Isolation and RAPD-PCR Characterization of New Entomopathogenic Nematode Strains From Palestine

By

MICHAEL AWAD MICHAEL SANSOUR

Under the Supervision
Of

Dr. Kamel Adwan, Dr. Nael Abu-Hasan
and Dr. Naim Iraki

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This Thesis was defended successfully on the 28th May 2000
and approved by

Committee Members	Signature
1. Kamel Adwan – Advisor	د.كامل عدوان
2. Dr. Nael Abu-Hasan - Advisor	د.نائل أبو حسن
3. Dr. Naim Iraki - Advisor	Naim د.نعيم عراقي
4. Prof. Adnan Shqueir – External Examiner	أ.د. عدنان شقير
5. Dr. Yahia Faydi – Internal Examiner	د. يحيى فيضي

TO

MY FATHER AND MOTHER FOR THEIR CONTINUOUS SUPPORT, TO MY SISTERS, TO MY BROTHERS IN LAW, AND TO MY BEST FRIEND ELI

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LIST OF ABBREVIATIONS

deoxyNucleoside TriPhosphate	dNTP
Entomopathogenic Nematodes	EPNs
Heat Shock Proteins	*HSP*
Infective Juveniles	IJs
Internal Transcribed Spacer	ITS
Juvenile	J
Lest Significant Difference	LSD
Lethal Dose	LD
Nutrient Bromothymol blue Triphpenyl tetrazolium chloride Agar	NBTA
Polymerase Chain Reaction	PCR
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment Length Polymorphism	RFLP
Scanning Electron microscopy	SEM
Thermus aquaticus	Taq
Unweight pair group Method Algorithm	UPGMA

Isolation and RAPD-PCR Characterization of New Entomopathogenic Nematode Strains from Palestine

Abstract

Entomopathogenic nematodes (EPNs) are being applied as biocontrol agents against soil-borne pests. Since they exhibit host specificity, and because of the need for improving their infectivity and tolerance to environmental conditions it is necessary to enlarge the number of available species and strains. As the number of isolated species and strains increases, there will be a great need for convenient and reliable methods of identification, genotyping and classification.

Two EPN strains (Bethlehem11 and Bethlehem22) were isolated by trapping with larvae of *Galleria mellonella* from the fields of Bethlehem district, Palestine. The novel strains were identified as *Heterorhabditis indica* based on the successful crossings with another *H. indica* strain (LN2). There were 66% successful crosses between each of the Bethlehem strains and LN2 strain, while the percentage of successful crosses between Bethlehem strains was 83%. The Bethlehem strains were more heat tolerance when compared with *H. bacteriophora* from temperate climatic region. The survival of the IJs of the local strains was about 80%, while that of the *H. bacteriophora* strain approached Zero, when incubated at 40°C for 4 hours. The infectivity of the local strains was higher than that of the *H. bacteriophora* strain tested. The LD_{50} values after 26 hours exposure to *G.*

mellonella larvae were 25 for Bethlehem11, 55 for Bethlehem22, and 160 IJs for *H. bacteriophora*. The novel strains are the first EPN strains that are isolated from Palestine. Studies on these strains are useful for the purpose to control local pests such as *Maladera matrida.*

The two local strains were examined for their genetic relatedness to one another and to other EPN strains by Randomly Amplified Polymorphic DNA markers (RAPD). Results obtained by using seven primers showed that DNA banding patterns from Bethlehem strains are at 96.42% similarity, which indicate that they are different strains. Also, results showed that these strains are different from another *H. indica* strain, isolated from India, where an average similarity of 58.66% was observed. Moreover, the results revealed low similarity between the *H. indica* strains on one hand, and another strain belonging to the *Steinernema* genus. This difference is expected since they belong to different genera.

The RAPD-PCR technique can not identify newly isolated strain at the species level. This is mainly because of the fact that this technique may show sometimes that the level of similarity between two species may be the same between two strains of the same species. On the other hand, this technique could be used to differentiate between two unknown strains.

Chapter I
General Introduction

1.1 Introduction

1.1.1 Biocontrol and chemical pesticides

Recently, it became generally accepted that biological control is a promising alternative for the synthetic chemical pesticides in agriculture (e.g., Driesche and Bellows, 1996). This modern pest control technology involves the action of natural enemies to suppress the population of a particular pest to a level below the harmful threshold.

Agricultural pests cause tremendous damage to economically important plants, either directly or indirectly by transmitting viral, fungal, and bacterial diseases. For example, the fungus *Phytophthora infesta* caused the epidemic that triggered the Irish Potato famine of the 1840s.

Synthetic chemical pesticides such as, organophosphates (Aliphatics), chlorinated hydrocarbons (DDT), carbamides (Naphthyl compounds) and fumigants (Methyl bromide) have been successfully used for controlling agricultural pests since long time ago. These compounds are very toxic not only to pests but also to humans and livestock. Extensive application of such toxic chemicals may pollute the environment and kill beneficial organisms including pest natural enemies. Because of the lack of natural degradation of some of these compounds, their remnants have polluted groundwater resources in areas of intensive agriculture (Dempster, 1987).

Moreover, the continuous application of these chemicals has caused development of new resistant pest strains (Brent, 1987). Because of these consequences and the high health risks imposed by these compounds, the use of some of them was banded in many countries in the world. The great environmental risks resulting from the use of these chemicals have led researchers and authorities to search for alternative means of insect control. During the last few decades, research work showed that application of some biocontrol agents might reduce the use of synthetic chemical pesticides. This is mainly because of the environmental safety of these agents, and their host specificity, with minimal chances for developing resistant pest strains.

1.1.2 Biological control

Biological control involves the action of natural enemies against a given target pest. Natural enemies may act against hosts in various ways such as predation, parasitism, and pathogenecity. In predation, the natural enemy kills and devours the pest, for example, the predacious coccinellids vedalia beetle (*Rodolia cardinalis*) was used to control the cottony cushion scale, *Icerya purchasi* in California (Caltagirone and Doutt 1989). In parasitism, the parasite lives in or on the pest and utilizes it for its own survival and proliferation leading to host death. For example, the encyrtid *Epidinocarsis lopezi* is a parasite that was successfully used to control the mealybug *Phenaccocus manihoti* in cassava in Africa (Neuenschwander *et al.*, 1989). In pathogenecity, natural enemies cause disease to their host by degradation and toxification of the host body. Biological agents that cause disease to insects are called entomopathogens. Entomopathogenic organisms include bacteria (e.g., *Bacillus thuringiensis*), fungi (e.g., *Beauveria bassiana*), viruses (e.g., polyhedrosis), and nematodes (e.g.,*Heterorhabditis* spp. and *Steinernema* spp.) (Driesche and Bellows, 1996). Nematodes from the families *Heterorhabditae* and *Steinernematidae* have been effectively used to control several insect pests, particularly soil-borne insects such as, black vine weevil (*Otiorrhynchus sulcatus*) and larvae of sciarid flies (Ehlers and Peter, 1995). These nematodes kill the insect by means of their symbiotic bacteria. In summary, in case of application of any of the above biological agents, the strategy is always to reduce the pest population level to a level below the harmful threshold.

Maintaining the pest level below the harmful level using biological agents is based on three major strategies. First, conserving the naturally occurring enemies of a target pest. Second, increasing the population of native natural enemies of a pest. Third, application of nonnative natural enemies from other areas.

Increasing the population of a natural enemy, a process known as augmentative biological control, can be achieved in two ways; inoculative and inundative release. The inoculative release involves the release of small number of individuals that will proliferate and cause pest suppression along the season. The inundative release, on the other hand, involves mass release of individuals with the aim of obtaining immediate pest control (Ehler, 1990). An example of inundative release is the application of entomopathogenic nematodes (EPNs) into the soil for controlling soil-born insects.

1.2 Entomopathogenic nematodes

Nematodes are roundworms belonging to the phylum Nematoda, and occupy a wide range of ecological niches as parasites in plants and animals including insects. They vary in size, from 0.01cm till 25cm. In general, members of the families; *Steinernematidae* and *Heterorhabditae* are entomopathogenic, soil-borne, and less than 1mm in length (Gaugler and Kaya, 1990). They carry, in their gut, insect-pathogenic bacteria (Thomas & Poinar, 1979).

Recently, the nematode-bacteria complex became a promising biological control agent against soil insects because of several reasons. First, the nematodes are able to move and locate their host. Second, techniques for mass production, formulation, and field application of this agent were developed during the last decade. Third, EPNs can tolerate some chemical pesticides and are compatible with other biocontrol agents, which allows their incorporation in integrated pest management (IPM) programs (Zimmerman and Cranshaw, 1990). Compared to other biocontrol agents, the fact that EPNs are motile and natural soil inhabitants makes them a unique biocontrol agent against soil-borne insects where no other biocontrol agent can act equally effective.

1.3 EPNs mode of action

The pathogenecity of EPNs involves penetration into the insect's body, and proliferation of their symbiotic bacteria on which they feed and reproduce. The penetration is accomplished by a special stage of EPN life cycle called infective juvenile (IJs). These juveniles release the symbiotic bacteria stored in their gut into the insect's haemolymph (Bird and Akhurst, 1983; Endo and Nickle, 1991). The bacteria multiply and kill the insect, by septicemia, within 24-48h. The proliferated bacteria constitute the food source for the infective juvenile, which recovers into adult to initiate the life cycle. The nematode life cycle (Figure 1.1), which starts with fertilized eggs laid by female, involves four juvenile stages. The first juvenile stage hatches from the egg and develops into the second juvenile stage, while feeding on bacteria. The latter juvenile stage develops in turn, into the third, fourth stage, and finally into adult male or female (Lunau *et al.*,

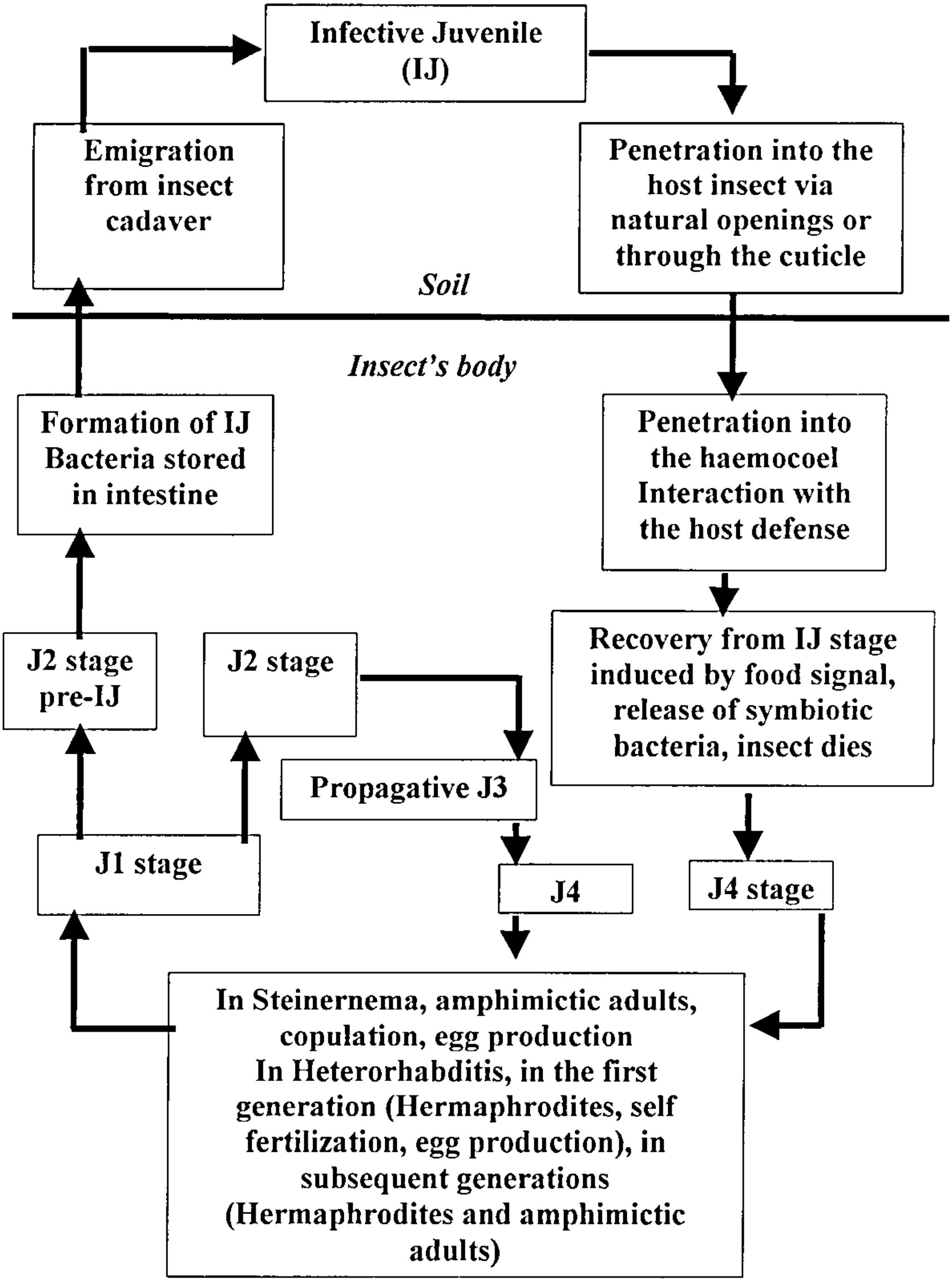

Figure 1.1: Life cycle of entomopathogenic nematodes from the *Steinernema* and *Heterorhabditis* genera.

1993). The cycle continues to run until the food source (bacteria) becomes limited. At this situation, the first juvenile stage develops through the second stage towards the infective juvenile. These juveniles store large amounts of food reserves in their body and small amount of symbiotic bacteria in their gut. They retain the second juvenile cuticle so their body becomes covered by double cuticle. In contrast to other stages, they do not feed, and move much faster (Campbell & Gaugler, 1991a).

Infective juveniles are morphologically and physiologically adapted to seek the host in the soil. Their bodies are covered by a double cuticle developed during the second and third stages juvenile (Campbell & Gaugler, 1991a). The double cuticle layers protect the infective juveniles from desiccation and other harmful environmental conditions such as, nematophagous fungi (Timper and Kaya, 1989). An infective juvenile is a non-feeding stage having the mouth and anus closed. Fat reserves are used for prolonged survival in the soil until the IJ reaches a host. In the gut, the IJs carry symbiotic bacteria, which play an important role in the pathogenecity to the host. The bacteria kill the insect by various toxins and hydrolytic enzymes and proliferate on its body whereby providing a mass of food to the nematodes. Infective juveniles are attracted to their hosts by several ways. For example, *S. carpocapsae* respond to chemical stimuli such as CO_2 concentration (Gaugler *et al.*, 1991; Gaugler *et al.*, 1980), host excretory products (Schmiege, 1963), and pH (Pye, and Burman, 1981). While IJs from the *Steinernematidae* family can penetrate the insect only through natural openings (mouth, anus, and spiracle) (Nguyen and Smart, 1991), their counterparts from the *Heterorhabditidae* family can penetrate directly through the cuticle in addition to their penetration through natural openings. This unique capability of the latter is attributed to the presence of an anterior tooth, which they use to rupture intersegmental areas of the insect cuticle (Bedding and Molyneux, 1982). The presence of such a tooth is a diagnostic character of the *Heterorhabditis* genus (Figure 1.2). During the penetration into the haemolymph, the infective juvenile loses its external cuticle (Campbell and Gaugler, 1991b) and cleans its body surface from any bacterial contaminants that might had been adsorbed from the soil. This event is particularly important because external contaminants may act as an antagonist against the symbiotic bacteria, resulting in inhibiting their proliferation inside the insect's body.

