3rd stage juvenile. The IJs is adapted both physiologically and morphologically for long term survival in extreme environmental conditions in the surrounding soil outside the host cadaver. Upon location of a potential host, the IJs of *Steinernema* penetrate into the insect body through natural body openings (the mouth, anus,

spiracles). In addition to these modes of entry, *Heterorhabditis* also gains entry by abrading the intersegmental membranes of the insect using a dorsal tooth (Stock *et al*., 1999). Once inside the host insect, the nematode invades the haemocoel and releases the symbiotic bacteria that are held in the nematode's gut. The bacteria cause a septicemia, killing the host within 24-48 hours after penetration (Hall & Menn, 1999).

The bacteria further contribute anti-immune proteins to assist the nematode in overcoming host defenses, and anti-microbial agents that suppresses colonization of the cadaver by competing secondary saprophytic microbail invaders. Conversely, the bacterium, which is an insect pathogen or primary virulent agent, lacks invasive powers and is dependent upon the nematode as a vector to locate and penetrate suitable hosts (Gaugler, 1998).

The IJs then feed on the rapidly multiplying bacterial cells, degrade host tissues and mature into adults. One or more adult reproductive generations may develop within the host cadaver, depending on available nutrient resources (Poinar, 1990b; Kaya & Gaugler, 1993). When food reserves are depleted, nematode reproduction ceases and the offspring develop into resistant IJs, corresponding to the stage 3 juvenile, that disperse from the dead host, and are able to survive in the environment, and to seek out new hosts (Hall & Menn, 1999). The life cycles of *Heterorhabditis* and *Steinernema* are similar, they have many features in common (i.e. association nematode and bacteria, single free-living stage) but, they also differ in their mode of reproduction (Poinar, 1993).

The IJs of *Steinernema* mature into amphimictic adults, having both males and females. In *Heterorhabditis* by contrast, the IJs mature to give first generation adult hermaphrodites, but these females give rise to a second generation of amphimictic males and females, plus self fertilizing hermaphrodites (Johnigk & Ehlers, 1999). They complete their reproductive cycle within the host from which thousands of new

IJs emerge and search for new hosts (Dix *et al*., 1992; Strauch *et al.,* 1994). Initially, eggs are laid into the host medium, but in *Heterorhabditis*, eggs hatch *in utero,* killing the female in a phenomenon known as *endotokia matricida*. It has been demonstrated that low food supply significantly promotes the induction of *endotokia matricida.*

1.1.6 Ecology of EPNs

Soil is an excellent habitat for nematodes, and 100 cm^3 of soil may contain several thousand nematodes. Their importance as biocontrol agents to agriculture depends on the IJ's ability to disperse and persist in the soil long enough until hosts can be located. Dispersion and persistence in the soil is crucial for the success of EPNs as biocontrol agents of insect pests in the soil, and it also important for the survival of naturally occurring populations of EPNs. Motility and persistence may be influenced by intrinsic factors (e.g. behavioral, physiological, and genetic characteristics) and extrinsic factors of abiotic (e.g. temperatures, soil moisture, soil texture, RH and UV radiation (Kaya, 1990; Smits, 1996) and biotic nature (antibiosis, competition, and natural enemies) (Koppenhofer& Kaya, 1996).

Soil moisture is the most important soil environment factor that affects nematode activity, because nematodes need a thin film of water for effective propulsion (Walace, 1971). In soil IJs move through the water film that coats the soil particles surrounding the interstitial spaces. If this film becomes too thin (in dry soil) or the interstitial spaces are completely filled with water (in water saturated soil), nematode movement can be restricted (Koppenhofer *et al*., 2000). Soil moisture content is closely linked with soil texture, another important factor, because soil particle size composition and organic matter content in soil strongly influence the availability of water in a given soil. Nematode motility generally decreases as soil pores become smaller (Kaya, 1990). Small soil pores, particularly in combination with higher soil moisture, will also limit oxygen levels and with that the activity and survival of

aerobically respiring EPNs (Burman & Pye, 1980; Kung *et al*., 1990). Consequently, nematode efficacy against soil-dwelling insect should generally decrease in finer-textured soils.

1.1.7 Bacterial symbionts

Xenorhabdus and *Photorhabdus*

The bacteria-nematode symbiotic association forms the basis for the nematode's pathogenicity and effectiveness as a biocontrol agent (Ehlers & Hokkanen, 1996). The bacterial symbiont facilitates the break down of the insect tissues thereby providing a rich food supply for the developing nematodes and produces antimicrobial substances to ward off other invading microorganisms (Hall & Menn, 1999). Furthermore, bacteria alone are incapable of penetrating the insects' alimentary tract and cannot independently gain entry to the host haemocoel. Thus nematodes act as vectors to transport the bacteria into a host within which they can proliferate, and the bacteria create conditions favourable for nematode survival and reproduction within the insect cadaver (Poinar & Thomas, 1966; Forst & Clark, 2002).

Photorhabdus and *Xenorhabdus* species are mutualistically associated with the IJs of heterorhabditids and steinernematids respectively. The life of both symbionts comprises a symbiotic stage in the nematode's gut and a virulent stage in the insect larvae, which it kills through toxemia and septicemia (Duchaud *et al.,* 2003). *Xenorhabdus* exist in a specific ventricular vesicle of the intestine of the free-living *Steinernema* IJ stage, while *Photorhabdus* are located in the esophagus and in the ventricular portion of the intestine of the free-living nematode IJ stage (Boemare *et al.,* 1996).

Xenorhabdus and *Photorhabdus* are motile, Gram-negative, non-spore forming facultative anaerobic rods, and members of the Enterobacteriaceae (Forst *et al,* 1997). Both bacteria are negative for nitrate reductase and *Xenorhabdus* is negative for catalase activity. Typical for symbionts of both the genera is the phenomenon of phase variation; the two extremes of which are the primary and secondary phase (Akhurst, 1980). Intermediate phase have been reported (Gerristen & Smits, 1997). Differences between the primary and secondary forms are mostly biochemical; the primary form produces antibiotics, adsorbs certain dyes, and develops large intracellular inclusions composed of crystal proteins, whereas the secondary form does not or only weakly produces antibiotics, and does not absorb dyes and produces intracellular inclusion bodies inefficiently. The primary form is superior to the secondary form in its ability to support nematode propagation *in vitro*. The reason for the occurrence of the 2 forms is unknown to date (Hazir *et al,* 2003). The primary phase is isolated from IJs or infected insects, their colonies are granulated, convex, opaque and circular with irregular margins, cells are small to middle sized, show positive antibiotic activity and they are red, bright pink or red-brown on MacConkey agar because, they absorb neutral red and on NBTA they absorb bromothymol blue and reduce triphenylterazolium chloride and exhibit strong bioluminescens in the dark. Secondary phase occurs either after *in vitro* sub culturing or *in vivo*, when the nematodes emigrate from the cadaver. Colonies are yellow-brown colour, flat may have translucent, rather flat, wide colonies and show very poor antibiotic activity. On NBTA colonies do not absorb the dye; the colours vary from blue to green to purple then red depending on the species for phase I colonies (Grunder, 1997). The secondary phase is not retained by the IJs of *Heterorhabditis bacteriophora* (*H. bacteriophora*) (Han & Ehlers, 2001). Some nematode species share the same bacterial species. For example, *Xenorhabdus bovienii* (*X. bovienii*) is associated with four species of *Steinernema,* and *X. Poinarii* is associated with two species of *Steinernema*. More rarely, some bacterial species share the same nematode species: for example, *Photorhabdus luminescens* (*P. luminescens*) and *Photorhabdus*

temperata (*P. temperata*) are both associated with the *H. bacteriophora* group (Grewal *et al.,* 1994).

1.1.8 Molecular and morphological characterization of EPNs

EPNs of the families Steinernematidae and Heterorhabditidae, together with their symbiotic bacteria *Xenorhabdus* sp. and *Photorhabdus* sp., respectively, are currently being mass produced commercially and used to control a variety of soil-dwelling insect pests in Europe, USA, Australia and China (Kaya & Gaugler, 1993; Ehlers, 1996). Discovery of new EPN species can substantially expand or improve the utility of these organisms in biological control. For example, discovery of *S. riobrave* and the recent discovery of *S. scarabaei* promise to expand nematode control of white grubs (Stock & Koppenhofer, 2003). Once a new species has been discovered, rapid and reliable diagnostic tests are required for species identification in such surveys and DNA fingerprint techniques are now becoming more widely used as a first screen to determine the species affiliation of newly isolated EPN collections. These molecular approaches can then be supplemented by morphological, morphometric and crossbreeding techniques to confirm the identification of putative new species (Hominick *et al*., 1997). *Steinernema* and *Heterorhabditis* share many similarities in their mode of life and morphology; these similarities result from convergent evolution and are not indicative of a close phylogenetic relationship between the two families (Poinar, 1993; Blaxter *et al*., 1998). *Steinernema* also appears to be more species rich than *Heterorhabditis*.

1.1.9 Motivation

- This project will increase our knowledge of the biology and systematics of indigenous EPNs in South Africa.

- Information arising out of this research will be helpful in advising farmers on the rational use of EPNs as biological control agents in agriculture.

1.1.10 The main objectives

- Characterization and identification of indigenous South Africa EPNs using molecular techniques such as 18S ribosomal DNA sequence analysis and morphological observation.
- Characterization of bacteria endosymbionts of the indigenous EPN isolates using 16S ribosomal sequence analyses.
- Application of bioassays to determine the virulence and infectivity of the indigenous EPN isolates. In these experiments the hosts for the virulence bioassays will be *Galleria mellonella*, *Tenebrio molitor* larvae and pupae.

1.1.11 Research strategy (Fig 1.2).

- EPNs were isolated from the collected soil samples by baiting the soil with larvae of *Galleria mellonella*.
- Re-infection of *Tenebrio molitor*, *Galleria mellonella* and pupae with IJs isolated in White traps from infected larvae was used to establish Koch's postulate.
- Putative EPNs were maintained by re-infecting larvae from White trap isolated IJs. All re-infected cycles involved an IJ recovery White trap step.
- Once consistency and reproducibility with regard re-infection efficacy and virulence was established, the EPNs status of the nematodes were verified by 18S rDNA sequence analysis.
- Isolated EPNs were characterized in terms of molecular systematic using 18S ribosomal DNA sequence data.

- Isolated bacterial endosymbionts were characterized with respect to the 16S ribosomal DNA sequence data.
- The virulence of isolated EPNs was determined by bioassays.

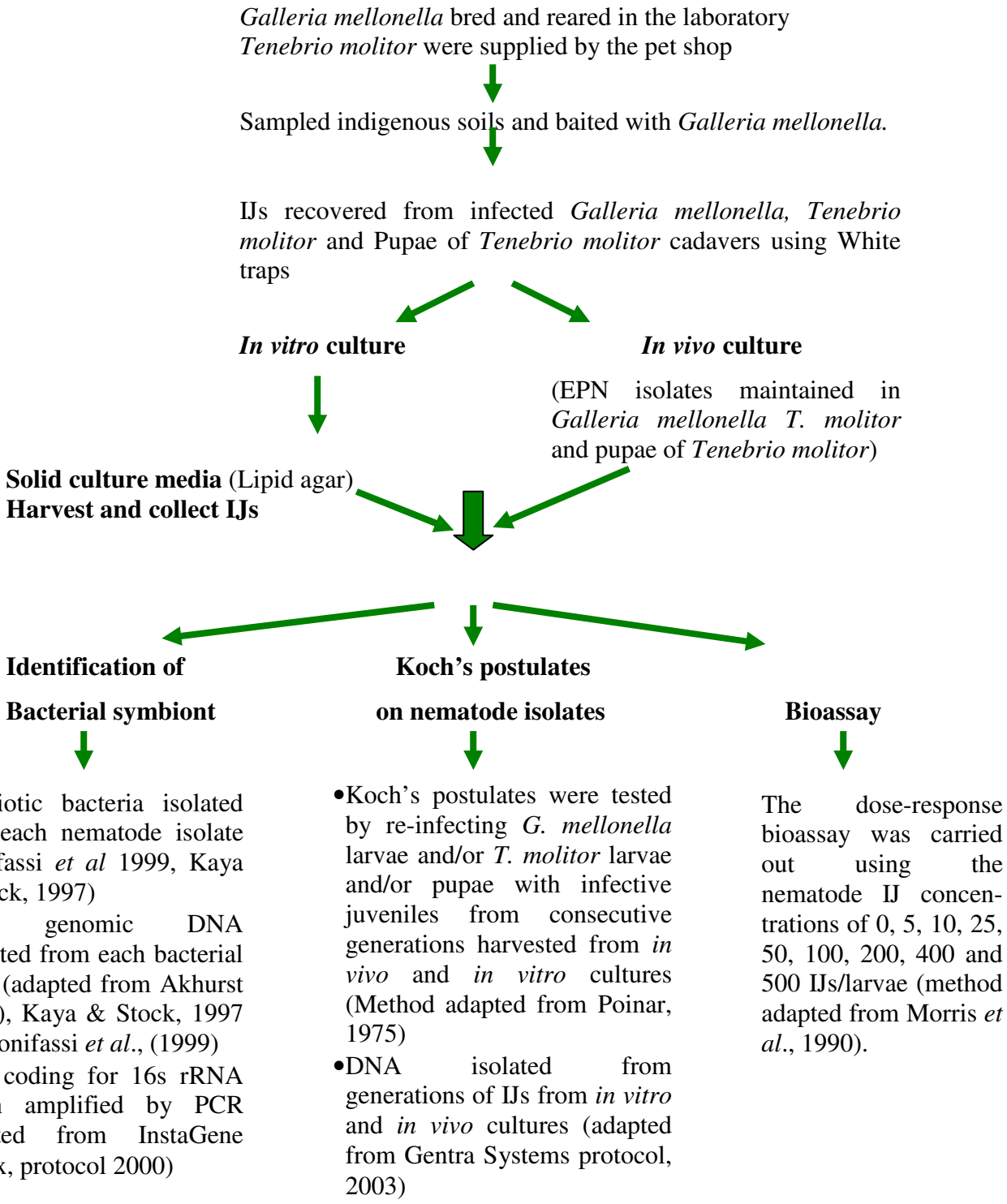


Fig 1.2: Overview of methodology used in this study.

Chapter 2
Materials and Methods

2.1 *Galleria. mellonella* (*G. mellonella*) breeding

Order: Lepidoptera
Family: Pyralidae
Scientific name: *G. mellonella*
Common name: Greater Wax Moth

The greater wax moth, *G. mellonella* is considered as a suitable host insect for propagating EPNs by nematologists because they are widely available, easily reared in the laboratory, highly susceptible to EPN's. Thus historically, *G. mellonella* larva has become the preferred host for the routine *in vivo* propagation of EPNs. However, in its natural state it is considered as a serious pest of honey-bee colonies (Williams, 1978). The greater wax larvae are used as a live-bait to isolate nematodes from soil sample and for culturing them *in vivo*. The adult moths and larvae are obtained from infested beehives and these moths were developed from eggs to adults in the same environment. Adult wax moths (male and female) were kept in 3 L glass bottles (Consol® glass jars) (11cm diameter and 23 cm height) at 25-28^{o}C on an artificial medium adapted from Woodring & Kaya (1988). The metal Consol ® jar lids were modified by cutting an 8 cm diameter circular opening into the lid (Fig 2.1). A 9 cm diameter circular stainless steel mesh screen (350µm) was placed on the inside or underside of the modified lid. The metal screen was clamped in place sealing off the opening by screwing the lid tightly onto the jar. The screen facilitated heat and air exchange while preventing larval or moth escape from the jar. Pleated or crumpled wax paper was inserted into the jar to be used as an oviposition site. Female moths

were allowed to mate and lay eggs. Eggs were also laid on the edge of the lid or on the paper and were collected by placing a razor blade gently under one side and lifting the eggs. Eggs were placed in another bottle filled with sterilized, cooled *Galleria* medium. Eggs hatched within 3-4 days. Larvae were fed weekly with fresh media.



Fig 2.1 Adult wax moths (male and female) and larva kept in 3L glass bottles

The *Galleria* medium (Appendix II) used in this study was adapted from Woodring & Kaya (1988). It consisted of a mixture of 500g of wheat cereal (Bokomo Pronutro^{TM}), 200 ml of glycerol, 200 ml of honey, 200 ml of boiled water, 5 tea spoons of yeast extract, 1.5 ml of IM HCl, and 1 g of sodium benzoate was added as a preservative to prevent growth of bacteria/fungal. The ingredients were first thoroughly mixed to form a dough-like mixture before being autoclaved at 121°C for 15 min. After 20 to 30 days, the larva reached the last instar and they were collected to be used in the experiments (to isolate EPNs from soil samples or for the *in vivo* culture of nematode isolates). Some larvae were left in containers to pupate. Two weeks later, adult moths emerged and females laid eggs (Fig 2.2).



Fig 2.2: A) The greater wax moth, *G. mellonella* insect. B) *G. mellonella* larvae

2.2 *Tenebrio. Molitor* (*T. molitor*)

T. molitor (Coleoptera: Tenebrionidae) (Fig 2.3) lay tiny, white, bean-shaped eggs are about 2 mm long by 9 mm wide. The larvae are dark yellow with brown bands; they are up to about 35 mm long, have a segmented body, six legs (towards the front of the body). The pupa is white/cream with a large head and a pointed tail (it darkens as it grows). The adult ranges in length from 12 to 25 mm and is dark brown. The mealworm larvae were used in various experiments (to isolate EPNs from the soil samples or for the *in vivo* culture of the nematode isolates). These larvae were obtained from commercial suppliers.



Fig 2.3: *T. molitor*

2.3 Sampling strategy.

Soil samples (Fig 2.4) were collected from ARC Roodeplaat experimental farm near Pretoria in Gauteng province (Fig 2.6) and Brits in North West Province (Fig 2.5). The collection sites were investigated over the first 3 months of the project. Soil samples for EPNs isolation were obtained according to standard principles, using appropriate sampling techniques. Random sampling was undertaken from each site to isolate, and identify EPNs. At each site soil samples were taken and mixed thoroughly in container plastic (170 x115 x 750 mm) to minimize moisture loss during transit to the laboratory and the following information was recorded at each site: date, name of field, and associated vegetation. The soil texture were classified as either sand, sandy loam, loam, clay, clay loam and the moisture content of each soil sample were approximated with wet, medium or dry according to a field soil texture procedure described by Fitzpatrick *et al.* (1999). Soil was taken from the top 15 cm of the soil profile using a stainless steel.

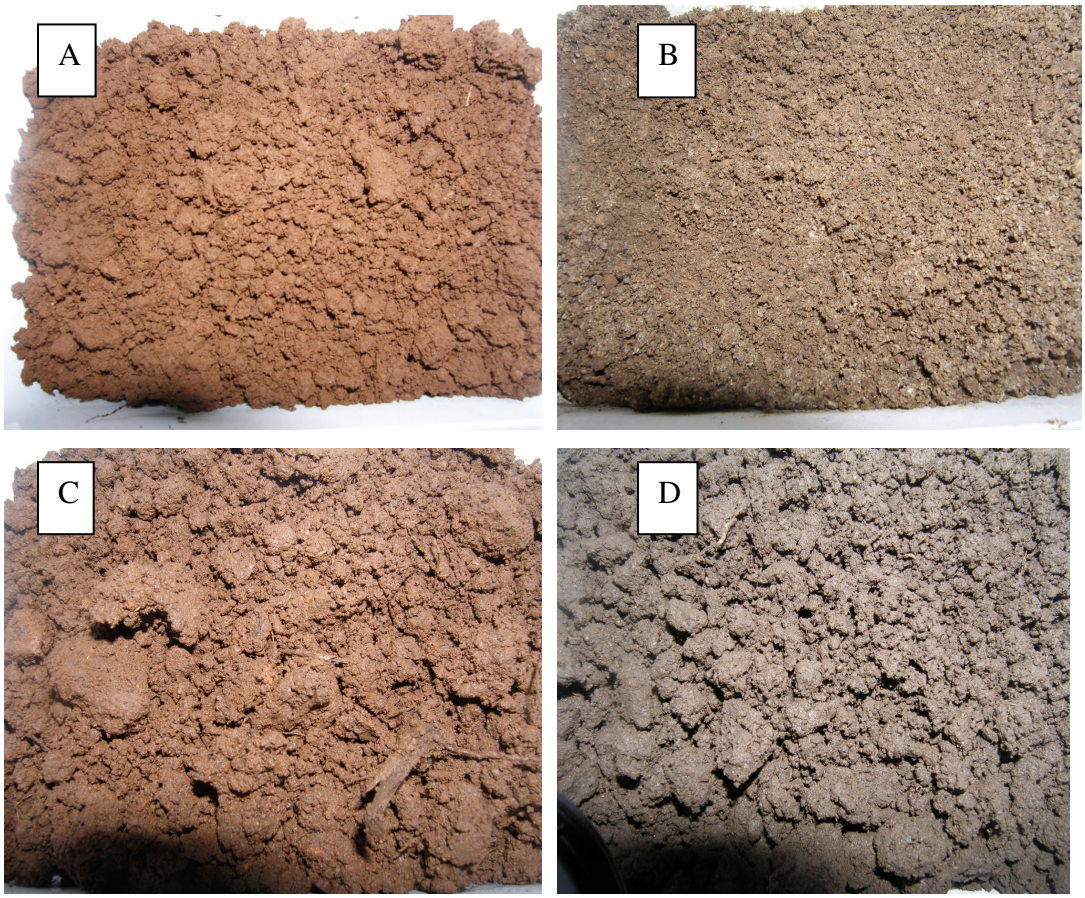


Fig 2.4: Type of soil samples: A): Clay soil, B): Sandy soil, C): Clay loam, D): Sandy loam.

2.4 Origin of the EPN isolates

2.4.1 Brits

Brit (Fig 2.5) is a large town and district situated in a fertile citrus producing area that is irrigated by the water of the Hartbeespoort Dam in the North West Province in South Africa. Brits is a important centre of agriculture, it is close to the City of Tshwane Metropolitan Municipality in Gauteng which includes Pretoria.

2.4.2 Agricultural research council (ARC)

ARC (Roodeplaat) (Fig 2.6) is situated about 25 km north of central Pretoria, on the Moloto/KwaMhlangu road. The farm covers approximately 4 000 ha, of which only 650 ha is irrigable land



Fig 2.5: Map of North West Province, South Africa-highlighting town around which soils were collected. (Source: www.stayinsouthafrica.co.za/images/northwest.jpg)

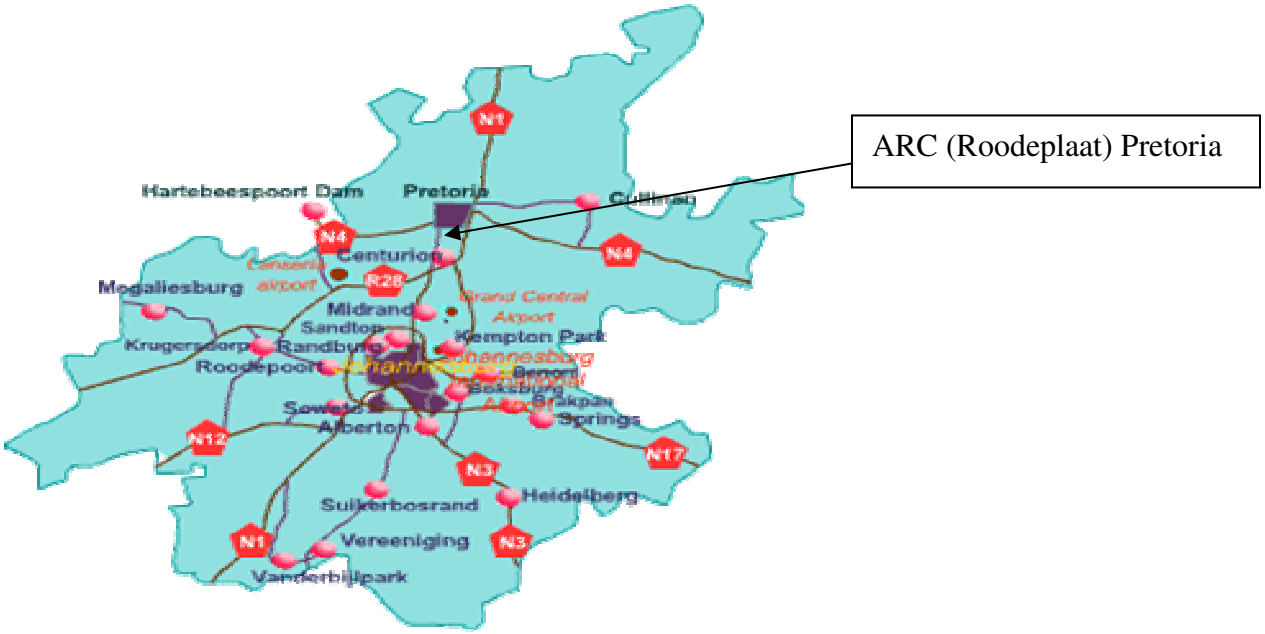


Fig 2.6: Map of Gauteng Province, South Africa-highlighting town around which soils were collected. (Source: www.africangamesafari.com/gauteng_map.gif)

2.5 EPNs isolation

2.5.1 Live-bait method

Although competition between different pathogenic nematode species or strains in a soil sample could still introduce bias into sampling with insect baits (Stuart & Gaugler, 1994; Sturhan & Mracek, 2000), the baiting technique with larvae of the greater wax moth, *G. mellonella* (Fig 2.2), was the method most commonly used for recovering infective-stage juvenile of EPNs from soil (Bedding & Akhurst, 1975; Mracek, 1980; Fan & Hominick, 1991; Ahmad & Hussain, 2002). Soil moisture was checked and adjusted by spraying with water if it was observed to be too dry for nematode movement; this was done as described by Kaya & Stock (1997). Ten last instar larvae of the wax moth *G. mellonella* were buried in approximately 1 kg of soil in 1 L plastic ice cream containers and covered with lids which have 20 small aeration holes, so as to facilitate infection by EPNs. While *G. mellonella* are highly

susceptible to insect pathogens, the bait insect method (Fig 2.7) could be biased for the isolation of lepidopteran-active EPNs (Barker & Barkr, 1998). Different baiting techniques were also conducted. For example, instead of burying the larvae, the larvae were placed on the top of the soil in the plastic tubs and the tubs covered with the lids. In other cases, after placing the larvae on top of the soil and closing with the lid, the tubs were then turned over so that the larvae were covered by the soil. All larvae baiting procedures gave positive results. In many instances, un-infected larvae survived being buried in the turned over tubs for up to 7 days. The containers were then held at room temperature (22-25°C) and checked at regular intervals for larvae mortality over a period of 7-14 days, after which time the soil sample was sieved to collect the larvae.

Dead larvae that exhibited signs of infection with EPNs were placed in modified White traps (Kaya & Stock, 1997) to collect the emerging IJs in the water moat. The IJs recovered from the moat of the White traps were then exposed to fresh *G. mellonella* (Fig 2.2) or *T. molitor* (Fig 2.3) larvae or pupae of *T. molitor* (Fig 2.3) in Petri dishes containing river sand (30 g) with 8% moisture to confirm pathogenicity by re-infection. Soil samples which were negative with respect to recovery of EPNs were baited with larvae for a second time to confirm the absence of EPNs in the first test. All negative soil samples were discarded after 3 months following collection.



Fig 2.7: *T. molitor* live bait method

2.5.2 White traps

The modified White traps (Fig 2.8) consisted of plastic Petri dish (9 cm diameter), filled with distilled water to a depth of 0.5 cm (White 1927). An inverted smaller plastic Petri dish cover (5 mm) or Syracuse watch glass (about 5×3.5 cm depending on plastic container) was placed in the water containing 9 cm Petri dish. A Whatman N°1 filter disk (5 cm) was placed on the Syracuse watch glass or on the inverted 5 mm Petri dish cover, so the edges of the filter paper disk come into contact with the water in the 9 cm Petri dish. The insect larvae infected with EPNs were placed on the moist filter paper disks on top of Syracuse watch glass or inverted 5 cm Petri dish cover, and the 9 cm Petri dish was then covered with its lid. White traps were incubated at room temperature (22-25°C). As IJ nematodes emerged from the insect cadaver, they migrated into the water moat surrounding the filter paper disk. The IJs in the distilled water were collected after 7-15 days following the application of infected larvae to the filter paper disk. Following removal of the water containing the IJs, fresh distilled added to the white traps until no more IJs could be observed (Kaya & Stock, 1997).

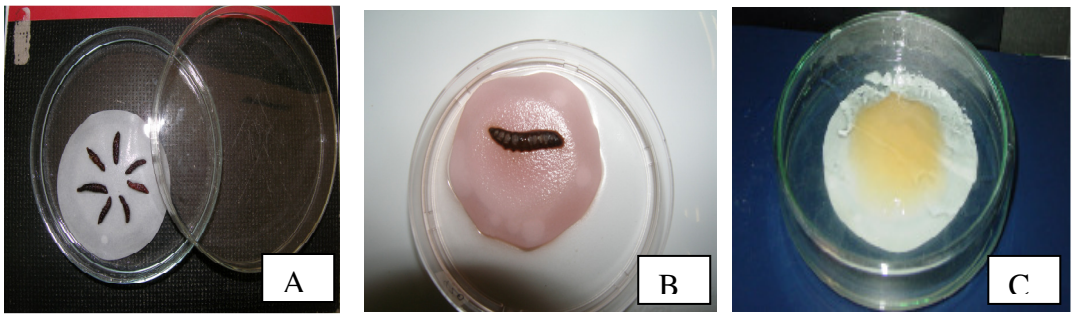


Fig: 2.8: A, B & C) illustration of White traps used to collect IJs from *G. mellonella*, *T. molitor* and lipid agar.

2.5.3 Harvesting of nematodes

Water samples containing IJs that had been collected from the White traps (section 2.5.2) were poured into 45 ml centrifuge tubes. Nematodes were left for about 30 minutes to settle at the bottom of the tube. After removing the supernatant the nematodes were washed by resuspending in fresh water. The washing process was repeated three times until the supernatant become clear. The nematodes were pooled for each sample and used to infect fresh *G. mellonella*, *T. molito*r larvae and pupae to produce nematodes for identification and establishment of cultures.

2.5.4 Maintenance of EPNs.

All nematode isolates were maintained by *in vivo* and *in vitro* culture procedures using methods adapted from Kaya & Stock (1997).

2.5.4.1 *In vivo* method

The greater wax moth (section 2.1) that has been the most common used in EPN studies, because of its susceptibility to nematodes, wide availability and its ability to produce high yields (Woodring & Kaya, 1988). *Tenebrio* larvae or pupae (section

2.2) were also used and treated in the same manner as the *G. mellonella* larvae. The *in vivo* system takes advantage of the IJs natural migration away from the host cadaver upon emergence. Five to ten wax moths *G. mellonella* or *T. molitor* or pupae of *T. molitor* were exposed to IJs in sterile Petri dishs lined with absorbent filter papers disc. (Whatmann 1, diameter 90 mm) or filled with 30 g sterilized sieved river sand (particle size 300 μm) containing 8% water. The IJs were transferred to each Petri dish in volume of 2 ml of distilled water containing about 100 active IJs. The Petri dishes were then stored at room temperature (25°C). These particular plates were chosen because the Petri dishes have a relatively small volume. The insect larvae, especially *G. mellonella* which often climbed onto the upper covering lid of the Petri dish, where thus forced to be in close proximity to EPNs. *G. mellonella* larvae that managed to cling onto upper lid were also infected, especially if 30 g of sand had been placed into the lower dish. After 2 to 7 days, the infected larvae were transferred to White traps (section 2.5.2) and the IJs were harvested. (Kaya & Stock, 1997).

2.5.4.2 *In vitro* solid method

EPNs were cultured on lipid agar adapted from Kaya & Stock (1997). The important factor seems to be monoxenicity, with the nematode and the associated bacterium as the only biotic agents present in or on the culture medium. With the *in vitro* solid agar method (see Appendix VI), symbiotic bacteria were introduced first and after a bacteria lawn become established; the Petri dishes were inoculated with the sterile nematodes IJs (Kaya & Stock, 1997). The symbiotic bacteria of the respective EPN isolate to be cultured was isolated and cultured aseptically for two days in nutrient broth and streaked onto NBTA or MacConkey agar plates to select for phase one colonies. Phase one bacteria were obtained by selecting green or blue-green colonies from NBTA, nutrient agar supplemented with 25 mg 1 bromothymol blue and 30 mg 1, 2, 3, 5-triphenyltetrazolium chloride or red colonies from MacConkey agar. Two milliliters of the phase one bacterial culture was added to sterilized lipid agar and incubated at room temperature (25°C) for 3 days. This incubation period allowed for

bacterial growth. Surface sterilized IJs of the isolate to be cultured were added to the lipid agar under sterile conditions and the lipid agar plate was incubated at 25°C. After three days of incubation the IJs were collected using White traps. In this procedure, squares of lipid agar were removed and placed directly onto the filter paper disk of the White trap.

Ingredients per 1 L of distilled water for the lipid agar medium:

- 10 g corn syrup
- 5 g yeast extract
- 25 g nutrient agar
- 2.5 ml cod liver oil
- 2 g $MgCl_2.6H_2O$

**10 g of honey was used in place of corn syrup*

2.6 Koch's Postulates

A modification of Koch's postulates adapted from Poinar (1975) was developed to test the entomopathogenic status of the nematode isolates. The nematodes apparently produced a disease in the larvae which killed insects. The following procedure was followed to determine the pathogenic status of the nematode isolates suspected of being entomopathogenic:

- The same nematode species should be found consistently associated with the diseased insect
- The nematode should be isolated and its infective stage obtained
- The infective stage should then be introduced into the external environment of the hast insect and produce the disease
- The same nematode isolates should be recovered from the experimentally infected insect.

2.7 Identification of Nematode Isolates

2.7.1 Molecular identification of EPNs

2.7.1.1 Extraction of DNA

Phylogeny in relation to 18S rDNA sequences was investigated. Total genomic DNA was extracted from several adult nematodes as outlined in Appendix III using methods adapted from Puregene DNA purification Kit, (# D-7000A Gentra system). The protocol was as follows: adult nematodes reared on sterile lipid agar plates (Kaya & Stock, 1997) were collected from White traps under sterile conditions under a laminar flow hood, and placed in 2 ml Eppendorf tubes. The 2 ml Eppendorf tubes were centrifuged at 12000 xg for 10 minutes to pellet the IJs. The excess water was removed and the tubes vortexed to re-suspend the pellets. The tubes containing the nematodes were frozen at (-194) in the liquid nitrogen and then the nematodes were ground up in the tube with a small nylon pestle. After which, 600 µl of cell lysis, 3 µl proteinase K, 3 µl RNase A, and 200 µl protein precipitation reagents were added in the tubes. Following this, the upper aqueous layer containing DNA was extracted in 100% isopropanol (2-propanol) and the Eppendorf tubes were micro-centrifuged for 10 minutes at 1400 xg to obtain a nematode pellet. The pellet was washed several times in 70% alcohol. The DNA pellet was dissolved in 100 µl DNA hydration solution (to give a concentration of 300 µg/ml if the total yield is 30 µg DNA) and used for PCR.