1.4 Pathogenecity is determined by symbiotic bacteria-EPNs complex

The symbiotic association between the nematodes and their bacteria is very specific and important for pathogenecity to the host (Akhurst & Boemare, 1988; Smits & Ehler, 1991). EPNs from the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with gram-negative

Figure 1.2: Scanning Electron Microscopy (SEM) of terminal tooth of *Heterorhabditis bacteriophora* D1. Photograph is courtesy of Bedding and Molyneux, 1982).

bacteria from the genera *Xenorhabdus* (Thomas & Poinar, 1979) and *Photorhabdus* respectively (Boemare *et al*, 1993). For example, *Steinernema glaseri* is associated with *Xenorhabdus poinarii* and cannot feed on other bacterial species. The main difference between *Xenorhabdus* and *Photorhabdus* bacteria is the unique characteristic of luminescence of the latter (Schmidt *et al.*, 1989), where dark adapted-eye can observe *Photorhabdus* cultures and insect cadaver infected with *Photorhabdus* bacteria. The mutual benefit existing between these two organisms is reflected in the protection of the bacteria by the nematodes from the external environment. These bacteria are non-sporeformers (Akhurst and Boemare, 1990; Poinar, 1990). In fact, free-living EPN bacteria have been isolated only from their symbiont EPNs and never from any other environment. On the other hand, the benefit of the EPNs from this symbiotic relationship is the fact that they feed on their symbiont bacteria (Knodo and Ishibashi, 1991). During pathogenecity development, the nematode acts as a vehicle that carries the bacteria into the insect's haemolymph (Milstead, 1979). Also the nematode may secret a factor, which inhibits the insect's immune system from acting against the bacteria (Götz, *et al.*, 1981; Burman, 1982). As a result of bacterial proliferation and secretion of exotoxins (protease, lecithinase, lipase) (Schmidt *et al.*, 1988) and endotoxins (polysaccharides), the insect dies. The proliferation of the symbiotic bacteria is insured by its secretion of antibiotic (Akhurst, 1982), which inhibits the growth of other competing microorganisms in the cadaver. Such antibiotic compounds include, the indole and *trans*-stilbene derivatives (Paul *et al.*, 1981).

1.5 Variations in life cycle, morphology and anatomy between the two EPN genera *Heterorhabditis* and *Steinernema*

There is one major difference in life cycle between nematodes of the two genera *Heterorhabditis* and *Steinernema.* In *Steinernema,* after the penetration of infective juveniles into the insect's body they always recover into females and males, while in *Heterorhabditis*, they recover into hermaphrodites only as shown in figure 1.3 (Poinar 1975). However, in subsequent generations of *Heterorhabditis,* individuals of the third stage may develop into males, females or hermaphrodites (Zioni *et al.*, 1992; Strauch *et al.*, 1994). In hermaphrodites there is self-fertilization of the eggs while males and females must mate. The mating pattern of *Heterorhabditis* individuals is of the -type while that of *Steinernema* is of the helix type as shown in figure 1.4 (Strauch *et al.*, 1994). Two or three generations can develop inside the insect body, depending on the size of the dead insect. In both genera, as the nematode population reaches to a certain density and the nutritional conditions become limited, the late second stage juveniles cease feeding and digestion, store about 100 bacterial cell in their intestine, and develop another cuticle under the

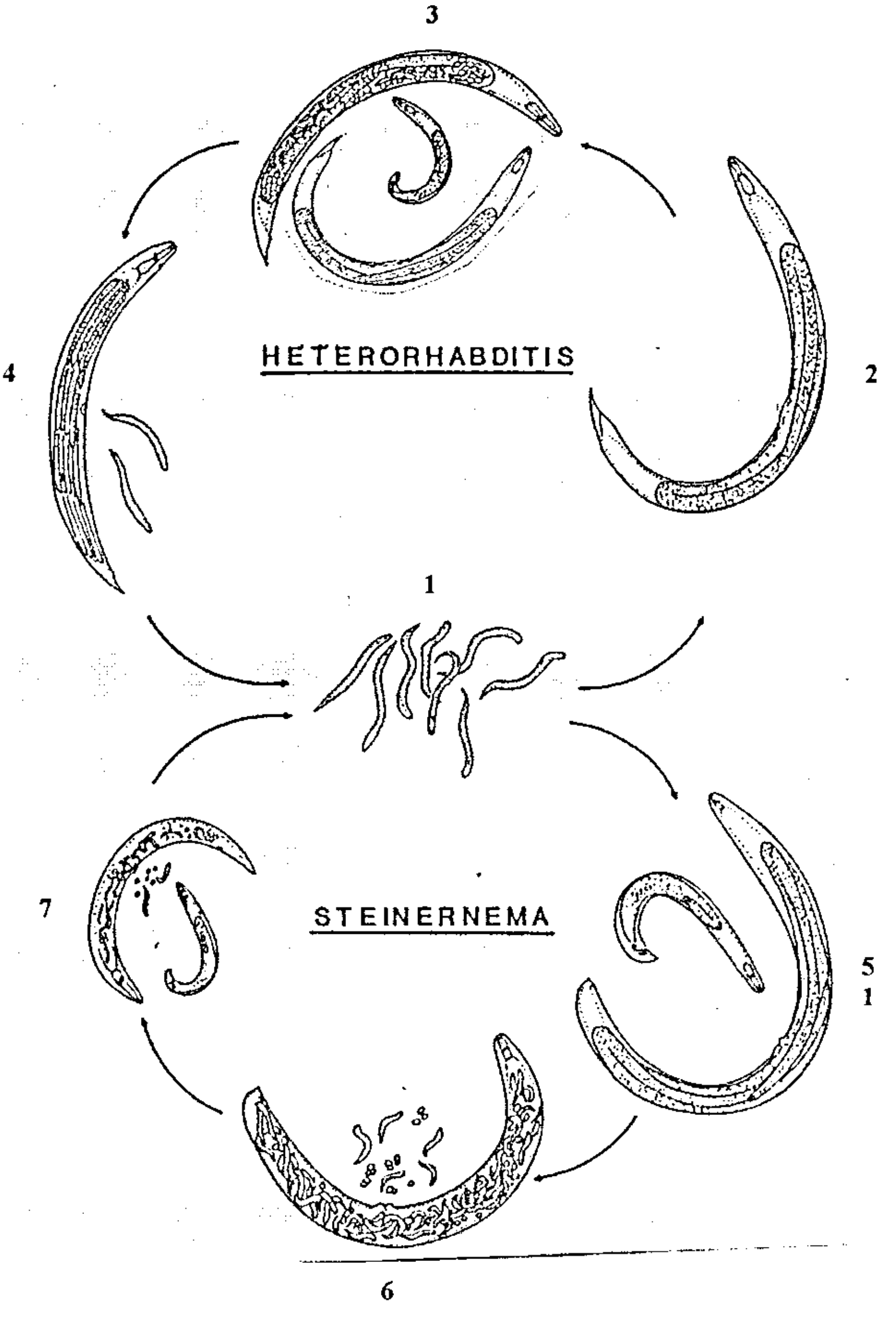

Figure 1.3: Generalized life cycle of EPNs; 1. Infective juveniles. In *Heterorhabditis*: 2. Hermaphrodites (first generation), 3. Females, males, and hermaphrodites (subsequent generations), 4. Females or hermaphrodites producing first juveniles. In *Steinernema*: 5. Females and males (first generation), 6. Juveniles emerging from females, 7. Females and males (subsequent generations). Modified from Poinar (1990).

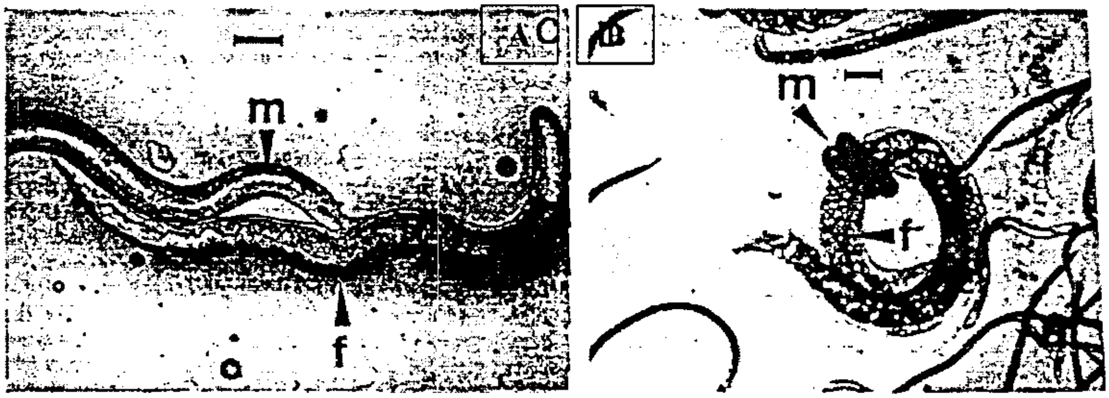

Figure 1.4: A: “ ” type copulation of female (f) and male (m) in *Heterorhabditis* spp.. B: Helix type copulation of female (f) and male (m) in *Steinernema* spp.. Photographs are courtesy of Strauch *et al.* (1994).

second stage cuticle. The latter cuticle, dose not molt and together with the former cuticle they constitute the double layer sheath. This unique stage is called, as was mentioned previously, infective juvenile. It leaves the insect cadaver searching for another host (Poinar, 1990).

There are several morphological differences that may distinguish between the two EPN genera. The IJ stage of heterorhabditids is characterized by the presence of anterior tooth, which is absent in Steinernematid counterpart. In the first generation, the female size in *Steinernema* is apparently larger than females and hermaphrodites of the *Heterorhabditis* genus. The female's tail in *Steinernema* is rounded, while it is pointed in *Heterorhabditis*. In contrast to males of *Steinernema,* males of *Heterorhabditis* have bursa, which is a brush-like structure located around the specula. The bursa aids in attaching the male to the female body during copulation.

1.6 Specificity of EPNs to target insects

The specificity of EPNs to a given target insect is determined by several factors. First, the nematode should locate its host in the soil (Lewis *et al.*, 1992, 1993). Second, the ability of the IJ to penetrate into the insect's haemolymph (Nguyen and Smart, 1991). Third, overcoming the insect's immune system (Van Sambeek *et al.*, 1999). The immune system of the insect may act against the invading IJs as well as against their symbiotic bacteria. The insect can encapsulate invading nematodes, by melanation and may synthesize toxic molecules with antibacterial activity against the symbiotic bacteria (Ehlers, and Beetz, 1998). However, some EPNs such as, *S. carpocapsae* could evade encapsulation by evasion (not recognized as non-self) or by overwhelming the insect's body with IJs (Dunphy and Webster, 1987; Molta and Hominick, 1989). Moreover, EPNs may produce extracellular proteins that suppress the bactericidal activity of the insect's immune system (Götz *et al.*, 1981). In general, for an EPN strain to be specific for a given insect, it should be able to find its host successfully, and to penetrate its body reaching the haemolymph, and to overcome its immune system. Hence, the specificity depends on all of these factors collectively, and all of them have to be possessed by a specific EPN strain. Because of this specificity, isolation of large number of EPN strains worldwide is important for selecting an appropriate strain against a given target insect.

1.7 Mass production, formulation, and field application

EPNs and their symbiotic bacteria were proven to be friendly to the environment, and have no health risks to plants, animals, and humans (Ehlers and Peter 1995). The United States Environmental Protection Agency (US EPA) assured their environmental safety and licensed their use as biocontrol agents in the field. Similar actions were undertaken in other countries such as Germany, China, and Japan. Today, several commercial products of EPNs are available for farmers in these countries. For example, in the United States various strains of *S. carpocapsae* are formulated as commercial products named ORTHO, BioSafe. BioVector, and Exhibit (Georgis, 1990a).

The availability of inexpensive mass production techniques is a prerequisite for introducing any biocontrol agent into commercial use. EPNs were known as potential biocontrol agent since 1930s (Glaser and Fox, 1931). However, the breakthrough in mass production was achieved only 1981 by Bedding (Bedding, 1981), who succeeded to produce them on solid and inexpensive medium. Since that time many other mass production technologies were developed, including production in fermentors (Friedman, 1989).

Mass production of EPNs requires establishment of monoxenic culture, which contains only EPNs and their symbiont bacteria. The EPN first juvenile stage is obtained from surface sterilized eggs, and then inoculated on medium containing its symbiont bacteria (Lunau *et al.*, 1993). These juveniles complete life cycle and produce large number of infective juveniles. The IJs can then be used as a stock for establishing commercial mass production culture. Commercial cultures could be of two types: Solid culture, using sponge as a substratum (Bedding, 1981, 1984) or liquid culture as reported by Lunau *et al.* (1993) and Fridman (1989). Culture media, in general, are designed to offer optimum growth conditions to the symbiotic bacteria, which in turn will provide the food source necessary for developing nematodes. The ingredients of these media include nutrient broth, yeast extract, vegetable oil and a protein source (Fridman, 1990). Furthermore, liquid culture requires continuous aeration, which is achieved either by shaking or continuous air bubbling and mixing of the liquid like in the case of fermentors. Mass production of EPNs using the solid medium technique usually requires relatively low costs of equipment (sponge and trays), while the required amount of labor is much greater compared to production in liquid culture. Therefore, it may be adopted in countries where labor costs are low. On the other hand, the liquid culture technique, which employs fermentors with sophisticated electronic control systems, demands high costs of equipment but smaller amount of labor. Hence, it is more economically feasible for industrialized countries, where labor costs are very expensive. In liquid culture, nematodes belonging to the *Steinernema* genus reproduce better than those of the *Heterorhabditis* (Strauch *et al.*, 1994). This is due to the fact that males and females of *Heterorhabditis* have the loose -type of copulation, which is difficult to maintain under continuous shaking, or bubbling and liquid mixing conditions. Therefore, the reproduction is solely dependent on hermaphrodites. In contrast, shaking or air bubbling does not affect the reproduction of steinernematids, which use the more stable helix type of copulation.

The aim of EPN formulation is to preserve the infectious state of IJs during shelf storage, in a medium that fits field application techniques. The formulation medium should provide the IJs with the required moisture and oxygen for their survival. EPNs have been formulated in polyacrylimide and alginate gels, or in clay, and kept at low temperatures (5-15°C) in order to prolong their shelf life (Georgis, 1990b). In general, steinernematids, especially *S. carpocapsae*, have a longer shelf life than that of heterorhabditids. Since various EPN strains show different shelf life lengths, it is important to select strains with long shelf life to scale up the efficiency of these nematodes as biocontrol agent. Field application of the formulated EPNs can be achieved in several ways (Georgis, 1990b). First, nematodes may be applied by ordinary agrochemical spraying equipment including mist blowers, electrostatic sprayers, fan sprayers, and helicopters. Second, nematodes can be introduced to the target soil by drip and sprinkler irrigation systems, this strategy provides sufficient moisture condition for the applied nematodes. Finally, in special cases such as wood-boring insects, nematodes can be delivered with syringe and cotton swab plug. An appropriate time of application is necessary for assuring a greater efficacy of EPNs. For example, early morning and preferably early evening or night applications are important for avoiding solar radiation, high temperatures, and low relative humidity. Furthermore, irrigation and conserving moderate soil moisture are essential for nematode movement, persistence, and pathogenecity.

The efficacy of EPNs depends on various factors, among which resistance to extreme environmental conditions is the most common one. Hence, isolation of additional new EPN strains from different climatic regions will enlarge the collection of strains with desired traits such as heat, desiccation, and solar radiation tolerance. These traits could be used for either selecting an appropriate strain to control a specific pest with high efficacy (Glazer *et al.*, 1996), or for transferring some desired traits to other EPN strains by conventional breeding (Shapiro *et al.*, 1997a) or genetic engineering techniques (Gaugler, 1987).

Adding more EPN strains will create problems of identification and classification. Accurate identification is also important for registration purposes before the release of any EPN strain to the field (Curran, 1990).

1.8 Characterization of EPNs

1.8.1 Classical characterization

Characterization of EPN species have been based so far on morphological characteristics combined with crosses with other identified species (Akhurst and Bedding, 1978; Poinar, 1990). Morphometry of the IJ stage is usually used for identification at the species level (Poinar, 1990). Once the identification at the species level is achieved, further tests involving crosses with the appropriate species are necessary for assuring the morphometric identification. However, both of these methods have remarkable limitations.

In many cases, EPNs show a little morphological variation within a given taxonomic group (mainly at the species level). For example, three species of *Heterorhabditis*; *H. bacteriophora*, *H. brevicaudis*, and *H. hawaiiensis* have overlapping in the ranges of IJ length and of specula (Nguyen and Smart, 1996). Unlike in *Steinernema*, in *Heterorhabditis* it is difficult to conduct crosses between individuals of the same species because of the presence of hermaphrodite stage. Hermaphrodites are morphologically very similar to virgin females, which makes it a difficult task to select microscopically virgin females for use in crosses (Curran and Webster, 1989). Because of the above limitations, molecular based techniques (e.g., based on DNA sequences) seem to offer a better choice for identification and studying the taxonomic relationships among EPNs, especially for the distinction between closely related strains. However the use of such techniques done not necessarily mean that they could totally replace the need for morphological and cross breeding data (Curran, 1990; Hominick *et al.*, 1997).

1.8.2 Molecular characterization

Several molecular techniques have been employed for the determination of genetic variability among EPNs. These techniques include; protein-based techniques such as isoenzyme patterns (Akhurst 1987), total protein patterns (Poinar and Kozodoi, 1988), and DNA-based techniques such as, restriction fragment length polymorphism (RFLP) detection within total genomic DNA (Curran and Webster, 1989), Random Amplified Polymorphic DNA (Hashmi *et al.*, 1996), and DNA sequence homology (Adams *et al.*, 1998).

The DNA-based techniques usually involve the use of the Polymerase Chain Reaction (PCR) tool (Hashmi *et al.*, 1996; Hominick *et al.*, 1997). PCR is a simple and rapid technique for DNA amplification. It requires relatively small amounts of DNA to start the reaction, as small as the DNA of a single IJ (Joyce *et al* 1994). The first step in standard PCR is the denaturation of the target DNA (template) by heating to 94°C which is followed by cooling (40°C-55°C) to facilitate the annealing of the different

DNA primers to complementary sequences on opposite strands. Then the temperature is raised to 72°C, and the polymerase Thermus aquaticus (Taq) starts sequence extension in the presence of deoxynucleoside triphosphate nucleotides (dNTPs). This round yields one copy of the target DNA sequence, and after several such rounds, millions of copies could be produced, which are sufficient for the required analysis.

PCR techniques have been applied in generating molecular markers of many organisms for species identification (Hominick *et al.*, 1997). For example, in PCR-RFLP method the DNA fragment length patterns resulting from restriction enzyme digestion of a specific DNA region amplified by PCR could be used as markers for identification and classification. Such specific regions include the internal transcribed spacer (ITS) located between the ribosomal 18 and 20S genes, and the region between the 16S gene and the cytochrome oxidase subunit II gene of the mtDNA (Hominick *et al.*, 1997; Joyce *et al.*, 1994).

1.8.2.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams *et al.*, 1990) is a PCR-based technique which could reveal molecular variations among organisms (Hashmi *et al.*, 1996; Hashmi and Gaugler, 1998; Shapiro *et al.*, 1997b). Unlike other PCR techniques, RAPD-PCR needs no previous knowledge about the sequence of the target DNA; this is because it detects DNA polymorphism by using a single primer of ten arbitrary oligonucleotides. If two of such a primer bind to the target DNA in the appropriate orientation and on opposite strands an amplified product could be generated. In general, one primer may produce 5-10 discrete DNA fragments (amplimers) of the size 30-3000bp that could be separated by agarose gel electrophoresis. Since the RAPD-PCR technique is sensitive to DNA concentration and quality, $MgCl_2$ concentration, number of reaction cycles, and the accuracy of the PCR machine, optimal reaction conditions should be developed.

RAPD analysis was used for rapid characterization of EPN strains. For example, Liu and Berry (1995,1996) found that RAPD profiles could be used for species characterization and for isolate identification within species. Also, RAPD was used together with morphological measures for the ~~for the~~ identification of a new species of *Heterorhabditis* from Hawaii (Gardner *et al.*, 1994), and for the identification of another *Heterorhabditis* spp from California (Stock *et al.*, 1996).

In conclusion, molecular characterization will allow us to distinguish between morphologically similar groups. This particularly important for identifying individual infective juveniles during the study of population dynamics, which is important in measuring the persistence of EPNs. Also, molecular taxonomy can be used for registration, and proprietary protection purposes, which are a prerequisite for the application of any biocontrol agent.

1.9 Characterization of the symbiotic bacteria

The characterization of the *Xenorhabdus* and *Photorhabdus* bacteria is usually based on some phenotypic characteristics (Boemare and Akhurst, 1988). These characteristics include cellular morphology, pigmentation, antimicrobial activity, bioluminescence, and catalase activity. However, these classical identification parameters are not sufficient to study the population diversity, since most of the strains are very similar in their phenotypic characteristics, and therefore, differences among the isolates of the same genus would be difficult to detect. Thus, DNA-based molecular techniques such as DNA/DNA hybridization and 16S rDNA sequencing were employed to detect differences among the *Xenorhabdus* as well as the *Photorhabdus* isolates (Rainey *et al.*, 1995). A PCR-based RFLP technique for rapid screening of large number of symbiotic bacteria isolates was employed by Brunel *et al.* (1997). They used the PCR amplification of the gene region coding for 16S rRNA (16S rDNA), and then digested by restriction endonucleases. Although the RAPD-PCR technique could be used to distinguish between symbiotic bacteria isolates, it has never been employed to study the detailed genetic diversity among *Xenorhabdus* as well as *Photorhabdus* isolates. This is probably because of the availability of more accurate and reproducible molecular techniques such as PCR-RFLP and sequence homology.

1.10 Objectives of the study

1. Isolation of novel strains of EPNs from Palestine's soil.
2. Isolation and culture of EPNs' symbiotic bacteria
3. Determination of heat tolerance and infectivity of the isolated EPN strains.
4. RAPD-PCR characterization of the isolated EPN strains.
5. RAPD-PCR characterization of the symbiotic bacteria of the isolated EPN strains

Chapter II

Materials and Methods

2.1 Collection of soil samples

During the period June 1997 - January 1998, a total of 78 soil samples were collected randomly from cultivated and regularly irrigated fields from various areas in the southern part of the West Bank and Gaza Strip, Palestine. The samples were from Al-Auja, north of Jerico (n=24), Hebron (n=25), Bethlehem (n=13), and Gaza (n=16). Each sample consisted of approximately 1L of soil obtained from 3-30cm depth. The material was placed in plastic pots of 13cm in height and 15cm in diameter and kept at 25°C till inspection of EPNs (Chandler *et al.*, 1997).

2.2 Rearing of *Galleria mellonella* larvae

Adults of *Galleria mellonella* (Lepidoptera: Pyralidae) were trapped in glass jar containing nutrient medium closed with metal net lined with tissue paper, where they lay eggs. Eggs were disinfected with 10% formaldehyde for one hour, followed by washing with running water for additional 1hour. The eggs were incubated at 33°C on nutrient medium containing autoclaved 200g honey mixed with 183g Glycerol, 47g Yeast Extract, and 4g of the fungicide Nipagine. The components were stirred at 50-60°C till softening, and then 320g of Wheat bran were added and mixed thoroughly. After 2-3 weeks of incubation, larvae hatched from eggs and they were allowed to develop into the last instar, which was used in all experiments.

2.3 Isolation of nematodes

Nematodes were isolated from soil samples by "baiting" with last instar larvae of the wax moth, *G. mellonella* (Bedding and Akhurst, 1975) which are highly susceptible to EPNs. Larvae (3-4) were trapped in a net, placed in each soil sample, and held at 25°C. Larvae survival was inspected every 5 days for 3 weeks. Dead, intact larvae were removed and surface-sterilized in 1.0 % Sodium hypochlorite for 3 min, then washed three times in sterile distilled water (Chandler *et al.*, 1997). Cadavers were placed on White trap (White, 1927), shown in figure 2.1, for collecting the emerging nematodes. The White trap consisted of an inverted petridish cover (60 mm diameter) placed inside larger petridish (90×15 mm). The cadavers were placed on with filter placed on the petridish cover and the outer petridish was filled with 20ml of sterile Ringer's solution (Woodring and Kaya, 1988) containing (g/L); 9.0 NaCl, 0.42 KCl, 0.48 $CaCl_2.6H_2O$, 0.20 $NaHCO_3$. After 2 weeks, the emerging nematodes were collected from the Ringer's solution, and tested for pathogenecity to *G. mellonella* using Koch's postulate. Each *G. mellonella* larvae was infected in one well (1.55cm in diameter and 1cm

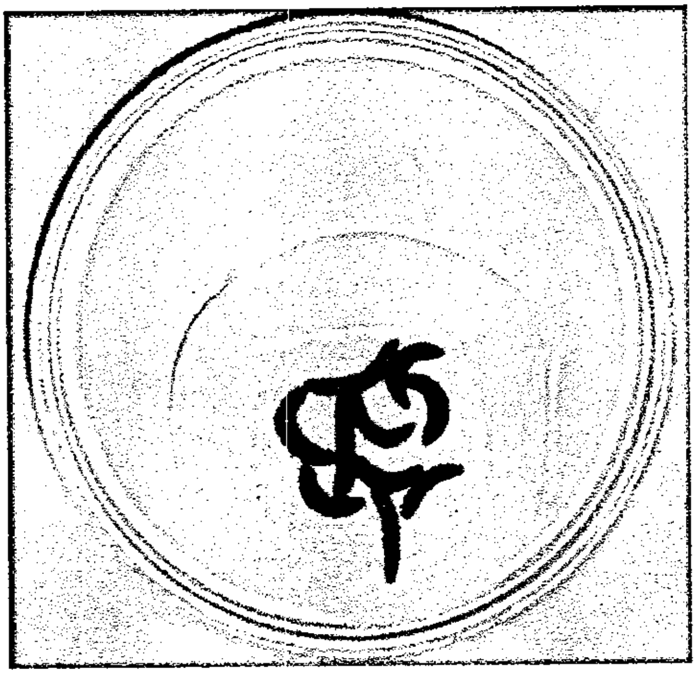

Figure 2.1: White trap method for collecting IJs from *G. mellonella* larvae cadaver.

height) of 24 multi-well plate (Costar ®, U.S.A.), filled with sterile sand wetted with 10% water. Each larva-containing well was supplemented with 100 µl Ringer's solution containing 100 IJs previously disinfected with 1% sodium hypochlorite and washed 3 times. Wells were covered with vented lid and incubated at 25°C. Recovered entomopathogenic nematodes were maintained and propagated on *G. mellonella* larvae.

2.4 Isolation of symbiotic bacteria

The symbiotic bacteria of EPNs were isolated from the haemolymph of infected *G. mellonella* larvae. Five larvae, each previously infected with about 100 infective juveniles, were used for this purpose. Immediately after larval death (48 hours), each larva was surface sterilized with 70% ethanol for two minutes, followed by a thorough wash in sterile water. Larvae were then transferred to 9 cm petridish containing NBTA medium (Akhurst, 1980). The NBTA medium contains (g/L); 37.0 Nutrient agar (Difco), 0.25 Bromothymol blue (Sigma), and 10 .0 2,3,5-Triphenyl tetrazolium chloride (Sigma), and solublized in tap water. Each larva was pricked, in the NBTA plate, with a surgical needle to release the haemolymph without rupturing the inner gut (Akhurst, 1980). A drop of the collected haemolymph was streaked on the NBTA medium. The streaked plates were incubated in the dark, at 25°C for 24 or 48 hours till the development of colonies.

Bacterial cultures were further purified by additional subculture of a single typical colony of the symbiotic bacteria on fresh NBTA plates. The pure culture, was transferred into BSA broth, and placed on a shaker at 260 rpm, in the dark at 25°C. The BSA broth contained (g/L); 10.0 Nutrient broth (Difco), 10.0 Tryptic soy broth (Difco), 5.0 Yeast extract (Difco), 5.0 Peptone (Difco), 5.0 NaCl, 0.35 KCl, 0.21$CaCl_2.2H_2O$, and 30 ml Corn oil, and solublized in tap water. Thirty six hours after incubation, the symbiotic bacteria were either used or stored at –80°C in sterile glycerol to a final concentration of 15%.