2.7.1.2 Polymerase Chain Reaction (PCR)

The PCR is an *in vitro* technique, where a given DNA sequence is identically copied. The number of copies rises exponentially, since every newly synthesized DNA-

sequence is also a template for the next copy (Blaxter *et al.*, 1998). For identification or establishing the taxonomic affinities of the unknown EPNs species that had been isolated a PCR based procedure was used. This procedure involved the PCR amplification and sequencing of the ITS 1 & 2 regions (internal transcribed spacers) of the ribosomal DNA. The PCR amplified region comprised the DNA flanking regions spanning from a 3' portion of 18 S rDNA to a 5' portion 28 S rDNA using universal primers as described by Powers *et al*., (1997). More specifically, the region of nuclear rDNA including the 18S 3'terminus, internal transcribed spacers (ITS-1, ITS-2), 5.8s subunit, and 28S 5'-terminus were amplified using universal primers for this region. The sequences of the ITS 1 & 2 regions can be used to establish the taxonomic affinities using the DNA of an unknown nematode isolate. In this case, it was the goal to see whether the isolates, whose EPN status had been confirmed by the infection experiments, belonged to the heterorhabditid or steinernematid group (Adams & Nguyen, 2002). For assigning the unknown isolates to one of the taxons, *Heterorhabditis* or *Steinernema*, the DNA sequences corresponding to the amplified internal transcribed spacer region of the ribosomal gene array (ITS rDNA) were aligned with the DNA sequences in GenBank (GenBank database of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/) by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The extracted DNA samples were quantified using NanoDrop ND-1000 ® spectrophotometer (Bio Rad) (Appendix XII) and stored in the fridge at 4°C. PCR amplification of the ITS region was carried out in a reaction volume of 50 µl for each strain, containing: 25µl Master Mix (# K 0171, Fermentas), 2.5µl template DNA 1.0µl primer forward, 1.0µl primer reverse and 20.5µl nuclease free water (# K 0171, Fermentas). The two primers used for amplifying the ITS regions were, TW81 consisted of the following sequences: 5'-GTTTCCGTAGGTGAACCTGC-3', Tm: 62ºC and AB28 5'-ATATGCTTAAGTTCAGCGGGT-3', Tm: 60ºC corresponding to the forward and reverse primers, respectively (Inqaba biotech). The templates were amplified using the GeneAmp PCR system 2700 ® thermocycler. PCR amplification involved an initial denaturation step at 94ºC for 3 minute, followed by 35 cycles consisting of a

30 second denaturation step at 94ºC for, a 45 second DNA strand annealing step at 53ºC, a 2 minute primer extension step at 72ºC and final primer extension step at 72ºC for 10 minute. At the end of the 35 cycles, the samples were stored at 4°C.

2.7.1.3 Agarose gel electrophoresis

This is a method used in biochemistry and molecular biology to separate DNA or RNA molecules by size. After PCR amplification the resulting amplified fragments were separated by electrophoresis using a 0.5% agarose gel containing ethidium bromide (0.2 %) in 0.5 x TBE buffer (54.0 g Tris base + 27.5 g Boric acid + 20 ml 0.5 M EDTA (pH 8.0). 5 µl of a DNA product was mixed with 3 µl loading suspension (# R0629, Fermentas) and transferred into the gel pockets (Appendix VII). One lane was loaded with 5 µl of a marker (# SM098, Fermentas) ranging from 100 to 1.000 base pairs, amplified DNA was subjected to 100 Volts for 35 minutes, and the fragments were photographed under ultraviolet light photographed using a Computer system (Bio Rad). The presence of a single bright band for each sample indicated successful amplification (Stock, 2003).

2.7.1.4 DNA sequencing

The DNA sequence of the genomic material in nuclei, mitochondria, and chloroplasts contains the genetic information that encodes the primary structure of proteins which constitutes the cellular machinery for development and metabolism (Adams *et al*., 1998; Blaxter *et al*., 1998, Szalanski *et al*., 2000). Sequencing of DNA PCR amplification products was done by Inqaba Biotechnical Industries (Pty) Ltd; South Africa. National Center for Biotechnology Information (NCBI-BLAST) results of the 18S rDNA sequence were used to assign or establish the affinities of the unknown nematodes to one of the EPNs taxons.

2.7.1.5 Sequence alignment and phylogenetic analysis

Multiple sequences alignment was done using DNAman (2006) and compared to EPN sequences derived from the Genbank data base using BLAST (http://www.ncbi.nlm.nih.gov/blast/). The Genbank sequences were used as references to establish the consensus sequences for the EPNs isolates. Only the alignments without ambiguous regions were used for phylogenetic analyses.

2.7.1.6 Morphological observations

Nematodes isolates from various soils were identified by morphological criteria using standard light microscopy and scanning electron microscopy according to Kaya & Stock (1997).

2.7.1.7 Light microscopy

For taxonomic studies, nematodes were reared on *G. mellonella, T. molitor* larvae or pupae of *T. molitor*. The IJs collected a week after their emergence from the insect cadavers was used; adults were obtained from sterile lipid agar (*in vitro*). Nematodes were killed and fixed in hot triethanolamine formalin (TAF) (60°C) (Courtney *et al*., 1955) and kept in this solution for 48h. Nematodes were transferred to glycerol according to (Steinhorst, 1959). Ten nematodes were mounted on a glass microscope slides with support of thin glass rods to avoid flattening (Fig 2.9). Observations were made with an Olympus BX60 light microscope equipped with differential interference contrast optics attached to color Olympus video camera (Appendix VIII).

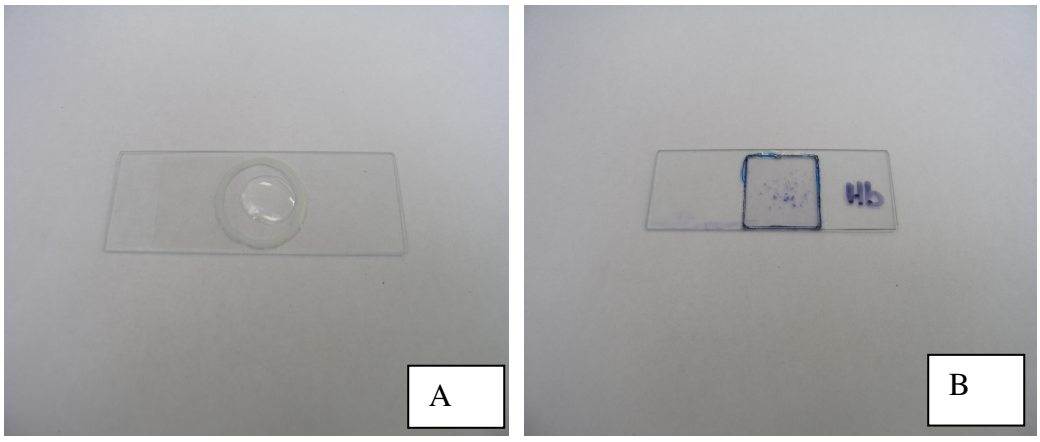


Fig 2.9: A); Illustration of the glass slide for permanent mount of EPNs after being processed through glycerol, and B): complete glass slide.

2.7.1.8 Scanning electron microscopy

Scanning electron microscopy (SEM) is very useful tool for the visualization and interpretation of certain nematode features that can not be appreciated with a light microscope but are important for taxonomic identification. The specimens from each stage (adults and IJs) were randomly collected from White traps or lipid agar. One ml of nematode samples containing about 15.000-20.000 IJs was placed in 7 ml tubes and killed by heating in water bath (60°C) for 2-3 minutes, rinsed three times (5 minutes each) in Ringer's solution (pH 7.3), and then fixed in 8% glutaraldehyde-25% EM grade (diluted in Ringer's solution pH 7.3) overnight. The nematodes were rinsed in sterile double distilled water three times, post fixed in 1% osmium tetroxide (OsO_4) for 2 hours at 4°C, rinsed in distilled water again and dehydrated at 10 minutes intervals through 30, 50, 70, 90, 95 and 100% ethanol, after which they were critical point dried with CO_2, mounted on SEM stubs, coated with gold and studied using JEOL JSM 840 scanning electron microscope equipped with digital Nikon F301 camera (Appendix IX)

2.8 Identification of symbiotic bacteria

2.8.1 Isolation of symbiotic bacteria

This method is modified from Akhurst (1980); Bonifassi *et al.,* (1999); Kaya & Stock (1997). One ml of infective juveniles samples were placed in 2 ml Eppendorf tube and surface sterilised by immersing them in 0.1% hyamine 1622 for 30 minutes, then rinsed three times in distilled water. The nematodes were concentrated and macerated in a sterile mortar and pestle. Thereafter, the cultures were streak plated onto NBTA (nutrient agar [Difco] with 25 mg bromothymol blue liter and 30 mg 1, 2, 3, 5-

triphenyltetrazolium chloride), MacConkey agar plates to obtain pure colonies. Phase I bacteria were obtained by selecting green or blue-green colonies from NBTA, nutrient agar supplemented with bromothymol blue liter and 30 mg 1, 2, 3, 5-triphenyltetrazolium chloride or red colonies from MacConkey agar and incubated at room temperature (25°C) for growing (Appendix VI).

2.8.2 Molecular identification of Symbiotic bacteria

2.8.2.1 Extraction of DNA

After growing (about one week) one colony of bacteria was used for DNA isolation using the InstaGene Matrix DNA Kit, (# 732-6030 Bio-Rad) isolation and purification protocol. In this protocol the following procedure was followed: A single colony was selected and resuspended in 1 ml of autoclaved water in an Eppendorf tube. The tube and contents were centrifuged for 1 minute at 10.000-12.000 xg. After centrifugation the supernatant were removed, 200 µl of InstaGene matrix was added to the pellet and incubated at 56°C for 15-30 minutes. The tubes were vortexed for 2-3 minutes and 20 µl of the resulting supernatant was used per 50 µl PCR reaction. The reminder of the supernatant was stored at -20°C (Appendix V).

2.8.2.2 PCR amplification of 16S rDNA

The bacterial isolates were identified using molecular techniques involving PCR amplification of a fragment of the 16S gene. Amplifications were carried out in a reaction volume of 50 µl, containing: 25 µl PCR Master Mix ((# K 0171, Fermentas), 1µl template DNA, 22 µl nuclease free water (# K 0171, Fermentas) and 1 µl of each oligonucleotide primer: The forward “sense (5 – AGAGTTTGATCCTGGCTCAG – 3, Tm: 60°C) and the reverse “antisense” (5 – AAGGAGGTGATCCACCGCA – 3, Tm: 60°C) (Inqaba biotech) were used in the PCR reaction for amplification of the

complete ITS region. The amplification profile was carried out using a Gene Amp PCR system 2700 ® thermocycler, which was using the following conditions: One cycle of 92°C for 2 min followed by 35 cycles of denaturation at 92°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 2 min, with a final cycle of extension at 72°C for 10 min.

2.8.2.3 Agarose gel electrophoresis

After DNA amplification, 5µl PCR products, 1.5µl of tracking dye (# R0629, Fermentas) and 5µl DNA ladder (# SM098, Fermentas) were loaded on a 1% agarose gel for DNA checking, separated by electrophoresis at 80 Volts for 4 hrs, stained with ethidium bromide, visualized with ultraviolet light and photographed with Polaroid camera. The presence of a single bright band for each sample indicated successful amplification (Stock, 2003). Sequencing of DNA amplification products was done by Inqaba Biotechnical Industries (Pty) Ltd; South Africa. NCBI BLAST results were used to establish the taxonomic affinities of the bacterial isolates with the previously published 16S gene sequences (Appendix VII)

2.8.2.4 Sequence alignment and phylogenetic analysis

Multiple sequences alignment was done using DNAman (2006) and compared to those of other organisms using BLAST (http://www.ncbi.nlm.nih.gov/blast/) and the resulting output was adjusted manually to improve homology statements. Only the alignment without ambiguous regions was used for phylogenetic analyses.

2.8.2.5 Scanning electron microscopy

Symbiotic bacteria were fixed in 8% glutaraldehyde-25% EM grade (diluted in Ringer's solution at pH 7.3 overnight. They were post-fixed with 1% osmium

tetroxide solution for 2 hrs at 4°C, dehydrated in a graded ethanol series, critical point dried with liquid CO_2, mounted and on SEM stubs, coated with carbon and gold palladium, scanned using a JEOL JSM 840 SEM and photographed with a Nikon F301 camera (Appendix IX).

2.9 Bioassays

South African EPNs that were isolated in the study were used in the present study. For IJs production EPNs were reared on *G. mellonella* and *T. molitor* larvae and on pupae in the case of *T. molitor* at 25°C, according to procedures in Woodring & Kaya (1998). The nematodes were isolated in the Nematology Laboratory at Wits University in the School of Molecular and Cell Biology from soil collected from Pretoria and Brits. Both isolate species were identified by using the molecular procedures already described in the previous sections. Harvested IJs were kept at ambient room temperature 25°C for less than 1 week before the tests.

2.9.1 Insects

Larvae of the *G. mellonella* were reared in the laboratory in 3 L glass bottles as described in section 2.1. Larvae of *T. molitor* were supplied by commercial mealworm breeders. All insects were maintained at a room temperature of 25°C. Insect larvae of the grater wax moth *G. mellonella* (0.25 to 0.50 g), *T. molitor* (0.20 to 0.50 g) and pupae (0.25g) were used throughout the study.

2.9.2 Nematode exposition assays including nematode concentration.

The dose-response assay (Glazer & Lewis, 2000) involved exposing insect to different EPN IJ concentrations over the range of 5 to 500 IJs/insect and insect mortality was recorded every 24 h for over a 96 h period. The effects of strains of EPNs on the mortality rate of infected larvae were determined in bioassays wells. The nematodes were dispensed as IJs into 3 cm-diameter wells (40-well plates) (Fig 2.10). Each well was filled with 0.5 g sterilized and sieved river sand (particle size: 355 µm). Each well was inoculated with 5, 10, 25, 50, 100, and 500 IJs in a 70 µl of distilled water. Counting large number of EPNs was impractical, so serial dilutions were used to generate the above IJ densities per 70 µl of distilled water. Nematode concentrations were prepared by allowing IJs to settle to the bottom of the 50 ml centrifuge tubes for at least 30 minutes, and then the excess water were removed. The nematodes were shaken well in its tube; 50 µl aliquot were withdrawn with a micropipette from each suspension and transferred to 5 cm Petri dish. Three such samples were taken from each suspension and placed into three different Petri dishes. The nematodes in the dishes were counted under a dissecting microscope. Nematode concentration per milliliter was calculated by multiplying the average of the three 50 µl count by 20. To adjust to any concentration the following formula was used: [(i/c)-1] x V=Va Where i= initial concentration 50 µl-1, C=final concentration 50 µl, V= volume of the suspension (ml) and Va= the amount of the water (ml) to be added if positive or to be removed if negative from the suspension. At low concentrations (< 50 IJs ml-1), counts of individual nematodes of each sample were used (Glazer& Lewis, 2000). At least 9 wells were used for each concentration of nematode, and one *G. mellonella* or *T. molitor* larva and pupa of *T. molitor* was transferred to each well. The control consisted only of sand soil and 70 µl of nematode-free water. The wells were sealed with Parafilm® and kept at room temperature (25°C). These particular plates were chosen because the wells have relatively small volume and the insect is

thus forced to be in close proximity to the nematodes. Larvae mortality was recorded 24, 48, 72 and 96h later. Upon insect death cadavers were transferred individually onto White traps. The emerging IJs were collected and counted under a dissecting microscope (Olympus) at 40X magnification and quantified using the above formula. The experiments were repeated three times.

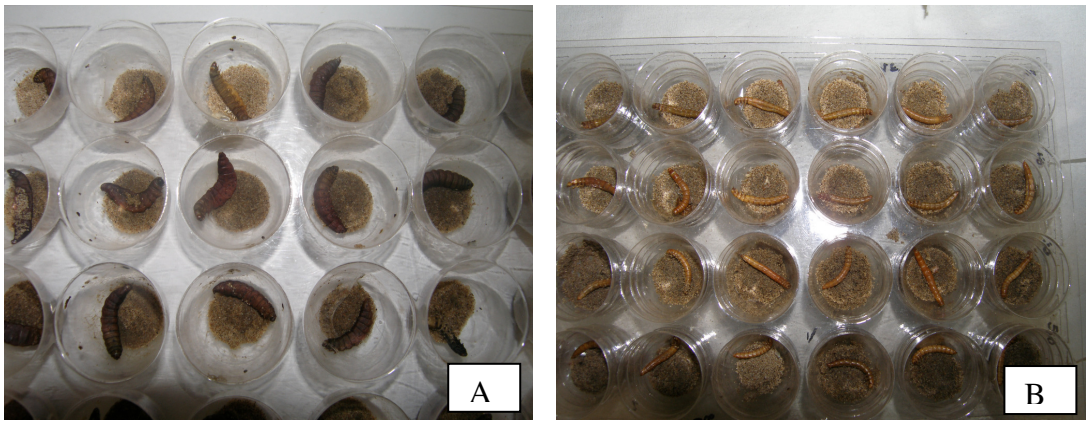


Fig 2.10: Bioassay set up in multi well plates. This dose-response bioassay was set up using A) *G. mellonella* and B) *T. molitor* larvae as hosts. The dead insects which are brick red in colour were infected by nematode isolate.

2.9.3 Data analysis

The percent mortality data as well as data on the mean number of progeny IJs emerging per cadaver were compared using one-way Analysis of Variance ANOVA and Tukey's multiple comparison tests (ANOVA, SAS, 2001). Differences with $P<$ 0.05 were considered significant. Mortality data that were expressed as percentages were transformed by Arcsin transformations. Control data were not included, the statistical analysis, since insect mortality did not result from any of the control treatments

Chapter 3

Entomopathogenic Nematode Isolation

3.1 Introduction

Nematology is regarded as a relatively young science (Chitwood, 1950). There has been an explosion in the discovery of new isolates, many of which are new species (Hunt, 1997). Nematology has only recently begun to receive the attention warranted by its wide scientific and academic impact (Hominick, 2002). Dr W.J. van der Linde was the pioneer of Nematology in South Africa in the early 1960s, when surveys were undertaken and a national collection of nematodes was established, though the collection was primarily plant parasitic nematode species. In 1971, Heyns provided a brief overview of Nematology in South Africa. Since plant parasitic nematodes had and still have a major economic impact on crop losses, Heyns' work was heavily based on plant parasitic nematodes with little emphasis on EPNs. Since 1972, little has been reported on EPNs. The discovery of EPN species and the rate at which they have been described in other parts of the world is correlated with the historical need for biological alternative to manage insect pest. Research on EPNs remained somewhat dormant as chemical-based pest control measures remained cheap, effective and relatively unregulated. Upon recognition of their negative environmental effects the search for biological alternatives to chemical-based pest management programs received renewed attention from scientists (Griffin *et al*., 2001). Soil is one of the natural habitats for EPNs. The dependence of the distribution of these nematodes on factors like habitat type, soil texture, soil pH, and altitude have been studied in some detail (Yoshida *et al*., 1998). Individual species of EPNs differ in their habitat preference (Mracek *et al*., 2005). Soil type may also be an important factor in EPN distribution (Miduturi *et al*., 1996; Georgis & Poinar, 1983) showed that soil texture plays an important role in the dispersal of EPNs and their persistence

(Kung *et al*., 1990). The objective of this research was to characterize, host bioassay, and *in vitro* culture of indigenous EPNs and their bacterial symbionts.

The nematode isolates were obtained by the live bait method (section 2.5.1). Although the nematodes were presumed to have infected and killed the insects, it is imperative to note that nematodes may also invade already dead insects that may have been killed by some other pathogen. Usually, black cadavers with associated putrefaction indicate that the host was not killed by an entomopathogenic species. Nematodes found within such cadavers tend to be free-living soil saprophages (Woodring & Kaya, 1988; Gaugler, 1998). It was therefore important to check that the IJs recovered from all cadavers be tested with Koch's postulates to ensure that the nematodes recovered from the dead insect were indeed responsible for killing it. Koch's postulates are four criteria published by Robert Koch, a German bacteriologist in 1884, which he said must be fulfilled in order to establish a causal relationship between a parasite and a disease. He applied these to establish the etiology of tuberculosis, but they have been generalized to other diseases (section 2.6). In this chapter, the processes that were involved in collection of soil samples, baiting and isolation of nemtodes are discussed.

3.2 Materials & Methods

3.2.1 Soil sampling

A total of 200 soil samples were randomly collected from 5 locations at the ARC Roodeplaat farm in Pretoria, Gauteng province (Fig 2.6) in April 2006; and from 5 locations in Brits, North West province (Fig 2.5) in March, 2006. At the different collection sites, soil samples were obtained from soils associated with various vegetation covers- [ARC Pretoria: (Sweet potato field, Peach trees, Alfalfa field, Grassland and Vegetable field)]; [Brits: (Mango field, Orange field, Sweet potato

field, Naartjie field, and vegetable within each habitat]. This aspect is described in more detail under materials and methods (section 2.3). Systematic sampling (Christopher & Schmitt, 1997) was used to guarantee complete coverage of an area or time. The towns from where the soil samples were collected are indicated in the maps (section 2.4).

3.2.2 Extraction of nematodes from soil

Soil samples were taken to a depth of between 15 and 20 cm. The samples were collected into plastic bags. At the collection site, the soil sample was immediately labeled with a number, collection site, and information on the type of vegetation. The soil type was classified as sandy, sandy loam, loam, clay, etc. Also, the soil moisture was estimated as wet, moist or dry (section 2.3). The live bait method (section 2.5.1), using lab-reared *G. mellonella* (section 2.1) was used to extract nematodes from the 200 soil samples collected from the different locations. The nematode isolates recovered from the baiting experiments were given numbers corresponding to those on the soil sample from which they were extracted. For example, nematodes extracted from soil sample number 135 were labeled isolate 135. In the laboratory, the soil samples were transferred to 2 L plastic ice cream containers and labeled according to the laboratory book. Dry soils were dampened by spraying sterile water and mixing (section 2.5.1). This was done to enhance nematode movement (if any were present in the soil sample). Ten *G. mellonella* larvae were then buried in each of the containers (section 2.5.1) to serve as bait to the nematodes. After 3 days, the soil samples were checked for dead larvae. Live larvae were re-buried. Dead larvae were checked for on a daily basis till all larvae had been recovered from the soil samples. Only single whole insect cadavers were mounted on White traps (section 2.5.2) to collect progeny nematodes. The offspring were then subjected to Koch's postulates to establish if they were entomopathogenic.

3.2.3 Koch's postulates (See section 2.6)

IJs were recovered from the dead insects on White traps (section 2.5.2), and used to reinfect healthy *G. mellonella* larvae or *T. molitor* larvae and/or pupae. Progeny IJs were recovered from the insects via the White trap and then reapplied to a third set of healthy insects. This process was repeated for all the nematode isolates recovered from the original infected larvae in the baiting experiment until reproducible and consistent infection results were obtained for isolates. In addition, the diagnostic method of Woodring and Kaya (1988) was used to determine the genus of the putative EPN that might have infected the dead insect. The authors explained that insects killed by most steinernematid nematodes become brownish-yellow, whereas insects killed by heterorhabditids become red and the tissue assume a gummy consistency. A dim luminescens given off by insects freshly killed by heterorhabditids is a foolproof diagnostic for this genus (the symbiotic bacteria provide the luminescens). Black cadavers with associated putrefaction indicate that the host was not killed by entomopathogenic species. Nematodes found within such cadavers tend to be free-living soil saprophages (Woodring & Kaya; 1988; Gaugler, 1998). The entire process (from baiting to White traps) was done over a period of about two months.

3.3 Results

3.3.1 Nematode isolation from soil samples

All buried insects died eventually but not necessarily from infection. Some had died from injury such as suffocation, weight of soil, mishandling, drowning etc. Many of the insects became infected by other soil pathogens, which caused the cadavers to disintergrate. Of the 2000 *G. mellonella* larvae buried in 200 soil samples over time,

only 863 whole cadavers (not disintergrated) were recovered and mounted on White traps. Progeny nemtodes were obtained from 513 of the White traps.

3.3.2 Koch's postulates

The entomopathogenic status of nematode isolates was tested using Koch's postulates. Only 2 of the isolates namely, 411 and 386 were putatively identified as entomopathogenic. Table 3.1 below shows the origin and characteristics of the soil samples from which the two nematode isolates were obtained. In addition to Koch's postulates, the colour cadavers infected by the putative EPNs were used as a diagnostic for categorizing the nematode isolates. Fig 3.1 (A & B) below shows *G. mellonella* larvae infected with nematode isolates 411 & 386 respectively, as well as isolate 386 emerging from a *T. molitor* larva as viewed under a light microscope.

Table 3.1: Results of soil samples

Soil sample	Sample site	Vegetation	Soil type	Soil moisture	Isolate Nº
411	ARC Roodeplaat experimental farm	Sweet potato field	Sandy soil	moist	411
386	Brits	Mango tree farm	Sandy soil	Cool moist	386



Fig 3.1: A, B, C & D): *G. mellonella* and *T. molitor*cadavers infected by different EPN isolates, those infected by nematode isolate 411 are brick red in colour, while those infected by nematode isolate 386 are green and brown in colour respectively (E & F) Light micrographs of nematode isolate 386 emerging from a *T. molitor* larvae and isolate 386 in a white trap respectively.

3.4 Discussion

Soil is an excellent habitat for nematodes; just 100 ml of soil may contain several thousand of them. Because of their importance to agriculture, the IJ's ability to disperse and persist until it can locate a host is crucial for the success of EPN applications for insect control in soil and the survival of naturally occurring EPNs population. The EPNs recovery of only 1% (only 2 EPN-positive samples out of 200 collected) was far less than the 3.8% reported in Northern Ireland (Blackshaw, 1988), 6.8% in the Hawaiian Islands (Hara *et al* ., 1991), and 10.5% in Ireland (Griffin *et al.,* 1991). Probable explanations for this observation may be due to either the susceptibility of insect hosts or the texture of soil sampled. The percentage of EPNs recovered may have been higher if more sites had been sampled. The widely used live bait method of Bedding & Akhurst (1975) for isolating EPNs from soil samples using *G. mellonella* was employed in this study. Nguyen & Smart (1992) showed that *G. mellonella* is not good bait for certain EPN species; e.g. the authors showed that *S. scapterisci* did not reproduce in *G. mellonella*. This may explain why EPNs recovery from the soil samples was quite low. It is possible that EPNs that do not infect *G. mellonella* were missed in the isolation process. Mracek & Webster (1993) found EPNs occurred more abundantly in sites where human impact had been substantial. They also found that insect pests are also plenty in cultivated land. The authors suggested that their presence may be the result of outbreaks of insect pests associated with intensive crop monoculture. It seems that the occurrences of EPNs in insect pest infested soils are higher because the EPNs depend on the insect pests as hosts. Mracek & Webster (1993) also put forward evidence that insecticides affected nematodes distribution. The two EPN isolates 386 and 411 were both recovered from cultivated soils. This observation supports those of Mracek & Webster (1993). In addition, both samples were obtained from moist sandy soils, suggesting that EPNs are more likely to occur in such moisture environment and soil type. An association of EPNs with sandy soils has also been demonstrated in surveys conducted in other

regions of the tropics: Hawaii (Hara *et al*., 1991), Sri Lank (Amarasingh *et al*., 1994) and Malaysia (Mason *et al*., 1996), and in subtropical Japan (Yoshida *et al*., 1998). Thus, soil texture also may be an important factor in determining the abundance of EPN species in the ARC Roodeplaat experimental farm and Brits. Nematodes are less common in clay soils than in sandy soil that reinforced the observation of Lee & Atkinson, 1976 that nematodes movement in soil is limited to available pore space and restricted by pore diameter. Clay content in some soils, such as in both sites may interfere with movement and parasitism of insects by infective juvenile nematodes and also may influence the presence of suitable insect hosts (Liu *et al*., 1997). In this study, isolates 411 and 386 were recovered from ARC Roodeplaat experimental farm (Fig 2.6) and Brits (Fig 2.5). Soils may provide material to select strains for controlling insect pest populations and may provide material for choosing more suitable native strains for use in biological control programs in some agriculture and forested regions. Recovery of EPNs may be influenced by interaction of soil temperature and moisture because the frequency varies with season (Akhurst & Bedding 1986; Blackshaw 1988; Griffin *et al*., 1991). Dry soil restricted movement of nematodes. The two EPN isolates 386 and 411 were both recovered in winter this observation has also been demonstrated in surveys conducted by Akhurst & Bedding (1986), nematode were more likely to be found from samples collected in autumn and winter than in spring and early summer.

Chapter 4
Molecular Characterization of Nematode Isolates 411 and 386

4.1 Introduction

EPNs of the genus *Steinernema* and *Heterorhabdtis* are being used as biological control agents for a number of economically important crop pests. Because of the morphological similarities of numerous strains, difficulties arise with the precise identification of species (Grenier *et al.,* 1996). Accurate identification of these nematodes is a prerequisite before any further research investigations can be undertaken (Stock & Reid, (2004). A molecular approach for rapid identification could go a long way in expediting EPN research. Tools such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) as well as other molecular evidence have been used to characterise steinernematids (Liu & Berry, 1995; Liu *et al*., 1997; Reid *et al*., 1997; Stock *et al*., 1998). DNA sequence analysis has been demonstrated to yield more information in biocontrol programmes to assist in the identification and monitoring of exotic, native and genetically engineered strains in the field (Curran et *al.,* 1984). In addition, sequence analysis has shown to be a more suitable tactic in assessing phylogenetic relationships at different taxonomic levels and as well as for species delineation (.Adams *et al*., 1998; Blaxter *et al*., 1998; Szalanski *et al*., 2000; Stock *et al*., 2001). However, before that, the pathogenic status of IJs recovered from all cadavers must be checked with Koch's Postulates (see chapter 3) to ensure that the nematodes recovered from the dead insect were indeed responsible for killing it.

4.2 Materials & Methods

The current study focused on obtaining rDNA sequence information of the nematode isolates 411 and 386 strains by using molecular method, to reveal sequence alignment and phylogenetic relationships from other nematodes from diverse taxa. To confirm the identification, Phylogeny in relation to 18S rDNA sequences of the nematodes isolated number 386 and 411 were investigated. The extraction of genomic DNA from infective juveniles was carried out using the purigene purification kit according to the manufacturer's instructions (appendix III). DNA was successfully extracted from two consecutive generations of IJs of isolate 386 (DNA samples 386A and 386B) and 411 (DNA samples 411A and 411B), (section 2.7.1.1) growth on sterile lipid agar and amplified as described in (section 2.7.1.2) using TW81 (5'-GTTTCCGTAGGTGAACCTGC-3', Tm: 62°C); and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3', Tm: 60°C) as forward and reverse primers, respectively (Inqaba Biotech).

4.3 Results

The analyses of 18S rDNA were used for assigning nematode material to particular genera as well as to species. The PCR amplied a DNA product of about 742 bp for sample from isolate number 386 and 780 for sample from isolate number 411. For both isolates, there was variation in size of the product on agarose gel (Fig 4.1). It is therefore likely that they are not the same species. However, size alone is not a reliable genetic marker for species–specific identification. NCBI BLAST results that were obtained for the 18S rDNA sequences confirmed the identity of the isolates 386 and 411 to be *Heterorhabditis zealandica* (*H. zealandica*) and *Heterorhabditis. bacteriopohora* (*H. bacteriopohora*) respectively. The two sequence data obtained from the two nematodes: *H. zealandica* and *H. bacteriophora* have not yet been allocated a Genbank accession number. The 18S sequence of *H. zealandica* and *H.*

bacteriophora isolate from South Africa were aligned using DNAman (2006) with various species of the genus *Heterorhabditis*: (EF690469, EF043440, AF029706, EF043441, AY321483, EF043442, AF548768, DQ665222, DQ372922, AY321478, AY321480, AY321477, EF043443, AF083004, AF029707, AY321481, EF408912, DQ121438) obtained from the Genbank database.Results based on the ITS sequence alignments shows that the South Africa *H. bacteriophora* isolate is closely related to *H. bacteriophora*, accession numbers EF690469 and the *H. zealandica* isolate was closely aligned to *H. zealandica*, accession numbers AY321481 (Fig 4.2 & 4.3)

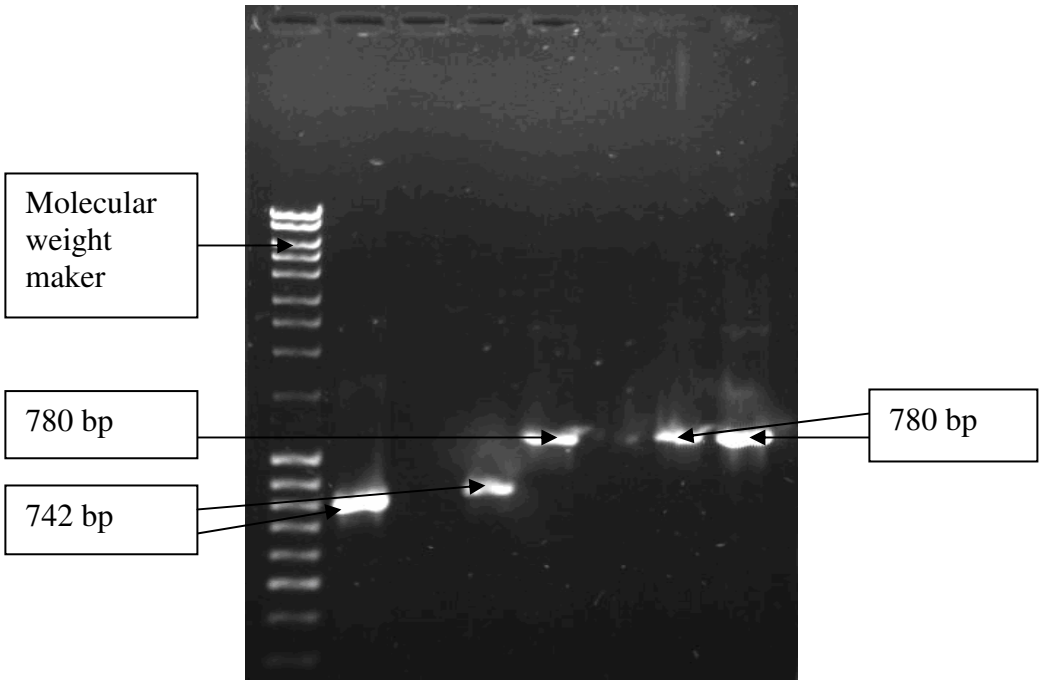


Fig 4.1: DNA fingerprints of nematodes isolates 411 and 386 amplified with TW81 and AB28 primers. Lane 1 (Molecular Weight Marker: # SM098, Fermentas) Lane 2-4 (386). Lane 5-6-7-8 (411).