2.5 Identification of EPN strains

Distinction between nematodes of the *Heterorhabditis* and those of the *Steinernema* genera was based on differences in the appearance of the infected larva, morphology, and life cycle of the nematode. Heterorhabditid nematodes were characterized based on the orange color and the luminescence of the infected *G. mellonella* larvae. Infected larvae were investigated for luminescence by observing dead larvae in the dark for 10 minutes. Infective juveniles of *Heterorhabditis* were also examined, under the microscope (400X), for the presence of a tooth on the head. The males of *Heterorhabditis* were identified based on the observation of the bursa microscopically (400X). Furthermore, *Heterorhabditis* were examined for the presence, in the first generation, of the hermaphrodite stage, which is distinguished from the Steinernema female by its pointed tail.

Identification at the species level involved crossbreeding with other reference strains (Dix, 1994). These strains were chosen based on morphological measures of the tested IJs compared with the reference strains. The selected reference strains were *H. bacteriophora* (strain hybrid) and *H. indica* (Type strain LN2). The former was obtained from R. Gaugler, Rudgers University, New Brunswick, NJ, USA, while the latter was obtained from Easwaramoorthy, Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India. Crossbreeding experiments were conducted in 24 multi-well plates filled with agar medium containing (g/L); 12.0 agar (Difco), 10.0 trypcase soy (API BioMerieux), 5.0 yeast (Merck), 5.0 nutrient broth (Difco), 1.0 NaCl, 0.5 g/L $MgSO_4 \times 6H_2O$, 0.2 $CaCl_2$ and 0.5% sunflower oil, adjusted to pH 7.0 with NaOH. Each well contained 5-10 amphimictic females (unfertilized) for each strain combined with approximately the same number of males from other strain and incubated at 25°C. Prior to the addition of nematodes, agar medium was seeded with 10μl of a 24 hour *Photorhabdus luminescens* culture in YS broth (Dye, 1968). The YS broth consisted of (g/L); 0.5 $NH_4H_2PO_4$, 0.5 K_2HPO_4, 0.2 $MgSO_4$, 5.0 NaCl, and 5.0 yeast extract (Difco). Controls contained males and females from the same strain, and additional control cultures contained only females of the same strain. All of the crosses between the strains were conducted in reciprocal patterns. The production of offspring was recorded five days later. After successful reproduction, IJs were then harvested and fertility of the offspring was tested by infestation of healthy Galleria larvae and recording propagation. The identification at the species level was carried out in collaboration with Mr. Stefan A. Yohnick at the laboratory of Dr. Ralf Udo-Ehlers at Kiel University, Germany.

2.6 Determination of heat tolerance

Samples of about 10000 IJs from about one week old stock, were incubated in a 35 ml glass vials containing 5ml deionized water and closed with cotton to allow ventilation. Vials were held in continuous shaking (70 rpm) at 37, 38, 39, or 40°C. Prior to the addition of the IJs, the water in the vials was allowed to equilibrate with the incubation temperature. At various time intervals, aliquots of 0.5ml IJs suspension were taken from samples at each incubation temperature and transferred to a 5.5 cm diameter petridish containing 7 ml deionized water at room temperature and left overnight at this temperature before determining viability under light microscope. Four viability counts were performed for each sampling aliquot. Each count involved forty infective juveniles per field. Motility and response to probing were used as indicators for live IJs. The viability at the various incubation temperatures was determined by three independent experiments.

2.7 Determination of infectivity

Twenty last instar larvae of *G. mellonella* of 0.164g weight in average were placed each in sand-containing well of a 24 multi-well as described in section 2.3. Larvae were exposed to different concentrations of infective juveniles (2-3 weeks old) in 100µl Ringer solution. The wells were closed with a vented lid and incubated in the dark at 25°C. Mortality of larvae was recorded after 26 hours. All experiments were repeated three times independently.

2.8 Statistical analysis

The collected data from the infectivity and heat tolerance experiments were statistically analyzed. Each experiment was set up as a two factor completely randomized design. Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. The data were analyzed using MSTATC software (Michigan State University, 1988).

2.9 Preparation of nematode and bacterial cultures for DNA extraction

The nematode strains that were used in this study included two locally isolated strains, *H. indica* (LN2 strain) isolated from India, *H.*

bacteriophora (HP88 strain) isolated from USA, and *S. feltiae* (EN0008 strain), which is a hybrid resulting from crosses of several northern Europe *S. feltiae* strains. Each isolate was reared on *G. mellonella*, and emerging infective juveniles were collected (Poinar, 1979). About 5000 of the infective juveniles were surface disinfected with 1% sodium hypochlorite for 4 minutes and washed 3 times with sterile Ringer's solution. The disinfected IJs were then inoculated on Wouts agar plates (5cm diameter) (Wouts, 1981) previously inoculated with few drops of 36 hours old symbiotic bacteria culture grown in BSA medium. Wouts media contained (g/L); 16.0 Bacto Nutrient Broth (Difco), 12.0 Bacto Agar (Difco), and 5.0 Corn oil. The nematode culture was incubated at 25°C in the dark for a period of one week to allow propagation. All nematode stages were collected from the Wouts medium with ringer's solution then centrifuged at 3000 rpm for 2 min and washed 3 times with sterile ringer solution. The washed nematodes were collected in 1.5ml Eppendorf tubes, and stored at -80°C until used for DNA extraction (Sulston and Hodgkin, 1988).

For DNA extraction from symbiotic bacteria, cultures were prepared by thawing a 1ml of bacterial stock stored at -80°C and culturing it on fresh BSA liquid medium. The culture was held on rotary shaker at 260 rpm in the dark at 25°C. After 36 hours, one ml of the culture was transferred to a sterile Eppendorf tube and centrifuged at 15000 rpm for five minutes and supernatant was discarded. One additional ml of the culture was added and centrifuged to increase the amount of bacterial cells in the sample. The supernatant was removed and the precipitated bacterial cells were used for DNA extraction.

DNA extraction from all stages of nematodes, and the precipitated symbiotic bacteria was performed using the Dnasy^{TM} System (QIAGEN) based on silica-gel-membrane technology (Cat.No. 69106).

2.10 RAPD-PCR runs and Data analysis

The PCR conditions and reagent concentrations were applied as described by Hashmi *et al.*, 1996. Total volume for each reaction was 20µl. Each reaction mixture contained 2µl of Promega (madison, WI) 10X assay buffer (500mM KCl, 100mM Tris-HCl, and 1% Triton X-100), 1.5mM $MgCl_2$ (Promega), 0.2mM of each of the four dNTPs (Promega), 1.5µM decamer primer, 0.5 unit Taq DNA Polymerase (Promega), and approximately 50ng of extracted DNA. Primers used in this study and their sequences are shown in table 2.1. All of the primers were purchased from GIBCO BRL. Amplifications were performed in MJ Research PTC100^{TM} temperature cycler, programmed as follows: one cycle of 94°C for 1 min, 40°C for 2 min, and 72°C for 3 min, 39 cycles of 94°C for 1 min, 40°C for 1.5 min, and 72°C for 2 min., and finally

Table 2.1: Ten-mer primers that were used in RAPD-PCR reactions

Code	***Sequence 5'→3'***
A01	CAGGCCCTTC
A02	TGCGGAGCTG
C01	TTCGAGCCAG
C02	GTGAGGCGTC
C09	CTCACCGTCC
S16	AGGGGGTTCC
H19	CTGACCAGCC

followed by 72°C for 10 min. The PCR products were electrophoresed in 3% w/v agarose gel (amresco ®) in 1X TBE buffer at 100 volt for 2 hours; 5X concentrated stock solution of TBE contains: 54.0g/L Tris base, 27.5g/L boric acid, and 20.0ml/L 0.5M EDTA, pH 8.0 (Sambrook *et al*, 1989). DNA fragments were stained with ethidium bromide, and photographed using Polaroid camera. RAPD reactions were repeated three times to assure reproducibility of DNA profiles.

The coefficient of similarity (F) between the generated DNA patterns generated was determined in a pair-wise comparison according to Nei and Li (1979) method. The F value was calculated using the formula ($F = 2\ C_{xy} / (T_x + T_y)$, where C_{xy} is the number of bands shared by both strains, and T_x and T_y are the total number of fragments produced by strain "*X*" and "*Y*", respectively. Calculated F values were averaged and then used for cluster analysis. Cluster analysis was performed using the unweight pair-group method algorithm (UPGMA) (Sneath and Sokal, 1973), and dendrograms (tree diagrams) were plotted.

Chapter III

Results and Discussion

3.1 Isolation and identification of EPNs

Of the total 78 soil samples tested, only two contained EPNs. One of them was collected from Battir and the other from Artas. Both locations are in Bethlehem district (Figure 3.1). The EPN strain isolated from Battir area was designated as Bethlehem11, and the one isolated from Artas was designated as Bethlehem22. Because of morphological similarity between EPNs and some saprophytic nematodes, there was a need to ascertain that the isolated nematodes are entomopathogenic and not saprophytic. This is usually done by conducting the Koch's postulate. In our case, the Koch's test was performed with the isolated EPNs on *G. mellonella* larvae including disinfecting the IJs before using them in the test. The disinfection with sodium hypochlorite kills the saprophytic nematodes but not the entomopathogenic ones due to the presence of thick cuticle coating their body. The fact that the IJs continued to kill the *G. mellonella* larvae and recover inside it suggested that these nematodes are entomopathogenic. This conclusion was supported by the typical orange color of the infected *G. mellonella* larvae (Figure 3.2) and the luminescence (Figure 3.3) that was observed in the dark. This light emission is typical to the *Photorhabdus* symbiotic bacteria associated

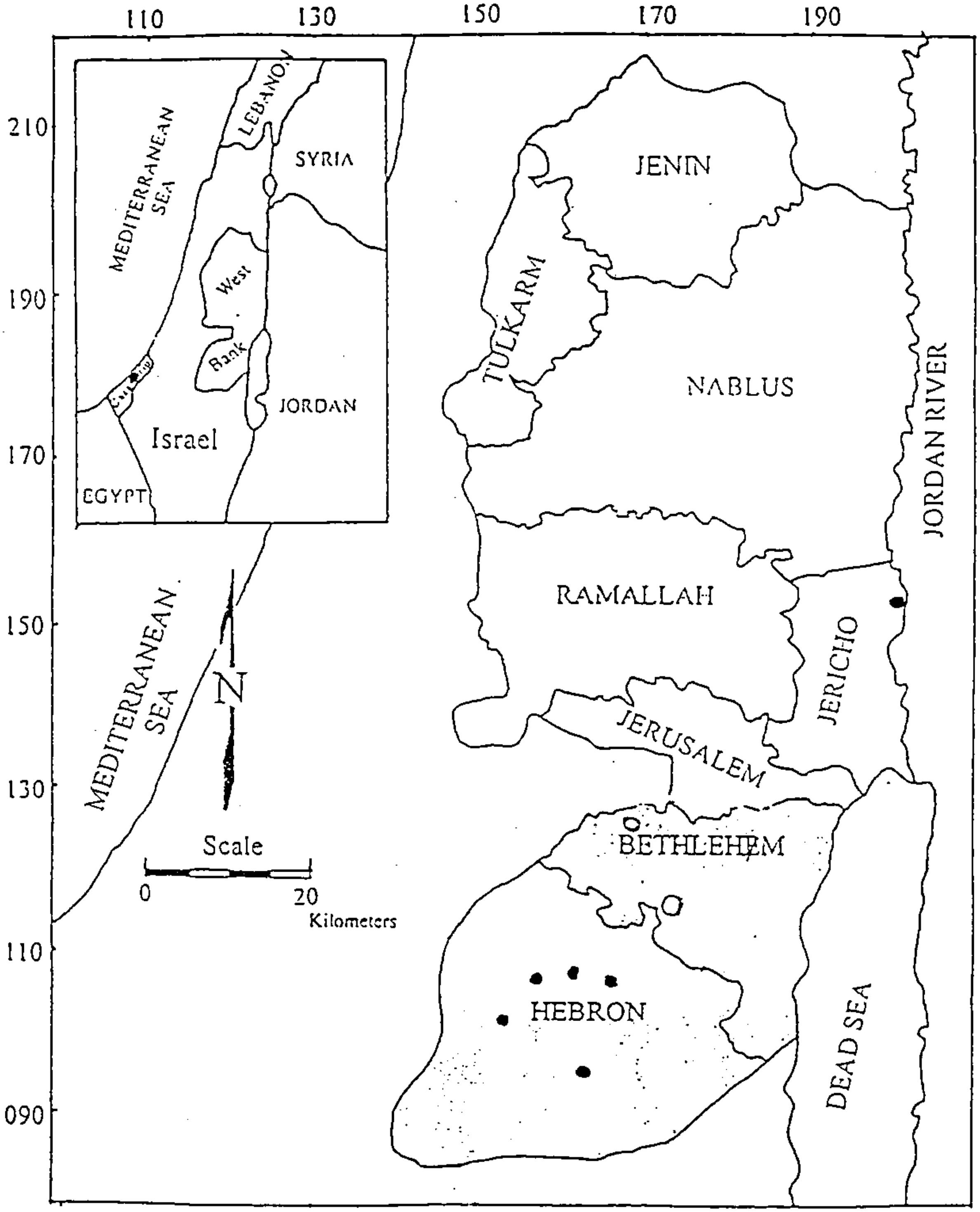

Figure 3.1: West Bank map showing location of soil sampling (dark dots). White circles indicate the locations from which entomopathogenic nematodes were isolated.

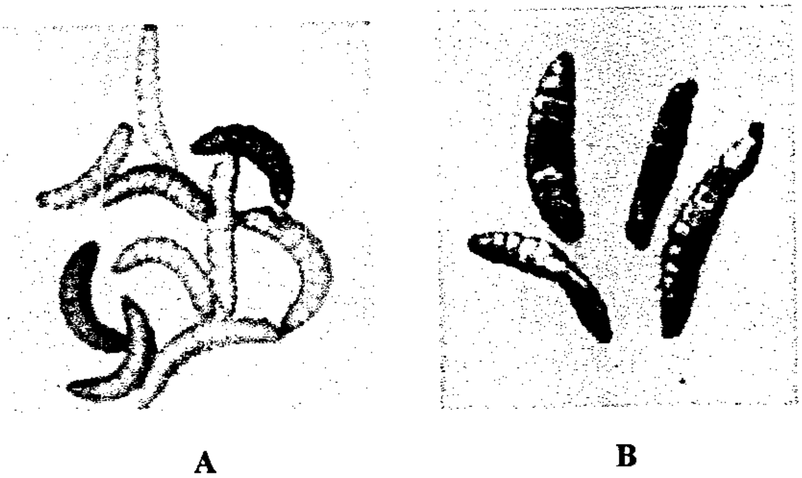

Figure 3.2: *G. mellonella* cadavers with orange color, which appeared after larvae infection with entomopathogenic nematodes, (A) Bethlehem11 strain, and (B) Bethlehem22.

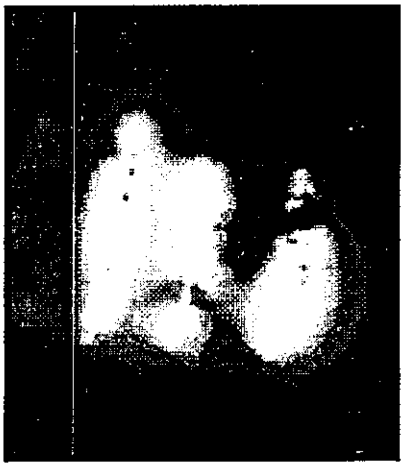

Figure 3.3: Luminescent *G. mellonella* larvae infected with the EPN, Bethlehem11 strain. The luminescence was also observed in *G. mellonella* larvae infected with Bethlehem22 strain.

with the genus *Heterorhabditis* and not to the *Xenorhabdus* bacteria associated with the steinernematids (Boemare *et al.*, 1993).

Further steps were conducted for the identification of the isolated EPN strains at the genus level. Microscopic inspection of the IJs of the two strains showed the presence of a tooth on the head, which is typical for the IJs of the *Heterorhabditis* genus (Figure3.4A). Further microscopic observation showed that males of the two strains possess a bursa (Figure 3.4C) which is typical for *Heterorhabditis* males. In both Bethlehem strains the hermaphrodite stage with its pointed tail, was observed in the first generation of their life cycle inside the *G. mellonella* larvae (Figure 3.5). This stage is found only in members of the *Heterorhabditis* genus and not in *Steinernema* (Poinar *et al.*, 1975).

All of the above morphological, anatomical, and physiological characteristics indicate that the newly isolated strains are of the *Heterorhabditis* genus. On the other hand, they do not indicate to which species the new strains belong.

The preliminary identification of the Bethlehem11 and Bethlehem22 strains at the species level was based on measurements of the IJs dimensions, while the final identification involved crosses with reference strains. The range of lengths of the IJs (n=25) of both new strains was 515-523 m. The mean length, on the other hand, was 519μm ± 1.88 for Bethlehem11 and 519 μ m ± 2.19 for Bethlehem22.

Both ranges were in agreement with the measures of the IJs of *H. indica* as reported by Poinar *et al.* (1992). They measured a length range of 479-573 μ m with a mean of 528 μ m. Furthermore, the range of the IJs length of the Bethlehem strains was within the range of IJs length in *H. bacteriophora*, 512-671 μ m, (Poinar *et al.*, 1975). Based on these findings, each of the Bethlehem strains was crossed with *H. indica* (strain LN2) and *H. bacteriophora* (strain hybrid). The results (Table 3.1) show 66% successful crosses of each of the Bethlehem strains with *H. indica* (strain LN2). A greater percent of successful crosses was obtained when the two Bethlehem strains were crossed with each other (83%). The progeny from all successful crosses were tested for fertility, and all of them were fertile. On the other hand, the crosses with *H. bacteriophora* gave no progeny. Based on the mean length of the IJs and the results of the crosses, we concluded that Bethlehem11 and Bethelehem22 strains are *H. indica*.

The absence of EPNs in the remaining soil samples, collected from Hebron and Gaza (Table 3.2), might be due to the time of collection. EPNs may be found at different depths in soil depending on the moisture and the presence of host insects. These two parameters vary along the year. For example, soil sampling is more suitable in spring because the soil is moister than that of summer, and the soil-borne insects are more

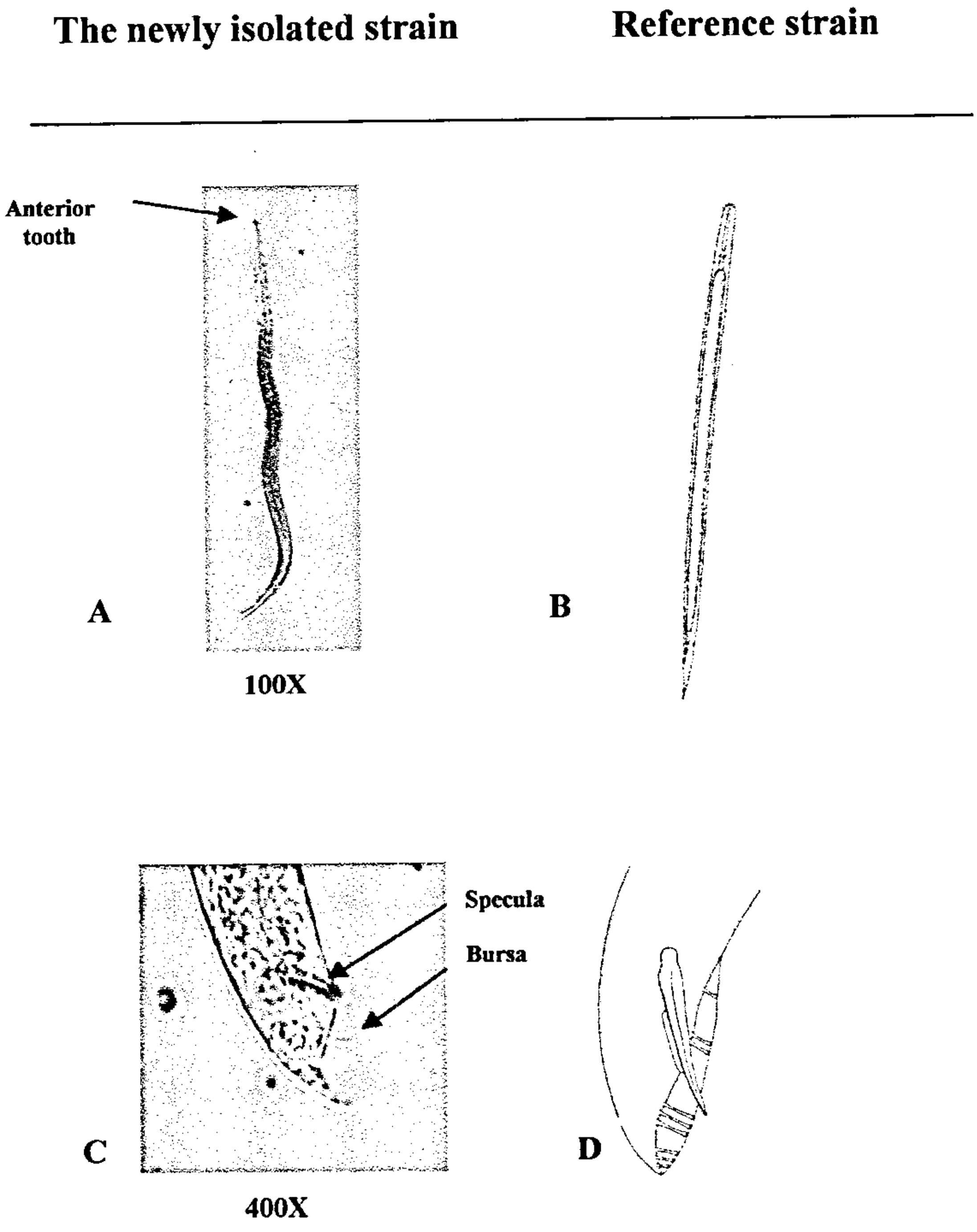

Figure 3.4: A: Infective juvenile of the newly isolated Bethlehem strain; the tooth appears on the anterior region. B: Reference infective juvenile strain (*Heterorhabditis* spp.) C: Male tail; specula and bursa structures. D: Reference male tail (*Heterorhabditis* spp.). The reference Photographs are courtesy of Liu and Berry (1996b). The same sturctures in A and C were observed in Bethelehem22 strain.

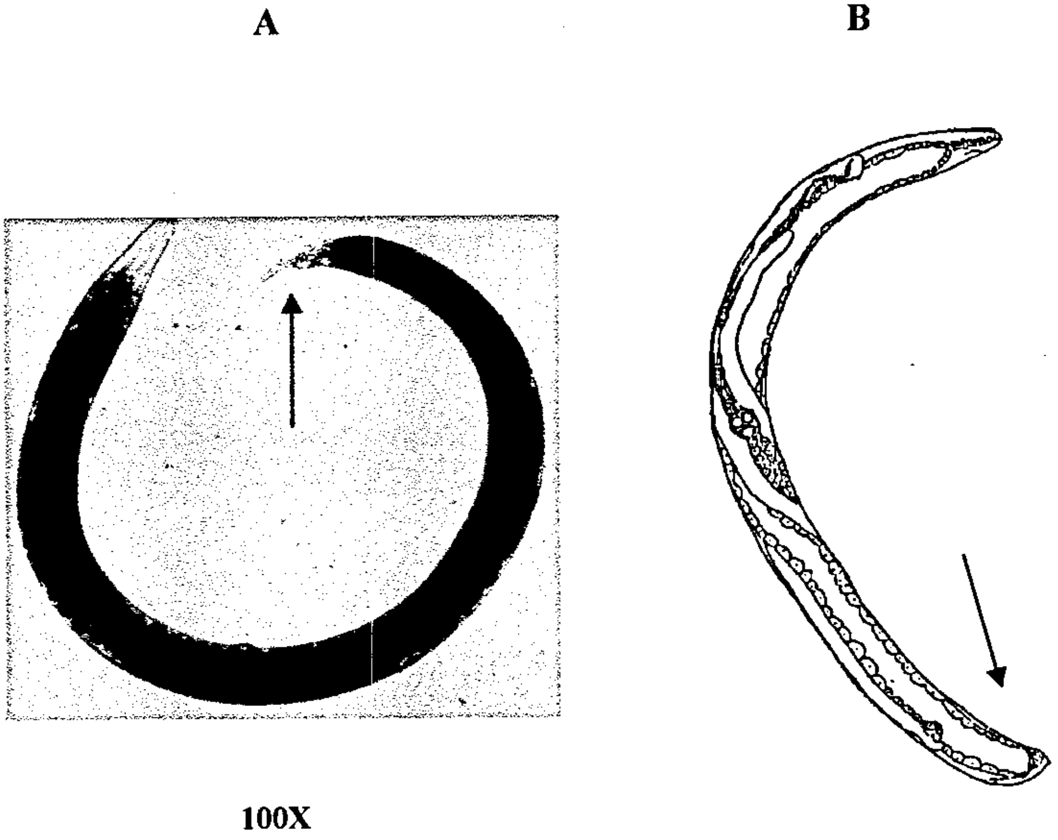

Figure 3.5: (A) hermaphrodite of the first generation from Bethlehem11 strain, which was isolated from infected *G. mellonella* larva. The arrow indicates the pointed tail, which is typical for *Heterorhabditis*. The tail morphology in (A) was observed in Bethlehem22 strain as well. (B) Steinernematid female (photograph is courtesy of Tallosi et al., 1995). The arrow indicates the rounded tail, which is typical for steinernematids

Table 3.1: Results of crosses of Bethlehem 11 and Bethlehem 22 strains with *Heterorhabditis bacteriophora* (Hybrid strain), and *Heterorhabditis indica* (LN2 strain), all progeny were tested for fertility and all of them were fertile.

Females	**Males**	**No. of plates with offspring (F1)**
H. bacteriophora	Bethlehem11	0 of 3
H. bacteriophora	Bethlehem22	0 of 3
H. bacteriophora	none (control)	0 3
H. indica	Bethlehem11	2 of 3
H. indica	Bethlehem22	2 of 3
H. indica	none (control)	0 of 4
H. indica	*H. indica*	1 of 1
Bethlehem11	*H. bacteriophora*	0 of 2
Bethlehem11	Bethlehem22	2 of 3
Bethlehem11	*H. indica*	2 of 3
Bethlehem11	none (control)	0 of 4
Bethlehem22	*H. bacteriophora*	0 of 2
Bethlehem22	Bethlehem11	3 of 3
Bethlehem22	*H. indica*	2 of 3
Bethlehem22	none (control)	1 of 4

Table 3.2: Occurrence of EPNs in soil samples collected from different areas in Palestine.

Area	**# of sites**	**# of samples**	**Occurrence of EPNs**	**Date**
Al Auja (Jordan Valley)	5	24	(-)	June 97
Hebron	5	25	(-)	October97
Gaza		16	(-)	January 98
Battir	1	6	(+)	November 97
Artas (Bethlehem district)	1	7	(+)	November 97

abundant. On the other hand, soil sampling may also be carried out in summer if the soil is regularly irrigated. This is due to the activity of insects in such soils where host crops are growing.

This study is the first attempt to isolate EPNs from Palestine. The two *H. indica* strains, which we isolated are the first *H. indica* strains that were isolated in this region (Palestine and Israel). In 1991 Galzer *et al.* reported the isolation of EPNs from the Negav region in Israel, which were later identified based on RFLP as *H. bacteriophora* (Glazer *et al.*, 1993). In 1996, one of the isolates (IS-5) was further characterized based on morphological measurements of the adults and IJs. These measurements did not distinguish the IS-5 isolate from the *H. bacteriophora* species (Glazer *et al.*, 1996). In 1998, Hashmi *et al.*, suggested that the IS-5 strain is an *H. indica* based on RAPD-PCR analysis. They found 89% genetic similarity between the IS-5 strain and another *H. indica* strain.

The interpretation of the RFLP and RAPD results may lead to the identification of EPNs (Hominick *et al.*, 1996). However, these methods may not separate between species in many cases. This is because of the possible existence of low variations among species and high variations among strains of the same species. For example, Hashmi *et al.* (1998) found 95% genetic similarity between two EPN species; *H. hepialius* and *H. marelatus.* On the other hand, low intra-species similarity was reported (Hashmi *et al.*, 1996), where 46.3% genetic similarity was observed between two *H. bacteriophora* strains (HP88 and IH273).

Although there are difficulties in conducting crosses among *Heterorhabditis* species due to the presence of hermaphrodites, crossing remains the best method for identification at the species level. In 1997, Shapiro *et al.* did genetic improvement of heat tolerance in *H. bacteriophora* through hybridization. The fact that they used the IS-5 strain for crossbreeding experiments with another *H. bacteriophora* strain (HP88), where fertile offspring were produced, confirms the identity of the IS-5 strain as *H. bacteriophora* and not as *H. indica* as was suggested by Hashmi et al. (1998).

Heterorhabditis indica is widely distributed in the tropics. It was first described from nematode populations recovered from the sugarcane top borer, *Scirpophaga excerptalis* (Pyralidae:Lepidoptera) in India (Poinar *et al.*, 1992). In the Guadeloupe islands, *H. indica* was found to be the dominant species. Out of 31 *Heterorhabditis* isolates, 27 where identified as *H. indica*, and three as *H. bacteriophora* (Le Saux *et al.*, 1999). Moreover, *H. indica* was found to be present in Srilanka, Japan, and Northern Australia (Hominick *et al.*, 1996).

Since EPNs exhibit host specificity, and because of the need for improving their infectivity and tolerance to environmental conditions it is necessary to enlarge the number of available species and strains. The climatic conditions in Palestine are widely diverse, and the probability of isolating EPN strains with desired characteristic such as tolerance to extreme environmental conditions (e.g, heat and desiccation tolerance), is relatively high. Therefore, we recommend of further isolation of EPN strains from Palestine to increase the collection from which one can select for an appropriate strain to control a given target insect with high efficacy.