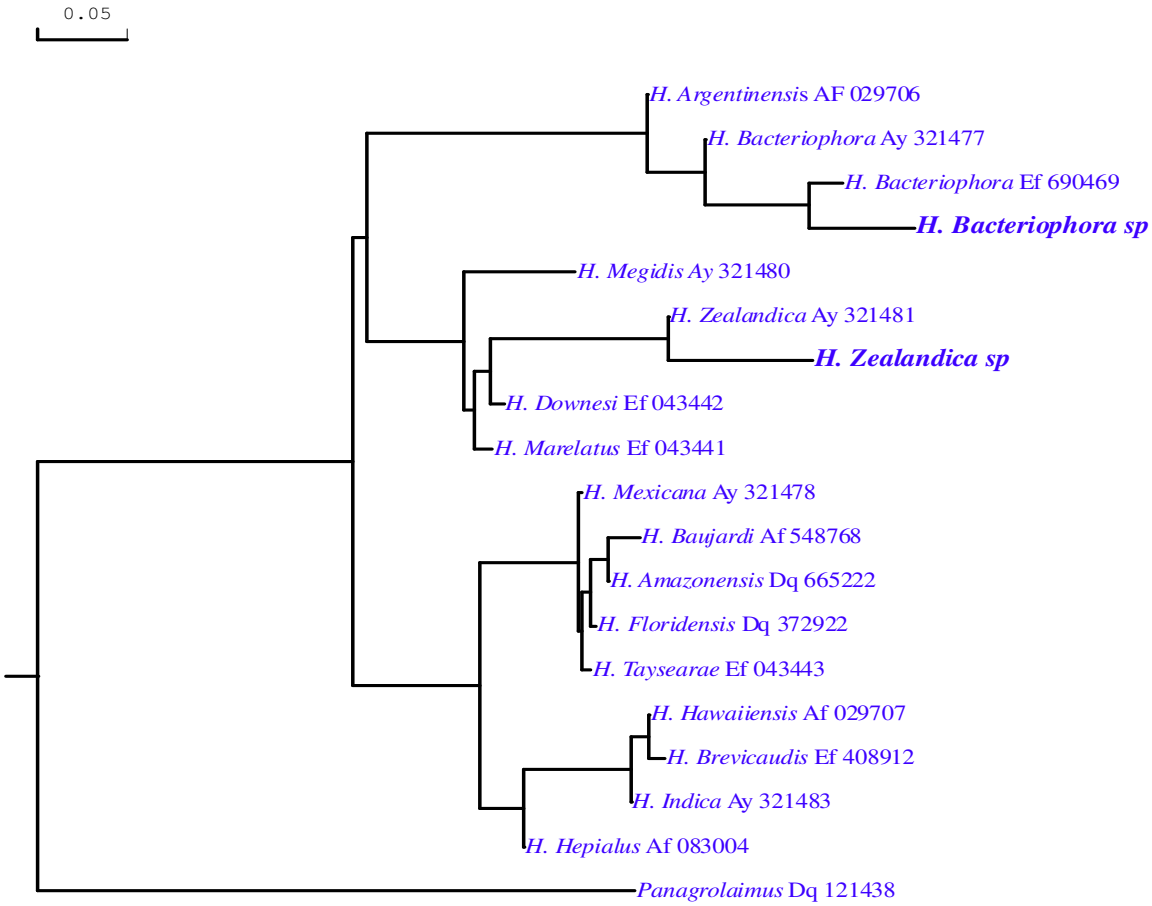


Figure 4.2: Phylogenetic tree resulting from existing *Heterorhabditis* nematode database sequences together with highlighting sequences discovered in this study.

FILE: MULTIPLE_SEQUENCE_ALIGNMENT
PROJECT:
NUMBER: 19
MAXLENGTH: 1858
NAMES: H.BAUJARDI AF548768 H.ARGENTINENSIS AF029706 H.HAWAIENSIS AF029707 H.HEPIALUS AF083004 H.BACTERIOPHORA AY321477 H.MEXICANA AY321478
H.MEGIDIS AY321480 H.ZEALANDICA AY321481 H.TAYSEARAE EF043443 H.INDICA AY321483 PANAGROLAIMUS DQ121438 H.BACTERIOPHORA SP
H.DOWNESI EF043442 H.BREVICAUDIS EF408912 H.BACTERIOPHORA EF690469 H.ZEALANDICA SP H.FLORIDENSIS DQ372922 H.AMAZONENSIS DQ665222
H.MARELATUS EF043441

MAXNAMELEN: 24
ORIGIN

H.BAUJARDI AF548768 ATAATCATGGAATCAAGCTT......GTTCTT.GATT.TCAGTCGGTGTCTCACC 96
H.ARGENTINENSIS AF029706 GTAccAATGGAATCAGGCTT......GTTCTT.GATT.TCAATCGGTtTCTCACC 256
H.HAWAIENSIS AF029707 ATAATCATGGAATCAGGCTT......GTTCTT.GGTT.CCAGTCGGTGTCTCACC 255
H.HEPIALUS AF083004 AGAGTGCTCAAAACAAGCGT......TTGCTTGAATGCTCGATCATGGAATAATA 756
H.BACTERIOPHORA AY321477 GTACCAATGGAATCAGGCTT......GTTCTT.GATT.TCAATCGGTTTCTCACC 256
H.MEXICANA AY321478 ATAATCATGGAATCAAGCTT......GCTCTT.GATT.TCAGTCGGTGTCTCACC 257
H.MEGIDIS AY321480 GTGCTACCGGAATCAGGCTT......GCTCCTCGATT.TCGATCGGTATCTCACC 257
H.ZEALANDICA AY321481 GTGCTCATGGAATCAGGCTC......ACGTTT.GATT.TCAATCGGTAGCTCACC 256
H.TAYSEARAE EF043443 GTAATCATGGAATCAAGCTT......GCTCTT.GATT.TCAGTCGGTGTCTCACC 278
H.INDICA AY321483 ATAATCATGGAATCAGGCTT......GTTCTT.GGTT.CCAGTCGGTGTCTCACC 255
PANAGROLAIMUS DQ121438 TTAGGTCGCGGCTCGTGTATCGAAATACTACA.CGTT.CCAGTTGAGATGCCGTT 115
H.BACTERIOPHORA SP GTACCATAGGTATACAGCTT......GTTCTT.GTAT.TCATACGGTTTCTCACC 108
H.DOWNESI EF043442 GTGCTACTGGAATCAGGCTT......GCTCTT.GATT.TCAATCGGTATCTCACC 277
H.BREVICAUDIS EF408912 ATAATCATGGAATCAGGCTT......GTTCTT.GGTT.CCAGTCGGTGTCTCACC 279
H.BACTERIOPHORA EF690469 GTACCAATGGAATCAGGCTT......GTTCTT.GATT.TCAATCGGTTTCTCACC 51
H.ZEALANDICA SP ..GCTCATGGATACAG.CTC......ACGTTT.GTAT.TCAATCGGTAGCTCACC 107
H.FLORIDENSIS DQ372922 ATAATCATGGGATCAAGCTT......GTTCTT.GATT.TCAGTCGGTGTCTCACC 283
H.AMAZONENSIS DQ665222 ATAATCATGGAATCAAGCTT......GTTCTT.GATT.TCAGTCGGTGTCTCACC 270
H.MARELATUS EF043441 GTGCTACTGGAATCAGGCTT......GCTCTT.GATT.TCAATCGGTATCTCACC 277

H.BAUJARDI AF548768 CC.ATCTAAGCTCTCGGAGANG.TGTCTATTCTTGATTGGAGCCGATT....TGA 145
H.ARGENTINENSIS AF029706 CC.ATCTAAGCTCATGGAGAGG.TGTCTAGTCCCAATTGGAGTCGCTT....TGA 305
H.HAWAIENSIS AF029707 CC.ATCTAAGCTCTCGGTGAGG.TGTCTATTCTTGATTGGAGCCGCTT....TGA 304
H.HEPIALUS AF083004 AA.AGAGGACTTCGGTTCTATT.TGTTGGTTCAGGAACTGAATTAATGGTTAAGA 809
H.BACTERIOPHORA AY321477 CC.ATCTAAGCTCATGGAGAGG.TGTCTAGTCCCAATTGGAGTCGCTT....TGA 305
H.MEXICANA AY321478 CC.ATCTAAGCTCTCGGAGAGG.TGTCTATTCTTGATTGGAGCCGATT....TGA 306
H.MEGIDIS AY321480 CCCATCTAAGCTCTCGGAGAGG.TGTCTAATCCCAATCGGAGTCGCTT....TGA 307
H.ZEALANDICA AY321481 CC.ATCTAAGCTCTTGGTGAGG.TGTCTATTCCCAATTGGAGTCGCTT....GGA 305
H.TAYSEARAE EF043443 CC.ATCTAAGCTCTCGGAGAGG.TGTCTATTCTTGATTGGAGCCGATT....TGA 327
H.INDICA AY321483 CC.ATCTAAGCTCTCGGTGAGG.TGTCTATTCTTGATTGGAGCCGCTT....TGA 304
PANAGROLAIMUS DQ121438 CA.ACAGGCGCTACTGATGGCT.GTTCGTTTGGAGTGTGACAGCTAAT....GGC 164
H.BACTERIOPHORA SP CC.ATCTAAGCTCATGGAGA.G.TGTCTAGTCCCAATTGGAGTCGCTT....TGA 156
H.DOWNESI EF043442 CC.ATCTAAGCTCTCGGAGAGG.TGTCTAATCGCAATCGGAGTCGCTT....TGA 326
H.BREVICAUDIS EF408912 CC.ATCTAAGCTCTCGGTGAGG.TGTCTATTCTTGATTGGAGCCGCTT....TGA 328
H.BACTERIOPHORA EF690469 CC.ATCTAAGCTCATGGAGA.GGTGTCTAGTCCCAATTGGAGTCGCTT....TGA 100
H.ZEALANDICA SP CC.ATCTAA.CTCTTGGTGAGG..GTCTATTCCCA.TTGGAGTCGCTT....GGA 153
H.FLORIDENSIS DQ372922 CC.ATCTAAGCTCTCGGAGAGG.TGTCTATTCTTGATTGGAGCCGATT....TGA 332
H.AMAZONENSIS DQ665222 CC.ATCTAAGCTCTCGGAGAGG.TGTCTATTCTTGATTGGAGCCGATT....TGA 319
H.MARELATUS EF043441 CC.ATCTAAGCTCTCGGAGAGG.TGTCTAATCCCAATTGGAGTCGCTT....TGA 326

```
H.BAUJARDI AF548768       GTGACGGC..A.ATGATAA.TTGGGTATG...CTCCCCGTA...AGA.GGTAGAG    189
H.ARGENTINENSIS AF029706 GTGACGGC..T.ATGAAAA.TTGGGTATG...TTCCCCGTG...AG..GGTCGAG    348
H.HAWAIENSIS AF029707    GTGACGGC..A.ATGATAG.TTGGGTATG...TTCCCCGTG...AG..GGTAGAG    347
H.HEPIALUS AF083004       GGGACAACTCG.GGGGCAT.TCGTATCCC...TGCGCGAGA...GG..TGAAATT    854
H.BACTERIOPHORA AY321477 GTGACGGC..T.ATGAAAA.TTGGGTATG...TTCCCCGTG...AG..GGTCGAG    348
H.MEXICANA AY321478       GTGACGGC..A.ATGATAA.TTGGGTATG...CTCCCGTTC...GG..ATAAGAG    349
H.MEGIDIS AY321480        GTGACGGC..A.ATGAGGA.TTGGGTGTGCCATACCCCATACGTGG..GGTAGAG    356
H.ZEALANDICA AY321481     GTGACGGC..A.GTGATGA.TTGGGTGTGTTG...CCCGTAT...G..GGTGGCG    348
H.TAYSEARAE EF043443      GTGACGGC..A.ATGATAA.TTGGATATG...CTCCCGTTC...GG..ATAAGAG    370
H.INDICA AY321483         GTGACGGC..A.ATGATAG.TTGGGTATG...TTCCCCGTG...AG..GGTAGAG    347
PANAGROLAIMUS DQ121438    AAAAAATC..ACGTAACGT.CTGGGCTTA...ATTGCCGCCGTACTTCGGTACTT    213
H.BACTERIOPHORA SP        GTGACGGC..T.ATGA.AA.TTGGGTATG...TTCCCCGTGAG.....GGTCGAG    198
H.DOWNESI EF043442        GTGACGGC..A.ATGAAGA.TTGTGTGTG...TTCCCCGTA...AG..GGTAGAG    369
H.BREVICAUDIS EF408912    GTGACGGC..A.ATGATAG.TTGGGTATG...TTCCCCGTG...AG..GGTAGAG    371
H.BACTERIOPHORA EF690469 GTGACGGC..T.ATGA.AAATTGGGTATG...TTCCCCGTGAG.....GGTCGAG    143
H.ZEALANDICA SP           .TGACGGC..A.GTGATGA.TTGGGTGTGTTG...CCCGTAT...G..GGTGGCG    195
H.FLORIDENSIS DQ372922    GTGACGGC..A.ATGATAA.TTGGGTATG...CTCCCGTTC...GG..ATAAGAG    375
H.AMAZONENSIS DQ665222    GTGACGGC..A.ATGATAA.TTGGGTATG...CTCCCCGTA...AG..GGTAGAG    362
H.MARELATUS EF043441      GTGACGGC..A.ATGAAGG.TTGGGTGTG...TTCCCCGTA...AG..GGTAGAG    369

H.BAUJARDI AF548768       CATAAGACTTAATGAGCTGATCTA..GGT..CTGTCGCCTCACC..AAAAAC.CC    237
H.ARGENTINENSIS AF029706 CATA.GACTTTATGAACAGTGCTG..GAG..CTGTCGCCTCACC..AAAAAA.TC    395
H.HAWAIENSIS AF029707     CATA.GACTTTATGAACAGAGCTG..GG...CTGTCGCCTCACC..AAAAAC.C.    392
H.HEPIALUS AF083004       CGTGGACCGCAGGGGGACGCCCTAAAGCGAAAGCA...TTTGCC..AAGAATGTC    904
H.BACTERIOPHORA AY321477 CATA.GACTTTATGAACAGTGCTG..GAG..CTGTCGCCTCACC..AAAAAA.TC    395
H.MEXICANA AY321478       CATAAGACTTAATGAGCTGATCTA..GGT..CTGTCGCCTCACC..AAAAAC.CC    397
H.MEGIDIS AY321480        CATA.GACGTTAGGAACAGTACTC..GGA..CTGTCGCCTCACC..AA.....CC    399
H.ZEALANDICA AY321481     CATA.GACTTAATGAACAGTACTCTCGGA..CTGTTGCCTCAGCCAAA.....CT    395
H.TAYSEARAE EF043443      CATAAGACTTAATGAGCTGATCTA..GGT..CTGTCGCCTCACC..AAAAAC.CC    418
H.INDICA AY321483         CATA.GACTTTATGAACAGAGCTG..GG...CTGTCGCCTCACC..AAAAAC.C.    392
PANAGROLAIMUS DQ121438    CGGCTGTCTCGACAGCCCGCTTTA..TGTGATACCTGTCGGATA..TGGTGG.CT    263
H.BACTERIOPHORA SP        CATA.GACTTTATGAACAGTGCTG..GAG..CTGTCGCCTCACC..AAAAAA.TC    245
H.DOWNESI EF043442        CATA.GACTTTAAGAACAGTGCTG..GGA..CTGTCCCCTCACC..AA.....CC    412
H.BREVICAUDIS EF408912    CATA.GACTTTATGAACAGAGCTG..GG...CTGTCGCCTCACC..AAAAAC.C.    416
H.BACTERIOPHORA EF690469 CATA.GACTTTATGAACAGTGCTG..GAG..CTGTCGCCTCACC..AAAAAA.TC    190
H.ZEALANDICA SP           CATA.GACTTAATGAA.AGTACTCTCGGA..CTGTTGCCTCAGCCAAA.....CT    241
H.FLORIDENSIS DQ372922    CATAAGACTTAATGAGCTGATCTA..GGT..CTGTCGCCTCACC..AAAAAC.CC    423
H.AMAZONENSIS DQ665222    CATAAGACTTAATGAGCTGATCTA..GGT..CTGTCGCCTCACC..AAAAAC.CC    410
H.MARELATUS EF043441      CATA.GACTTTATGAACAGTGCTG..GGA..CTGTCGCCTCACC..AAAAAC.TC    416

H.BAUJARDI AF548768       ATCGATAGTTGGTGGCTAAGTGATGAGACT.TTGTCAAAATCACTA.ATC...TG    287
H.ARGENTINENSIS AF029706 ATCGATAACTGGTGGCTATGTGT...GACA.TTAGTCACATAGGTA.TCT...GC    442
H.HAWAIENSIS AF029707     ATCGATAACTGGTGGCTGAGTGA.............GAAATCACTGGATC...TG    431
H.HEPIALUS AF083004       TTCATTAATCAAGAACGAAAGTCAGAGGTT.CGAAGGCGATTAGAT.ACC...GC    954
H.BACTERIOPHORA AY321477 ATCGATAACTGGTGGCTATGTGT...GACA.TTAGTCACATAGGTA.TCT...GC    442
H.MEXICANA AY321478       ATCGATAGTTGGTGGCTAAGTGATGAGACT.TTGTCAAAATCACTG.ATC...TG    447
H.MEGIDIS AY321480        GTCGATAACTGGTGGCTA..TGA.GTGACT.TCGGTCAC.TCGA.G.ATC...TG    444
H.ZEALANDICA AY321481     ATCGTGGACCGGTGGCTG..TGATGTGACT.TCGGTCACATCGA.G.ATC...CG    442
H.TAYSEARAE EF043443      ATCGATAGTTGGTGGCTAAGTGATGAGACT.TTGTCAAAATCACTA.ATC...TG    468
H.INDICA AY321483         ATCGATAACTGGTGGCTGAGTGA.............GAAATCACTGGATC...TG    431
PANAGROLAIMUS DQ121438    ATACGCAACACGGGACCTCTCGGGGTCGCTGTTGATAGTCTTAATG.ATCGGATG    317
H.BACTERIOPHORA SP        ATCGATAGCTGGTGGCTATGTG...TGACA.TTAGTCACATAGGTA.TCT...GC    292
H.DOWNESI EF043442        GTCGATTACTGGTGGCTA..TGA.GTGACT.TCGGTCAC.TCGA.G.ATC...TG    457
H.BREVICAUDIS EF408912    ATCGATAACTGGTGGCTGAGTGA.............GAAATCACTGGATC...TG    455
H.BACTERIOPHORA EF690469 ATCGATAACTGGTGGCTATGTG...TGACA.TTAGTCACATAGGTA.TCT...GC    237
H.ZEALANDICA SP           ATCGTGGACCGGTGGCTG..TGATGTGACT.TCGGTCACATCGA.G.ATC...CG    288
```

```
H.FLORIDENSIS DQ372922   ATCGATAGTTGGTGGCTAAGTGATGAGACT.TTGTCAAAATCACTA.ATC...TG    473
H.AMAZONENSIS DQ665222   ATCGATAGTTGGTGGCTAAGTGATGAGACT.TTGTCAAAATCACTA.ATC...TG    460
H.MARELATUS EF043441     ATCGATAACTGGTGGCTA..TGATGTGACT.TCGGTCGCATCGA.G.ATC...CG    463

H.BAUJARDI AF548768      CTATGCGGGGAGCCTTAATG.AGTTGT..TCGTGTCAC.CTGACCGAG.ACAACC    337
H.ARGENTINENSIS AF029706 TGATGCAGAGAGCCTTAATG.AGTTGT..TCGTGTCAT.CTGACCTA...CAACC    490
H.HAWAIENSIS AF029707    CTATGCAGGGAGCCTTAATG.AGTTGG..T.......C.TTCACCGAC.ACAACC    474
H.HEPIALUS AF083004      CCTAGTTCTGACCGTAAACT.ATGCCA..TCTAGCGAT.CCAATGGGG.TATTGT   1004
H.BACTERIOPHORA AY321477 TGATGCAGAGAGCCTTAATG.AGTTGT..TCGTGTCAT.CTGACCTA...CAACC    490
H.MEXICANA AY321478      CTATGCGGGGAGCCTTAATG.AGTTGT..TCGTGTCAC.TTGGCCGAG.ACAACC    497
H.MEGIDIS AY321480       CTATGCAGAGAGTCTCAATG.AGTTGTGTTCGTC.............CAACC    482
H.ZEALANDICA AY321481    CTATGCGGAGAGCCTCAATG.AGTTGT.TCCGTC.............AACCG    479
H.TAYSEARAE EF043443     CTATGCGGGGAGCCTTAATG.AGTTGT..TCGTGTCAC.TTGGCCGAG.ACAACC    518
H.INDICA AY321483        CTATGCAGGGAGCCTTAATG.AGTTGG..T.......C.TTCACCGAC.ACAACC    474
PANAGROLAIMUS DQ121438   CTGACCTAAAAAGCAGTGCT.CGGCGC..CAACAACAC.GTAAGCACCTGTTCTT    368
H.BACTERIOPHORA SP       TGATGCAGAGAGCCTTAATGGAGTTGT..TCGTGTCCTTCTGACCT...ACAACC    342
H.DOWNESI EF043442       CTACGCAGAGAGCCTCAATG.AGTTGT..TCGTC.............CAACC    493
H.BREVICAUDIS EF408912   CTATGCAGGGAGCCTTAATG.AGTTGG..T.......C.TTCACCGAC.ACAACC    498
H.BACTERIOPHORA EF690469 TGATGCAGAGAGCCTTAATG.AGTTGT..TCGTGTCAT.CTGACCT...ACAACC    285
H.ZEALANDICA SP          CTATGCGGAGAGCCTCAATG.AGTTGT.TCCGTC.............AACCG    325
H.FLORIDENSIS DQ372922   CTATGCGGGGAGCCTTAATG.AGTTGT..TCGTGTCAC.TTGACCGAG.ACAACC    523
H.AMAZONENSIS DQ665222   CTATGCGGGGAGCCTTAATG.AGTTGT..TCGTGTCAC.TTGACCGAC.ACAACC    510
H.MARELATUS EF043441     CTTTGCGGCTAGCCTCAATG.AGTTGT..TTGCT.............CAACC    499

H.BAUJARDI AF548768      GCCAGTATCGGTA.AATCTC.....TTCCCAATTAACTTGTTTCTAG..TGAAG.    383
H.ARGENTINENSIS AF029706 GCCAGTATCGGTA.AATCAA.......CCCAATTAACTTGTTTCTTG..TGTCGT    535
H.HAWAIENSIS AF029707    GCCACTATCGGTA.A.TCTA.....TTCCCAATTAACTTGTTTCTAG..TAAAA.    519
H.HEPIALUS AF083004      AGCCTTGTTGGGG.AGCTTC.....CCGGAAACGAAAGTCTTTCGGTTCCT.GG.   1051
H.BACTERIOPHORA AY321477 GCCAGTATCGGTA.AATCAA.......CCCAATTAACTTGTTTCTTG..TGTCGT    535
H.MEXICANA AY321478      GCCAGTATCGATA.AATCTC.....TTCCCAATTAACTTGTTTCTAG..TAAAG.    543
H.MEGIDIS AY321480       GCCGGTGTCGATAGAAATTT.....TTTCCTATTAACTTGTTTCCTG.ATTCGT.    530
H.ZEALANDICA AY321481    CCTTGCGATATTTGATTTTTGGGTACGTATCGTTATCTTGTCTCTTGGATACGT.    533
H.TAYSEARAE EF043443     GCCAGTATCGATA.AATCTC.....TTCCCAATTAACTTGTTTCTAG..TAAAG.    564
H.INDICA AY321483        GCCACTATCGGTA.A.TCTA.....TTCCCAATTTACTTGTTTCTAG..TAAAA.    519
PANAGROLAIMUS DQ121438   ATTTGATTCAGTA.CACTGT.....TGCCAAAAGCTTCGGCTTGCGG..TGATT.    414
H.BACTERIOPHORA SP       GCCACTATCGGTA.AATCA.......ACCCAATTAACTTGTTTCTTG..GGTCGT    387
H.DOWNESI EF043442       GCCGGTGTCGATAGAAATTT.....TTTCCTATTAACTTGTTTCTTG.ATTCGT.    541
H.BREVICAUDIS EF408912   GCCACTATCGGTA.A.TCTA.....TTCCCAATTAACTTGTTTCTAG..TAAAA.    543
H.BACTERIOPHORA EF690469 GCCAGTATCGGTA.AATCA.......ACCCAATTAACTTGTTTCTTG..TGTCGT    330
H.ZEALANDICA SP          CCTTGCGATATTTGATTTTTGGGTACGTATCGTTATCTTGTCTCTTGGATACGT.    379
H.FLORIDENSIS DQ372922   GCCAGTATCGGTA.AATCTC.....TTCCCAATTAACTTGTTTCTAG..TAAAG.    569
H.AMAZONENSIS DQ665222   GCCAGTATCGGTA.AATCTC.....TTCCCAATTAACTTGTTTCTAG..TAAAG.    556
H.MARELATUS EF043441     GCCGGTGTCGATA.AATCTT.....ATCTCAATTAACTTGTTTCTTG.ATTCGT.    546

H.BAUJARDI AF548768      GCT..ATTGAGTGT.TAGTGGA....A.........CATTAGCCTT.AGCGATGG    421
H.ARGENTINENSIS AF029706 GTT..AATACATACCTGGCAAA....GTG.......TATTAGCTTT.AG         570
H.HAWAIENSIS AF029707    GGCTAAATTAGT...CAGTGGA....A.........AAT.AGCCTT.AG         550
H.HEPIALUS AF083004      GGT..AGTATGG...TTGCAAA....G.CTGAAACTTAAA........GAAATTG   1088
H.BACTERIOPHORA AY321477 GTT..AATACATAC.TGGCAAA....GTG.......TATTAGCTTT.AGCGATGG    575
H.MEXICANA AY321478      GCT..ATTGAGT...TAGTGGA....G.........CATTAGCCTT.AGCGATGG    579
H.MEGIDIS AY321480       GTT..AATACAT...TTTGGCACAATG.........TATTAGCTTC.AGCGATGG    570
H.ZEALANDICA AY321481    GTT..AATACAT...TTTGGCACAATG.........TATTAGCTTC.AGCGATGG    573
H.TAYSEARAE EF043443     GCT..ATTGAGT...TAGTGGA....A.........CATTAGCCTT.AGCGATGG    600
H.INDICA AY321483        GGCTAAATTAGT...CAGTGGA....A.........AAT.AGCCTT.AGCGATGG    556
PANAGROLAIMUS DQ121438   GCT..GTTGAGTTTCCCTAGGGATCTCGATAATACGTACTAGCTTC.AGCGATGG    466
H.BACTERIOPHORA SP       GTT..AATACATAC.TGGGCAAAATGTAT.........TTAGCTTTTAGCGATGG    430
```

```
H.DOWNESI EF043442       GTT..AATACAT...TTTGGCACAATG.........TATTAGCTTC.AGCGATGG    581
H.BREVICAUDIS EF408912   GGCTAAATTAGT...CAGTGGA....A.........AAT.AGCCTT.AGCGGTGG    580
H.BACTERIOPHORA EF690469 GTT..AATACATAC.TGG.CAAAGTGTAT.........T.AGCTTT.AGCGATGG    370
H.ZEALANDICA SP          GTT..AATACAT...TTTGGCACAATG.........TATTAGCTTC.AGCGATGG    419
H.FLORIDENSIS DQ372922   GCT..ATTGAGT...TAGTGGA....A.........CATTAGCCTT.AGCGATGG    605
H.AMAZONENSIS DQ665222   GCT..ATTGAGTAG.TTGTGGA....A.........CATTAGCCTT.AGCGATGG    594
H.MARELATUS EF043441     GTT..AATACAT...TTTGGCACAATG.........TATTAGCTTC.AGCGATGG    586

H.BAUJARDI AF548768      ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.GC    471
H.HEPIALUS AF083004      ACGGAAGGGCA.CCACCAGGAGTGGAGCC.TGCGGCTTAATTTGACTCAACA.CG   1140
H.BACTERIOPHORA AY321477 ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.AC    625
H.MEXICANA AY321478      ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.GC    629
H.MEGIDIS AY321480       ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.AC    620
H.ZEALANDICA AY321481    ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..TAGCTGCGTTATTT.AC    623
H.TAYSEARAE EF043443     ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.GC    650
H.INDICA AY321483        ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..TAGCTGCGTTATTT.AC    606
PANAGROLAIMUS DQ121438   ATCGGTTGCAT.CGAGTATCGATGAAGAA.CGCAGC..TTGTTGCGTTACTT.AC    516
H.BACTERIOPHORA SP       ATCGGTTGATTTCGCGGATCGATGAAAAAACGCAGC..AAGCTGCGTTATTTTAC    483
H.DOWNESI EF043442       ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.AC    631
H.BREVICAUDIS EF408912   ATCATCGGACT.CGTAA                                          596
H.BACTERIOPHORA EF690469 ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.AC    420
H.ZEALANDICA SP          ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..TAGCTGCGTTATTT.AC    469
H.FLORIDENSIS DQ372922   ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.GC    655
H.AMAZONENSIS DQ665222   ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.GC    644
H.MARELATUS EF043441     ATCGGTTGATT.CGCGTATCGATGAAAAA.CGCAGC..AAGCTGCGTTATTT.AC    636

H.BAUJARDI AF548768      CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAATTTT.GAACGCACAG    515
H.HEPIALUS AF083004      GGAAAA.CTCACCCGGCCCGGACACCGT.TAGGATTGACAGATT.GAAAGCTCTT   1192
H.BACTERIOPHORA AY321477 CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    669
H.MEXICANA AY321478      CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAATTTT.GAACGCACAG    673
H.MEGIDIS AY321480       CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    664
H.ZEALANDICA AY321481    CACGAA.TTG.........CAGACGCTTA.GAGTGGTAAAGTTTT.GAACGCACAG    667
H.TAYSEARAE EF043443     CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAATTTT.GAACGCACAG    694
H.INDICA AY321483        CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    650
PANAGROLAIMUS DQ121438   CACGAA.TTG.........GAGACGCTTA.GAGTGGGGAAATTTC.GAACGCCTAG    560
H.BACTERIOPHORA SP       CACGAAATTG.........CAGACGCTTAAGAGTGGGGAAGTTTTTGAACGCACAG    530
H.DOWNESI EF043442       CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    675
H.BACTERIOPHORA EF690469 CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    464
H.ZEALANDICA SP          CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    513
H.FLORIDENSIS DQ372922   CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAATTTT.GAACGCACAG    699
H.AMAZONENSIS DQ665222   CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAATTTT.GAACGCACAG    688
H.MARELATUS EF043441     CACGAA.TTG.........CAGACGCTTA.GAGTGGTGAAGTTTT.GAACGCACAG    680

H.BAUJARDI AF548768      CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    564
H.HEPIALUS AF083004      TCTCGATTTGGTGGTTGG..TGGTGCATGGCCGT.TCTTAGTTGGTGGA..GCGA   1242
H.BACTERIOPHORA AY321477 CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAA    718
H.MEXICANA AY321478      CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    722
H.MEGIDIS AY321480       CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTATATAGAGT    715
H.ZEALANDICA AY321481    CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGCTTA..AAGT    716
H.TAYSEARAE EF043443     CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    743
H.INDICA AY321483        CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    699
PANAGROLAIMUS DQ121438   CACCG.ACTGGGTCTCCA..GCCGGTACGTCTGG.TTCAGGGTTGTTTA..ACTC    609
H.BACTERIOPHORA SP       CGCCG.TTGGGTTTTTCCCTTTCGGCACGTCTGGGCTCAGGGTTGTTTA..ATAA    582
H.DOWNESI EF043442       CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..AAGT    724
H.BACTERIOPHORA EF690469 CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAA    513
H.ZEALANDICA SP          CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGCTTA..AAGT    562
```

```
H.FLORIDENSIS DQ372922   CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    748
H.AMAZONENSIS DQ665222   CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..ATAG    737
H.MARELATUS EF043441     CGCCG.TTGGGTTTTCCC..TTCGGCACGTCTGG.CTCAGGGTTGTTTA..AAGT    729

H.BAUJARDI AF548768      ACT.TCGGTATT....GCTAGGA....AGGCGGCAATACCGTGAACCAAACGGTG    610
H.HEPIALUS AF083004      TTTGTCTGGTTT....ATTCCGA....TAACGAGCGAGACTCTAGCCTGCTAAC.   1288
H.BACTERIOPHORA AY321477 GCG.AAAGTGTTGAAAGTTCATT....AAACGAGAGTTCGGTGATACTGACAACA    768
H.MEXICANA AY321478      ACT.TCGGTATT....GCTAGGA....AGGCGGCAATACCGTGAACCAAACGGTG    768
H.MEGIDIS AY321480       ACT.GCGGTGTT....GCTCCGA....GAGTAGCAATGTCGAGTGTCGAACGGCG    761
H.ZEALANDICA AY321481    ACT.GCGGTGTT....GCTGCGA....AAGTGGCAACGCCGAATGTCGAGCGGCG    762
H.TAYSEARAE EF043443     ACT.TCGGTATT....GCTTGGA....AGGCAGCAATACCGCGAACCAAACGGTG    789
H.INDICA AY321483        ACT.TCGATATT....GCTAGGA....AGGCAGCAATATCGTGCACCGAACGGTG    745
PANAGROLAIMUS DQ121438   ACT.GCCCAACT..ATTGCAGTTGTGGC.........TTGTCGGATGACATTGAG    652
H.BACTERIOPHORA SP       GCG.AAAGTGTT....GAAAGTTCATTAAACGAGAGTTCGGTGATACTGACAACA    632
H.DOWNESI EF043442       ACT.GCGGTGTT....GCTGCGA....AAGTAGCAACACCGAATGTCGAACGGCG    770
H.BACTERIOPHORA EF690469 GCG.AAAGTGTT....GAAAGTTCATTAAACGAGAGTTCGGTGATACTGACAACA    563
H.ZEALANDICA SP          ACT.GCGGTGTT....GCTGCGA....AAGTGGCAACGCCGAATGTCGAGCGGCG    608
H.FLORIDENSIS DQ372922   ACT.TCGGTATT....GCTAGGA....AGGCGGCAATACCGTGAACCAAACGGTG    794
H.AMAZONENSIS DQ665222   ACT.TCGGTATT....GCTTGGA....AGGCGGCAATACCGTGAACCAAACGGTG    783
H.MARELATUS EF043441     ACT.GCGGTGTT....GCTGCGA....AAGTGGCAACACCGATTGTCGAACGGCG    775

H.BAUJARDI AF548768      AT......A..GTGCCTAAAAT....GTG.GTGCATGC...CCCGTTACAGGGGA    649
H.HEPIALUS AF083004      .T......A..GTGTCCAGA.TA.T.GTA.GGTCTGGCTTACTTCTTAGAGGGAT   1330
H.BACTERIOPHORA AY321477 CT......ACGTCGAGCGGTGTACT.GTT.GAAAGTAC...CCCGTTCAAGTATC    812
H.MEXICANA AY321478      AT......A..GTGTCTAGAATA.T.GTG.GTGCATGC...CCCGTTACAGGGGA    809
H.MEGIDIS AY321480       TT......AGTGT..............TGGTTACTGGC...CCCGTTCTAGTTCC    793
H.ZEALANDICA AY321481    TT......AGTGT..............TA.CTACTGGC...CCCGTTCTAGTTCC    793
H.TAYSEARAE EF043443     AT......A..GTGTCTAGAATA.T.GTG.GTGCATGC...CCCGTTACAGGGGA    830
H.INDICA AY321483        AT......A..GTGTCTATAAAA.TAGTG.GTGCATAC...CCCGTTTTAGGGTA    787
PANAGROLAIMUS DQ121438   AT......A..CTATCGATCAG....GCA.ACTGATTG...TAGGTGACTCGATC    691
H.BACTERIOPHORA SP       CTGCGTCGA..TCGGGTGTACT....GTT.GAAAGTAC...CCCGTTCAAGTATC    677
H.DOWNESI EF043442       TT......AGTGT..............TA.CTACTGGC...CCCGTTCTAGTTCC    801
H.BACTERIOPHORA EF690469 CTACGTCGA..GCGG.TGTACT....GTT.GAAAGTAC...CCCGTTCAAGTATC    607
H.ZEALANDICA SP          TT......AGTGT..............TA.CTACTGGC...CCCGTTCTAGTTCC    639
H.FLORIDENSIS DQ372922   AT......A..GTGTCTAGAATA.T.GTG.GTGCATGC...CCCGTTACAGGGGA    835
H.AMAZONENSIS DQ665222   AT......A..GTGTCTAAATT....GTG.GTGCATGC...CCCGTTACAGGGGA    822
H.MARELATUS EF043441     TT......AGTGT..............TA.CTACTGGC...CCCGTTCTAGTTCC    806

H.BAUJARDI AF548768      GAATGGTGGTTAAATGAATT.T.....NTCTTAACGCCG....GAGAAATATTAG    694
H.HEPIALUS AF083004      AAGCGGTGTTTAGCCGCACG.A.....GATTGAGCGATA....ACAGGTCTGTGA   1375
H.BACTERIOPHORA AY321477 TTTATGGGGCAACATGTCTT.C.....TATATGGAGACAT...GAAAGATATTAA    858
H.MEXICANA AY321478      GAATGGTGGTTAAATGGACT.T.....CTCTTAATGCCG....GAGAAGTATTAG    854
H.MEGIDIS AY321480       GAATATTGGCAACATGTCAT.CACGTGTTCGTGACGACGCCGTAGAGAGTATAGG    847
H.ZEALANDICA AY321481    GAATATTGGCAACATGTCTT.C.....TTCGTGAAGACGCCGTAGAGAGTATAAG    842
H.TAYSEARAE EF043443     GAATGGTGGTTAAATGAACT.T.....CTCTTAACGCCG....GAGAAGTATTAG    875
H.INDICA AY321483        AAATAAAGGTCGTAACTGAA.A.....CTTCTTCCGCCC....GAGAAGTTATAG    832
PANAGROLAIMUS DQ121438   TCGTTCTAGT..ACCCGAAG.C.....ATCACAAGTC......GAGACCCGACAG    732
H.BACTERIOPHORA SP       TTTATGGGGCAACATGTCTTCT.....ATACGGAGACAT....GAAAGATATTAA    723
H.DOWNESI EF043442       GAATATTGGCAACATGTCTT.C.....TACGTGAAGACGCCGTAGAGAGTATAAG    850
H.BACTERIOPHORA EF690469 TTTATGGGGCAACATGTCTTCT.....ATATGGAGACAT....GAAAGATATTAA    653
H.ZEALANDICA SP          GAATATTGGCAACATGTCTT.C.....TTCGTGAAGACGCCGTAGAGAGTATAAG    688
H.FLORIDENSIS DQ372922   GAATGGTGGTTAAATGAATT.T.....CTCTTAAAGCCG....GAGAAATATTAG    880
H.AMAZONENSIS DQ665222   GAATGGTGGTTAAATGAACT.T.....CTCTTAAAGCCG....GAGAAGTATTAG    867
H.MARELATUS EF043441     GAATATCGGCAACATGTCTT.C.....TTCGTGAAGACGCCGTGGAGAGTATAAG    855

H.BAUJARDI AF548768      GTTT...CACTTATGGATATG.CCATGTATGAAATACGACGTGGT.TATATACAT    744
```

```
H.HEPIALUS AF083004       TGCC...CTTAGATGTTCGGGGCTGCACGCGCGCTACACTGGAAG.AATCAGCTG   1426
H.BACTERIOPHORA AY321477 GAGTATATACCTGTGGATGC..CCACGTATGAAATATGACGTGTCGTATACACGG    911
H.MEXICANA AY321478       GTTT...CACTTATGGATATG.CCATGTATGAAATACGACGTGGT.TATATACAT    904
H.MEGIDIS AY321480        TCTG...TACCTGTGGATGTG.TAGCGTATGAAATATGATGCT.T.CCCATACAT    896
H.ZEALANDICA AY321481     TCTG...TACCTATGGATGTG.TCGCGTATGAAATATGATGCT.T.CCCATACAT    891
H.TAYSEARAE EF043443      GTTT...CACTTATGGATATG.CCATGTATGAAATACGACGTGGT.TATATACAT    925
H.INDICA AY321483         GTAA...TACTTATGGATGTG.CCATGTATGAAATATGACGTGGT.TATATACAT    882
PANAGROLAIMUS DQ121438    TTCT...TAGTAATTGTCGAC.TCGTA.ATGTGACGCGCTACTCT.TATAT.CAT    780
H.BACTERIOPHORA SP        GAGTATATACCTGTGGATGC..CCACGTATGAAATATGACGTG.T.CGTATACAC    774
H.DOWNESI EF043442        TCTG...TACCTATGGATGTG.TCGCGTATGAAATATGATGCT.T.CCCATACAT    899
H.BACTERIOPHORA EF690469 GAGTATATACCTGTGGATGC..CCACGTATGAAATATGACGTG.T.CGTATACAC    704
H.ZEALANDICA SP           TCTG...TACCTATGGATGTG.TCGCGTATGAAATATGATGCT.T.CCCATACAT    737
H.FLORIDENSIS DQ372922    GTTT...CACTTATGGATATG.CCATGTATGAAATACGACGTGGT.TATATACAT    930
H.AMAZONENSIS DQ665222    GTTT...CACTTATGGATATG.CCATGTATGAAATACGACGTGGT.TATATACAT    917
H.MARELATUS EF043441      TCTG...TACCTGTGGATGTG.TCGCGTATGAAATATGATGCT.T.CACATACAT    904

H.BAUJARDI AF548768       ....AAAT....CGTGTTTCCTATA...AGTCTCATTATGCAACCTGAGCTCAGT    788
H.HEPIALUS AF083004       ..GTCC.T....TCCATTGCCGAAA...GGTATTGGTAAACCGTTGAAACTCTTC   1471
H.BACTERIOPHORA AY321477 ....CTAGGAGGTATGTCTCAGATG...AATTTGTTTATGCAACCTGAGCTCAGT    959
H.MEXICANA AY321478       ....AAAT....CGTGTTTCCTATA...AGTCTCATTATGCAACCTGAGCTCAGT    948
H.MEGIDIS AY321480        AGCGAGGA....GGTGCCTCTTATACGTATCTTGCTTATGCAACCTGAGCTCAGT    947
H.ZEALANDICA AY321481     GGCTAGGA....GGTGCTTCTTATGC.TATCTTACTTGTGCAACCTGAGCTCAGT    941
H.TAYSEARAE EF043443      ....AAAT....CGTGTTTCCTATA...AGTCTCATTATGCAACCTGAGCTCAGT    969
H.INDICA AY321483         ....AAAT....AGTATTTCCTTGA...AGTCTCATTATGCAACCTGAGCTCAGT    926
PANAGROLAIMUS DQ121438    C                                                         781
H.BACTERIOPHORA SP        ....GGCTAG                                                780
H.DOWNESI EF043442        AGCGAGGA....GGTGCTTCTTATGC.TATCTTGCTTATGCAACCTGAGCTCAGT    949
H.BACTERIOPHORA EF690469 ....GGCTAGGAGGTATGTCTCAGATGAATTTGTTTATGCAACCTGAGCTCAGTC    755
H.ZEALANDICA SP           GGCTA                                                     742
H.FLORIDENSIS DQ372922    ....ATAT....CGTGTTTCCTATA...AGTCTCATTATGCAACCTGAGCTCAGT    974
H.AMAZONENSIS DQ665222    ....AAAT....CGTGTTTCCTATA...AGTCTCATTATGCAACCTGAGCTCAGT    961
H.MARELATUS EF043441      GGCGAGGA....GGTGCTTCTTGTAC.TATCTTGCTTATGCAACCTGAGCTCAGT    954
```