3.2 Determination of heat tolerance

The local *H. indica,* Bethlehem11 and Bethlehem22 strains were significantly more heat tolerant at 37°C, 38°C, 39°C, and 40°C than the *H. bacteriophora* strain tested (See appendix for analysis of variance, ANOVA, tables). IJs of the Bethlehem strains showed about 100% survival compared to about 90% survival of IJs of *H. bacteriophora* strain when incubated for 6 hours at 37°C (Figure 3.6a). At 38°C, more than 90% of IJs of Bethlehem11 and Bethlehem22 strains survived, compared to about 70% survived of the IJs of *H. bacteriophora* when incubated at the same conditions (Figure 3.6b). Moreover, when strains were incubated at 39°C for 4 hours, Bethlehem11 and Bethlehem22 IJs showed 93% survival compared to 58% survival of *H. bacteriophora* (Figure 3.6c). More dramatic differences was observed between the tolerance of the strains at 40°C for 4 hours of incubation, the survival of the local strains was 80%, while that of the *H. bacteriophora* strain approached zero (Figure 3.6d). This indicates that Bethlehem11 and Bethlehem22 are more tolerant to elevated temperatures than the *H. bacteriophora* strain, which is a hybrid strain of different *H. bacteriophora* originating from temperate climatic regions. This tolerance of the local strains might be attributed to an evolutionary adaptation to the local climatic conditions of the Middle East compared to other nematodes from temperate regions.

EPNs show different tolerance to low and high temperatures as they are isolated from different geographic origins. Griffin and Downes (1991) found that several Heterorhabditid strains isolated from Ireland were active at low temperature. On the other hand, Glazer et al (1996) examined the tolerance to elevated temperatures (>30°C) for two Heterorhabditid strains; HP88, isolated from Logan, Utah, USA (Poinar and Georgis, 1990), and IS-5, isolated from the Negev region of Israel. They found that the incubation of IJs for one hour at 40°C killed all the IJs of the HP88 strain compared to 74% death of the IS-5 strain. They suggested that this may be related to the fact that IS-5 was isolated from a semi-arid climate with summer average soil temperatures between 32°C and 35°C (Glazer *et al.*, 1991).

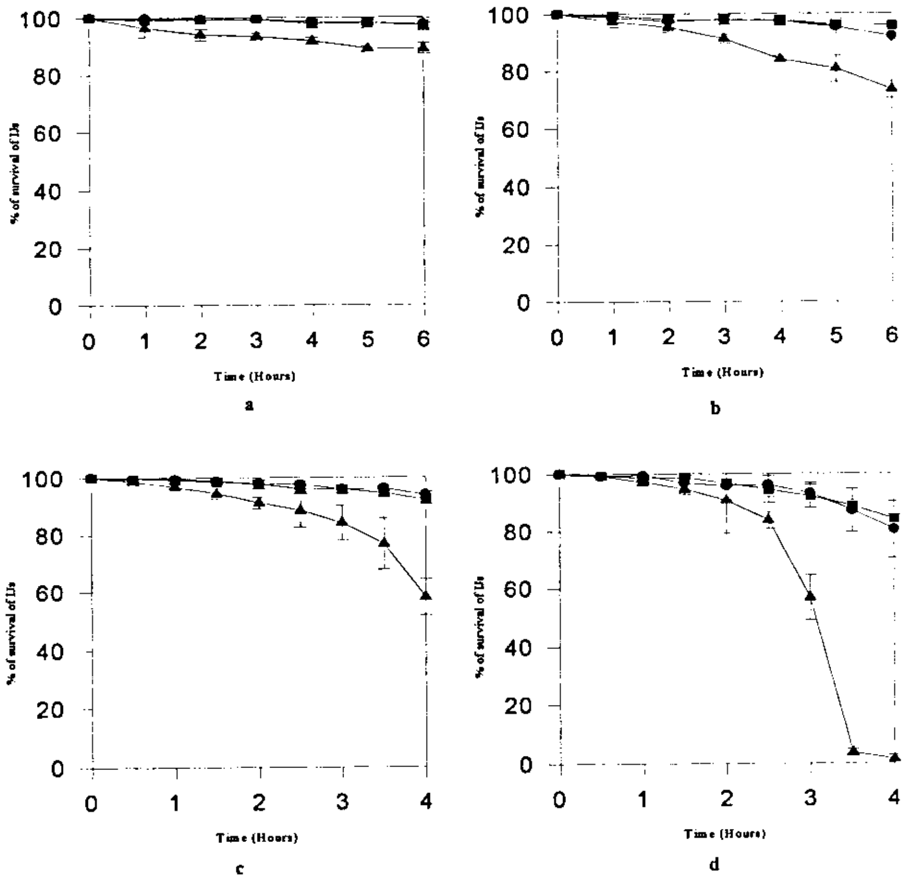

Figure 3.6: Survival (%) of infective juveniles of Bethlehem11 (—•—), Bethlehem22 (—■—), and Hybrid (—5—) strains at 37°C (a), 38°C (b), 39°C (c), and 40°C (d). n=3. Bars indicate ± SD. Means were separated by LSD at 0.05 level of probability (See appendix)

Some researchers suggest that the tolerance of EPNs is correlated with the production of heat shock proteins (*hsps*) encoded by heat shock genes (e.g., Selvan *et al.*, 1996). Heat shock proteins are a set of proteins (especially, the 70-kD family), which help organisms to survive at temperature above their normal growth temperature. For example, heat shock pretreatment for 2 hours at 36°C increased the heat tolerance of the IJs of the IS-5 strain at 40°C when incubated for one hour. The survival of the IJs increased from 26% (without pretreatment) to 43% after pretreatment (Glazer *et al.*, 1996). Variations in thermotolerance among EPNs could be correlated in part with the polymorphism in the heat shock protein gene (*hsp*70) as was reported by Hashmi *et al.* (1997). Difference in heat tolerance may also be attributed to the differences in the expression of the *hsp*70 gene. Because of the relatively high tolerance of the local Bethlehem11 and Bethlehem22 strains to elevated temperature, studies on their *hsp70* gene in relation to other EPN strains should be examined and evaluated.

Temperature affects nematode efficacy when they are applied in the field. For example, it may affect their infectivity and persistence. Bethlehem11 and Bethlehem22 may be applied as biocontrol agents in warm environment, where the activity of commercial nematode strain is very low. The local strains may also be used for hybridization with other heterorhabditid strains to transfer their beneficial trait.

3.3 Determination of infectivity

The newly isolated Bethlehem strains showed an elevated infectivity compared to the *H. bacteriophora* strain against *G. mellonella* larvae (Figure 3.7). Of the two local strains, Bethlehem11 was significantly more infective than the other one. While for the H. *bacteriophora* strain, 400 IJs were required for causing 100% mortality after 26h of incubation, only a 100 and 140 IJs of Bethlehem11 and Bethlehem22 respectively were needed to cause the same mortality at the end of the same incubation period.

The values of the lethal dose needed to kill 50% of the population (LD_{50}) were calculated for each strain from the data in figure 3.7. The LD_{50} of Bethlehem11 was one sixth of that of *H. bacteriophora,* while the LD_{50} of Bethlehem22 was one third of that of *H. bacteriophora* (Table 3.3). Indicating that the infectivity of Bethlehm11 and Bethlehem22 strains is much greater than that of the *H. bacteriophora* strain. Whether this more virulence against *G. mellonella* larvae is related to the high ability of penetration of Bethlehem strains or whether it is due to the rapid proliferation of their symbiotic bacteria, when compared to the *H. bacteriophora* strain cannot yet be concluded and needs further investigation.

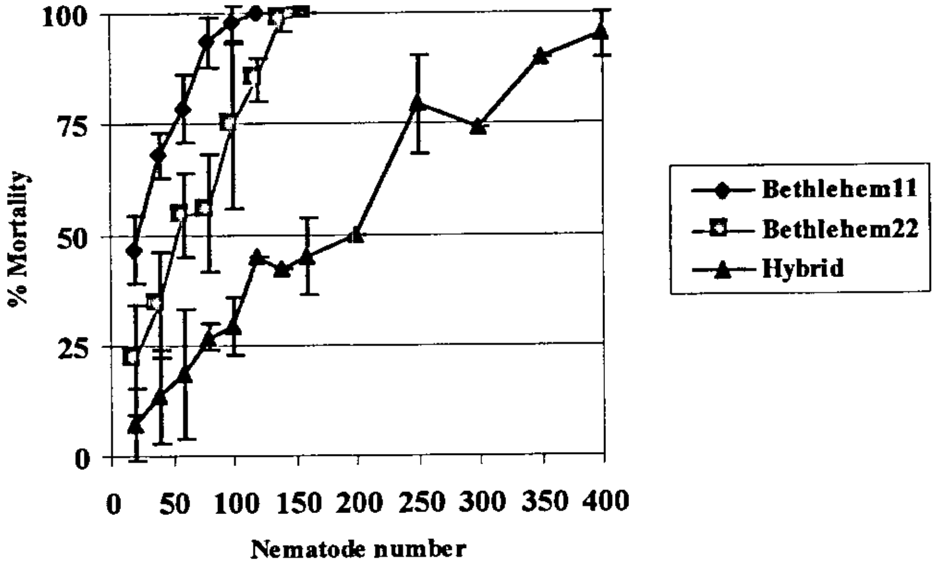

Figure 3.7: Percent of mortality of *G. mellonella* infected with different concentrations of IJs from *H. indica*; Bethlehem11and Bethlehem22 strains, and *H. bacteriophora*; Hybrid strain. Twenty last instar larvae of *G. mellonella* were infected with various concentrations of IJs and incubated in wet sand wells at 25°C. Mortality was recorded after 26 hours of incubation. LSD=10.98 at 0.05 level of probability.

Table 3.3: LD_{50} values of Bethlehem11, Bethlehem22, and Hybrid as calculated from data in figure 3.7.

STRAIN	LD_{50} VALUE
Bethlehem11	25
Bethlehem22	55
Hybrid	160

Variations were found in the pathogenecity of different EPN strains to specific insects (e.g, de Doucet *et al.*, 1999). These differences might be measured by comparing the LD_{50} values of different strains. For example, de Doucet *et al.*, (1999) found that the *H. bacteriophora* (Rio Negro) was a virulent isolate against the *G. mellonella* larvae, its LD_{50} value was one third of that of *S. feltiae* (Filopjev). On the other hand, Glazer (1991) compared the LD_{50} values of different strains against *G. mellonella* larvae as well as against different insects (e.g., *Spodoptera littoralis* and *Heliothis armigera*). The LD_{50} value obtained against the *G. mellonella* larvae of *H. bacteriophora* HP88 strain was approximately four folds of that of *Heterorhabditis* spp. IS strain. Also, the infectivity of the same strains was tested on the insect *Spodoptera littoralis*, the LD_{50} value of the HP88 strain was ten folds of that of IS strain. In both cases, the LD_{50} values indicate the poor infectivity of the former strain against both insects compared to the IS strain. Although, in our study, promising results of higher infectivity of Bethlehem strains were obtained, further experiments on the infectivity of Bethlehem strains against a specific target insect (e.g. *Maladera matrida*) have to be conducted in order to tests their specificity and virulence against local insects.

In Palestine, soil–borne insects cause a remarkable damage to several crops especially in Gaza strip where intensive irrigated agricultural regimes are employed. For example, the larva stage of the insect *Maladera matrida,* causes a great damage to summer crops, such as sweet potato (*Ipomoea batatas*), potatoes, and carrots. The only mean currently available for controlling this insect is the use of methyl bromide, which is harmful not only to the beneficial microbial population in soil but also to the environment. Glazer and Gol'berg (1989 and 1993) reported promising control results against these grubs using *Heterorhabditis bacteriophora* (strain from North Carolina, USA) in the laboratory and field trials. However, three consecutive applications were necessary to reach results that are comparable to the chemical control. One of the advantages of the application of EPNs is their potential to establish and survive in the soil. This persistence will provide long term effect on the pest population (e.g., Ehlers and Peters, 1998). However, the persistence of nematodes may be affected by extreme conditions such as high temperatures and desiccation, which are prevalent in the Middle East. For example, the failure to control the *M. matrida* after a single application of *H. bacteriophora* (Glazer and Gol'berg, 1989 and 1993) might be attributed to its temperate origin. This is probably because it is less adapted to encounter the climatic conditions in the Middle East region, and to less specific to control local insects. Therefore, local EPNs might be better adapted to control local pests. Further work on testing the Bethlehem strains in the field against *M. matrida* should be carried out. This will answer whether they will be able to establish and persist in the soil successfully. Long-term persistence will provide sustainable pest suppression, which will be of a great value in respect to crop productivity and environmental safety.