Fig 4.3: DNAman multiple sequence alignment of the ITS1 - rDNA partial sequence obtained for *H. zealandica* and *H. bacteriophora*, South African isolate

4.4 Discussion

The recent development of PCR and DNA sequencing has been shown to be a powerful tool to interpret phylogenetic relationships at different taxonomic levels and as well as for species delineation. The phylogeny of the genus *Heterorhabditis* has been reconstructed based on the analyses of sequences from the partial 18S gene of rDNA (Liu *et al*., 1997) .The survey on the occurrence of EPNs in the ARC Roodeplaat experimental farm and Brits, revealed the presence of two species distributed within this region. According to molecular observations (PCR and DNA sequencing), the first nematodes was identified as *H. bacteriopohora* (Poinar, 1976) and the second as *H. zealandica* (Poinar, 1990a). This species was only isolated from cultivated sandy soil collected from the ARC Roodeplaat experimental farm and Brits respectively. In spite of these efforts, the ITS region seems to be only useful for resolving relationships among closely related *Heterorhabditis* species, and is perhaps too variable to reliably infer relationships among all species in the genus. *H. bacteriophora* isolates from South Africa was most similar to *H. bacteriophora*, accession numbers EF690469 and the *H. zealandica* was closely related to the *H. zealandica*, accession numbers AY321481 (Fig 4.2). The resulting phylogenetic tree was congruent with most implicit and explicit expectations of relatedness based on overall morphological similarity or evolutionary interpretations of particular characters. (.Adams *et al*., 1998; Blaxter *et al*., 1998; Szalanski *et al*., 2000; Stock *et al*., 2001). But both approaches are complementary and valuable for identifying and species of *Heterorhabditis* and confirm conclusion made by Stock & Kaya (1996).

Chapter 5
Morphological Classification of Nematode Isolates 411 and 386

5.1 Introduction

Morphological identification using standard light microscopy, confocal microscopy and scanning electron microscopy according to Kaya & Stock (1997) appears to be the most widely accepted and reliable method. Nguyen & Smart (1996) have published taxonomic keys to aid the identification of both *Heterorhabditis* and *Steinernema* species. Identification based solely on infective juveniles may not be accurate because there are few differentiating morphological characteristics between species and morphormetrical ranges of several species overlap. Characteristics of males and females must be used for accurate identification of most species. Recently, Nguyen & Smart (1996) reported that morphometrics of *Heterorhabditis* and *Steinernema* species vary depending on the time of harvest and whether the nematodes are reared *in vitro* or *in vivo*. These differences complicate identification in some cases.

5.2 Materials & Methods

In general, *H. zealandica* and *H. bacteriophora* were identified using a key provided by Adams & Nguyen (2002). The following morphological features that are examined for their identification including structures of stomal opening, the position and number of papillae, the presence and size of phasmids and/or amphids; presence/absence of dorsal teeth, the morphology of the vulva and shape of the tail tip. Morphological identification of *H. zealandica* and *H. bacteriophora* were examine both first and second generation, carried out using scanning electron microscopy (section 2.7.1.8) and light microscopy (section 2.7.1.7)

5.3 Results

5.3.1 *H. zealandica*

The light microscope shows that, *H. zealandica* is characterized by the following combination of characters: The hermaphroditic female has a smooth cuticle, no lateral lines. In small females the lip region shows pronounced labial papillae projecting. In large females, the lip frequently shows wide shoulders surrounding stoma (Fig 5.1). The labial papillae are large. The cheilostom is present, but wide. The sclerotized protostom are not present. The telostom is present, initially as wide as cheilostome. The esophagus is short in relation to total length narrowing nerve ring (Fig 5.1). Basal pharyngeal bulb is pyriform moves the food from the pharynx to the intestine and is connected to pharynx by the isthmus (Lee& Atkinson, 1976; Kaya & Stock, 1997) (Fig 5.2). The excretory pore is situated at level of basal bulb. The genital tract is found paired and reflexed. The rectum and anus are distinct (Fig 5.4). Rectum is the short portion of the digestive tract at the end of the intestine .It opens into anus. Reproductive system opens through the vulva (Fig 5.6). The tail is conical with very narrow pointed terminus (Fig 5.3). The male has a cuticle smooth, with no lateral

lines. The lip region and stoma are similar to pre-adult female. The esophagus is more than half body width wide narrowing distinctly at level of nerve ring. The basal bulb is pyriform with distinct valve. The excretory pore is located posterior to level of base of esophagus. The testis is present, transparent and reflexed. The spicules are slender and paired. The gubernaculum is small and single. The tail is tapering. The tail is enveloping by the bursa. In most IJs the cuticle is easily seen by scanning electron microscopy with large number of longitudinal lines starting short distance behind the head and terminating on the tail (Fig 5.7). The nerve ring is distinct but the excretory pore is obscure. The anus is distinct. The tail is similar to the male.

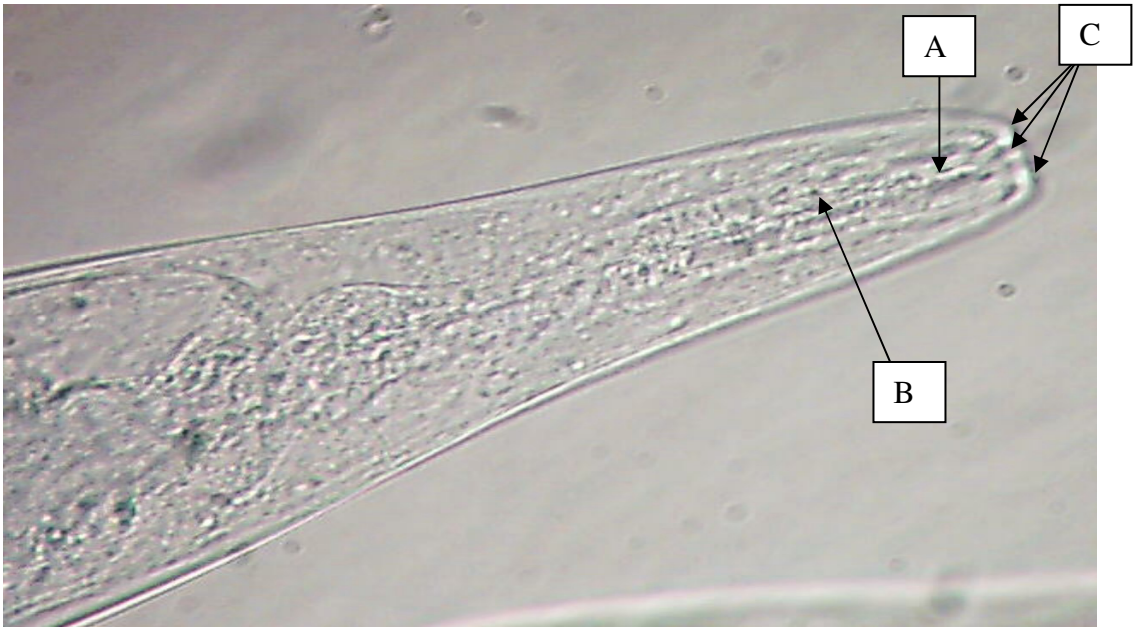


Fig 5.1: Light micrograph of anterior region of female showing A) stoma, B) corpus of pharynx and C) lips.

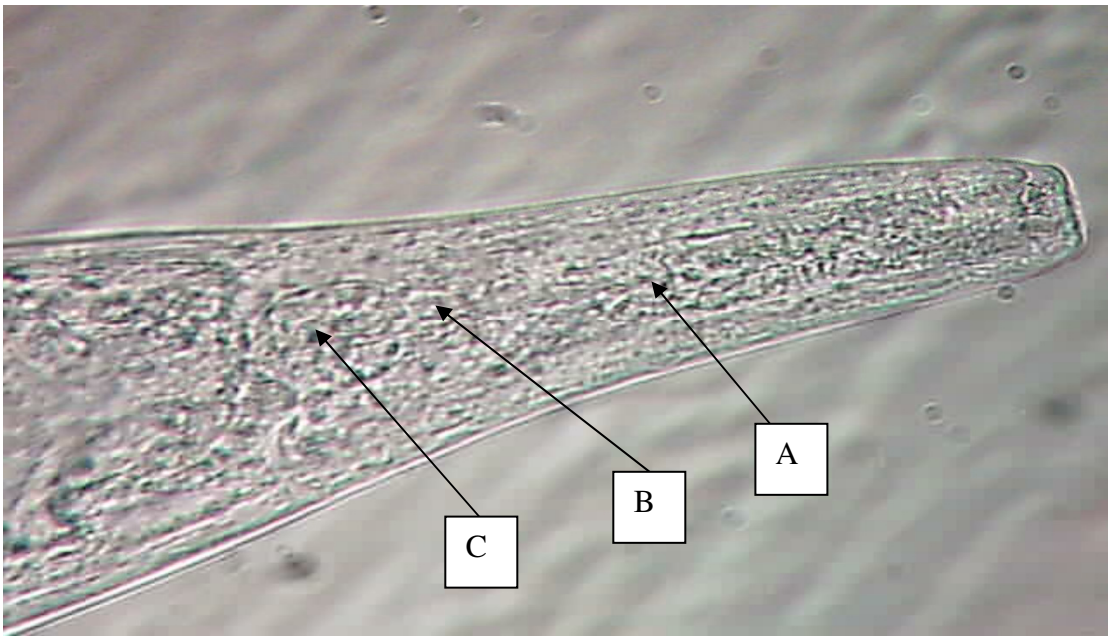


Fig 5.2: Light micrograph of pharyngeal region of adult female showing A) nerve ring B) isthmus C) and basal pharyngeal bulb



Fig 5.3: Light micrograph of young female posterior showing the conical tail with narrow pointed terminus

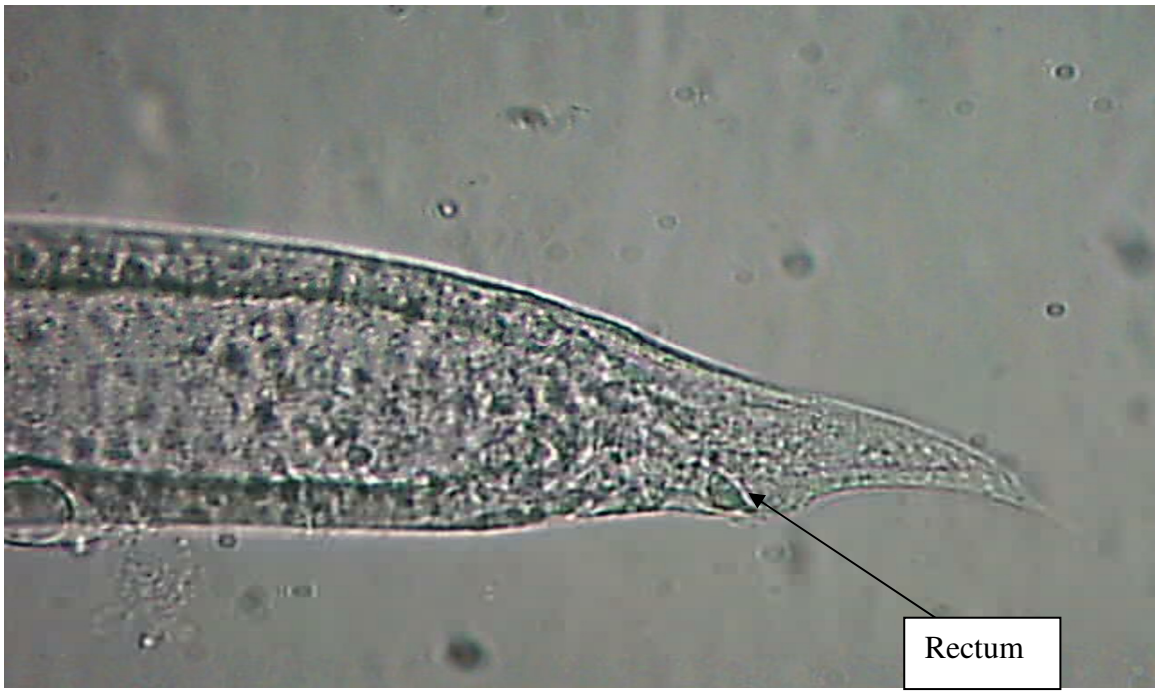


Fig 5.4: Light micrograph of young female posterior showing the rectum

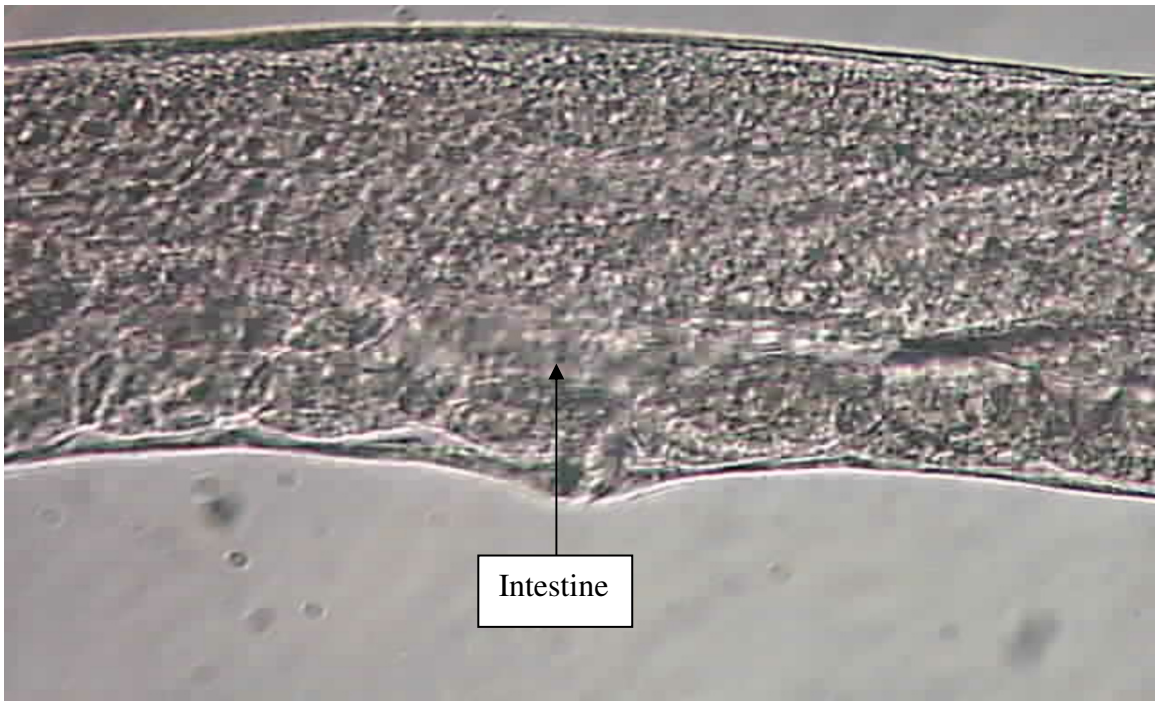


Fig 5.5: Light micrograph: Female mid region showing the intestine.

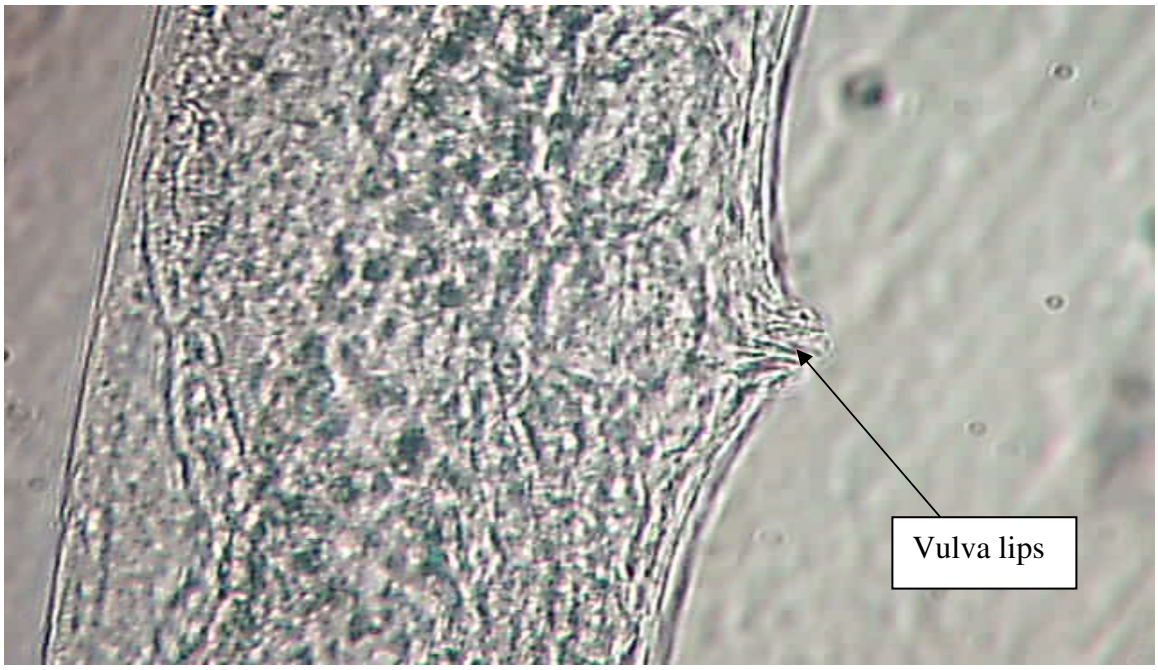


Fig 5.6: Light micrograph: Female mid region showing vulva lips protruding.

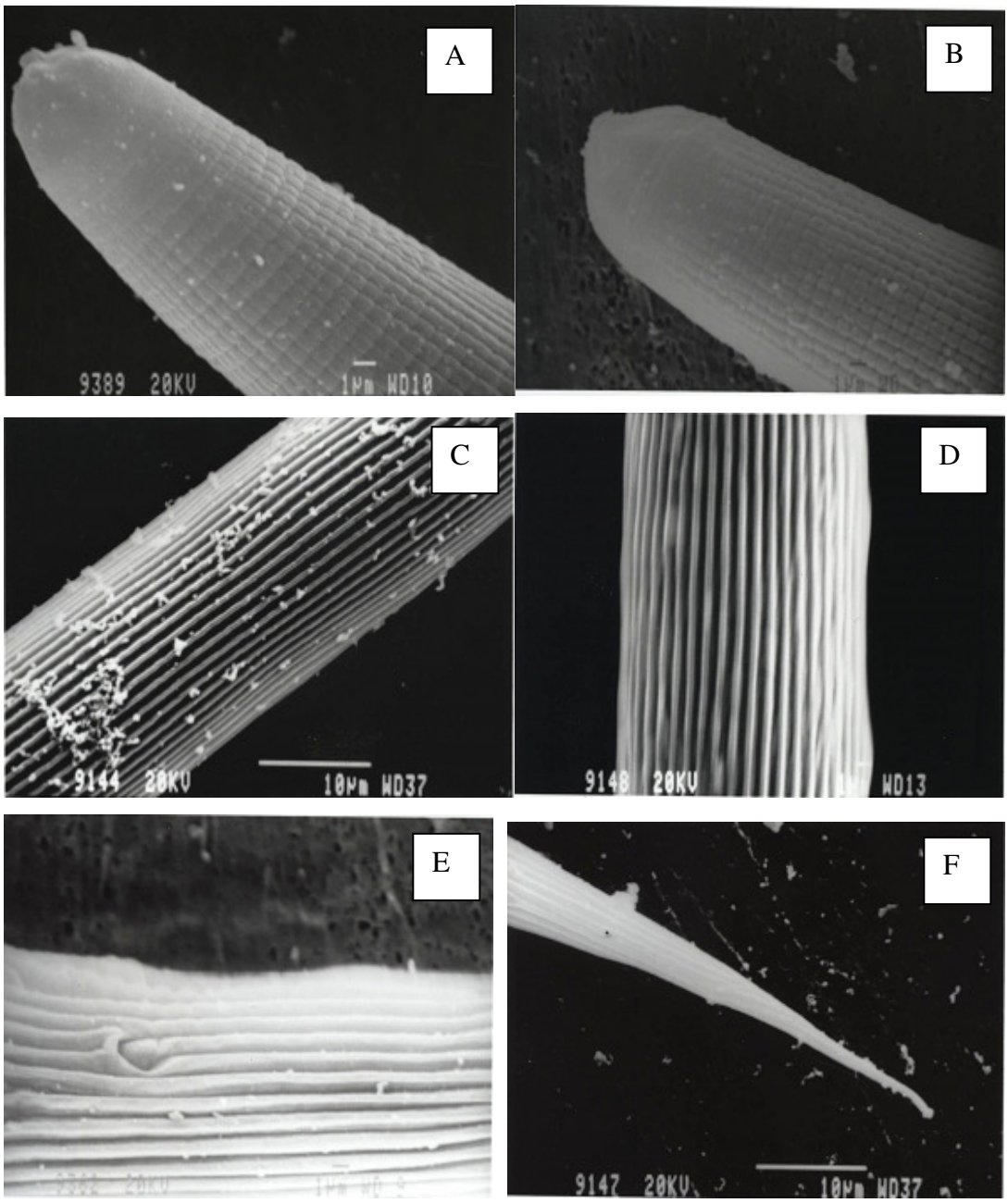


Fig 5.7: *H. zealandica*. Scanning electron micrographs of IJs. A and B) anterior region showing head with tessellate pattern; C) showing position of vulva; D) Portion of body showing tessellate pattern and longitudinal ridges; E) Posterior region showing anus position and; E) the conical tail with narrow pointed terminus

5.3.2 *H. bacteriophora*

The head is truncate or slightly rounded and is situated at the anterior region of the nematode body. This has six distinct protruding lips surrounding the mouth with six labial papillae on top. The mouth is situated on the head and opens into the buccal cavity. Which open into the pharynx and is connected to intestine by the isthmus (Lee & Atkinson, 1976) (Fig 5.8). The amphids are chemoreceptors located on or just behind to labial papillae. The cheilorhabdions is present as lightly refractile areas lining the anterior portion of the stoma (Fig 5.9). The posterior portion of the stoma collapse, with reduced pro-, meso- and metarhabdions. Each metarhabdial segment bears a small tooth. The telorhabdions are absent. The basal bulb is often surrounded by the anterior portion of the intestine (Fig 5.8). The nerve ring can be seen usually surrounding isthmus and he is just positioned anterior to the basal bulb (Fig 5.8). The excretory pore, ventral opening in the cuticle usually located posterior to the basal bulb, serves as a means to eliminate the waste products of the excretory system from the nematodes body (Kaya & Stock, 1997). The intestine is present (Fig 5.10). The phasmids are inconspicuous. The vulval is near mid-body (Fig 5.11). The gonads are amphidelphic and reflexed. The anus is visible and situated anterior to the tail (Fig 5.13). The female tail is pointed (Fig 5.12). Male has a single reflexed testis; head similar to female but short. The rectum is located at the end of the intestine. It opens to cloaca. This is common opening of reproductive and digestive system. Cloaca, anus and rectum are lined with cuticle. The bursa is present, peloderan and open with normally nine pairs of genital papillae. The gubernaculum is present. The IJs are small with excretory pore situated posterior to nerve ring. The tail is pointed. The mouth and anus are closed. The pharynx and intestine are shorts. Scanning electron micrographs of IJs shows anterior region with tessellate pattern. The cuticle frequently shows tessellate pattern and longitudinal ridges. The posterior region shows position of anus and tail.

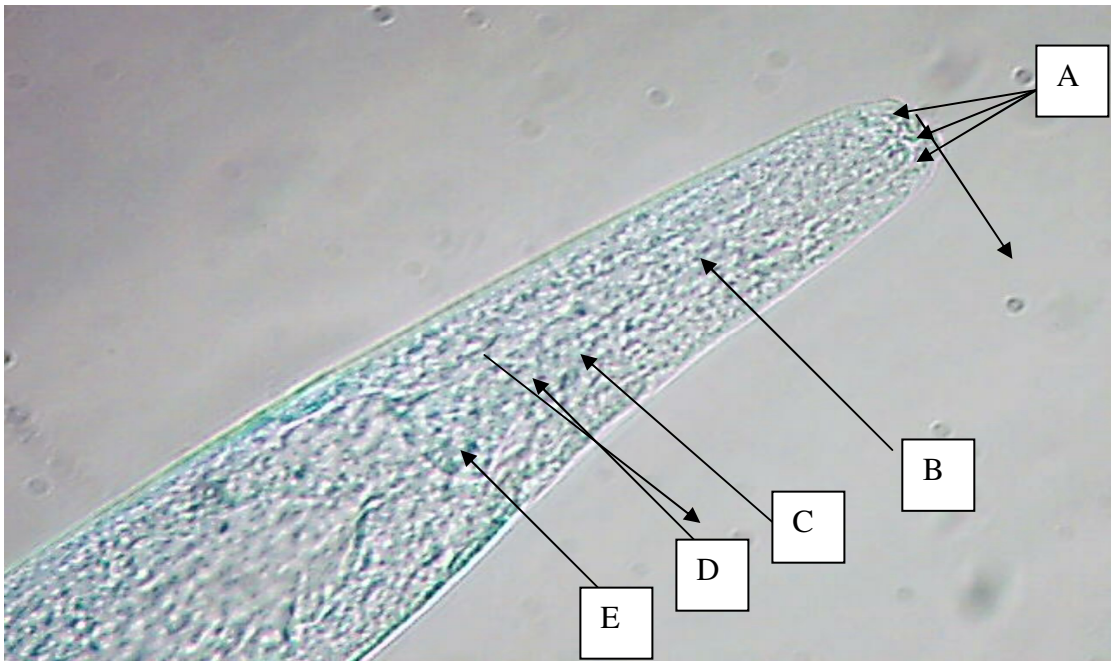


Fig 5 8: Light micrograph of anterior region of young female showing A) transparent lips B) corpus of pharynx, C) nerve ring, D)) isthmus, E) basal pharyngeal bulbe

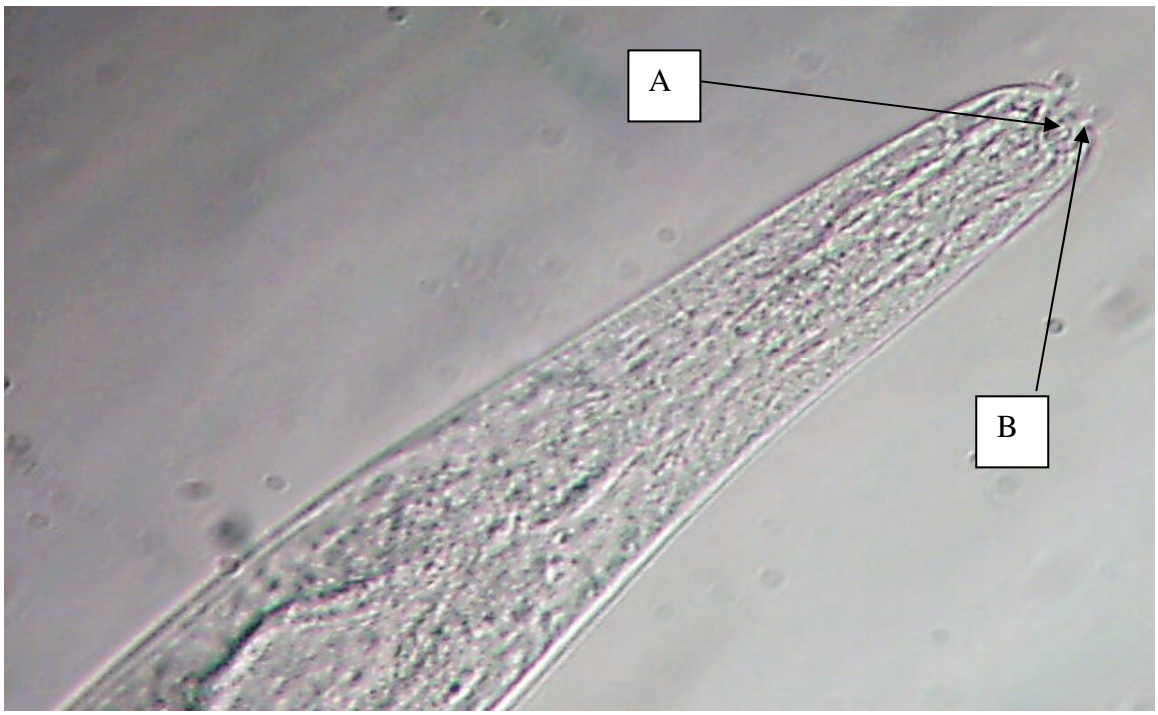


Fig 5 9: Light micrograph of pharyngeal region of adult female showing A) stoma and B) lips

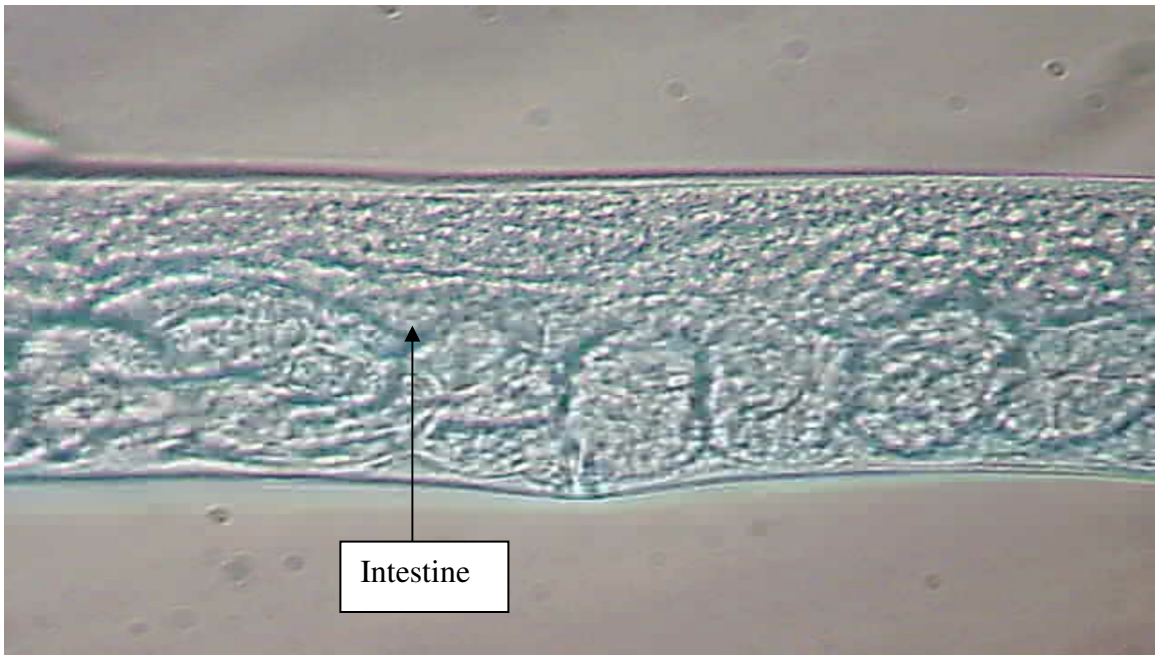


Fig 5.10: Light micrograph: Female mid region showing the intestine.

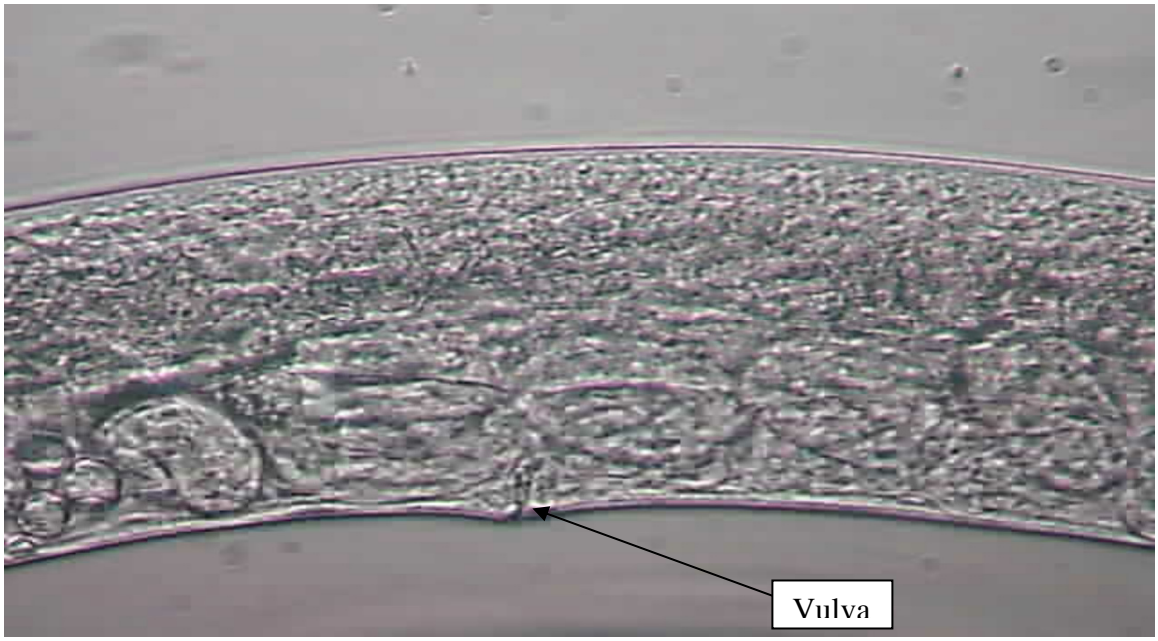


Fig 5.11: Light micrograph: Female mid region showing vulva.

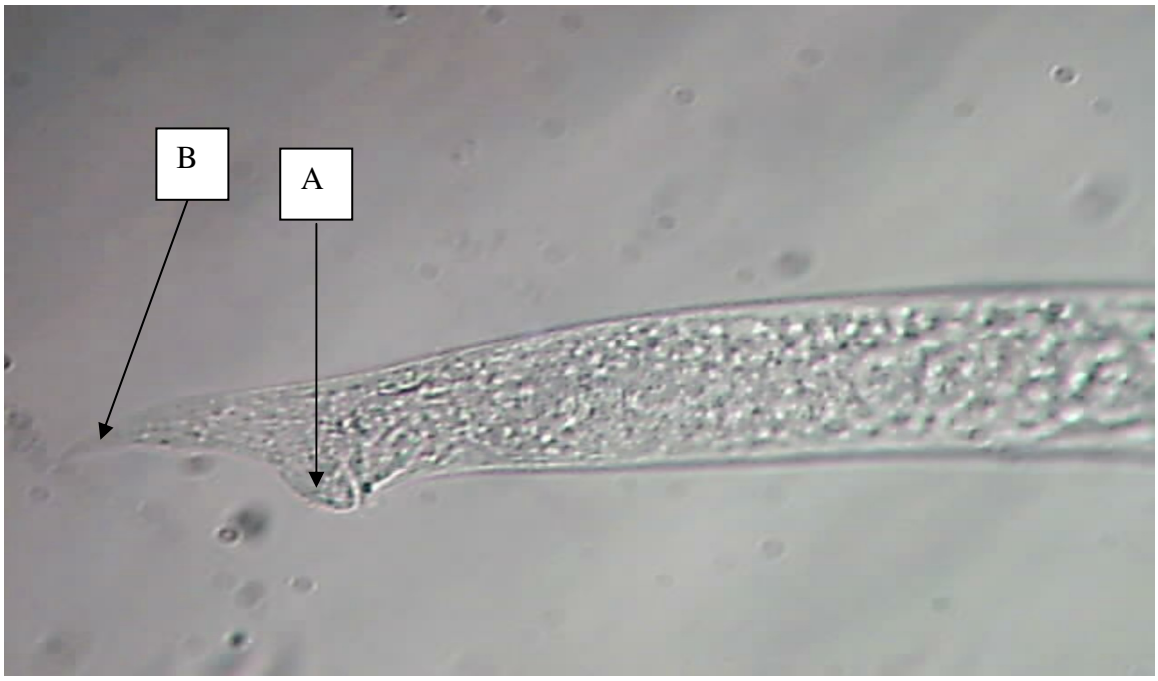


Fig 5 12: Light micrograph of young female posterior showing A) anal region slightly protruding and B) tail pointed

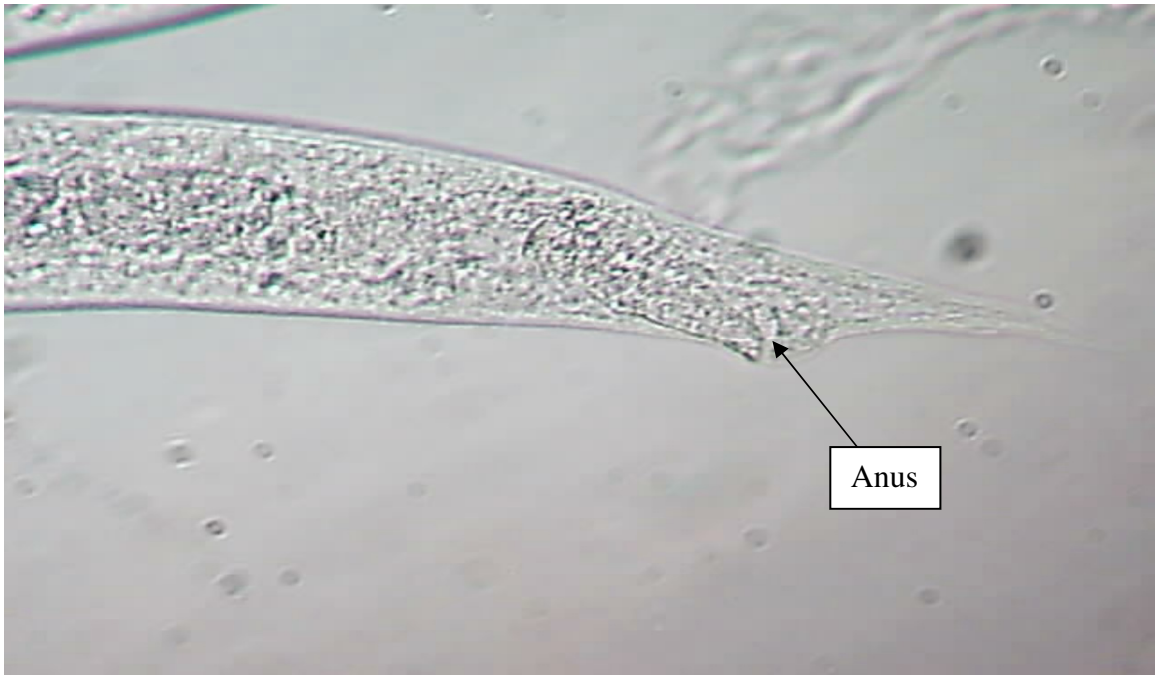


Fig 5 13: Light micrograph of young female posterior showing visible anus and situated anterior to the tail

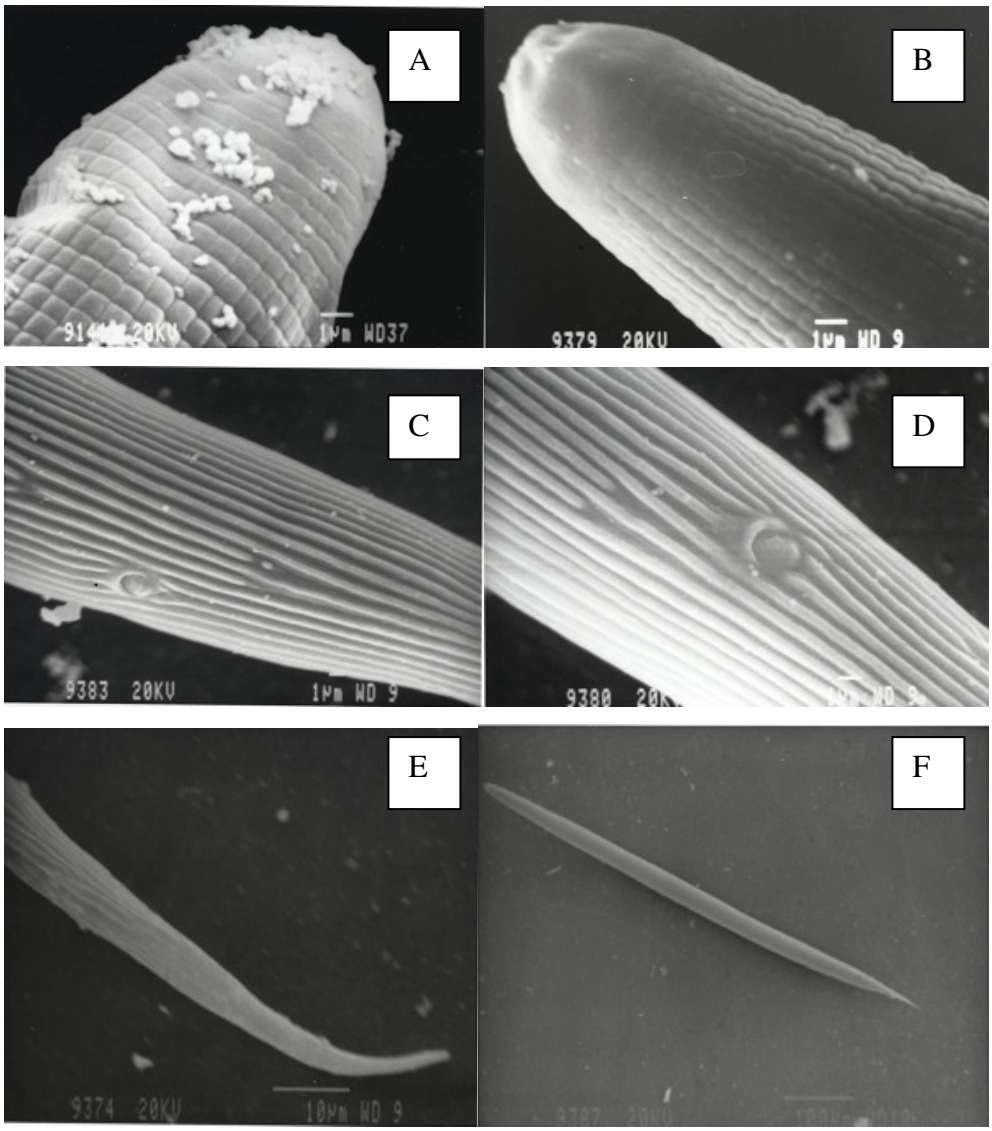


Fig 5.14: *H. bacteriophora*. Scanning electron micrographs of IJs. A and B) anterior region showing head with tessellate pattern; C) Portion of body showing tessellate pattern and longitudinal ridges; D) Posterior region showing anus position and E) tail pointed; F) Whole IJs.

D

5.4 Discussion

The results obtained by morphological characteristics appears to be the most widely accepted and reliable method. Nguyen & Smart (1996) have published taxonomic keys to aid the identification of both *Heterorhabditis* and *Steinernema* species. Observations of both adults and IJs of *H. bacteriophora* and *H. zealandica* were carried out using scanning electron and light microscopy in an attempt to obtain morphological data. The results indicate that the nematodes *H. bacteriophora* and *H. zealandica* are present in South Africa. Despite the importance of nematodes of the genus *Heterorhabditis* in biological control, few studies of morphological and genetical variation in *Heterorhabditis* have been published (Akhurst, 1987; Curran, 1990). Usually, some knowledge of the extent of morphological or genetical of the population and the species is required before a specimen can be assigned to its proper species. As it has been stated by several authors (Smits *et al.,* 1991; Dix *et al.,* 1992; Griffin *et al.,* 1994), morphological characterization of *Heterorhabditis* species is difficult to interpret, especially for untrained biologists and the lack of comparative data makes it difficult to assign individuals of these nematodes to correct species using only morphological characters. However, the developments of molecular techniques (such as allozyme electrophoresis, DNA sequencing, and RAPD analysis) provide useful tools for diagnostic studies at the population and species level (Smits *et al.,* 1991; Gardner *et al.,* 1994).

Chapter 6

Isolation, Characterization and Identification of Endosymbiotic Bacteria Associated with EPNs *H. zealandica* and *H. bacteriophora*

6.1 Introduction

The nematodes and bacteria have a mutualistic relationship. The relationship between nematode and bacteria is highly specific. Steinernematids are associated with *Xenorhabdus* spp. and heterorhabditids are associated with *Photorhabdus* spp (Akhurst & Boemare. 1990). EPNs and their endosymbiotic bacteria are potent bioinsecticides that can control a wide variety of economically important agricultural pests (Shapiro-Ilan & Gaugler 2002). To complete the study of *H. zealandica* and *H. bacteriophora* isolates; the endosymbiotic bacteria associated with these isolates were isolated from surface sterilized IJs method (Akhurst 1980; Kaya & Stock (1997). This method minimizes potential bacterial contamination. The contamination occurs regularly when the bacteria are isolated from the insect hemolymph. The pathogenic status of the bacteria was confirmed using a modified approach to confirm Koch's postulates (see chapter 3) (Poinar, 1975). The objectives of this study were to obtain partial 16S rRNA sequence from the symbiotic bacteria isolates from *H. bacteriophora* and *H. zealandica* and to use these sequence data to determine how similar these bacteria are to previously described species of *Photorhabdus*.

6.2 Materials & Methods

Endosymbiotic bacterial associated with *H. zealandica* and *H. bacteriophora* were successfully isolated and cultured *in vitro* on liquid culture medium (LCM), NBTA and MacConkey agar (section 2 8 1). Phase I and phase II colony variants were obtained by selecting green or blue-green colonies from NBTA, nutrient agar supplemented with bromothymol blue or red colonies from MacConkey agar. Both endosymbiotic bacteria are distinctly rod-shaped, as viewed with the scanning microscope. The bioluminescens was determined visually in the dark room. Some characteristics of endosymbiotic bacteria from isolates *H. zealandica* and *H. bacteriophora* are outlined in (table 6.1). Genomic DNA was extracted from the symbiotic bacteria associated with two consecutive generations of *H. zealandica* and *H. bacteriophora* (section 2 8 2 1). The 16S rDNA genes of both strains were amplified by using primers representing regions of the 16S rDNA conserve in the bacteria. The forward “sense (5 – AGAGTTTGATCCTGGCTCAG – 3, Tm: 60°C) and the reverse “antisense” (5 – AAGGAGGTGATCCACCGCA – 3, Tm: 60°C) (Inqaba biotech) (section 2.8.2.2).

6.3 Results

Table 6.1: Characteristics of *Photorhabdus sp* and *P. luminescens laumondii*

Characteristic	***Photorhabdus* sp**	***P. luminescens laumondii***
Host nematode	*H. zealandica*	*H. bacteriophora*
Colony diameter (mm)	1-3 mm	2-5 mm
Colony morphology on NBTA at room temperature phase I	Circular, convex, opaque, with irregular marginal and sticky consistency	Circular, convex opaque, with irregular marginal and sticky consistency
Colony morphology on NBTA at room temperature phase II	Flat with irregular marginal	Flat irregular marginal
Pigmentation on NBTA	Blue-green with red centre	Green with a red centre
Pigmentation on MacConkey agar	Deep green-brown	Deep red
Pigmentation on nutrient broth	Cream coloured with a shade of orange	Red
Bioluminescens	None	Strong luminescens

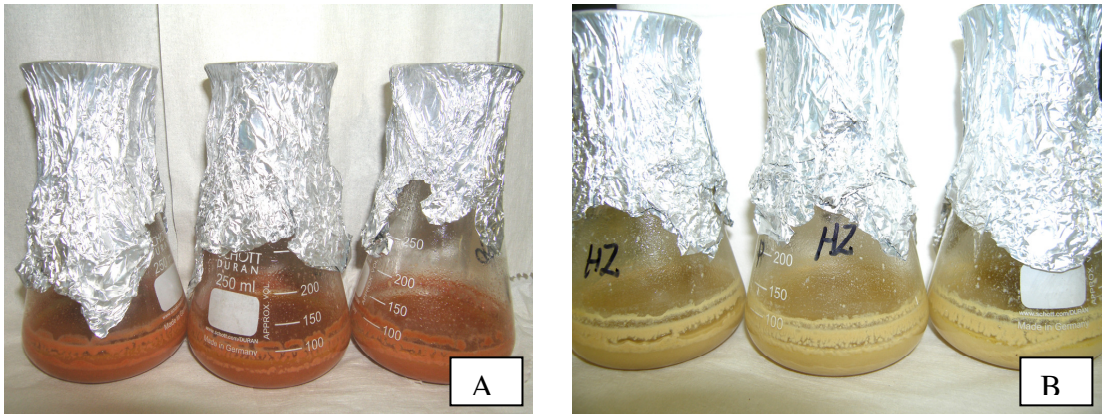


Fig 6.1: Flasks A and B) had been inoculated with a 24 h symbiotic bacterium and incubated on a shaker for 72 h. The different colours are attributed to the symbiotic bacteria. A) Symbiotic bacteria from *H. bacteriophora* and B) Symbiotic bacteria from *H. zealandica*.

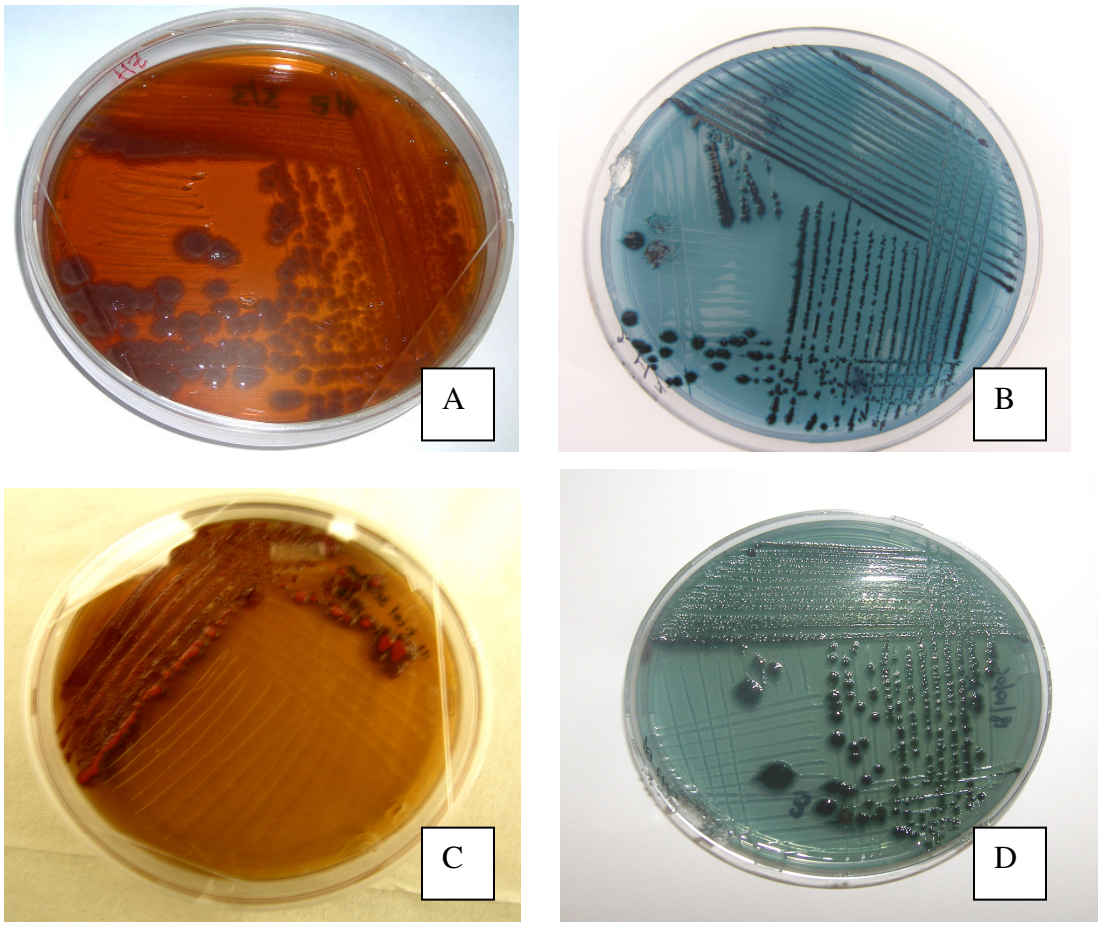


Fig 6.2: EPN symbiotic bacteria growing in MacConkey and NBTA plates. The different colours are characteristic of the bacteria. A and B): Symbiotic bacteria isolated from *H. zealandica*, colonies are deep green-red on MacConkey agar and blue-green with a red centre on NBTA, C and D) Symbiotic bacteria isolated from *H. bacteriophora*; colonies are deep red on MacConkey agar and green with a red centre on NBTA.

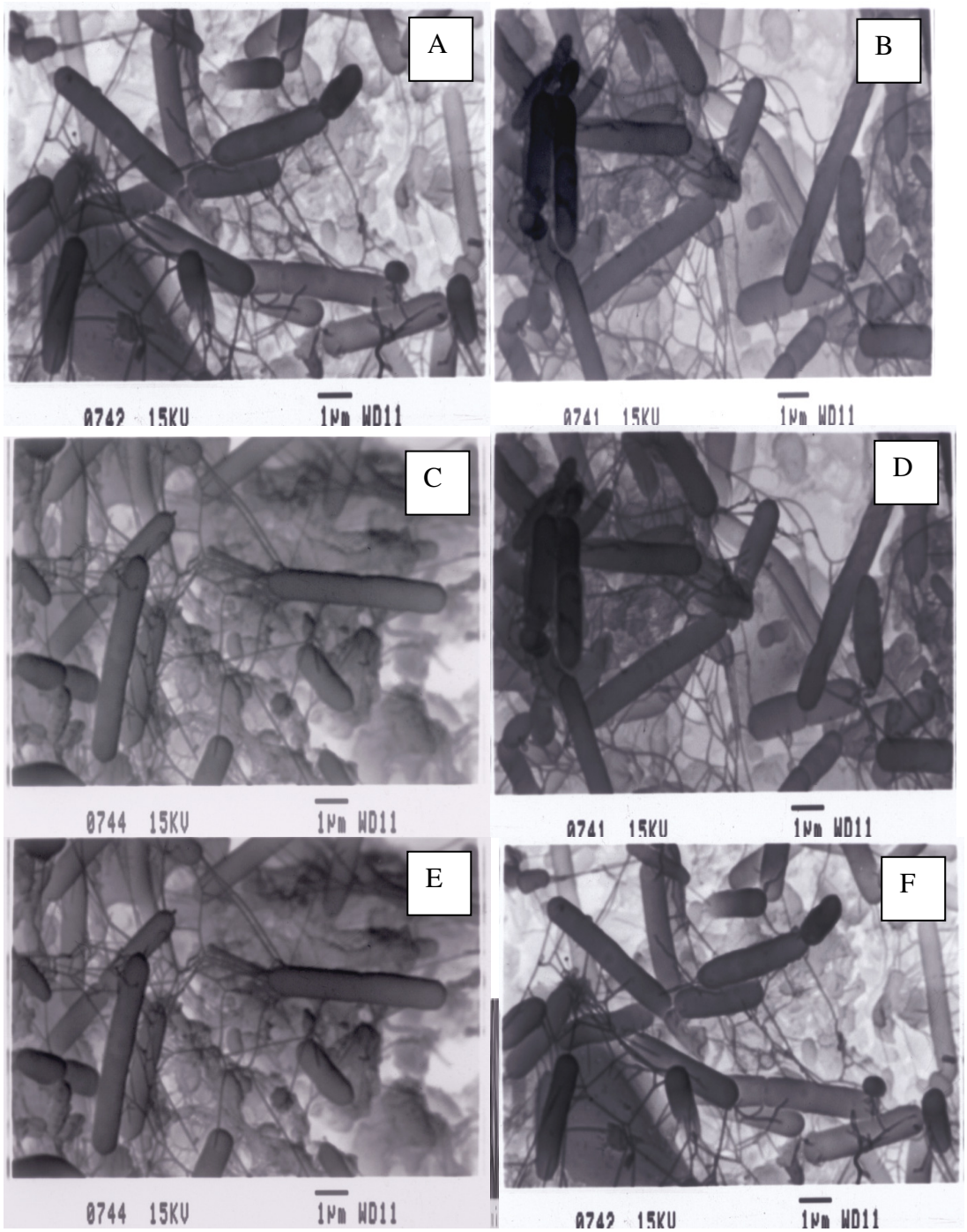


Fig 6.3: Scanning electron micrograph showing the bacterial cells associated with (A, B, C) Symbiotic bacteria isolate from *H. zealandica* and (D, E, F). Symbiotic bacteria isolate from *H. bacteriophora*.

6.3.1 Identification of endosymbiotic bacteria from *H. zealandica* and *H. bacteriophora* based on 16S rDNA sequence

Each bacteria produced a single band of approximately 700 base pairs (Fig: 6.4). Sequencing of DNA amplification products of both symbiotic bacteria were done by Inqaba Biotechnical Industries (Pty) Ltd; South Africa. The 16S rDNA sequences obtained were compared to published sequences in the NCBI database by using the algorithm BLAST, in order to identify the most similar 16S rDNA sequences. Blast results of the 16S rDNA sequence confirm the status of the bacteria to be *Photorhabdus luminescens laumondii* (*P. luminescens laumondii*) (*H. bacteriophora*) and *Photorhabdus* sp (*H. zealandica*).

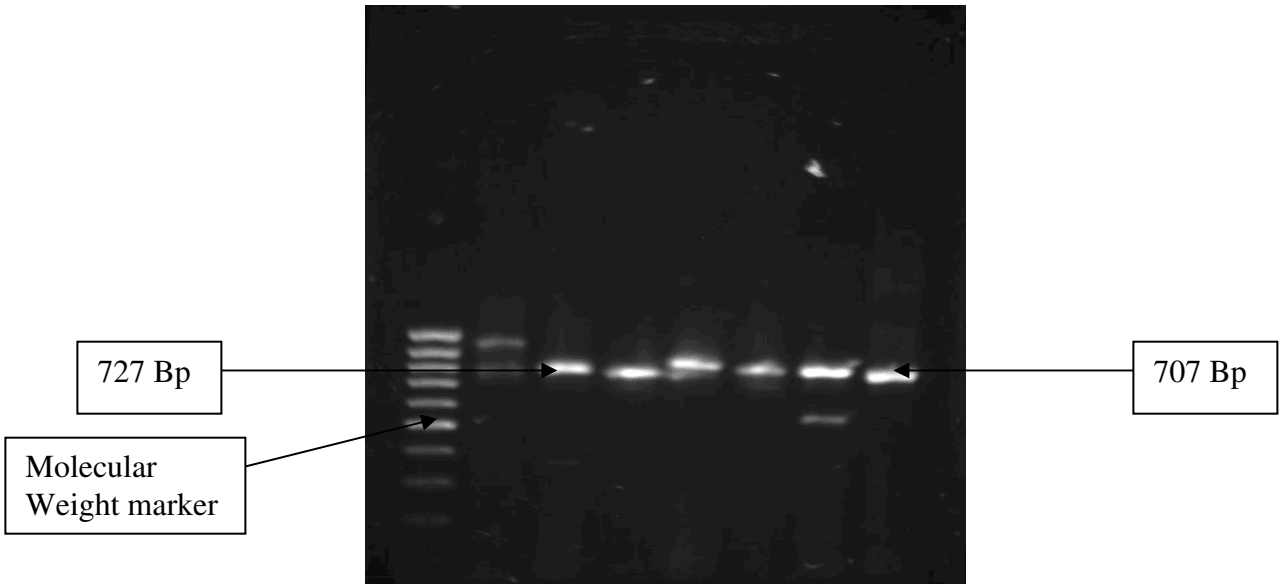


Figure: 6.4: PCR amplification of 16S rDNA. Lane 1: Molecular Weight marker (# SM098, Fermentas). Lanes: 2-3-4 bacterial strain isolated from *H. zealandica*. Lanes 5-6-7-8: Bacterial strain isolated from *H. bacteriophora.*

6.3.2 Phylogenetic tree and sequence Alignments

The two sequence data obtained from the two symbiotic bacteria *P. luminescens laumondii* and *Photorhabdus* sp are still under process to the Genbank for accession number. The *Photorhabdus* sp analysis was based on a total of 727 bp of clean sequence, and the *P. luminescens laumondii* analysis was based on 707 bp. Both sequences were aligned using DNAman (2006) with various species of the genus *Photorhabdus* (*P. luminescens* Ehlers x 82250, *P. temperate* strain *p7* Ay 278666, *P. temperate* strain *heliothidis* Ay 278658, *P. asymbiotica* Ay 278672*, Photorhabdus luminescens* subsp *luminescens* strain *hm* Ay 278641*, P.* sp *Q 614* Ay 216500*, P. asymbiotica* subsp *australis* Ay 280574*, P.* sp *Hit* Ay 278671*, P.* sp *Jun* Ay 278670*, P. luminescens* strain *brecon* Z76744*, P. luminescens* subsp *laumondii* strain *TT01* Ay 278646, *P. luminescens* subsp *kayaii* strain *DSM 15197* Aj 560632, *P. luminescens* Ay 17605 *P. luminescens* subsp *akhurstii* strain *W 14* Ay 278642, *P. luminescens* subsp *thracensis* strain *Iran 3 EU* 122952 and one out-group *E.coli* strain *ATCC 25922* Dq 360844) obtained from the Genbank database. The ITS region seems to be only useful for resolving relationship among closely related EPNs (Stock & Reid, 2004). In this chapter the result show that isolate from *H. bacteriophora* appear closely related to *P. luminescens* subsp *laumondii* strain *TT01* Ay 278646. The isolates from *H. zealandica* appear most closely related to the known *P. sp Q 614* Ay 216500 (Fig 6.5 & 6.6).



Fig 6.5: Phylogenetic tree of 16S rDNA of *P. luminescens* subsp *laumondii and Photorhabdus* sp, symbionts *of H. bacteriophora and. H. zealandica* respectively isolated from South Africa soil.