3.4 RAPD-PCR analysis of EPNs

RAPD-PCR was used in this study to determine the genetic relatedness of two *H. indica* strains (Bethlehem11, and Bethlehem22) to one another and their relatedness to other reference EPN strains. The seven ten-mer primers used in this study were able to discriminate between all strains at least by a single band difference. Representative banding patterns are shown in figure 3.8. Percent of similarity was calculated based on common DNA fragments detected per primer and the results were averaged (Table 3.4). DNA banding patterns from *H. indica* Bethlehem11 and Bethlehem22 strains were at 96.42% similarity. The similarity of Bethlehem11 to *H. indica* strain LN2 isolated from India was 59.44% while that of Bethlehem22 was 57.78% (an average of 58.66%). The similarity of each of the Bethlehem strains to *H. bacteriophora* strain HP88 isolated from USA was 61.32% for Bethelehem11 and 59.78% for Bethelehem22 (an average of 60%). Similarity of 79.16% was observed between LN2 and HP88 strains. On the other hand, when all of the *Heterorhabditis* strains used in this study (Bethlehem11, Bethlehem22, LN2, and HP88) were compared

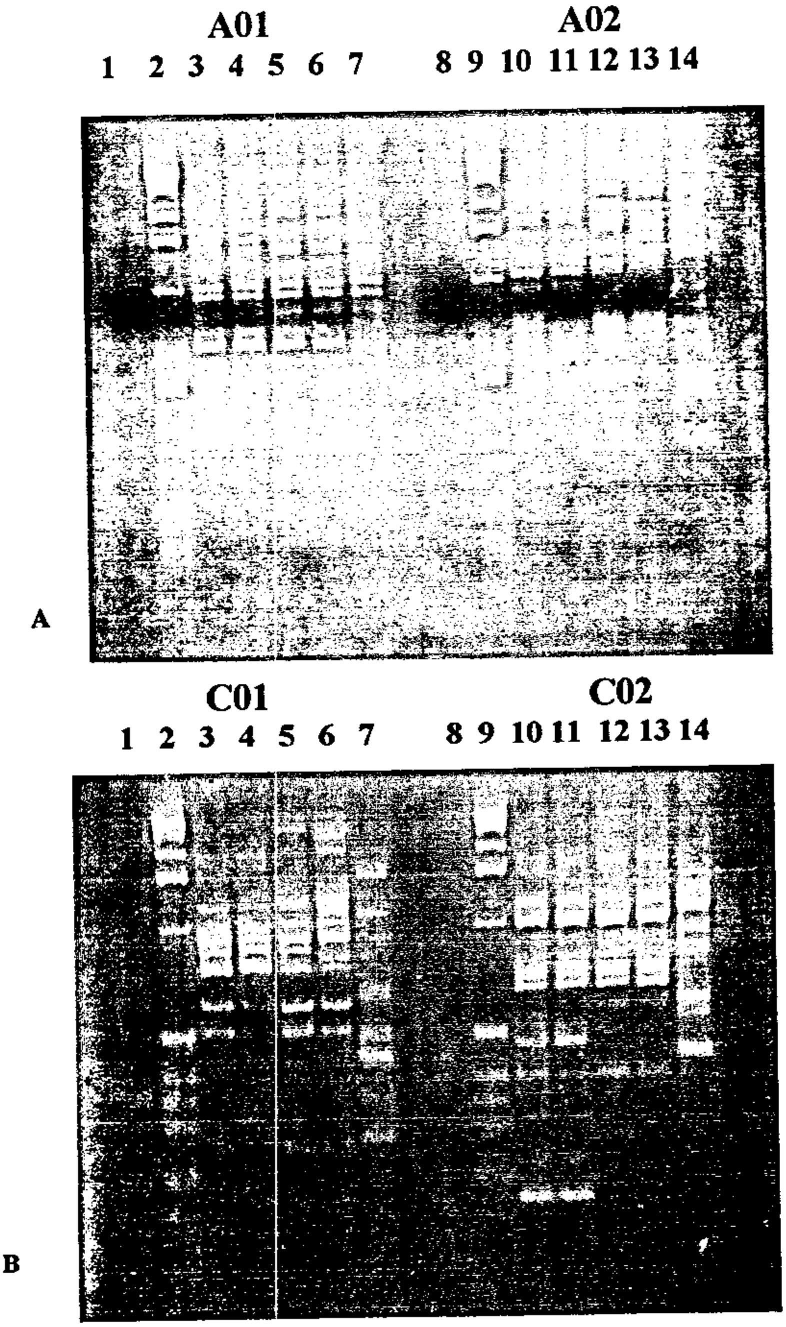

Figure 3.8: RAPD amplifications of five different EPN strains using four primers (A: A01 and A02. B: C01 and C02), where lanes 1 and 8 are control (No DNA); lanes 2 and 9: 1 Kb DNA ladder (GIBCO BRL); lanes 3 and 10: *H. indica* Bethlehem11; lanes 4 and 11: *H. indica* Bethlehem22; lanes 5 and 12: *H. indica* LN2; lanes 6 and 13: *H. bacteriophora* HP88; lanes 7 and 14: *S. feltiae* EN0008.

Table 3.4: Average percent similarity among different entomopathogenic nematode strains analyzed with seven primers.

Strain	Bethlehem11	Bethlehem22	LN2	HP88	EN0008
Bethlehem11	100				
Bethlehem22	96.42	100			
LN2	59.44	57.88	100		
HP88	61.32	59.78	79.16	100	
EN0008	6.06	5.99	5.19	6.25	100

with the *S. feltiae* (strain EN0008), very low similarity was observed which ranged from 5.19% to 6.25%, an average of 5.9% (Table 3.4).

The cluster analysis, using the unweight pair-group method algorithm (UPGMA) analysis of the five EPN strains (shown in figure 3.9) resulted in dividing the tested strains into two major clusters at the highest of divergence. The first *Heterorhabditis* spp. major cluster included two subclusters; one of Bethlehem11 and Bethlehem22 strains, and the other of LN2 and HP88 strains. The second major cluster included the species *feltiae* from the genus *Steinernema*.

In RAPD analysis, the proportion of shared DNA fragments between two genotypes is ought to be correlated with the degree of genetic relatedness between them (Hashmi *et al.*, 1996; Hashmi and Gaugler, 1998; Shapiro *et al.*, 1997). In our study, the RAPD analysis revealed very low similarity between the two genera; *Heterorhabditis* and *Steinernema* (an average of 5.9%), as expected from the fact of their being different genera. Similar results were reported by Hashmi *et al.* (1996), where similarity of 0-6% was observed between these two genera.

When the intra-species variation was examined, high and intermediate levels of similarity were observed. A high degree of similarity was observed between the two *H. indica* strains, Bethlehem11

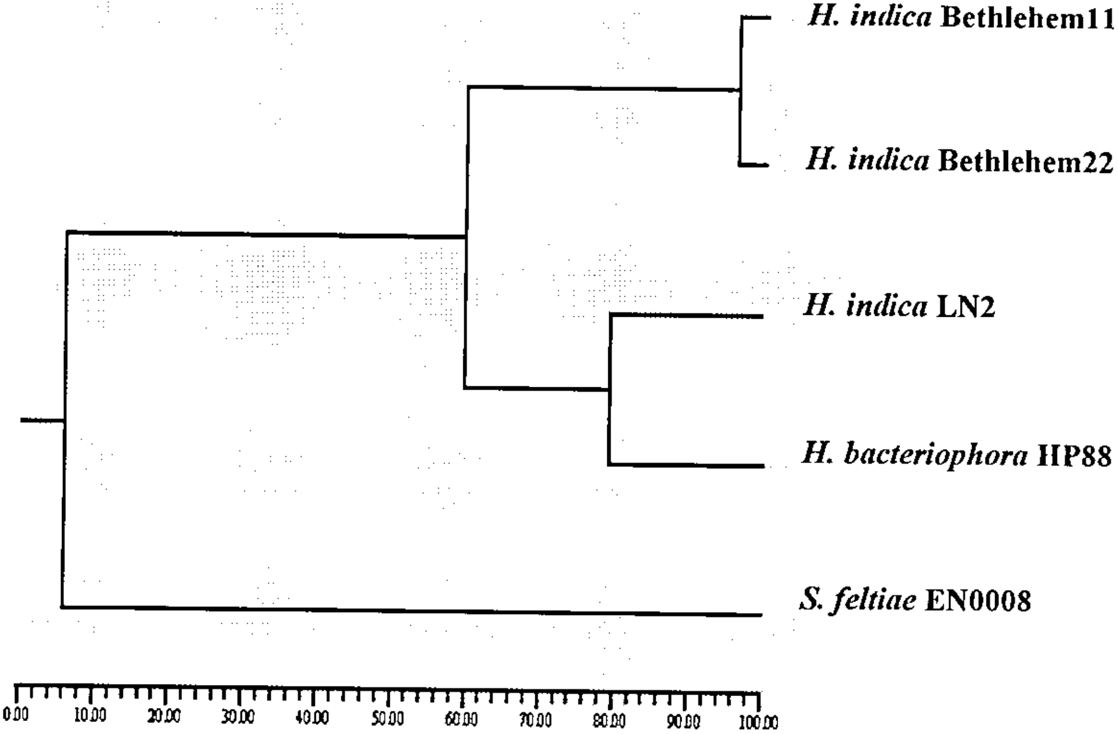

Figure 3.9. UPGMA dendrogram showing the average genetic similarity among different EPN strains based on polymorphisms generated by RAPD-PCR. Similarity matrix was generated on the basis of shared DNA fragments as described in materials and methods chapter. Average linkage cluster analysis was performed on the values of F.

and Bethlehem22 (96.42%), which indicates that both strains are genetically close. The same level of similarity (96%) between two strains of *H. bacteriophora* (HP88 and HB1) was reported by Hashmi *et al.* (1996). This close genetic relatedness between the Bethlehem strains might be correlated with a very similar heat tolerance of both strains as was observed in heat tolerance experiments. Furthermore, crosses between the two strains were at 83 % of success, which are supported with the RAPD analysis.

On the other hand, an intermediate intra-species similarity between the Bethlehem strains and the *H. indica* (strain LN2) was observed (58.66%). Similar intraspecies variation (46.3%) was documented by Hashmi *et al.* (1996) when they compared two *H. bacteriophora* strains (HP88 and IH273). The relatively low similarity between the Bethlehem strains and LN2 is also reflected in a lower percentage (66%) of successful crosses between them.

Investigating the inter-species variation between the *H. indica* (Bethlehem11, Bethlehem22, and LN2) and the *H. bacteriophora* (HP88) strains showed an average of 66.75% similarity as calculated from table2. This similarity is higher than the interspecies similarity (22.2%) reported by Hashmi & Gaugler (1998). This difference could be explained by the fact that they used different strains of *H. indica* as well as different primers.

In summary, the data of RAPD-PCR analysis showed intermediate similarities between species (66.75%), and intermediate to high levels of similarities among strains of the same species (58.66-96.42%). These inter and intra-species similarities between the tested EPNs may be due to the taxonomic relationship and the geographical distribution of these nematodes. The information that has been obtained from RAPD analysis could also be used for the rapid characterization and studying the genetic variability of EPNs.

3.5 RAPD-PCR analysis of the symbiotic bacteria

In this study, RAPD-PCR was used to characterize the *Photorhabdus luminescens*, the symbiotic bacteria of the local EPNs. Genetic differences within the local strains and other *Photorhabdus luminescens* bacteria isolated from *H. indica* (LN2), and *H. bacteriophora* (HP88), as well as *Xenorhabdus bovienii* isolated from the *S. feltiae* EN0003 strain, were studied. RAPD banding patterns generated by the seven primers used in the study were able to characterize these bacterial strains. Representative examples of RAPD-PCR products are shown in figure 3.10. Genetic similarities between the strains were calculated based on the number of shard DNA fragments generated by each primer. The results of the seven primers were averaged and a similarity matrix was produced (Table 3.5). No differences were observed between the *Photorhabdus luminescens* strains of Bethlehem

EPN strains. The DNA banding patterns of the *Photorhabdus luminescens* of Bethlehem strains showed 77.76% similarity with another *Photorhabdus luminescens* strain isolated from the *H. indica* (LN2 strain). The similarity of *Photorhabdus luminescens* isolated from the H. bacteriophora strain HP88 to other *Photorhabdus luminescens* isolated from H. indica strain was for 47.52 for Bethlehem strains and 49.33% to LN2 strain (an average of 48.12%). On the other hand, very low similarities, which ranged from 2.42% to 5.56% (an average of 4.43%), were observed when the *Photorhabdus luminescens* strains were compared with the *Xenorhabdus bovienii* isolated from the *S. feltiae* EN0008 strain.

Based on the UPGMA analysis of the symbiotic bacteria tested, a dendrogram tree was plotted (Figure 3.11). The *Xenorhabdus bovienii* and the *Photorhabdus luminescens* bacteria formed two major clusters at the highest divergence. The *Photorhabdus luminescens* strains isolated from the *H. indica* nematodes formed one distinct subcluster, while the *Photorhabdus luminescens* isolated from the *H. bacteriophora* HP88 strain formed another subcluster.

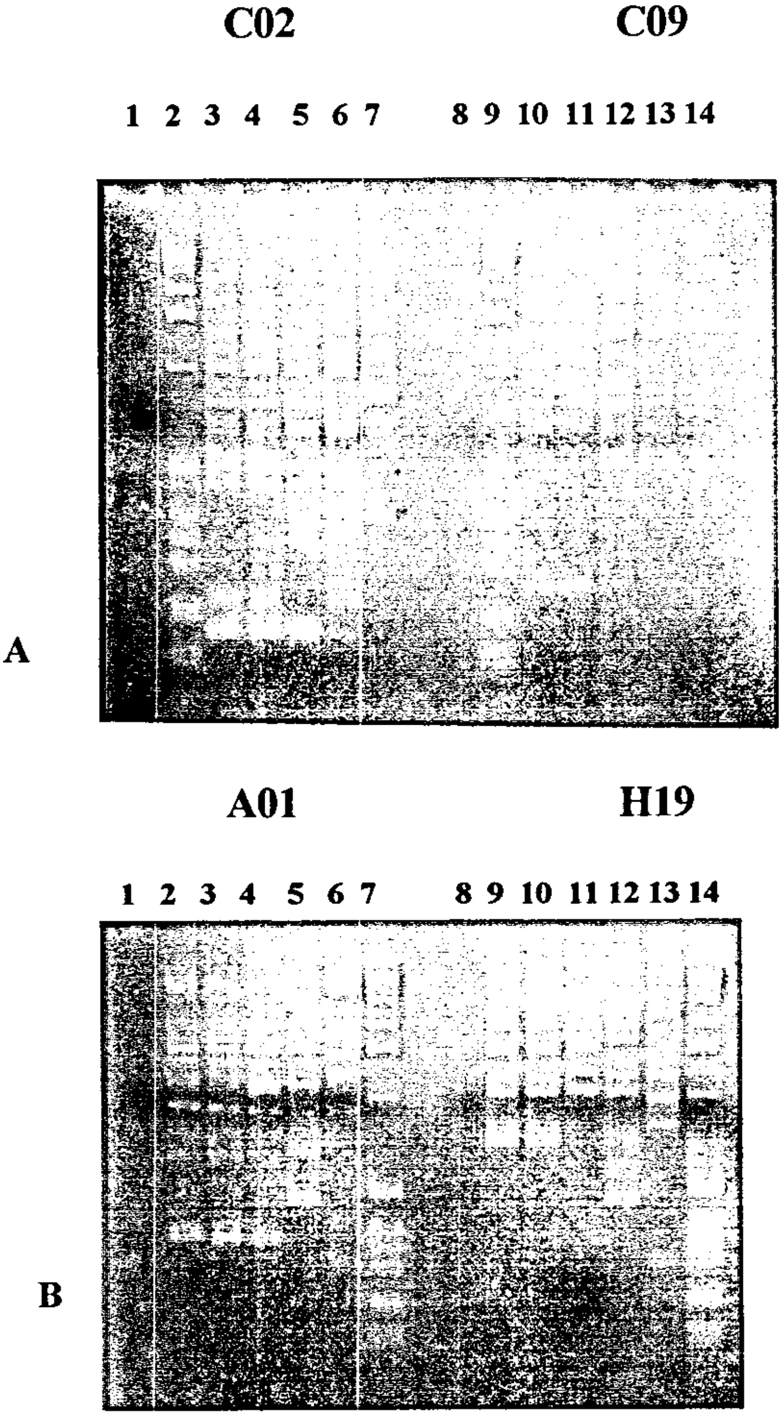

Figure 3.10: RAPD amplifications of *Photorhabdus* and *Xenorhabdus* bacteria isolated from five different EPN strains using four primers (C02, C09, A01, and H19). **A:** Lanes 1 and 8 are control (No DNA); lanes 2 and 9: 1 Kb DNA ladder (GIBCO BRL); lanes 3 and 10: *P. luminescens* isolated *H. indica* Bethlehem11; lanes 4 and 11: *P. luminescens* from *H. indica* Bethlehem22; lanes 5 and 12: *P. luminescens* from *H. indica* LN2; lanes 6 and 13: *P.luminescens* from *H. bacteriophora* HP88; lanes 7 and 14: *X. bovienii* from *S. feltiae* EN0008. **B:** Lanes 1 and 8 are control (No DNA); lanes 2 and 9: *P. luminescens* isolated *H. indica* Bethlehem11; lanes 3 and 10: *P. luminescens* from *H. indica* Bethlehem22; lanes 4 and 11: *P. luminescens* from *H. indica* LN2; lanes 5 and 12: *P.luminescens* from *H. bacteriophora* HP88; lanes 6 and 13: *X. bovienii* from *S. feltiae* EN0008; lanes 7 and 14: 1 Kb DNA ladder (GIBCO BRL).