FILE: Multiple_Sequence_Alignment
PROJECT:
NUMBER: 18
MAXLENGTH: 1565
NAMES: P.luminescens HSH 2 x 82250 (Ehlers) P.sp Ay 216500 P.luminescens subsp luminescens strain Hm Ay 278641 P.sp Hit Ay 278671 P.sp Jun Ay 278670 E.coli strain ATCC 25922 Dq 360844

P.luminescens strain brecon Z 76744 P.temperata strain P7 Ay 278666 P.luminescens subsp akhurstii strain W14 Ay 278642 P.luminescens subsp kayaii strain DSM 15197 Aj 560632 P.luminescens subsp thracensis strain Iran 3 EU 122952 P.temperata strain heliothidis Ay 278658

P.asymbiotica subsp australis Ay 280574 P.luminescens subsp laumondi strain TT01 P. sp P.luminescens Y 17605 P.asymbiotica Ay 278672 P.luminescens subsp laumondi strain TTO1 Ay278646

MAXNAMELEN: 54
ORIGIN

```
P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGTGCTTGCACTT    74
P.SP AY 216500                                          CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGCGCTTGCGCTT    87
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGCGCTTGCGCTT     6
P.SP HIT AY 278671                                      CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGCGCTTGCGTTT    66
P.SP JUN AY 278670                                      CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGCGCTTGCGTTT    66
E.COLI STRAIN ATCC 25922 DQ 360844                      CCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCT    84
P.LUMINESCENS STRAIN BRECON Z 76744                     CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGCGCCTGCGCTT    50
P.TEMPERATA STRAIN P7 AY 278666                         CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGTGCTTGCGCTT    66
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGCGCTTGCGCTT    66
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGTGCTTGCGCTT    74
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 C.TAACACATGCAAGTCGAGCG.TAACAGGAAAATGCTTGCATTT    84
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGCGCTTGCGCTT    66
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 CTTAACACATGCAAGTCGGGCGGTAACAGGGAGGCGCTTGCGCCT    90
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                ............................................      0
P. SP                                                   ..................................TATAGGTG      8
P.LUMINESCENS Y 17605                                   CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGCGCTTGCGCTT    68
P.ASYMBIOTICA AY 278672                                 CCTAACACATGCAAGTCGAGCGGTAACAGGAAAGTGCTTGCACTT    66
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       CCTAACACATGCAAGTCGGGCGGTAACAGGAAAGTGCCTGCACTT    66

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTCC   118
P.SP AY 216500                                          TTGCTGACGAGCGGCGGACGGGTGAGT.AAAGTCTGGGGATCTGC   131
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110
P.SP HIT AY 278671                                      TTGCTGACGAGCGGCGGACGGGTGAGT.AAAGTCTGGGGATCTGC   110
P.SP JUN AY 278670                                      TTGCTGACGAGCGGCGGACGGGTGAGT.AAAGTCTGGGGATCTGC   110
E.COLI STRAIN ATCC 25922 DQ 360844                      TTGCTGACGAGTGGCGGACGGGTGAGT.AATGTCTGGGAAACTGC   128
P.LUMINESCENS STRAIN BRECON Z 76744                     TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC    94
P.TEMPERATA STRAIN P7 AY 278666                         TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   118
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   128
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 TTGCTGACGAGCGGCGGACGGGTGAGT.AAAGTCTGGGGATCTGC   134
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                ...GTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC    41
P. SP                                                   TAGCTGACGAGCGGCGGACGGGTGAGTTAAAGTCTGGGGATCTGC    53
P.LUMINESCENS Y 17605                                   TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   112
P.ASYMBIOTICA AY 278672                                 TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       TTGCTGACGAGCGGCGGACGGGTGAGT.AATGTCTGGGGATCTGC   110

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT   163
P.SP AY 216500                                          CTGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT   176
```

```
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.SP HIT AY 278671                                     CTGAGGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.SP JUN AY 278670                                     CTGAGGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
E.COLI STRAIN ATCC 25922 DQ 360844                     CTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCAT  173
P.LUMINESCENS STRAIN BRECON Z 76744                    CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  139
P.TEMPERATA STRAIN P7 AY 278666                        CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  163
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  173
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                CTGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  179
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               CCGATGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT   86
P. SP                                                  CTGATGGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT   98
P.LUMINESCENS Y 17605                                  CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  157
P.ASYMBIOTICA AY 278672                                CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      CCGAGGGCGGGGGATAACCACTGGAAACGGTGGCTAATACCGCAT  155

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   AATGTCGCGAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  207
P.SP AY 216500                                         GAAGTCGCAAGACCAAAGTGGGGGACCTGC..G.GGCCTCACGCC  218
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    AATGTCGCGAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  199
P.SP HIT AY 278671                                     AAAGTCGCAAGACCAAAGTGGGGGACCTTC.G..GGCCTCACGCC  197
P.SP JUN AY 278670                                     AAAGTCGCAAGACCAAAGTGGGGGACCTTC.G..GGCCTCACGCC  197
E.COLI STRAIN ATCC 25922 DQ 360844                     AACGTCGCAAGACCAAAGAGGGGGACCTTC..G.GGCCTCTTGCC  215
P.LUMINESCENS STRAIN BRECON Z 76744                    AATGTCGCGAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  183
P.TEMPERATA STRAIN P7 AY 278666                        AATGTCGCAAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  199
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     AATGTCGCAAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  199
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  AATGTCGCGAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  207
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 AATGTCGCAAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  217
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               AATGTCGCAAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  199
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                GATGTTGCGAGACCAAAGTGGGGGACCTGA.GAGGGCCTCACGCC  223
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               AATGTCGCGAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  130
P. SP                                                  GAAGTCCCGAGACCAAAGTGGGGGACCTGC..G.GGCCTCACGCC  140
P.LUMINESCENS Y 17605                                  AATGTCGCNAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  201
P.ASYMBIOTICA AY 278672                                GATGTCGCGAGACCAAAGTGGGGGACCTGAAAG.GGCCTCACGCC  199
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      AATGTCGCGAGACCAAAGTGGGGGACCTGA.AAGGGCCTCACGCC  199

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   CTCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAATGG  251
P.SP AY 216500                                         ATCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAATGG  262
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    GTCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAAAGG  243
P.SP HIT AY 278671                                     TTCGGATGAACCCA.GATGGGATTAGCTAGTCGGTAGGGTAATGG  241
P.SP JUN AY 278670                                     TTCGGATGAACCCA.GATGGGATTAGCTAGTCGGTAGGGTAATGG  241
E.COLI STRAIN ATCC 25922 DQ 360844                     ATCGGATGTGCCCA.GATGGGATTAGCTAGTAGGTGGGGTAAAGG  259
P.LUMINESCENS STRAIN BRECON Z 76744                    GTCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  227
P.TEMPERATA STRAIN P7 AY 278666                        CGTGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  243
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     GTCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  243
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  ATCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAACGG  251
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 CGCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  261
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               CGTGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAATGG  243
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                ATCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAATGG  267
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               ATCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  174
P. SP                                                  ATCGGATGAACCCACGATGGGATTAGCTGGTAGGTAGGGTAATGG  185
P.LUMINESCENS Y 17605                                  GTCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  245
P.ASYMBIOTICA AY 278672                                CTCGGATGAACCCA.GATGGGATTAGCTAGTAGGTAGGGTAATGG  243
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      GTCGGATGAACCCA.GATGGGATTAGCTGGTAGGTAGGGTAATGG  243

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   CCTACCGAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC  296
```

```
P.SP AY 216500                                          CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   307
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   288
P.SP HIT AY 278671                                      CCTACCGAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   286
P.SP JUN AY 278670                                      CCTACCGAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   286
E.COLI STRAIN ATCC 25922 DQ 360844                      CTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   304
P.LUMINESCENS STRAIN BRECON Z 76744                     CCTACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGACCAGC   272
P.TEMPERATA STRAIN P7 AY 278666                         CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   288
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   288
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   CCTACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGACCAGC   296
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952  CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   306
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   288
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   312
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                CCTACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGACCAGC   219
P. SP                                                   CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   230
P.LUMINESCENS Y 17605                                   CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   290
P.ASYMBIOTICA AY 278672                                 CCTACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC   288
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       CCTACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGACCAGC   288

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   341
P.SP AY 216500                                          CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   352
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333
P.SP HIT AY 278671                                      CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   331
P.SP JUN AY 278670                                      CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   331
E.COLI STRAIN ATCC 25922 DQ 360844                      CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA   349
P.LUMINESCENS STRAIN BRECON Z 76744                     CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   317
P.TEMPERATA STRAIN P7 AY 278666                         CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   341
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952  CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   351
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   357
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   264
P. SP                                                   CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGTAGCT   275
P.LUMINESCENS Y 17605                                   CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   335
P.ASYMBIOTICA AY 278672                                 CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA   333

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   386
P.SP AY 216500                                          GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   397
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
P.SP HIT AY 278671                                      GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   376
P.SP JUN AY 278670                                      GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   376
E.COLI STRAIN ATCC 25922 DQ 360844                      GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAACCATGCC   394
P.LUMINESCENS STRAIN BRECON Z 76744                     GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   362
P.TEMPERATA STRAIN P7 AY 278666                         GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   386
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952  GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   396
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 GTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCCATGCC   402
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                GTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCCATGCC   309
P. SP                                                   GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   320
P.LUMINESCENS Y 17605                                   GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   380
P.ASYMBIOTICA AY 278672                                 GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC   378
```

```
P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  431
P.SP AY 216500                                          GCGTGTATGAAGAAGGCTTTCGGGTTGTAAAGTACTTTCAGCGGG  442
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423
P.SP HIT AY 278671                                      GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  421
P.SP JUN AY 278670                                      GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  421
E.COLI STRAIN ATCC 25922 DQ 360844                      GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  439
P.LUMINESCENS STRAIN BRECON Z 76744                     GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  407
P.TEMPERATA STRAIN P7 AY 278666                         GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  431
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952  GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  441
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  447
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  354
P. SP                                                   GCGTGAATGAAGAAGGCTTTCGGGTTGTAAAGTACTTTCAGCGGG  365
P.LUMINESCENS Y 17605                                   GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  425
P.ASYMBIOTICA AY 278672                                 GCGTGTATGAAGCAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGG  423

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    GAGGAAGGGTTTAGCCTGAACAGGGTTGAATTTTGACGTTACCCG  476
P.SP AY 216500                                          GAGGAAGGGTGTGGCCTGAATAGGGTTGTGCTTTGACGTTACCCG  487
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     GAGGAAGGGTTCAGCTTGAACAGAGCTGGATTTTGACGTTACCCG  468
P.SP HIT AY 278671                                      GAGGAAGGGTTTGACCTGAAGAGGGTTAAATTTTGACGTTACCCG  466
P.SP JUN AY 278670                                      GAGGAAGGGTTTGACCTGAAGAGGGTTAAATTTTGACGTTACCCG  466
E.COLI STRAIN ATCC 25922 DQ 360844                      GAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCG  484
P.LUMINESCENS STRAIN BRECON Z 76744                     GAGGAAGGGTTTAGCCTGAAGAGGGTTAGATTTTGACGTTACCCG  452
P.TEMPERATA STRAIN P7 AY 278666                         GAGGAAGGGTATGGCCTGAAGAGGGCGATAGCTTGACGTTACCCG  468
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      GAGGAAGGATTTAACTTGAACAGAGTTGGACTTTGACGTTACCCG  468
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632   GAGGAAGGAATTGGCTTGAAGAGAGTTAATTTTTGACGTTACCCG  476
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952  GAGGAAGGATATCGCTTGAACAGAGCGGTATGTTGACGTTACCCG  486
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                GAGGAAGGGTATGGCCTGAAGAGGGCGATAGCTTGACGTTACCCG  468
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 GAGGAAGGGTTTGGCCTGAAGAGGGTTAAATTTTGACGTTACCCG  492
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                GAGGAAGGGTTTAGCCTGAAGAGGGTTGGACTTTGACGTTACCCG  399
P. SP                                                   GAGGAAGAGTGCGGCCTGAATAGGGCTGGACTTTGACGTTACCCG  410
P.LUMINESCENS Y 17605                                   GAGGAAGGGTTGAGCCTGAACAGGGCTGGGCCTTGACGTTACCCG  470
P.ASYMBIOTICA AY 278672                                 GAGGAAGGGTTTGGCCTGAAGAGGGTTAAATTTTGACGTTACCCG  468
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       GAGGAAGGGTTTAGCCTGAAGAGGGTTGGACTTTGACGTTACCCG  468

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                    CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  521
P.SP AY 216500                                          CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  532
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641     CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
P.SP HIT AY 278671                                      CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  511
P.SP JUN AY 278670                                      CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  511
E.COLI STRAIN ATCC 25922 DQ 360844                      CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  529
P.LUMINESCENS STRAIN BRECON Z 76744                     CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  497
P.TEMPERATA STRAIN P7 AY 278666                         CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642      CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632 CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC   521
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  531
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                 CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  537
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  444
P. SP                                                   CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  455
P.LUMINESCENS Y 17605                                   CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  515
P.ASYMBIOTICA AY 278672                                 CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646       CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC  513
```

P.LUMINESCENS HSH 2 X 82250 (EHLERS) GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 566
P.SP AY 216500 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 577
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558
P.SP HIT AY 278671 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 556
P.SP JUN AY 278670 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 556
E.COLI STRAIN ATCC 25922 DQ 360844 GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG 574
P.LUMINESCENS STRAIN BRECON Z 76744 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 542
P.TEMPERATA STRAIN P7 AY 278666 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 566
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 576
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574 GGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGGGCACG 582
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01 GGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 489
P. SP GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 500
P.LUMINESCENS Y 17605 GGAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 560
P.ASYMBIOTICA AY 278672 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646 GGAGGGTGCAAGCGTTAATCGGAATGACTGGGCGTAAAGCGCACG 558

P.LUMINESCENS HSH 2 X 82250 (EHLERS) CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 611
P.SP AY 216500 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 622
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 603
P.SP HIT AY 278671 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 601
P.SP JUN AY 278670 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 601
E.COLI STRAIN ATCC 25922 DQ 360844 CAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTG 619
P.LUMINESCENS STRAIN BRECON Z 76744 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 587
P.TEMPERATA STRAIN P7 AY 278666 CAGGCGGTCAATTAAGTTGGATGTGAAATCCCCGGGCTTAACCTG 603
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 603
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632 CAGGCGGTGAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 611
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 621
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658 CAGGCGGTCAATTAAGTTGGATGTGAAATCCCCGGGCTTAACCTG 603
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 627
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 534
P. SP CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTG 545
P.LUMINESCENS Y 17605 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 605
P.ASYMBIOTICA AY 278672 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 603
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646 CAGGCGGTCAATTAAGTTAGATGTGAAATCCCCGGGCTCAACCTG 603

P.LUMINESCENS HSH 2 X 82250 (EHLERS) GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 655
P.SP AY 216500 GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 666
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641 GGAATGGCATCTAAGACTGGTTGGCTGGA.GTCTCGTAGAGGGGG 647
P.SP HIT AY 278671 GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 645
P.SP JUN AY 278670 GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 645
E.COLI STRAIN ATCC 25922 DQ 360844 GGAGCTGCATCTGATACTGGCAAGCTTGA.GTCTCGTAGAGGGGG 663
P.LUMINESCENS STRAIN BRECON Z 76744 GGAATGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG 631
P.TEMPERATA STRAIN P7 AY 278666 GGAACGGCATCCAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 647
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642 GGAACGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG 647
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632 GGAATGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG 655
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 665
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658 GGAACGGCATCCAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 647
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574 GGAACGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 671
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01 GGAATGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG 578
P. SP GGAACGGCATCTAAGACTGGTTGGCTAGATGTCTCGTAGAGGGGG 590
P.LUMINESCENS Y 17605 GGAACGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG 649
P.ASYMBIOTICA AY 278672 GGAATGGCATCTAAGACTGGTTGGCTAGA.GTCTCGTAGAGGGGG 647

```
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      GGAATGGCATCTAAGACTGGTTGACTGGA.GTCTCGTAGAGGGGG   647

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   694
P.SP AY 216500                                         GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   705
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   686
P.SP HIT AY 278671                                     GTA.GAATTCCACGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   684
P.SP JUN AY 278670                                     GTA.GAATTCCACGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   684
E.COLI STRAIN ATCC 25922 DQ 360844                     GTA.GAATTCCAGGTGTA.GCGGTG.AAATGCGTA.GAGAT..CT   702
P.LUMINESCENS STRAIN BRECON Z 76744                    GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   670
P.TEMPERATA STRAIN P7 AY 278666                        GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.AAGAT..GT   686
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   686
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   694
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   704
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   686
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   710
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   617
P. SP                                                  GTAAGAATTCCATGTGTATGCGGTGTAAATGCGTATAAGATTAGT   635
P.LUMINESCENS Y 17605                                  GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   688
P.ASYMBIOTICA AY 278672                                GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   686
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      GTA.GAATTCCATGTGTA.GCGGTG.AAATGCGTA.GAGAT..GT   686

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   736
P.SP AY 216500                                         GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   747
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728
P.SP HIT AY 278671                                     GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   726
P.SP JUN AY 278670                                     GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   726
E.COLI STRAIN ATCC 25922 DQ 360844                     GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   744
P.LUMINESCENS STRAIN BRECON Z 76744                    GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACAAA.AACT   712
P.TEMPERATA STRAIN P7 AY 278666                        GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   736
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   746
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   752
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               GGAGGAATACCGGGTGGCGAAGGCGGCCCCCT.GGACGAA.GACT   660
P. SP                                                  GGAGGAATACCGGGTGGCGAAGGCGGCCCCCTTGGACGAAAGACT   680
P.LUMINESCENS Y 17605                                  GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   730
P.ASYMBIOTICA AY 278672                                GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646      GGAGGAATACCGG.TGGCGAAGGCGGCCCCCT.GGACGAA.GACT   728

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                   GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   778
P.SP AY 216500                                         GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   789
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641    GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770
P.SP HIT AY 278671                                     GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   768
P.SP JUN AY 278670                                     GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   768
E.COLI STRAIN ATCC 25922 DQ 360844                     GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   786
P.LUMINESCENS STRAIN BRECON Z 76744                    GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   754
P.TEMPERATA STRAIN P7 AY 278666                        GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642     GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632  GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   778
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952 GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   788
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658               GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                GACGCTGA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   794
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01               GACGCTCA.GGTGCGAAATGCGTGGGGAGCAAACATGGATTAGAT   704
P. SP                                                  GACGCTCAAGGTGCGAAAAGCGTGGGGAGCAAACATGGATTATAT   725
P.LUMINESCENS Y 17605                                  GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   772
```

```
P.ASYMBIOTICA AY 278672                                    GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646          GACGCTCA.GGTGCGAAA.GCGTGGGGAGCAAACA.GGATTAGAT   770

P.LUMINESCENS HSH 2 X 82250 (EHLERS)                       ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTT   823
P.SP AY 216500                                             ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTT   834
P.LUMINESCENS SUBSP LUMINESCENS STRAIN HM AY 278641        ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTG   815
P.SP HIT AY 278671                                         ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTT   813
P.SP JUN AY 278670                                         ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTT   813
E.COLI STRAIN ATCC 25922 DQ 360844                         ACC.TGGTAGTCCACGCCGTAAACGATGTCGACCTGGAGGTTGTG   830
P.LUMINESCENS STRAIN BRECON Z 76744                        ACCCTGGTAGTCCACGCTGTAAACGATGTCAATTTGGAGGTTGCG   799
P.TEMPERATA STRAIN P7 AY 278666                            ACCCTGGTAGTCCACGCGGTAAACGATGTCGATTTGGAGGTTGTT   815
P.LUMINESCENS SUBSP AKHURSTII STRAIN W14 AY 278642         ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTG   815
P.LUMINESCENS SUBSP KAYAII STRAIN DSM 15197 AJ 560632      ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGCG   823
P.LUMINESCENS SUBSP THRACENSIS STRAIN IRAN 3 EU 122952     ACCCTGGTAGTCCACGCGGTAAACGATGTCGATTTGGAGGTTGTT   833
P.TEMPERATA STRAIN HELIOTHIDIS AY 278658                   ACCCTGGTAGTCCACGCGGTAAACGATGTCGATTTGGAGGTTGTT   815
P.ASYMBIOTICA SUBSP AUSTRALIS AY 280574                    ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTG   839
P.LUMINESCENS SUBSP LAUMONDI STRAIN TT01                   ACC                                             707
P. SP                                                      TC                                              727
P.LUMINESCENS Y 17605                                      ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGCG   817
P.ASYMBIOTICA AY 278672                                    ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTG   815
P.LUMINESCENS SUBSP LAUMONDI STRAIN TTO1 AY278646          ACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGCG   815
```

Fig 6.6: DNAman multiple sequence alignment of the 16S - rDNA partial sequence obtained from *P. luminescens* subsp *laumondii* and, *Photorhabdus* sp, symbionts of *H. bacteriophora* and *H. zealandica respectively* isolated from South Africa soil.

6.4 Discussion

P. luminescens lamaundii and *Photorhabdus* sp associated consecutive generations of infective juveniles of *H. bacteriophora* and *H. zealandica* were successful isolated, cultured aseptically in *vitro* on NBTA, MaCconkey agar and Nutrient broth using the grinding IJs method at 25°C. Although, the cultures were purified several times using the streak plate technique to get pure single colonies. Nevertheless, the purified colonies had the exact microbiologically characteristics same as those found documented by. Kaya & Stock, (1997); Forst *et al*., (1997). The occurrence of primary and secondary forms of *P. luminescens* is well described by Forst & Nealson. (1996). Phase I of the *P. luminescens laumondii* isolated from *H. bacteriophora* possess all characteristics which have been described for other *P. luminescens* isolates (table 6.1). They absorb dyes from NBTA and MacConkey agar, produce pigments. The phase II lost these characteristics. The bioluminescens was not visible,

even after adaptation of the eyes to dark for 20 min. The measurable bioluminescens was significantly less than in phase I. *Photorhabdus* sp was without most of these primary form characteristics (Fig 6.1). The bioluminescens was not visible in comparison with *P. luminescens laumondii* and absorbed dyes slowly, especially neutral red. Although a secondary form was not detected in this study. Different *Photorhabdus* spp. show differences in their luminescens intensity (Gerritsen & Smits, 1997).

To increase the accuracy and precision of the results it is recommended that all available techniques be used to characterize the symbiont. The partial 16 S rRNA gene was amplified by PCR for each bacterial strain to confirm the result. Phylogenetic tree and sequence alignment showed the relationships among *P. luminescens laumondii* and *Photorhabdus* sp isolate from *H. bacteriophora* and *H. zealandica* respectively from South Africa and those from the Genbank. It has been confirmed that sequencing only a part of the 16S rRNA gene can be sufficient to establish phylogenetic relationships (Lane *et al*., 1985; Yong *et al*., 1991). In the present study sequencing genes and combine phenotypic, molecular and DNA sequence data have been used to provide a definitive taxonomy of the genera *Photorhabdus.* The scanning electron microscopy revealed that both bacteria are rod shaped. The biochemical properties of both bacteria were not investigated in this study. This was not considered as limitation in this study, since the objective was to isolate and identify the bacteria.

Chapter 7
Culture and Maintenance of Putative EPN Isolates

7.1 Introduction

EPNs have been successfully used against numerous soil-inhabiting insect pests (Georgis & Manweiler, 1994; Shapiro & Gaugler 2002); but their poor survival when stored at room-temperature is one of the main factors that prevent them from realizing their full potential as bioinsecticides (Grewal, 2002). IJs of EPNS depend solely on stored reserves for their energy supply as they do not feed. Therefore, energy conservation is vital in prolonging the survival of IJs, as well as extending the shelf live of these bioinsecticides (Patel *et al*., 1997). IJs can be kept alive in aerated water for several months or in refrigerated tanks for extended periods of time, but their pathogenicity progressively deteriorates during storage. Certain factors accelerate this reduction in pathogencity; these include: (i) prolonged storage, (ii) suboptimal storage temperature, and (iii) reduced lipid reserves (Gaugler & Georgis, 1991). It is imperative to culture EPNs in the laboratory with the objective to maintain their pathogenicity and minimize nematode mortality. Hence, the culture of EPNs by *in vitro* or *in vivo* methods presents a better alternative because the techniques provide optimal conditions that guarantee high levels of IJ infectivity as well as survival (Kaya & Stock, 1997). Also, these culture techniques are useful for laboratory and small field trials, though it is not practical for large-scale nematode production. This chapter examines the processes involved in maintaining the nematode isolates putatively identified as EPNs, *in vivo* in insect hosts and *in vitro* in an artificial medium.

7.2 Materials & Methods

7.2.1 *In vivo* culture of putative EPNs

The EPNs were cultured *in vivo* according to the method of Woodring & Kaya (1998) as explained in section 2.5.4.1, in *G. mellonella* larvae along with *T. molitor* larvae or pupae. The progeny IJs were collected in White traps and used in experiments or to reinfect more insect hosts.

7.2.2 *In vitro* culture of putative EPNs

In vitro culture of the EPNs was carried our in lipid agar, which is an artificial medium (section 2.5.4.2).

7.3 Results

Most steinernematids and heterorhabditids can be produced in *G. mellonella* larvae or other suitable insect hosts such as *T. molitor* larvae or pupae. The isolates obtained from the soil samples (see chapter 3) during the study, only two EPNs confirmed Koch's postulates (Chapter 3).

Table 7.1: Results of in *vivo* culture of 2 nematode isolates.

Isolate	*In vivo culture*
411	Culture in *G. mellonella* for 12 months, *T. molitor* for 4 months and Pupae of *T. molitor* for 2 months
386	Culture in *G. mellonella* for 12 months, *T. molitor* for 4 months and Pupae of *T. molitor* for 2 months

For large scale nematode production steinernematids and heterorhabditids have been cultured on variety of substrates (Woodring & kaya, 1988; Friedman, 1990). The ingredients for nematodes culture including a source of nutrients for symbiotic bacteria and sterol source for the nematodes were mostly a major step toward commercial production of the nematodes for insect control (Surrey & Davies, 1996). *In vitro* solid method, (lipid agar), the two isolates nematodes number 411 and 386, were tested for two weeks in order to obtain a large amount of IJs for DNA extraction and bacteria isolation. The growths of these isolates were successful. Alternative, nematodes were harvested from lipid agar by setting up a White trap (section 2.5.2).

Table 7.2: Results of in *vitro* culture of 2 nematode isolates.

Isolate	*In vitro culture*
411	2 weeks in lipid agar
386	2 weeks in lipid agar

7.4 Discussion

Since EPNs entered commercial use for biological control of insect pests, the problem of short shelf life has inhibited the expansion in the use of EPNs (Grewal, 2002). To maintain survival, it was necessary to keep a balance between reduction of metabolism and water availability. Several storage media have been developed to improve the survival of storage nematodes such as IJs enclosed in calcium alginate granules were important to achieve a shelf life at room temperature, and this led to the increased acceptability of nematodes in high-value niche markets (Grewal, 1998). However, in *vitro* solid method (lipid agar) and *in vivo* production (insects) technique provides a ready source of nematodes in adequate quantities for laboratory and small scale field investigations. The *in vivo* maintenance culture of isolate 386 and 411

using *G. mellonella*, *T. molitor* and Pupae as insect hosts were successful. After research (2 years) both isolate 411 and 386 cultured *in vivo* were still pathogenic, effectively killing *G. mellonella*, *T. molitor* and Pupae within 48 hours of infection. Entomopathogenic nematodes at least partly depend on the survival of the symbiotic bacteria; therefore, it is imperative that they are cultured to maintain their higher original virulence. Compared to *in vitro* culture, *in vivo* culturing is an important factor because of the preservation of the nematode's virulence. Although completely reliable, assumptions were made that all 2 nematode isolates belong to the genus *Heterorhabditis* especially as their physical appearance suggested the same. Furthermore, their pathogenicity was confirmed using Koch's postulates (section 2.6). The IJs of both isolates were maintained in *vitro* solid method and *in vivo* usisng insects *G. mellonella*, *T. molitor* and Pupae in the laboratory at room temperature (25°C).

Chapter 8

Bioassay Studies

8.1 Introduction

Nematodes are currently used commercially as biological control agents of insect pests (Aydin & Susurluk, 2004). The degree of parasite virulence to a specific host depends upon the characteristic of the parasite-host interaction as well as upon environmental factors. Numerous studies with other arthropods, has demonstrated wide variations in the virulence of EPNs, as shown by the rate and degree of mortality of their hosts (Bedding & Molyneux, 1982; Glazer *et al*., 1991; Glazer *et al*., 2001). It has been shown that the variation in virulence of nematode strains to insects, as expressed by the degree or rate of mortality, is attributed to (i) the ability of the IJs to locate and invade a host, (ii) the ability of the nematodes and their symbiotic bacteria to overcome the immune response, and (iii) the propagation rate of the symbiotic bacteria (Glazer, 1992; Caroli *et al*., 1996). Susceptible insects are often killed within 1-3 days (Caroli *et al*., 1996). With the expansion of commercial interest in EPNs, the susceptibility of many economically important insect pests has been tested in a wide range of laboratory assays (e.g. Glazer, 1992; Caroli *et al*., 1996; Ricci et al, 1996). The dose response bioassay is one of the most commonly used bioassays (Morris *et al*., 1990). It is used to determine the IJ lethal dose required to cause death in 100% of the test insects. In this study the dose response bioassay was carried out on the two indigenous South African EPN species, namely; *H. bacteriophora* and *H. zealandica*. Furthermore, a study was carried out to determine if there were differences in the number of IJs emerging from the different insect hosts infected at different IJ doses.

8.2 Materials & Methods

Individual *G. mellonella* larvae, and *T. molitor* larvae and pupae were exposed to 5, 10, 25, 50, 100, 200, 400 and 500 IJs of *H. bacteriophora* and *H. zealandica* IJs applied in 100 µl of sterile distilled water on filter paper or in sterilized sand as described in section 2.9.2. Control larvae received water only, but were exposed in a similar maner. Mortality data were recorded every 24 h for 96 h following exposure of the IJs. Following the bioassay, the cadavers were individually set up on white traps to collect progeny IJs, which were later on counted (section 2.9.2) to determine if there were differences between the numbers of IJs emerged. SAS (SAS institute, 2001) was used to analyse all data. Mortality data and IJs emerging per cadaver were analysed in a one-way analysis of varaince (ANOVA). Mortality data expressed as percentages were Arcsin transformed before statistical analysis, since insect mortality did not result from any of the controls. Means for percentage mortality were seperated using Tukey's multiple comparison tests. Differences with $P< 0.05$ were considered significant. The experiment was performed three times.

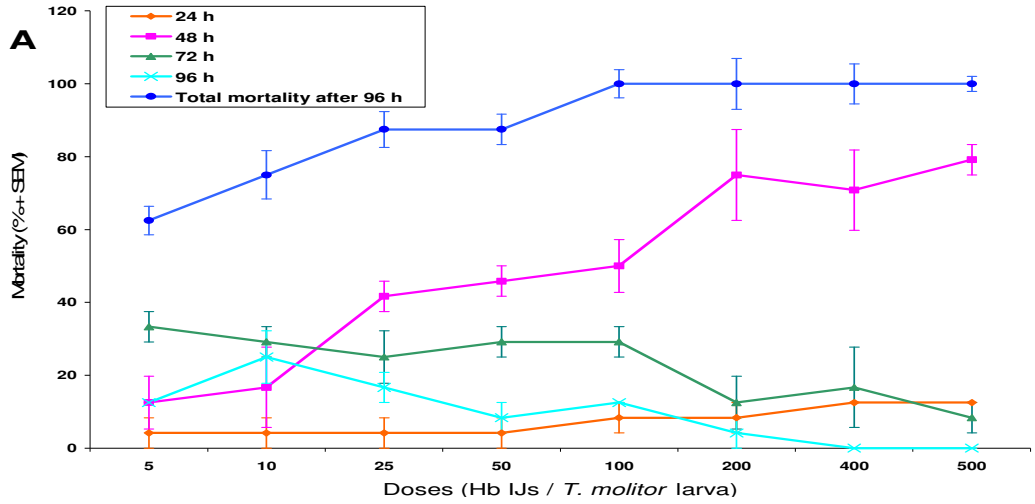
8.3 Results

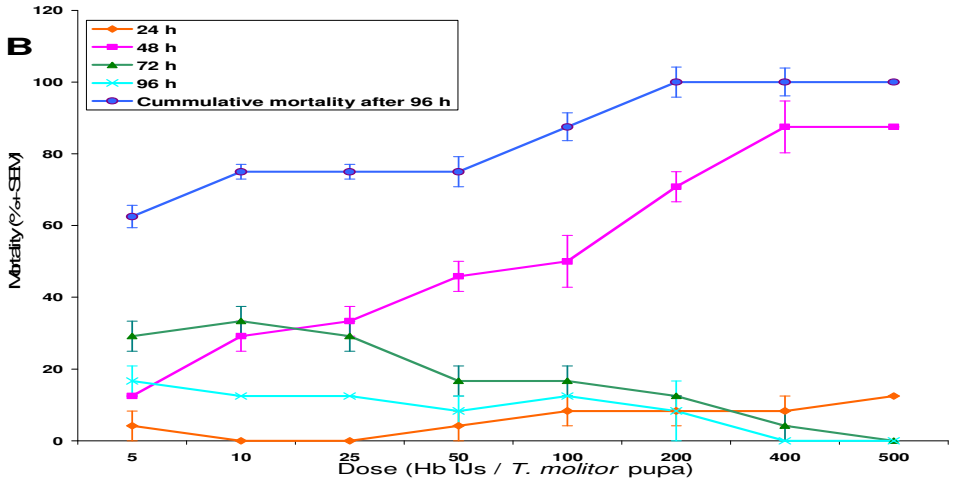
In the first part of the dose-response assay, the virulence of *H. bacteriophora* and *H. zealandica*, were evaluated on the basis of their ability to infect and kill the insects at different doses. Data was not collected for control insects since none of them experienced mortality. Figs 8.1 (A-C) and 8.2 (A-C) below shows the mortality trend caused by *H. bacteriophora* and *H. zealandica* respectively in *G. mellonella* larvae as well as *T. molitor* larvae and pupae, at 24, 48, 72 and 96 h.

Insect mortality increased over time up to 48 h at all dosages for both *H. bacteriophora* and *H. zealandica*. Insect mortality was highest at 48 h for all insects for both *H. bacteriophora* and *H. zealandica.* After 48 h, the trend in mortality is

more consistent for insects that were exposed to >100 IJs of *H. bacteriophora* or *H. zealandica.* Also, it seems that larvae exposed to IJs of *H. zealandica* experienced higher levels of mortality compared to those exposed to *H. bacteriophora*. Less than 10% of the larvae were killed when exposed to 100 IJs of *H. bacteriophora* but mortality levels for larvae were generally higher than 15% for *H. zealandica* (Fig 8.2 A-C). *T. molitor* larvae and pupae were far more susceptible to the *H. zealandica* than to *H. bacteriophora*. Mortality was particularly high among pupae exposed to the *H. zealandica* with >20% dying after 24 h of exposure. At IJ concentrations of 5-50 IJs /larvae there was a similar trend in mortality for all larvae exposed to either *H. bacteriophora* or *H. zealandica*. However, mortality was higher at smaller doses (i.e. at 5 and 10 IJs *G. mellonella* and *T. molitor* larvae) exposed to *H. zealandica* compared to those exposed to *H. bacteriophora*.

When mortality data 24 h post exposure to IJs of *H. bacteriophora* and *H. zealandica* were analysed, ANOVA showed no differences in mortality between larvae exposed to different *H. bacteriophora* IJ doses (*H. bacteriophora*: $F = 2.15$; $df = 2$; $P = 0.1379$) for *G. mellonella* larvae as well as *T. molitor* larvae and pupae (Fig 8.1 A-C). However there were significant differences in mortality between larvae exposed to different IJ doses of *H. zealandica* (*H. zealandica*: $F = 7.28$; $df = 2$; $P=0.0034$) for *G. mellonella* larvae as well as *T. molitor* larvae and pupae (Fig 8.2 A-C). ANOVA revealed no significant differences in mortality between insects exposed to different doses of IJs of both *H. bacteriophora* and *H. zealandica* at 48 h: (*H. bacteriophora*: $F = 0.23$; $df = 2$; $P = 0.7975$; | *H. zealandica*: $F = 0.25$; $df = 2$; $P = 0.7846$); 72 h: (ANOVA, *H. bacteriophora*: $F = 1.49$; $df = 2$; $P = 0.2444$; | *H. zealandica*: $F = 6.50$; $df = 2$; $P = 0.1555$); and also at 96 h: (*H. bacteriophora*: $F = 0.79$; $df = 2$; $P = 0.4662$; | *H. zealandica*: $F = 2.74$; df 2.74; $P = 0.0850$).