Table 3.5: Average percent similarity among different *Xenorhabdus* and *Photorhabdus* strains isolated from different entomopathogenic nematode strains analyzed with seven primers.

Strain	Bethlehem11	Bethlehem22	LN2	HP88	*X. bovienii*
P. luminescens					
Bethlehem11	100				
Bethlehem22	100	100			
LN2	77.76	77.76	100		
HP88	47.52	47.52	49.33	100	
X. bovienii	5.56	5.56	4.2	2.42	100

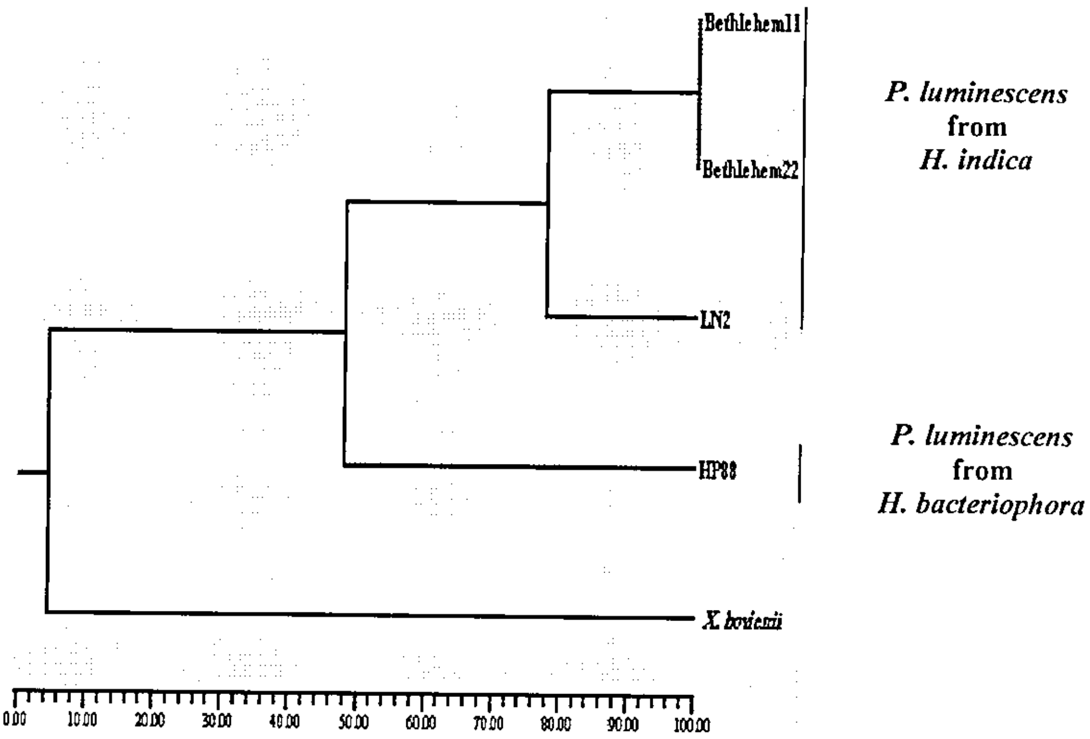

Figure 3.11. UPGMA dendrogram showing the average genetic similarity among different *Xenorhabdus* and *Photorhabdus* bacteria based on polymorphisms generated by RAPD-PCR. Similarity matrix was generated on the basis of shared DNA fragments as described in materials and methods chapter. Average linkage cluster analysis was performed on the values of F.

RAPD-PCR was able to characterize the bacterial symbionts of the entomopathogenic nematodes at the genus and species levels. Genetic relatedness between *Xenorhabdus* and *Photorhabdus* was very low (4.43%) as indicated by RAPD analysis (Table 3.5). Our findings were in accordance with the DNA/DNA hybridization results of Boemare *et al.* (1993), and the results obtained by Brunel *et al.* (1997). Both of these studies were able to show low genetic similarities between the two EPNs genera. Our genetic findings are also in agreement with the phenotypic differences between the *Xenorhabdus* and *Photorhabdus*, where the later is bioluminescence and produce catalase, and the former lack these features. On the other hand, genetic differences at the species level detected between the *Photorhabdus luminescens* bacteria isolated from the *H. indica,* Bethlehem strains might be due to the close geographic origin of their symbiont nematodes. The finding of 77.76% similarity between the symbiotic bacteria of Bethlehem strains and other symbiotic bacteria of *H. indica* LN2 strain, and the finding of 48.12% similarity between *Photorhabdus luminescens* of *H. indica* strains and *H. bacteriophora* HP88 strain, indicate intra-specific variations in the *Photorhabdus luminescens* species. These RAPD-PCR variations might be attributed to the taxonomic and geographic distribution of their nematode partners.

Two subclusters were identified within the *Photorhabdus* major cluster (Figure 3.11); these were the bacterial symbionts of *H. indica*, and *H. bacteriophora*. This indicates the existence of a close association between the bacterial genotypes and their nematode species, as reported by Le Saux et al (1999), who applied the PCR-RFLP of the 16S rDNA to study the symbionts of the EPNs. They found close relationship between the *Photorhabdus-Heterorhabditis* complex, where two bacterial genotypes where associated to *H. indica* and two other to *H. bacteriophora*. Reported studies on the close relationship between the bacteria-nematode complex, *Xenorhabdus* and *Steinernema* (Akhurst and Boemare, 1988), adds further support for the possibility of the existence of a similar association between *Photorhabdus-Heterorhabditis* complex, although only one species of *Photorhabdus* is defined at the present time.

In conclusion, the RAPD-PCR data obtained are correlated with the *Xenorhabdus* and *Photorhabdus* taxonomic relationships. Moreover, intra-specific variations within the *Photorhabdus luminescens* were documented. Hence, other molecular techniques such as complete sequencing of the 16S rDNA may clarify the diversity between different bacterial strains, and their relationship to their symbiont nematodes. This is particularly important when it is needed to make combinations of nematode-bacteria complexes to increase their infectivity against a given target insect.

3.6 Summary and conclusions

This study is the first attempt to isolate EPNs from Palestine. Two novel strains, Bethlehem11 and Bethlehem22, were isolated from Bethlehem district, and they were identified as *H. indica.* Bethlehem strains showed higher heat tolerance at 40°C and higher infectivity to *Galleria mellonella* larva, than the *H. bacteriophora* strain (Hybrid). However, this not necessary means that Bethlehem strains are more able to control target insects such as *Maladera matrida.* This is mainly because of the specificity of the nematodes to their target hosts, moreover, although Bethlehem strains showed heat tolerance, we do not know if their infectivity is affected at higher temperatures. Therefore, further work should be done to test these nematodes on target insects.

The RAPD-PCR technique was able to differentiate among the tested EPNs, as well as among their symbiotic bacteria. However, RAPD-PCR can not identify newly isolated strain at the species level. This is mainly because of the fact that this technique may show sometimes that the level of similarity between two species is similar between two strains of the same species. On the other hand, this technique could be used to differentiate between two unknown strains.

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Appendix

Tables of analysis of variance (ANOVA)

Table 1. ANOVA for the effect of temperature (37°C) on the survival of IJs from the Bethlehem11, Bethlehem22, and Hybrid strains for a period of 6 hours of incubation.

K value	Source	Degree of freedom	Some of square	Mean square	F value	Probability
2	Strain	2	477.815	238.907	127.7327	0.0
4	Time	5	107.870	21.574	11.5347	0.0
6	Interaction	10	46.185	4.619	2.4693	0.0229
-7	Error	36	67.333	1.870		
	Total	53	699.204			

Coefficient of variation: 1.42%
Least Significant Difference Test (LSD): LSD = 2.264 at alpha = 0.05

Table 2. ANOVA for the effect of temperature (38°C) on the survival of IJs from the Bethlehem11, Bethlehem22, and Hybrid strains for a period of 6 hours of incubation.

K value	Source	Degree of freedom	Some of square	Mean square	F value	Probability
2	Strain	2	1188.111	594.056	156.4829	0.0
4	Time	5	819.722	163.944	43.1854	0.0
6	Interaction	10	590.333	59.033	15.5502	0.0
-7	Error	36	136.667	3.796		
	Total	53	2734.833			

Coefficient of variation: 2.08%
Least Significant Difference Test (LSD): LSD = 3.226 at alpha = 0.05

Table 3. ANOVA for the effect of temperature (39°C) on the survival of IJs from the Bethlehem11, Bethlehem22, and Hybrid strains for a period of 4 hours of incubation.

K value	Source	Degree of freedom	Some of square	Mean square	F value	Probability
2	Strain	2	1905.528	952.764	101.7789	0.0
4	Time	7	2202.667	314.667	33.6142	0.0
6	Interaction	14	1760.917	125.780	13.4364	0.0
-7	Error	48	449.333	9.361		
	Total	71	6318.444			

Coefficient of variation: 3.28%
Least Significant Difference Test (LSD): LSD = 5.023 at alpha = 0.05

Table 4. ANOVA for the effect of temperature (40°C) on the survival of IJs from the Bethlehem11, Bethlehem22, and Hybrid strains for a period of 4 hours of incubation.

K value	Source	Degree of freedom	Some of square	Mean square	F value	Probability
2	Strain	2	13882.583	6941.292	2056.6790	0.0
4	Time	7	17927.208	2561.030	758.8236	0.0
6	Interaction	14	18868.083	1347.72	399.3245	0.0
-7	Error	48	162.000	3.375		
	Total	71	50839.875			

Coefficient of variation: 2.15%
Least Significant Difference Test (LSD): LSD = 3.179 at alpha = 0.05

Table 5. ANOVA for the effect of the concentration of IJs from the Bethlehem11, Bethlehem22, and Hybrid strains on the % of mortality of *G. mellonella* larvae after 26 hours of incubation.

K value	Source	Degree of freedom	Some of square	Mean square	F value	Probability
2	Strain	2	39714.724	19857.362	434.9174	0.0
4	Concentraion	12	57951.398	4829.283	105.7713	0.0
6	Interaction	24	11616.853	484.036	10.6014	0.0
-7	Error	78	3561.307	45.658		
	Total	116	112844.281			

Coefficient of variation: 9.34%
Least Significant Difference Test (LSD): LSD = 10.98 at alpha = 0.05

فصل عزلات جديدة من النيماتودات الممرضة للحشرات من فلسطين وتشخيصها بواسطة RAPD-PCR

الخلاصة

تستخدم النيماتودا الممرضة للحشرات لمكافحة الآفات التي تسكن في التربة. بما أن هذه النيماتودا تظهر خاصية بينها و بين الحشرة وبسبب الحاجة لتحسين فعاليتها ومقاومتها للظروف البيئية فإنه من الضروري زيادة عدد العزلات والأنواع منها. مع ازدياد عدد الأصناف والعزلات تزداد الحاجة لطرق سهلة ومعتمدة لتعريف وتصنيف هذه الكائنات.

تم فصل عزلتين (Bethlehem11 , Bethlehem22) من مناطق بيت لحم في فلسطين باستخدام طريقة الطعوم "trapping" لعثة الشمع. تم تعريف العزلتين الجديدتين *Heterorhabditis indica* اعتماداً على تهجين ناجح crossing مع العزلة LN2 للنوع *H.indica*. كانت نسبة التهجين الناجح لكل من العزلتين الجديدتين ٦٦% مع العزلة LN2 new paras . أظهرت كل من العزلتين الجديدتين مقاومة للحرارة أكثر من *H.*

bacteriophora المعزولة من مناطق مناخية معتدلة. حيث وصلت نسبة صمود الطور المعدي "IJs" في درجة حرارة ٤٠° لمدة اربع ساعات إلى حوالي ٨٠% لكل من العزلتين الجديدتين. بينما إقتربت الى الصفر نسبة صمود الطور المعدي للنوع *H. bacteriophora* ، عندما مكث لنفس الفترة في نفس درجة الحرارة. كانت قدرة إحداث المرض "Infectivity" لكل من العزلتين الجديدتين أعلى بالمقارنة مع *H. bacteriophora* ، حيث كانت مقادير الـ LD_{50} للعزلة Bethlehem11 ٢٥ نيماتودا وللعزلة Bethlehem22 ٥٥ نيماتودا وذلك بالمقارنة مع ١٦٠ نيماتودا من نوع *H. bacteriophora* وذلك عند كشف يرقات عث الشمع للنيماتودا لمدة ٢٦ ساعة. العزلتان الجديدتان هما أول محاولة لعزل نيماتود تمت في فلسطين. يمكن دراسة هذه العزلتين بهدف ملاءمتها لمكافحة الآفة المحلية *Maladera matrida* .

تم فحص التقارب الجيني بين العزلتين الجديدتين مقارنة مع عزلات أخرى بطريقة RAPD-PCR. أظهرت نتائج هذه الطريقة أن التشابه بين العزلتين الجديدتين هو ٤٢, ٩٦%. وهذا يدل على كونهما عزلتين منفصلتين. هذا وقد أظهرت الطريقة أن هاتين العزلتين تختلفان عن عزلة أخرى من نفس النوع (*H.indica*, LN2) حيث يصل معدل تشابه الى ٦٦, ٥٨%. بالإضافة الى ذلك أظهر الفحص اختلافا كبيرا نسبيا بين جميع عزلات *H.indica* المفحوصة من

ناحية وعزلة من الفصيلة *Steinernema* من ناحية أخرى. يعتبر هذا الاختلاف طبيعياً لأن العلاقة بين المجموعتين هي بمستوى الفصيلة فقط.

طريقة ال RAPD-PCR لوحدها لا تكفي لتشخيص نوع أو عزلة جديدة غير معروفة. ويعود ذلك الى الحقيقة أن هذه الطريقة يمكن أحياناً أن تظهر مستوى التشابه القائم بين نوعين مختلفين بنفس مستوى التشابه القائم بين عزلتين من نفس النوع. من ناحية أخرى يمكننا استعمال هذه التقانة للتمييز بين عزلتين غير معروفتين.