A
24 h
48 h
72 h
96 h
Total mortality after 96 h
Mortality (%+ SEM)
120
100
80
60
40
20
0
5
10
25
50
100
200
400
500
Doses (Hb IJs / T. molitor larva)


B
24 h
48 h
72 h
96 h
Cummulative mortality after 96 h
Mortality (%+ SEM)
120
100
80
60
40
20
0
5
10
25
50
100
200
400
500
Dose (Hb IJs / T. molitor pupa)

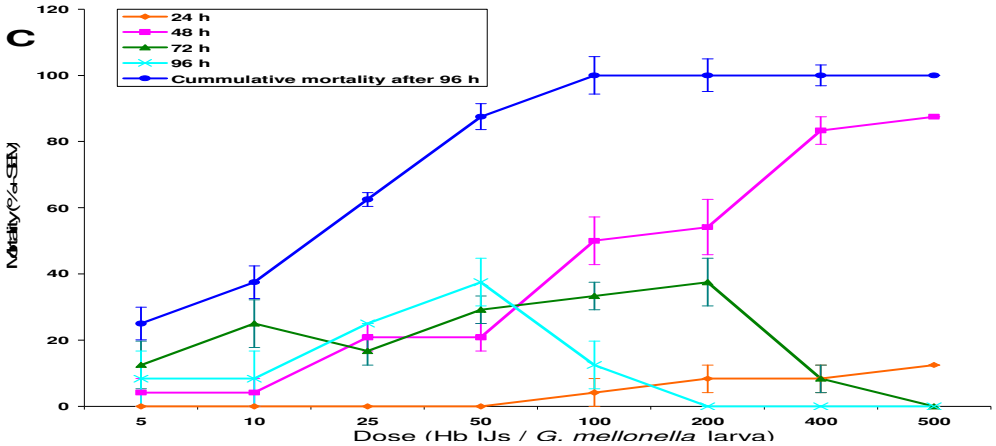
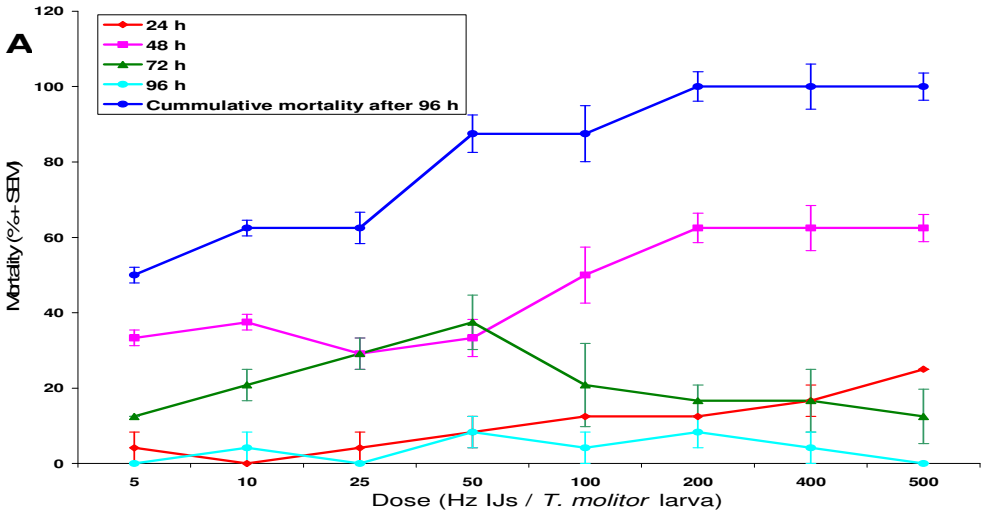
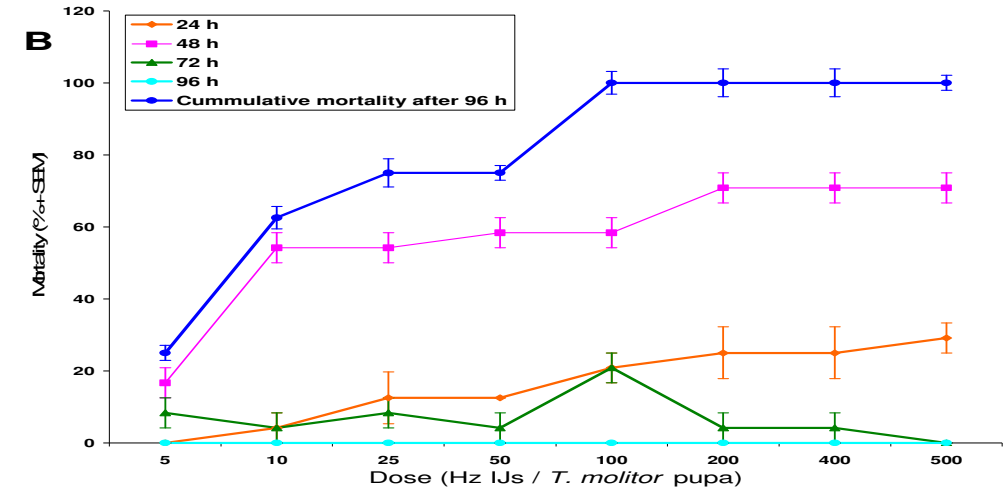


Figure: 8.1: A, B, C The percentage mortality of *T. molitor* larvae, pupae and *G. mellonella* larvae following exposure to different concentrations (5, 10, 25, 50, 100, 200, 400, and 500) of IJs of *H. bacteriopohora* in the dose response assay for 24 h, 48 h, 72 h and 96 h of exposure represents the cumulative mortality after 96 h. Bars represent ± standard error of the mean.





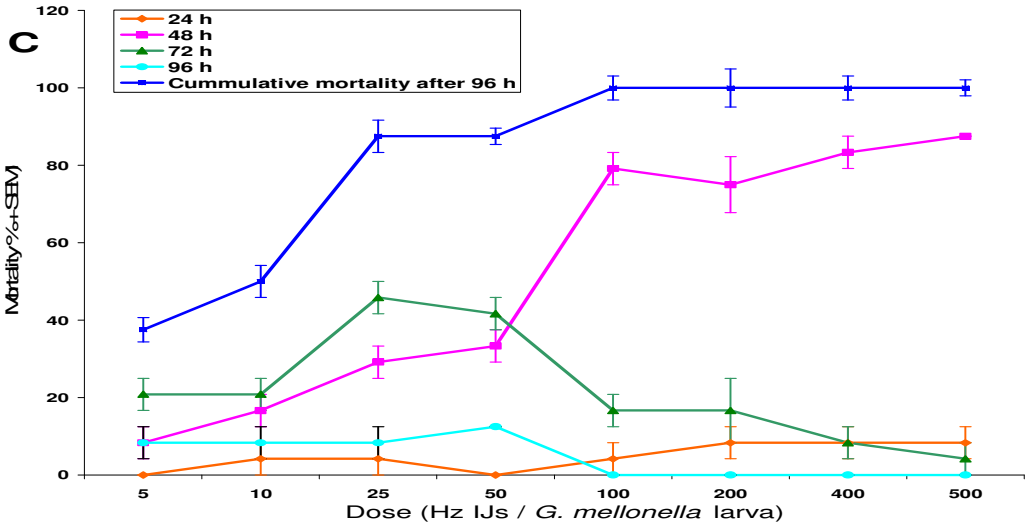


Figure: 8.2 A, B, C: The percentage mortality of *T. molitor* larvae, pupae and *G. mellonella* following exposure to different concentrations (5, 10, 25, 50, 100, 200, 400, and 500) of IJs of *H. zealandica* in the dose response assay for 24 h, 48 h, 72 h and 96 h of exposure represents the cumulative mortality after 96 h. Bars represent ± standard error of the mean.

The second part of the bioassay sought to determine if there were differences in the number of emerging progeny IJs from *G. mellonella* larvae, and *T. molitor* larvae and pupae, which had been exposed to different IJ doses (5, 10, 25, 50, 100, 200, 400, and 500) of *H. bacteriophora* and *H. zealandica*. The results are presented in Figs 8.3 & 8.4 below.

The results indicate that IJ progeny production differed among the three larvae hosts used, the IJ doses they were exposed to, as well as the EPN species (Figs 8.3 & 8.4). The highest number of emerged IJs of *H. zealandica* was produced by *G. mellonella* (mean ± SEM: 220500 ± 133933 IJs), followed by *T. molitor* larvae (mean ± SEM: 152133 ± 45466 IJs) and the lowest then *T. molitor* pupae (mean ± SEM: 103366 ± 56933 IJs). In the case of *T. molitor*, more progeny IJs were produced by pupae which had been exposed to lower doses of *H. zealandica* IJs (<50 IJs/ pupa), but the IJ production was reduced in pupae that had been exposed to higher doses of *H. zealandica* IJs. The afore-described trend for *T. molitor* pupae is the reverse in the larvae (fig 8.3). For *H. bacteriophora* infected cadavers, the highest number of emerged IJs was observed in *T. molitor* larvae which had been exposed to 500 IJs, produced on average 197666.6 IJs/ cadaver. Also, *G. mellonella* insects which had been individually exposed to 10 *H. bacteriophora* IJs produced on average 147933.333 IJs/ cadaver. The least number of progeny IJs was produced by *T. molitor* pupae (13533.33 IJs). IJ production is reduced in *G. mellonella* exposed to higher IJ doses of *H. bacteriophora*, while the numbers produced by *T. molitor* larvae seem to increase as the IJ dose increases.

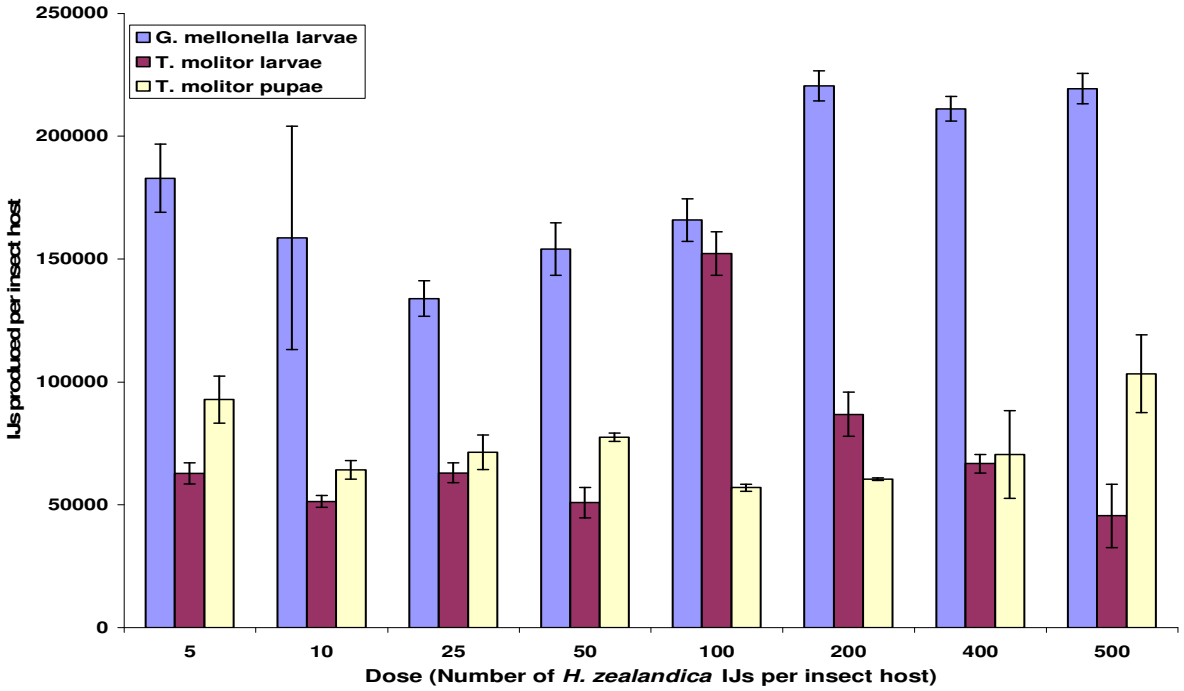


Fig 8.3: Mean number of progeny IJs emerged from *G. mellonella* and *T. molitor* larvae and pupae that were exposed to different doses of *H. zealandica* nematodes. Bars are ± standard error of the mean.

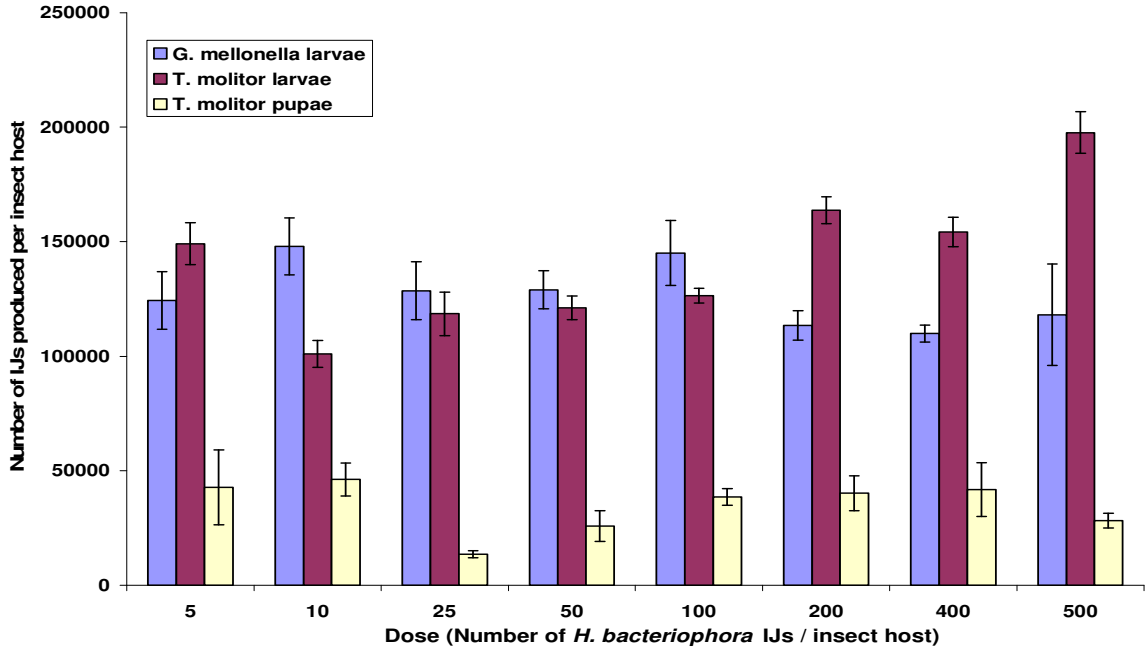


Fig 8.4: Mean number of progeny IJs emerged from *G. mellonella* and *T. molitor* larvae and pupae that were exposed to different doses of *H. bacteriophora* nematodes. Bars are ± standard error of the mean

ANOVA revealed no significant differences in the number of emerged IJs among *H. bacteriophora* and *H. zealandica* in the three larvae hosts (*H. bacteriophora*: $F = 0.22$; $df = 7$; $P = 0.9799$| *H. zealandica:* $F = 0.54$, $df = 7$, $P = 0.8024$). Also, there were no significant differences between the number of emerged IJs between *H. bacteriophora* nematode and *H. zealandica* nematode at all doses: [(5 IJs/larvae: $F = 0.2924$; $df = 16$; $P = 0.3869$); (10 IJs/larvae: $F = 0.2654$; $df = 16$; $P = 0.6029$); (25 IJs/larvae: $F = 0.1154$; $df = 16$; $P = 0.4588$); (50 IJs/larvae: $F = 0.0905$; $df = 16$; $P = 0.4645$); (100 IJs/larvae: $F = 0.8862$; $df = 16$; $P = 0.1943$); (200 IJs/larvae: $F = 0.5431$; $df = 16$; $P = 0.2973$); (400 IJs/larvae: $F = 0.4776$; $df = 16$; $P = 0.3197$); (500 IJs/larvae: $F = 0.2205$; $df = 16$; $P = 0.4141$)].

8.4 Discussion

The dose response assay measured the nematodes ability to locate and to infect insect pests. This assay has been used many times previously (Morris *et al*., 1990; Mannion & Janson, 1992) Each insect host, *G. mellonella*, *T. molitor* larva and Pupae, were exposed to various dosages (5, 10, 25, 50, 100, 200, 400, 500,) of IJs of *H. bacteriophora* and *H. zealandica* in the laboratory for 24 h, 48, 72h and 96 h. In the first part of the bioassay, mortality data was collected for both *H. bacteriophora* and *H. zealandica* nematodes. Both high and low nematode inoculums were effective in causing insect mortality but few differences in infectivity were found among nematodes within insect hosts; however, infectivity differed between host for *H. bacteriophora* and *H. zealandica*. At 24 h IJs of *H. zealandica* showed a relatively higher virulence than *H. bacteriophora* as it caused 20% mortality to *G. mellonella* and *T. molitor* larvae, while *H. bacteriophora* caused less than 5% mortality in *T. molitor* pupae.

At 48 h, both nematodes killed great numbers of larvae hosts when exposed to IJ doses even as low as 50 IJs/larvae. Apparently, a low IJ dose results in a low host

susceptibly, while a higher IJ concentrations result in higher levels of failed infections due to competition with secondary invaders (Woodring & Kaya, 1988). However, this was not the case in this study. This reinforces the view that local populations of EPNs can have important biological traits. Bedding *et al*., (1983) suggested testing EPNs at a concentration of 100 IJs/larvae as a preliminary assessment of host susceptibility when beginning the process of testing a nematode species or strain as a potential biological control agent. In this study, at 72 and 96 h post larvae exposure to IJs, mortality was still being recorded at lower IJ doses. This observation suggests that longer exposure times make it possible for more nematodes to penetrate their larvae hosts. Caroli *et al*. (1996) observed that for susceptible larvae such *G. mellonella* and other lepidopterans, complete mortality was reached within 24-72 h of exposure to nematodes at concentrations similar to those used in this study. Still on the study by Caroli *et al*. (1996), the time necessary for *H. bacteriophora* and *H. zealandica* to cause 50 and 90% mortality was determined after 24 hr of exposure to higher concentrations of nematodes. In most other treatments, complete mortality was obtained after 96 hrs. These delays could be attributed to the amount and rate of nematodes and their symbionts penetrating the host hemocoel of the larvae hosts. However both Heterorhabditid nematodes tested in this study were virulent enough to kill over 85% of the insect host they were applied to.

Following the dose-response bioassay, a study was carried out to determine if there was an association between the number of progeny IJs produced by the larvae cadavers and the IJ dosages they were exposed to. According to Poinar (1979), entomopathogenic nematodes can be reared by *in vivo* methods; with yields of 100,000-200,000 IJs per *G. mellonella*. In this study similar numbers of IJs were observed in *G. mellonella* exposed to IJs of *H. zealandica.* In addition, the results indicated that IJ progeny production differed among the three larvae hosts, IJ doses they were exposed to, as well as the EPN species (Figs 8.3 & 8.4). Infact, compared to *T. molitor* larvae and pupae, IJ progeny production *G. mellonella* was consistently higher (220500-133933 IJs/*G. mellonella* larva) than in the two followed (152133-

45467/ *T. molitor* larva) respectively. But, in the case of *H. bacteriophora,* progeny IJ production was similar in the *G. mellonella* and *T. molitor* larvae (all on average regardless of IJ dose larvae was exposed to, 197666-101033/ *G. mellonella* 147933-109900/ *T. molitor* larvae. IJ production in was greatly reduced in both *H. bacteriopohora* and *H. zealandica* infected cadavers at all exposure doses reduced and *T. molitor* larvae than in the *T. molitor* pupae (46200-13533 and 103366-56933) respectively per *T. molitor* pupa. These observations concur with previous studies by Carissimi-Boff (2001). The author showed that the body size of the host, host species and nematode dose applied definitely influences the total number of IJs developing inside the cadaver. This might explain the observation in this study. Moreover, *in vivo* production yields are dependent on nematode dosage (Zervos *et al*., 1991; Boff *et al*., 2000). In this study, *G. mellonella* and *T. molitor* larvae consistently produced more progeny nematode IJs (both *H. zealandica* and *H. bacteriophora*) than *T. molitor* pupae. However there was no relationship between Progeny number and dosage. Apparently, all things being equal, total progeny production per larvae increases almost proportionately with the increasing host size (Carissimi-Boff, 2001).

Also, the host larvae morphological characteristics could enhance or adversely affect nematode penetration, e.g. *G. mellonella* larvae have a softer cuticle, more spiracles and a large surface area that aids nematode penetration; *T. molitor* pupae do not move around as much as the larvae, they have a softer cuticle especially when they are newly moulted, and have a more rugged body structure compared the larval stage, features that are favorable to nematode penetration. In addition, the *T. molitor* larvae have a waxy cuticle with more chitin compared to the *G. mellonella* and *T. molitor* pupae which makes nematode attachment and penetration more difficult compared to the other insects used in this study. These characteristics may explain the variation in mortality patterns observed in this study. Also, it was noticeable that the lowest mortalities were amongst insects exposed to fewer IJs, implying that the more IJs are applied or available to invade larvae, the greater chance of killing the larvae, in that more IJs can successfully penetrate the larvae. According to Kaya (1987), the

continued association of certain nematode species with the same insect species may reduce virulence rather than enhance it. Since *H. bacteriophora* and *H. zealandica* were reared *in vivo* in *T. molitor* throughout the duration of this study, this may be a likely explanation for the observation.

In summary, the differences in *H. bacteriophora* and *H. zealandica* infectivity demonstrated great variation in their ability to reduce insect pest populations. From this bioassay, it was obvious that the susceptibility of *G. mellonella* and *T. molitor* larva and pupae to *H. bacteriophora* and *H. zealandica* was different, thus, suggesting that each complex presents different virulence degrees. This finding is profusely documented in the literature (Bedding *et al*., 1983; Forschler & Nordin, 1988; Fuxa *et al*., 1988; Glazer & Navon, 1990; Molyneux *et al*., 1983; Morris *et al*., 1990). Differences between the reproduction potential of EPNs may be related to the isolates, species, and host susceptibility, number of bacteria per infective stage, invasion rate, temperature and humidity. It is possible that differences in virulence between species and isolates might be greater for less susceptible hosts. Prior to biocontrol applications, bioassays should be performed against the target pest insect (Stilling, 1992).

The factors that influence the rate of larvae mortality include the rate at which nematodes and their bacterial symbionts penetrate the host hemocoel (Glazer, 1992; Caroli *et al*., 1996; Ricci *et al*., 1996); their rate of proliferation within the host; and the host's efficiency in protecting itself against the nematode's symbiont's deleterious secretions (Wang & Bedding, 1996). It seems that the age of the IJs can also influence nematode infection and resulting host mortality (Perez *et al*., 2003); i.e. the younger or newly emerged IJ carries more potent bacteria cells than older IJs.

According to (Glazer *et al*., (2001), the degree of parasite virulence to a specific host depends upon the characteristic of the parasite-host interaction as well as upon environmental factors. In determining a good biocontrol agent it is necessary to

investigate its physiological, ecological and behavioral response to different insect pests. It is also important to acquire information about different phases in their interaction with their host. Many studies have demonstrated the efficacy of EPNs in controlling insect pests reducing significantly the rate of economic damage (Glaser *et al*., 1992; BenYakir *et al*., 1998). The efficacy of various nematode species or strains for controlling a particular insect pest may differ significantly. Efficacy is influenced by the rate of IJ penetration into the insect, the time it takes to release the symbiotic bacteria, and the virulence of the latter. Moreover, these indigenous isolates may provide a more suitable alternative for ununditative release against a variety of native and/or introduced pests because of their adaptation to local climate conditions and population regulations. Current research efforts are focused on selecting native entomopathogens, which are highly virulent to arthropod pests, for developing efficient and minimizing current usage of chemical pesticides, therefore providing a more environmentally friendly alternative for the management of the crop (pathogen and .parasitic nematode).

It has been recognized that a laboratory bioassay that predicts performance of entomopathogenic nematodes in the field is needed to facilitate selection of nematodes in biological control programs (Hominick & Briscoe, 1990; Mannion, 1992). Mannion, (1992) conducted Petri dish, sand, soil, and simulated field bioassays to select suitable entomopathogenic nematodes for the biological control of insect pests and consistently found that *H. zealandica* were superior to *H. bacteriophora* in all bioassay systems tested. The present bioassay system may also have potential for selecting suitable entomopathogenic nematodes, especially heterorhabditid nematodes, for insects. Morris *et al*., (1990) noted that both infectivity and reproduction within hosts were important attributes of nematodes capable of reinfesting new hosts in the field. The present study demonstrated that *H. zealandica* reproduced better in *G. mellonella* while *H. bacteriophora* reproduced similarly in both *G. mellonella* and *T. molitor* larvae. It is suggested that this nematodes be cultured in vivo in either of these hosts insects based on their

reproduction capacity within. The fact that IJ production did not correlate with the IJ doses the insects were exposed to, suggest that they may be no association whatsoever. What may have an effect is the time taken for the progeny IJs to emerge from the insect cadavers. Although not properly tested in this study, it was observed that IJs emergence time was negatively correlated with II dose the cadaver had been exposed to prior to becoming infected and dying. It is recommended that further studies be carried out to confirm this observation and to test if this correlation is descriptive of an association. Collectively, these data suggests that the use of the dose response bioassay is a useful tool for determining the lethal dose necessary for achieving certain levels of mortality in specific host populations. However, it is not useful identifying potential candidate heterorhabditid nematodes in biological control programs for target insect pests.

References

Adams, B.J. & Nguyen, K.B. (2002) Taxonomy & Systematic. In: *Entomopathogenic Nematology.* Ed. Gaugler, R CABI Publishing, Wallingford, pp 1:34.

Adams, B.J., Burnell, A.M. & Powers, T.O. (1998) A phylogenetic analysis of *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA Sequence data. *J. Nematol*. **30**:22-39.

Ahmad, S. & Hussain, Z. (2002) Entomopathogenic Nematodes Associated with Soil Types and Vegetation Cover in Pothwar Region of Pakistan. *Pakistan Journal of Biological Sciences* **5(6)**: 640-642.

Akhurst, R. J. (1987) Use of starch gel electrophoresis in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). *Nematologica* **33**, 1–9.

Akhurst, R.J. & Bedding, R.A. (1986) Natural occurrence of insect pathogenic nematodes (Heterorhabditidae and Steinernematidae) in soil in Australia. *Journal of the Australian Entomological Society* **25**,241-244.

Akhurst, R.J. & Boemare, N.E. (1990) Biology and taxonomy of *Xenorhabdus* .In: Gaugler, R. & Kaya, H.K. (eds) *Entomopathogenic Nematodes in Biological Control*. CRC Press, Boca Raton, Florida, PP. 75-90.

Akhurst, R.J. (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis. Journal of General microbiology* **121**, 303-309.

Amarasinghe, L.D., Hominick, W.M., Briscoe, B.R. & Reid, A.P. (1994) Occurrence and distribution of entomopathogenic nematodes in Sri Lanka. *Journal of Helminthology* **68**, 277-286.

Aydin H, Susurluk. (2004) A. Competitive abilities of entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora* in the same host at different temperatures. *Turk J Biol*, **29**: 35-39, 2005.

Barker, C.W. & Barkr, G. M. (1998) Generalist Entomopathogens as Biological Indicators of Deforestation and Agricultural Land Use Impacts on Waikato Soils. *New Zealand Journal of Ecolog*y **22(2)**: 189-196.

Bathon, H. (1996) Impact of entomopathogenic nematodes on on-target hosts. *Biocontrol Science and Technology* **6**, 421-434.

Bedding, R.A. & Akhurst, R.J. (1975) A simple techniques for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica* **21**,109-110.

Bedding, R.A., & Molyneux, A.S. (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematoda). Nematologica **28,** 354–359.

Bedding, R.A., Molyneux, S. & Akhurst, R.J. (1983) *Heterorhabditis spp., Neoaplectana spp.,* and *Steinernema kraussei:* Interspecific and intraspecific differences in infectivity for insects. *Experimental Parasitology* **55**, 249–257.

Ben-Yakir, D., Efron, D., Chen, M. & Glazer, I. (1998) Evaluation of entomopathogenic nematodes for biocontrol of the European corn borer, *Ostrinia nubilalis*, on sweet corn in Israel. Phytoparasit. 26: 1-8.

Blackshaw, R.P. (1988) A survey of insect parasitic nematodes in Northern Ireland. *Nematologica* **21**,109-110.

Blaxter, M.L.; De Ley, P.; Garey, J.R.; Liu, L.X.; Scheldeman, P.; Vierstraete, A.; Vanfleteren, J.R.; Mackey, L.Y.; Dorris, M.; Frisse, L.M.; Vida, J.T. & Thomas, W.K. (1998) A Molecular Evolutionary Framework for the Phylum Nematoda. *Nature* **392**, 71-74.

Boemare, N.E., Laumond, C. & Mauleon, H. (1996) The nematode-bacterium complexes: biology, life cycle, and vertebrate safety. Bio.control Science and Technology **6**, 333-345.

Boff, M.I.C., Wiegers, G.L. & Smits, P.H. (2000) Influences of host size and host species on the infectivity and development of *Heterorhabditis megidis* (strain NLH-E87.3). *Biocontrol* **45**, 469–482.

Bonifassi, E., Saux, M.F., Boemare, N., Lanois, A., Laumond, C. & Smart, G. (1999) Gnotobiological study of infective juveniles and symbionts of *Steinernema scapterisci:* A model to clarify the concept of the natural occurrence of monoxenic associations in entomopathogenic nematodes. *Journal of Invertebrate Pathology* **74**, 164-172.

Boucias, D. & Pendland, J., (1998) Principles of Insect Pathology: Insect-Pathogen Relationships. Chapter 8 General Properties or Fungal Pathogens. Kluwer Academic Publishers.

Burges, H. & Hussey, N. (1971) Microbial Control of Insects & Mites. Academic Press.

Burman, M. & Pye, A.E. (1980) *Neoaplectana carpocapsae*: Movements of nematodes populations on a thermal gradient. *Exp. Parasitol*., 49,258-265.

Cabanillas, H.E., Poinar, G.O & Raulston, J.R. (1994) *Steinernema riobravis* sp. Nov (Rhabditida: Steinernematidae) from Texas. *Fundamental and Applied Nematology*, **17**, 123-131.

Carissimi Boff, M.I. (2001) The entomopathogenic nematode *Heterorhabditis megidis*: host searching behaviour, infectivity and reproduction. (An MSc dissertation). University of Wageningen, U.S.A.

Caroli, L., Glazer, I., & Gaugler, R. (1996) Entomopathogenic nematode infectivity assay: multi variable comparison of penetration into different hosts *Biocontrol Science and Technology* **6**, 227-233.

Chitwood, B.G. & Chitwood, M.B. (1950). Introduction to Nematology. University Park Press. Baltimore.

Christopher, H. & Schmitt, D. (1997) Environmental Sampling and Monitoring Primer- Regional Sampling Methods. http://ewr.cee.vt.edu/environmental/teach/smprimer/design/Gilbert#Gilbert

Courtney, W. D., Polley, D. & Miller V. L. (1955). TAF, an improved fixative in nematode technique. Plant Disease and Reproduction **39**: 570-571.

Curran, J. 1990. Molecular techniques in taxonomy. *In* entomopathogenic nematodes in biological control. R. Gaugler & H.K. Kaya (eds). CRC Press, Boca Raton. Florida. P 63-74.

Curran, J., Baillie & Webster, J.M. (1984) Use of genomic DNA restriction fragment length differences to identify nematode species. *Biological Sciences* **90,** 137-144.

Dent, D.R. (1997) integrated pest management and microbial insecticides In *Microbial Insecticides*: Novelty or necessity? British crop protection council proceeding/ Monograph series N° **68**, 127-138.

Dix, I., Burnell, A.M., Griffin, C.T., Joyce, S.A, Nugent, J.M & Down M.J. (1992) The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross breeding second-generation amphimictic adults. *Parasitology* **104**,509-518.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourist, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.-F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin,A. & Kunst, F. (2003) The genome sequence of the entomopathogenic bacterium Photorhabdus luminescens Nature Biotechnology **21**,1307-1313.

Ehlers, R-U. & Hokkanen, M.T. (1996) Insect biocontrol with non-endemic entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* sp.): OECD and COST workshop on scientific and regulation policy issue. *Biocontrol Science and Technology* **6**, 295-302.

Ehlers, R-U. (1990) Some contemporary issues in biological control of insects and their relevance to the use of entomopathogenic nematodes, in *Entomopathogenic nematodes in biological control* (Gaugler, R. and Kaya, H.K. Eds), CRC Press, Boca Raton, USA, pp.215-231.

Ehlers, R-U. (1996) Current and future use of nematodes in biocontrol: Practice and commercial aspects with regard to regulatory policy issues. *Biocontrol Science and Technology* **6**, 303-316.

Emeden, V H. F. & Service, M.W. (2004) Pest &Vector Control Cambridge *University Press*.

Fan, X. & Hominick, W.M. (1991) Efficiency of the *Galleria* (Wax Moth) Baiting Technique for Recovering Infective Stages of Entomopathogenic Rhabditids (Steinernematidae and Heterorhabditidae) from Sand and Soil. *Revue Nematologica* **14(3):** 381-387.

Ferron, P & Deguine, J., P. 2005 Crop protection, biological control, habitat management and integration farming. A review. *Agron. Sustain. Dev*. **25**: 17–24 17.

Fitzpatrick, R.W., McKenzie, N., & Maschmedt, D.J. (1999) Soil Morphological Indicators and their Importance to Soil Fertility, in *Soil analysis: an interpretation manual* (Peverill, K.I., Sparrow, L.A. and Reuter, D.J. Eds) CSIRO Publishing, Australia.

Forschler, B.T., & Nordin, G.L. (1988) Comparative pathogenicity of selected entomogenous nematodes to the hardwood borers, Prionoxystus robiniae (Lepidoptera: Cossidae) and Megacyllene robiniae (Coleoptera: Cerambicidae). *J. Invertebr. Pathol*. **52**, 343–347.

Forst, S. & Nealson, K.H. (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiological Review* **60**, 21-43.

Forst, S. & Clarke, D.J. (2002) Bacteria-nematode symbiosis. In: Gaugler, R. (ed.0 Entomopathogenic Nematology. CAB international, walling-ford, UK, pp. 57-77.

Forst, S., Dowds, B., Boemare, N., & Stackebrandt, E. (1997) *Xenorhabdus* and *Photorhabdus* spp.: Bugs that kill bugs. *Annual Review of Microbiology* **51**, 47-72.

Friedman, M.J. (1990) Commercial production and development, in *Entomopathogenic Nematodes in Biological Control*. (Gaugler, R. and Kaya, H.K. Eds), CRC Press, Boca Raton, USA.

Fuxa, J.R., Richter, A.R., & Agudelo-Silva, F. (1988) Effect of host age and nematode strain on susceptibility of Spodoptera frugiperda to Steinernema feltiae. *J. Nematol*. **20**, 91–95.

Gardner, S.L., Stock, S.P. & Kaya, H.K. (1994) A new species of *Heterorhabditis* from Hawaiian Islands. *Journal of Parasitology* **80**, 100-106.

Gaugler, R. & Georgis, R. (1991) Culture method and efficacy of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae). *Biological Control* **1**, 269-274.

Gaugler, R. (1998) Rhabditida: Steinernematidae & Heterorhabditidae, in Biological control: a guide to natural enemies in North America (Weeden, C.R., Shelton, A.M. & Hoffmann, M.P., Eds), Cornell University [online] cited: 4th December, 2006. Available at http://www.nysaes.cornell.edu/ent/biocontrol/pathogens/nematodes.html

Georgis, R. & Manweiler, S.A. (1994) Entomopathogenic nematodes: A developing biological control technology. *Agric. Zool. Rev.* **6**: 63-94.

Georgis, R. & Poinar Jr., G.O., (1983) EVect of soil texture on the distribution of *N. carpocapsae* (Nematoda: Steinerenematidae). *J. Nematol*.**15**, 308–311.

Gerritsen, L.J.M. & Smits, P.H. (1997) The influence of *Photorhabdus luminescens* strains and form variants on the reproduction and bacterial retention of *Heterorhabditis megidis*. *Fundamental Applied Nematology* **20**, 317-322.

Glazer, I., Galper, S. & Sharon, E. (1991) Virulence of the nematode *(Steinernematids* and *Heterorhabditis): bacteria (Xenorhabdus* spp.) complex to the Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae). Journal of Invertebrate Pathology **57**: 94-100.

Glazer, I. & Lewis, E.E. (2000) Bioassays of entomopathogenic nematodes, in; *Bioassays of Entomopathogenic Microbes and Nematodes* (Navon, A. & Ascher, K.R.S., Eds.) Bet Dagan, Israel pp 229-245.

Glazer, I. (1992) Invasion rate as a measure of infectivity of Steinermatid and Heterorhabditid nematodes to insects. *Journal of Invertebrate Pathology* **59**, 90–94.

Glazer, I. & Navon, A. (1990) Activity and persistence of entomogenous nematodes used against *Heliothis armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **83**:1795-1800.

Glazer, I., Klein, M., Navon, A. & Nakache, Y. (1992) Comparison of efficacy of entomopathogenic nematodes combined with antidesiccants applied by canopy against three cotton pests (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **85**:1636-1641.

Glazer, I., Alekseev, E. & Samish, M. (2001) Factors affecting the virulence of entomopathogenic nematodes to engorged female *Boophilus Annulatus* ticks. *J Parasitol,* **87(4)**, 808-812.

Grenier, E., Laumond, C. & Abad, P. (1996) Molecular characterization of two species-specific tandemly repeated DNAs from entomopathogenic nematodes Steinernema and Heterorhabditis (Nematoda.: Rhabditid). *Molecular and bio-chemical Parasitology* **83**, 47-56.

Grewal, P.S. (2002) Formulation and application technology. In:Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, Oxfordshire, UK, pp. 265–287.

Grewal, P.S., (1998) Formulations of entomopathogenic nematodes for storage and application. Jpn. *J. Nematol*. **28**, 68-74.

Grewal, P.S., Lewis, E.E. Gaugler R. & Campbell, J.F. (1994) Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes, *Parasitology* **108**, 207-215

Griffin, C.T, O'Callaghan, KM. & Dix, I. (2001) A self-fertile species of Steinernema from Indonesia further evidence of convergent evolution amongst entomopathogenic nematodes. Parasitology **122**:181-186.

Griffin, C.T., Joyce, S.A., Dix, I., Burnell, A.M. & Downes, M.J. (1994) Characterisation of the entomopathogenic nematode *Heterorhabditis* (Nematoda: Heterorhabditidae) from Ireland and Britain by molecular and crossbreeding techniques, and the occurrence of the genus in these islands. *Fundamental and Applied Nematology* **17**, 245-253.

Griffin, C.T., Moore, J.F. & Downes, M.J. (1991) Occurrence of insect parasitic nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. *Nematologica* **37**, 92-100.

Grunder, J.M. (1997) *Photorhabdus luminsecens* als symbiont insektenpathogener Nematoden. PhD thesis, ETH, Zurich.

Gullan, P. J., & P. S. Cranston. 1994. The Insects: An outline of entomology. Pp 23-422. Chapman and Hall. London.

Han, R & Ehlers, R-U. (2001) Effect of *Photorhabdus luminescens* phase variants on the *in vivo* and *in vitro* development and reproduction of entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae. FEMS Microbiology Ecology* **35**, 239-247.

Hall, F.R. & Menn J.J. (1999) Biopesticides Used & Delivery. Humana Press Inc New Jersey pp 271-299.

Hara, A.H., Gaugle, R., Kaya, H.K. & Lebeck, L.M. (1991) Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiian Islands. *Environmental Entomology* **20**,211-216.

Harris, J. & Dent, D. (2000) Priorities in Biopesticide Research & Development in Developing Countries. CABI Publishing.

Hazir, S., Kaya, H.K., Stock, P.S. & Keskin, N. (2003) Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests turk.j.biol.pp27.

Heyns, J. (1971) A Guide to the Plant & Soil Nematodes of South Africa. A.A. Balkema, Cape Town.

Hominick, W.M. (2002) Biogeography. In: *Entomopathogenic Nematology*. Ed.: Gaugler, R. CABI Publishing, Wallingford, Pp. 115-143.

Hominick,W. M. & Briscoe, B. R. (1990) Survey of 15 sites over 28 months for entomopathogenic nematodes (Rhabditida: Steinernematidae). *Parasitology* **100**: 289-294.

Hominick,W.M., Briscoe, B.R., Pino, F.G. Del, Jian, H., Hunt, D.J., Kozodoy, E., Mracek, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Sturhan, D., Waturu, C. & Yoshida, M. (1997) Biosytematics of entomopathogenic nematodes: current status, protocol & definitions. *Journal of Helminthology* **71**,271-298.

Hoy, M.A. & D.C. Herzog. (1985) Biological Control in Agricultural IPM Systems. (eds.)Academic Press, Inc., Orlando. pp 589.

Hunt, D.J. (1997) Nematode Species:Concepts and Identification Strategies Exemplified by the Longidoridae, Steinernematidae and Heterorhabditidae. In: *Species. The Units of Biodiversity*. Eds.: Claridge, M.F.; Dawah, H.A. & Wilson, M.R. Chapman & Hall, London. pp 221- 245.

Johnigk, S-A, & Ehlers, R-U., (1999). Juvenile development and life cycle of *Heterorhabditis bacteriophora* and *H. indica* (Nematoda: *Heterorhabditidae*). *Nematology* **1(3),** 251-260.

Kadir, A.S.A. & Barlow H.S. (1992) Pest management & the environment in 2000. C. A. B. International.

Kaya, H.K. (1990) Soil ecology, in *Entomopathogenic nematodes in biological control* (Gaugler, R. and Kaya, H.K. Eds), CRC Press, Boca Raton, USA.

Kaya, H.K. (1987). Diseases caused by Nematodes, PP. 453-470 in J.R. Fuxa & Y. Tanada (eds), Epizootiology of insect diseases. J. Wiley, New York.

Kaya, H.K. & Gaugler, R. (1993) Entomopathogenic Nematodes. *Annual Review of Entomology* **38**, 181-206.

Kaya, H.K., & Stock, S.P. (1997) Techniques in insect Nematology, in *Manual of Techniques in Insect Pathology*. Academic Press Limited, NY. Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G. & Ellar, D.J. (1989) A cytolytic δ-endotoxin from *Bacillus thuringiensis* var *isrealensis* forms cation-selective channels in planar lipid bilayers. *FEBS Letters* **244**, 259-262.

Koppenhofer, A.M. & Kaya, H.K. (1996) Coexistence of Two Steinernematid Nematode Species (Rhabtida: Steinernematidae) in the Presence of Two Host Species. *Applied Soil Ecology* **4**: 221-230.

Koppenhöfer, A.M., Brown, I.M., Gaugler, R., Grewal, P.S., Kaya H.K. & Klein, M.G. (2000) Synergism of entomopathogenic nematodes and imidacloprid against white grubs: greenhouse and field evaluation. *Biological Control* **19,** 245–251.

Kung, S.P., R. Gaugler & H.K. Kaya. (1990) Influence of soil pH and oxygen on persistence of *Steinernema* sp. *J. Nematol*., **22:** 440-445.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin. & N. R. Pace. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82**:6955–6959.

Lee, D.L. & Atkinson, H.J. (1976) Physiology of nematodes 2nd edition. The MacMillan Press, Ltd., London.

Liu, J. & Berry, R.E. (1995) Determination of PCR conditions for RAPD analysis in entomopathogenic nematodes (Rhabditida: Steinernematidae & Heterorhabditidae).*Journal of Invertebrate Pathology* **65**, 79-81.

Liu, J., Berry, R.E. & Moldenke, A.F. (1997) Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rDNA gene sequences. *Journal of Invertebrate Pathology*, **69**, 246–252.

Mannion, C.M. (1992) Selection of suitable entomopathogenic nematodes for biological control of *Cylas formicarius* (Coleoptera: Apionidae). P.h.D. Diss., University of Florida, Gainesvilee, FL.

Mannion, C.M. & Jansson, R.K. (1992) Comparison of ten entomopathogenic nematodes for control of sweetpotato weevil (Coleoptera: Apionidae). *J. Econ. Entomol.* **85**: 1642-1650.

Mason, J.M., Razak, A.R. & Wright, D.J. (1996) The recovery of entomopathogenic nematodes from selected areas within Peninsular Malaysia. *Journal of Helminthology* **70**, 303-307.

Matthews, G. A. (1984) Pest management. Longman Group Limited.

Miduturi, J.S., Moens, M., Hominick, W.M., Briscoe, B.R. & Reid, A.P. (1996) Naturally occurring entomopathogenic nematodes in the province of West-Flanders, Belgium. J. Helminthol. **70**, 319–327.

Molyneux, A.S., Bedding, R.A. & Akhurst, R.J. (1983) Susceptibility of larvae of the sheep blowfly Lucilia cuprina to various *Heterorhabditis spp*., *Neoaplectana spp*., and an undescribed steinernematid (Nematoda). *J. Invertebr. Pathol*. **42**, 1–7.

Morris, O.N., Converse, V. & Harding, J. (1990) Virulence of entomopathogenic nematode–bacteria complexes for larvae of noctuids, a geometrid, and a pyralid. *Canadian Entomology*. **122**, 309–319.

Mracek, Z., & Webster, J.M. (1993) Survey of Heterorhabditidae and Steinernematidae (Rhabditida: Nmeatoda) in Western Canada. *J. Nematol*. **25**, 710-717.

Mracek, Z. (1980) The Use of 'Galleria Traps' for Obtaining Nematode Parasites of Insects in Czechoslovakia (Lepidoptera: Nematoda, Steinernematidae). *Acta Entomologia Bohemoslovakia* **77**: 378 -382.

Mracek, Z., Becvar, S., Kindlmann, P. & Jersakova, .J. (2005) Habitat preference for entomopathogenic nematodes, their insect hosts and new faunistic records for the Czech Republic. *Biological Control* **34,** 27–37.

Nguyen, K.B. & Smart, G.C. (1992) Life cycle of *Steinernema scapterisci*. *Journal of Nematology* **24(1)**, 160-169.

Nguyen, K.B. & Smart, G.C. (1996) Identification of entomipathogenic nematodes in the Steinernematidae and Heterorhabditidae (Nemata: Rhabditida) *Journal of Nematology* **28 (13),** 286-300.

O'Brien, R. D. (1967) Insecticides. *Action and Metabolism*. Academic Press, Inc.

Patel, M.N., Perry, R.N. & Wright, D.J. (1997) Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). *International Journal of Parasitology* **27**, 61-70.

Perez, E.E., Lewis, E.E. & Shapiro-Ilan. D.I. (2003) Impact of host cadaver on survival and infectivity of entomopathogenic nematodes (Rhabdita: *Steinernematidae* and *Heterorhabditae*) under desiccating conditions. *Journal of Invertebrate Pathology* **82**, 111–118.

Pimentel, D. & Hanson, A.A. (2000a) CRC Handbook of Pest Management in Agriculture 2nd edition, Volume II, CRC Press, Inc.

Pimentel, D. & Hanson, A.A. (2000b) CRC Handbook of Pest Management in Agriculture 2nd edition, Volume III, CRC Press, Inc.

Poinar, G.O. (1976) Description and biology of a new insect parasite rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida; Heterorhabditidaen. fam.). *Nematologica* **21**: 463-470.

Poinar, G.O. (1990a) Entomopathogenic nematode. *Heterorhabditis Zealandica*. Description and diagnosis. kbn.ifas.ufl.edu/ZEALANDI.htm - 7k.

Poinar, G.O. Jr. (1993) Origins and Phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* **16(4),** 333-338.

Poinar, G.O. Jr. (1979) Nematodes for Biological Control of Insects. CRC Press, Boca Raton, FL., 270 pp.

Poinar, G.O., Jr. (1975) Entomogenous nematodes: A manual and host list of insect-nematode associations. E.J. Brill, Netherlands.

Poinar, G.O., Jr. & Thomas, G.M. (1966) Significance of *Achromobacter nematophilus* Poinar and Thomas (Achronobacteriaceae: Eubacteriales) in the development of the nematode, DD-136 (*Neoaplectana* sp., Steinernematidae). *Parasitology* **56**, 385-390.

Poinar, G.O.Jr. (1990b) biology and taxonomy, PP. 23-61.*In* R. Gaugle and H.K.Kaya (eds.), Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL.

Powers, T.O.; Todd, T.C.; Burnell, A.M.; Murray, P.C.; Fleming, C.C.; Szalanski, A.L.; Adams, B.A. & Harris, T.S. (1997) The rDNA Internal Transcribed Spacer Region as a Taxonomic Marker for Nematodes. *Journal of Nematology* **29(4):** 441 – 450.

Reid, A.P., Hominick, W.M. & Briscoe, B.R. (1997) Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of rDNArepeat unit. *Syst. Parsitol*. **37**: 182-193.

Ricci, M., I. Glaser, J.F. Campbell. & R. Gaugler. (1996) Comparison of bioassays to measure virulence of different entomopathogenic nematodes. *Biocontrol Sci. Technol*. **6**: 235–245.

Robert, L. M. & William, H. L. (1982) Introduction to insect pest management, 2nd edition John Wiley & Sons, Inc. 420-421.

Ruberson, J. R., Nechols, J. R. & Tauber, M. J. (1999) Biological Control of Arthropod Pests. In *Handbook of Pest Management*. (J. R. Ruberson ed): 417-448. Marcel Dekker Inc.

SAS, (2001). SAS Software: Version 8.2. SAS Institute, Cary, NC.

Seinhorst, J. W. (1959) A rapid method for the transfer of nematodes from fixative to anhydrous glycerine. Nematologica **4:** 117-128.

Shapiro-Ilan, D.I. & Gaugler, R. (2002) Production technology for entomopathogenic nematodes and their bacterial symbionts. *Journal of Industrial Microbiology and Biotechnology* **28**, 137-146

Smart, G.C.Jr. (1995) Entomopathogenic nematodes for the biological control of insects supplement to the journal of nematology **27**:529-534.

Smits, P.H. (1996) Post-application persistence of Entomopathogenic nematodes. Biocontr. Sci. Technol. **6**:379-387.

Smits, P.H., Groenen, T.M. & DE Raay, D.G. (1991) Characterisation of *Heterorhabdditis* isolates using DNA restriction fragment length polymorphism. *Revue de Nématologie***14**, 445-453.

Stiling, P.D, (1992) Introductoy Ecology, Prentice Hall Engewood Cliffs, NJ.

Stock, S. P. & Kaya, H. K. (1996) A Multivariate Analysis of Morphometric Characters Of *Heterorhabditis* Species and the Role of Morphometrics in the Taxonomy of the Species of the Genus. *Journal of Parasitology* **82**: 806-813.

Stock, S. P.; Pryor, B. M. & H. K. Kaya. (1999) Distribution ff Entomopathogenic Nematodes (Steinernematidae and Heterorhabditidae) in Natural Habitats in California. *Biodiversity and Conservation* **8**: 535-549.

Stock, S.P. & Koppenhofer, A.M. (2003) Steinernema scarabaei n. sp. (Rhabditida; Steinernematidae), a natural pathogen of scarab beetle larvae (Coleoptera: Scarabaeidae) from New Jersey, USA. *Nematology* **5**, 191-204.

Stock, S.P. & Reid, A.P. (2004) Biosystematics of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae): current status and future directions. In: Cook, R. & Hunt, D.J. (Eds). *Proceedings of the Fourth International Congress of Nematology, 8-13 June 2002, Tenerife, Spain. Nematology Monographs and Perspectives* **2**, 435-446.

Stock, S.P., Campbell, J.F. & Nadler, S.A. (2001) Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology*, **87**, 877–889.

Stock, S.P., Somsook, V. & ReidD, A.P. (1998) *Steinernema siamkayai* n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. *Systematic Parasitology*.

Stock, S.P. (2003) New trends in entomopathogenic nematode systematic: Impact of molecular biology and phylogenetic reconstruction monduzzi editore S.P.A. medimond Inc USA pp 3.

Strauch, O., Stoessel, S. & Ehlers, R-U. (1994) Culture conditions define automictic or amphimictic reproduction of entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. *Fundamental and Applied Nematology* **17,** 575-582.

Stuart, R.J. & Gaugler, R. (1994) Patchiness in Populations of Entomopathogenic Nematodes. *Journal of Invertebrate Pathology* **64**: 39-45.

Sturhan, D. & Mracek, Z. (2000) Comparison of the Galleria baiting technique and a direct extraction method for recovering Steinernema (Nematoda: Rhabditia) infective stage juveniles from soil folia parasitological **47**:315-318.

Surrey, M.R. & Davies, R.J. (1996) Pilot-Scale Liquid Culture and Harvesting of and Entomopathogenic Nematode, *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* **67**, 92-99.

Szalanski, A. L., Taylor, D. B. & Mullin, P. G. (2000) Assessing nuclear and mitochondrial DNA sequence variation within Steinernema (Rabditida: Steinernematidae). *Journal of Nematology* **32**, 229 - 233.

Travasoss, L. & Sobre O. (1927) Genera *Oxysomatium.* Bol. Biol. Soa Paolo, **5**: 20-21.

Waage, J. (1999) Beyond the realm of conventional biological control harnessing bioresources and developing biologically-based technologies for sustainable pest management, pp: 5-17. *In:* L. W. Hong, S. S. Sastroutomo, I. G. Caunter, A. Jambari, L. K. Yeang, S. Vijaysegaran, and Y. H. Sen. [eds.], Biological control in the tropics. CABI Publishing. U. K.

Wallace, H.R. (1971) Abiotic influences in the soil environment in *Plant Parasitic Nematodes, vol. I.* (Zuckerman, B.M., Mai, W.F. and Rohde, R.R., Eds.), Academic Press, New York, pp 257-280.

Wang, J.X. & Bedding, R.A. (1996) Population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in the larvae of *Galleria mellonella*. Fundamental and Applied Nematology **19**: 363-367.

Webster, H. S. Jr. (1982) Plant Protection. *An Integrated Interdisciplinary Approach*. The Iowa State University Press.

Weinzierl, R. & Henn, T. (1991) Alternatives in insect management: Biological and biorational approaches. North Central Regional Extension Publication 401, Univ. of Illinois at Urbana-Champaign, Cooperative Extension, 73 pp.

White, G.F. (1927) A method for obtaining infective nematode larvae from cultures. *Science* **66**, 302-303.

Williams, J.L. (1978) Insects: Lepidoptera (moths), in *Honey bee pests, predators and diseases* (Morse, R.A., Ed.) Cornell University Press, London.

Woodring, J.L. & Kaya, H.K. (1988) Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques. Southern Cooperative Series Bulletin, Arkansas Agricultural Experiment Station. Fayetteville, Arkansas **331**, 1–17.

Wright, R.J. (2006) Biological Control of Mite Pests. The Board of Regents of the University of Nebraska on behalf of the University of Nebraska–Lincoln Extension. All rights reserved.

Wyniger, R. (1962) Pest of crops in warm climates and their control. Friedrich Reinhardt AG., Basel clichés Steiner & co., Basel.

Yong, J. P. W., Downer, H. L. & Eardly, B.D. (1991) Phylogeny of the phototrophic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. **173**:2271–2277.

Yoshida, M., Reid, R.P., Briscoe, B.R. & Hominick, W.M. (1998) Survey of entomopathogenic nematodes (Rhabditida: Steinernematidae & Heterorhabditidae) In Japan. *Fundamental and Applied Nematology* **21**,185-198.

Zervos, S., Johnson, S. C. & Webster, J. M. (1991) Effect of temperature and inoculum size on reproduction and development of *Heterorhabditis heliothidis* and *Steinernema glaseri* (Nematoda: Rhabditoidea) in *Galleria mellonella. Can. J. Zool.* **69**, 1261–1264.

Appendix I

Source of chemicals and supplies

Chemical/Supply	Source
Agarose	Saarchem
Agar	BioLab
Boric Acid	Saarchem
Calcium Chloride (CaCl2)	Saarchem
Canola oil	Epic Foods
Casein	Oxoid
Chicken fat	Rainbow chickens
Chloroform	Saarchem
Cod liver oil	The Clicks organisation
Corn flour	Tiger Milling (Tiger Brands)
EDTA (ethylene diamine tetracetic acid)	Saarchem
Ethanol (95% and 70%)	Saarchem
Ethidium Bromide	Saarchem
Glycerol	Merck
Honey	Gold Crest foods
Hydrochloric acid	Saarchem
JIK®	Reckitt and Benckiser
MacConkey agar	Merck
NBTA	Merck

Nutrient Broth	Biolab
Nutrient Agar	Oxoid
Parafilm ® M	Sigma
Polyurethane foam	Unilever
Potassium Chloride	Saarchem
ProNutro	Bokomo foods
Ringer’s solution	Merck
Sodium Chloride	Saarchem
Spray dried egg yolk	Sigma
Safflower oil	Massmart Holdings
TAF	Saarchem
Tris base	Roche
Whole milk powder	Nestle foods
Yeast extract	Merck

Appendix II

Galleria Medium

(Method adapted from (Woodring and Kaya, 1988)

The following changes were made:

- ProNutro original whole wheat was used instead of multivitamin bran
- Calcium Propionate was replaced with 1M HCl.

The recipe:

500g	ProNutro (original whole wheat)
200ml	Glycerol
200ml	Honey
200ml	Boiled distilled water
5 teaspoon	yeast extract
1.5ml	1M HCl

The yeast extract and HCl are dissolved in the water. Honey is warmed to thin. The glycerol and honey are added to the water solution. The ProNutro is then added to the total mixture and thoroughly mixed. Mixture is then placed in heavy duty aluminum foil and autoclaved for 20 minutes at 121^{o}C and 15psi

Appendix III

Nematode genomic DNA extraction

(Method adapted from Fermentas Life Sciences Puregene DNA purification Kit, Gentra systems 2003

[(# D-7000A)]

1) Wash nematodes 3 times in sterile double distilled water
2) Pellet nematodes in a microfuge tube by spinning at 14000 rpm for 10 minutes. Remove excess water. Drop microfuge tube in liquid nitrogen and pulverize frozen nematode pellet with a mini pestle.
3) Resuspend pulverized nematodes in 200µl of TE buffer
4) Mix 200µl sample with 600µl lysis solution (Fermentas kit) and invert several times to mix.
5) Add 3 µl Protinase K solution and mix by inverting 2.5 times. Incubate at 55ºC for 3 hours to overnight.
6) Add 3µl RNase a solution to the cell lysate.
7) Mix the sample by inverting the tube 25 times and incubate at 37ºC for 15-60 minutes.
8) Cool sample to room temperature.
9) Add 200 µl Protein Precipitation Solution to the RNase A- treated cell lysate.
10) Vortex vigorously at 6.000 x g for 3 minutes high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate.
11) Centrifuge at 13.000-16.000 xg for 3 minutes. The precipated proteins should form a tight pellet.
12) Pour the supernatant containing the DNA (leaving behind the precipited protein pellet) into a 1.5 ml centrifuge tube containing 600 µl 100% Isopranol (2- propanol).
13) Mix the sample by inverting gently 50 times.

14) Centrifuge at 13.000-16.000 xg for 1 minute; the DNA will be visible as white pellet.
15) Pour off supernatant and drain tube on clean absorbent paper.
16) Add 600 µl of 70% Ethanol and invert tube several times to wash the DNA pellet.
17) Centrifuge at 13.000-16.000 xg for 1 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.
18) Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.
19) Add 100 µl Hydration solutions.
20) Dehydrated DNA by incubating samples 1 hour at 65ºC or overnight at room temperature.
21) Store DNA at 4 ºC. For long-term storage, store at -20ºC or -80ºC.

Appendix VI

Protocol for the isolation of symbiotic bacteria associated with EPNs

(Adapted from Akhurst, 1980; Bonifassi *et al,* 1999; Kaya and Stock, 1997)

1) Surface sterilize infective juveniles by immersing then in 0.1% sodium hypochlorite (JIK® contains 3.5% sodium hypochlorite) for 1 hour.
2) Transfer the infective juvenile in fresh 0.1% sodium hypochlorite for a further 3 hours.
3) Rinse nematodes twice with Ringer's solution under a laminar flow
4) Suspend surface sterilized IJ in a small volume of nutrient broth
5) Homogenate suspension in a sterile mortar and pestle
6) Aseptically transfer the homogenate to a sterile McCartney bottle
7) Allow bacteria to multiply in the dark on a shaker for 24-48 hours at 25ºC
8) Streak onto MacConkey agar and NBTA and incubate in the dark for 24 hours at 25ºC to allow colony growth.

Appendix V

Isolation of genomic DNA from bacterial cells associated with EPNs

(Method from InstaGene matrix. (Catalog # 732-6030)

1) Pick an isolated bacterial colony and resuspend it in 1 ml of autoclaved water in a microfuge tube
2) Centrifuge for 1 minute at 10,000 – 12,000 rpm. Remove the supernatant.
3) Add 200µl of InstaGene matrix to the pellet and incubate at 56ºC for 15-30 minutes. **Note**: InstaGene matrix mix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. The pipette tip to be used should have a large bore, such as a 1,000µl pipette tip (Bio-Rad's catalog # 223-9378).
4) Vortex at high speed for 10 seconds. Spin at 10,000-12,000 rpm for 2-3 minutes
5) Use 20µl of the resulting supernatant per 50µl PCR reaction. Store the remainder of the supernatant at -20ºC. Repeat step 5 when reusing the InstaGene DNA preparation.

Appendix VI

Recipes for *in vitro* culture media

1 Solid culture media

1.1 Lipid agar

[Method adapted from Woodring and Kaya (1997)]

*Corn syrup was replaced with honey

10g honey

5g yeast extract

25g nutrient agar

2.5ml cod liver oil

2g $MgCl_2.6h_20$

Everything is mixed together, autoclaved at 121^oC and 15psi for 20 minutes and aseptically poured into Petri dishes.

2. Liquid culture media

2.1 Medium adapted from Han & Ehlers, (2001)

The nematode liquid medium is made up of the following components (in g/L):

10 g Trypcase soy broth,

10 g nutrient broth,

5 g yeast extract,

5 g casein peptone,

0.35g KCl,

0.21g CaCl2,

5.0g NaCl,

30ml vegetable oil

These are all mixed together in IL of distilled water. 10ml of the resultant mixture is added into 250ml Erlenmeyer flasks, stopped with cotton wool and autoclaved at 121^{o}C for 20minutes and 15psi.

Appendix VII

Gel Electrophoresis

Agarose Gel (50ml)

Agarose (0.4g for 0.8% and 0.5g for 1%)

10ml 5X TBE

40ml distilled water

Heat until agarose has completely dissolved

Add 1 µl Ethidium Bromide

5X TBE

54g Tris base

27.5g Boric acid

20ml 0.5M EDTA pH 8.0

Make up to 1L with distilled water and autoclave at 121ºC at 15psi for 20 minutes

Electrophoresis buffer

100ml 5X TBE

900ml sterile distilled water

2.5µl Ethidium Bromide

Appendix VIII

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

Killing and fixing nematodes

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

Processing nematodes to pure glycerine

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

Solutions

TAF

8 ml 35% formaldehyde

2.28 ml triethanolamine

104 ml distilled water

Double-strength TAF

8 ml 35% formaldehyde

2.28 ml triethanolamine

52 ml distilled water

Solution I

20 ml 95% ethanol

1 ml glycerine

79 ml distilled water

Solution II

5 ml glycerine

95 ml 95% ethanol

Appendix IX

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

1. Kill nematodes by placing them in a water bath at 60ºC for 2 minutes.

2. Rinse three times (5 minutes each) in Ringer's solution (pH 7.3).

3. Prefix in 8% gluteraldehyde (25% EM grad gluteraldehyde diluted in Ringer's Solution (pH 7.3) overnight.

4. Rinse three times (5 minutes each) in Ringer's solution (pH 7.3).

5. Rinse once, for 5 minutes, in sterile double distilled water.

6. Post-fix in 1% osmium tetroxide for 2 hours at 4ºC.

7. Rinse three times (5 minutes each) in sterile double distilled water.

8. Dehydrate with a series of ethanol washes (30%, 50%, 70%, 90%, 95% for 30 minutes each.

9. 9. Finally wash in several changes of 100% ethanol over 30-60 minutes.

10. Dry nematodes to critical point with liquid CO_2.

11. Mount specimens immediately onto SEM stubs and coat with carbon and gold Palladium.

Solutions:

Ringer's solution pH 7.3

9g NaCl

0.4g KCl

Appendix X

Heterorhabditis Zealandica 18S rDNA gene partial sequence

TAT GGT TAT GCT TTG GTC ACG AGC TAT CGG TGC TCA TGG AAT CAGGCT
CAC GTT TGA TTT CAA TCG GTA GCT CAC CCC ATC TAA GCT CTT GGTGAG
GTG TCT ATT CCC AAT TGG AGT CGC TTG GAG TGA CGG CAG TGA TGATTG
GGT GTG TTG CCC GTA TGG GTG GCG CAT AGA CTT AAT GAA CAG TACTCT
CGG ACT GTT GCC TCA GCC AAA CTA TCG TGG ACC GGT GGC TGT GATGTG
ACT TCG GTC ACA TCG AGA TCC GCT ATG CGG AGA GCC TCA ATG AGTTGT
TCC GTC AAC CGC CTT GCG ATA TTT GAT TTT TGG GTA CGT ATC GTTATC
TTG TCT CTT GGA TAC GTG TTA ATA CAT TTT GGC ACA ATG TAT TAGCTT
CAG CGA TGG ATC GGT TGA TTC GCG TAT CGA TGA AAA ACG CAG CTAGCT
GCG TTA TTT ACC ACG AAT TGC AGA CGC TTA GAG TGG TGA AGT TTTGAA
CGC ACA GCG CCG TTG GGT TTT CCC TTC GGC ACG TCT GGC TCA GGGTTG
CTT AAA GTA CTG CGG TGT TGC TGC GAA AGT GGC AAC GCC GAA TGTCGA
GCG GCG TTA GTG TTA CTA CTG GCC CCG TTC TAG TTC CGA ATA TTGGCA
ACA TGT CTT CTT CGT GAA GAC GCC GTA GAG AGT ATA AGT CTG TACCTA
TGG ATG TGT CGC GTA TGA AAT ATG ATG CTT CCC ATA CAT GGC TA

Heterorhabditis bacteriophora 18S rDNA gene partial sequence

GGG GTT AGA CTT TGT ACA CAG AGA TAC GGT ACC ATA GGA TAC AGCTTG
TTC TTG TAT TCA TAC GGT TTC TCA CCC CAT CTA AGC TCA TGG GAGAGT
GTC TAG TCC CAA TTG GAG TCG CTT TGA GTG ACG GCT ATG AAA ATTGGG
TAT GTT CCC CGT GAG GGT CGA GCA TAG ACT TTA TGA ACA GTG CTGGAG
CTG TCG CCT CAC CAA AAA ATC ATC GAT AGC TGG TGG CTA TGT GTGACA
TTA GTC ACA TAG GTA TCT GCT GAT GCA GAG AGC CTT AAT GAG TTGTTC
GTG TCA TCT GAC CTA CAA CCG CCA CTA TCG GTA AAT CAA CCC AATTAA

CTT GTT TCT TGT GTC GTG TTA ATA CAT ACT GGC AAA GTG TAT TAGCTT
TAG CGA TGG ATC GGT TGA TTC GCG TAT CGA TGA AAA ACG CAG CAAGCT
GCG TTA TTT ACC ACG AAT TGC AGA CGC TTA GAG TGG TGA AGT TTTGAA
CGC ACA GCG CCG TTG GGT TTT CCC TTC GGC ACG TCT GGC TCA GGGTTG
TTT AAT AAG CGA AAG TGT TGA AAG TTC ATT AAA CGA GAG TTC GGTGAT
ACT GAC AAC ACT GCG TCG ATC GGT GTA CTG TTG AAA GTA CCC CGTTCA
AGT ATC TTT ATG GGG CAA CAT GTC TTC TAT ACG GAG ACA TGA AAGATA
TTA AGA GTA TAT ACC TGT GGA TGC CCA CGT ATG AAA TAT GAC GTGTCG
TAT ACA CGG CTA GGA GGT

Appendix XI

Photorabdus luminescens laumondii, 16S rDNA gene partial sequence.

GTG ACG AGC GGC GGA CGG GTG AGT AAT GTC TGG GGA TCT GCC CGATGG
CGG GGG ATA ACC ACT GGA AAC GGT GGC TAA TAC CGC ATA ATG TCGCGA
GAC CAA AGT GGG GGA CCT GAA AGG GCC TCA CGC CAT CGG ATG AACCCA
GAT GGG ATT AGC TGG TAG GTA GGG TAA TGG CCT ACC TAG GCG ACGATC
TCT AGC TGG TCT GAG AGG ATG ACC AGC CAC ACT GGG ACT GAG ACACGG
CCC AGA CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA CAA TGGGCG
GAA GCC TGA TGC AGC CAT GCC GCG TGT ATG AAG AAG GCC TTC GGGTTG
TAA AGT ACT TTC AGC GGG GAG GAA GGG TTT AGC CTG AAG AGG GTTGGA
CTT TGA CGT TAC CCG CAG AAG AAG CAC CGG CTA ACT CCG TGC CAGCAG
CCG CGG TAA TAC GGA GGG TGC GAG CGT TAA TCG GAA TGA CTG GGCGTA
AAG CGC ACG CAG GCG GTC AAT TAA GTT AGA TGT GAA ATC CCC GGGCTC
AAC CTG GGA ATG GCA TCT AAG ACT GGT TGA CTG GAG TCT CGT AGAGGG
GGG TAG AAT TCC ATG TGT AGC GGT GAA ATG CGT AGA GAT GTG GAGGAA
TAC CGG GTG GCG AAG GCG GCC CCC TGG ACG AAG ACT GAC GCT CAGGTG
CGA AAT GCG TGG GGA GCA AAC ATG GAT TAG ATA CC

Photorabdus sp 16S rDNA gene partial sequence.

TAT AG: GTG TAG CTG ACG AGC GGC GGA CGG GTG AGT TAA AGT CTGGGG
ATC TGC CTG ATG GAG GGG GAT AAC CAC TGG AAA CGG TGG CTA ATACCG
CAT GAA GTC CCG AGA CCA AAG TGG GGG ACC TGC GGG CCT CAC GCCATC
GGA TGA ACC CAC GAT GGG ATT AGC TGG TAG GTA GGG TAA TGG CCTACC
TAG GCG ACG ATC CCT AGC TGG TCT GAG AGG ATG ACC AGC CAC ACTGGG

ACT GAG ACA CGG CCC AGA CTC CTA CGG GAG GTA GCT GTG GGG AATATT
GCA CAA TGG GCG CAA GCC TGA TGC AGC CAT GCC GCG TGA ATG AAGAAG
GCT TTC GGG TTG TAA AGT ACT TTC AGC GGG GAG GAA GAG TGC GGCCTG
AAT AGG GCT GGA CTT TGA CGT TAC CCG CAG AAG AAG CAC CGG CTAACT
CCG TGC CAG CAG CCG CGG TAA TAC GGA GGG TGC AAG CGT TAA TCGGAA
TGA CTG GGC GTA AAG CGC ACG CAG GCG GTC AAT TAA GTT AGA TGTGAA
ATC CCC GGG CTT AAC CTG GGA ACG GCA TCT AAG ACT GGT TGG CTAGAT
GTC TCG TAG AGG GGG GTA AGA ATT CCA TGT GTA TGC GGT GTA AATGCG
TAT AAG ATT AGT GGA GGA ATA CCG GGT GGC GAA GGC GGC CCC CTTGGA
CGA AAG ACT GAC GCT CAA GGT GCG AAA AGC GTG GGG AGC AAA CATGGA
TTA TAT TC

Appendix XII

Spectrophotometer Determination of DNA concentration

1.5 µl of DNA was diluted to 1500 µl with distilled deionized water and absorbance read at A230, A260, and A280. The A260/A280 ratio provides an estimate of the purity of the nucleic acid.

In a pure DNA sample this ratio should be around 1.8; lower values indicate protein or phenol contamination.

A230 should be less than A260 and may be the same as the A280. High A230 readings indicate that residual phenol remains in the preparation.

An A260 of 1 corresponds to approximately 50 µg/ml of double stranded DNA in a 1cm quartz cuvette. The nucleic acid concentration can be calculated as follows:

A260 X 50mg/Ol X 0.001 µl /ml X dilution factor (1500Ol/1.5Ol). Doing this you get the concentration of your DNA in µg/ µl.