Fall 2007

Evaluation of a putative entomopathogenic Caenorhabditis (Nematoda: Rhabditid) and associated Serratia (Proteobacteria: Enterobacteriaceae)

Kaitlin M. Bonner
University of New Hampshire, Durham

EVALUATION OF A PUTATIVE ENTOMOPATHOGENIC *CAENORHABDITIS* (NEMATODA: RHABDITID) AND ASSOCIATED *SERRATIA* (PROTEOBACTERIA: ENTEROBACTERIACEAE)

BY

KAITLIN M. BONNER

B.S., ALLEGHENY COLLEGE, 2001

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the requirements for the Degree of

Master of Science

in

Zoology

September 2007

This thesis has been examined and approved.

Thesis Director, William Kelley Thomas,
Associate Professor of Biochemistry and Molecular Biology

Co-Thesis Director, Thomas D. Kocher,
Professor of Zoology

Donald S. Chandler, Professor of Zoology

August 15, 2007
Date

ACKNOWLEDGMENTS

I began my master's work at UNH in the Ocean Processes Analyses Laboratory with Ann Bucklin. I would like to thank Ann for her insightful advice and her belief in my capabilities as a graduate student, as well as the members of the Bucklin Lab for their continual support and camaraderie. At the start of my second year, I was very fortunate to have been adopted by Kelley Thomas and Tom Kocher. They graciously took me in after Ann took a new position at the University of Connecticut. I am very grateful because they have not only made me a better student, scientist and researcher, but they also taught me invaluable lessons along the way.

This project would not have been possible without many people: M. Jumba made the discovery of the nematode and associated bacterium; E. Abebe did the morphological identification; K. Morris performed the initial molecular identification; A. Tucker provided the ND5 primers; the microsatellite perl script was written by W. Sung; expert technical support was provided by J. Anderson; F. Abebe-Akele for helpful discussions; J. Ser and D. Bauer provided valuable comments. The Zoology Department and the Graduate School provided financial support. My committee members, Kelley Thomas, Tom Kocher and Don Chandler, imparted invaluable advice and support throughout this project. I would also like to thank the members of the Thomas and Kocher labs for support and friendship. Finally, I would like to thank all my family and friends that have supported me especially my parents, and my husband, Travis Godkin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

EVALUATION OF A PUTATIVE ENTOMOPATHOGENIC *CAENORHABDITIS* (NEMATODA: RHABDITID) AND ASSOCIATED *SERRATIA* (PROTEOBACTERIA: ENTEROBACTERIACEAE)

by

Kaitlin M. Bonner

University of New Hampshire, September 2007

A major goal of modern biology is to extend our understanding of biological systems investigated in the laboratory to their ecological and evolutionary context. With extensive understanding of the function, development and genetics of *C. elegans*, exploring the link between this laboratory model and the environment in which this system evolved would be very interesting because very little is known about the natural history of this premier model organism.

A new strain of *Caenorhabditis briggsae* (SoAf) with an associated bacterium, *Serratia* sp. SCBI, has been isolated from South Africa using common entomopathogenic nematode trapping methods. This study taxonomically identified and evaluated entomopathogenic behavior of *C. briggsae* SoAf and *Serratia* sp. SCBI. Hemocoelic injections of *Serratia* sp. SCBI demonstrated pathogenicity in insect larvae, *Galleria mellonella*. *Caenorhabditis briggsae* SoAf and other *Caenorhabditis*, including *C. elegans*, were capable of infection, growth and reproduction within the insect larvae in the presence of *Serratia* sp. SCBI. Identification of a putative entomopathogenic *C. briggsae* expands on our

understanding of *Caenorhabditis* ecology and provides a framework for further investigation of entomopathogensis in other free-living nematodes.

CHAPTER I

INTRODUCTION

Background

Nematodes are among the most diverse phyla in the animal kingdom. They are bilaterally symmetric microscopic, roundworms that lack segments and a true coelom. Life history traits of nematodes, including reproductive strategies, morphology, locomotion, foraging strategies, and food preference vary immensely, even within closely related species. They can survive in both extreme and temperate conditions and inhabit terrestrial, marine, and freshwater environments. The diversity of nematode interactions within their ecosystems varies from free-living to predaceous behavior, and associations with other organisms range from ephemeral phoresy to obligate parasitism. (see reviews by Fitch 2005; De Ley 2006; Kiontke and Sudhaus 2006)

The evolutionary origin of the phylum Nematoda remains controversial, and our understanding of its phylogenetic position has been dominated by two competing hypotheses (Fitch and Thomas 1997; Fitch 2005). Historically, the phylogenetic framework has placed greater emphasis on the Coelomata hypothesis, which was based on morphological characters and placed nematodes basal to both arthropods and deuterostomes, thus following the traditional trend of evolutionary progression in development of the coelom.

Research by Sidow and Thomas (1994) supported this theory through sequencing the RNA polymerase II gene in an extensive survey of nematode species. The competing hypothesis, Ecdysozoa, initially supported by Aguinaldo *et al.* (1997) through sequencing the complete 18S rRNA gene, placed nematodes as most closely related to arthropods, suggesting that the development of molting was a single evolutionary event and all organisms that undergo molting form a single monophyletic clade, referred to as the Ecdysozoa. While recent studies have offered support for both hypotheses, most current research supports the Coelomata hypothesis (Hausdorf 2000; Wolf *et al.* 2004; Longhorn *et al.* 2007; Rogozin *et al.* 2007) rather than the Ecdysozoa hypothesis (Adoutte *et al.* 2000; Philippe *et al.* 2005).

Nematode parasitism in both plants and animals has evolved independently many times through history (Fig 1) (Blaxter 2003). Parasitic nematodes play a major role in regulating ecosystem community structure through host interaction, utilizing other invertebrates, vertebrates and plants as opportunistic or obligate host organisms. Parasitic nematodes have a significant impact on agriculture through both plant parasitism and vertebrate parasitism in domesticated animals. Plant parasitic nematodes have been blamed for yearly crop losses of up to $80 billion dollars through infestation and destruction of many economically valuable crops worldwide (Divergence 2006). While animal parasitic nematodes contribute to economic losses in the livestock industry and domestic pets through costly infections and sometimes causing animal death. Animal parasitic nematodes also cause many human diseases, such as

trichinosis (*Trichinella sprialis*), elephantiasis (*Wuchereria bancrofti* and *Brugia malayi*), river blindness (*Onchocerca volvulus*), intestinal dysfunction (*Ascaris lumbricoides*, among others), and eosinophilic meningitis (*Angiostrongylus catonenesis*). They are transmitted through ingestion of contaminated livestock or water, contact with invertebrate vector intermediate hosts or through topical exposure, in which the nematodes burrow directly though the skin (Poinar 1983). While there are many nematodes associated with detrimental impacts on both human health and agriculture, some entomopathogenic species of nematodes are considered beneficial. Nematode insect pathogens can aid in pest management in agriculture by parasitizing common insect pests. The entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema glaseri* are commonly sold as natural alternatives to chemical insecticides.

Evolution of Parasitism in Nematodes

Symbiotic associations between organisms are classified using two major characteristics: type of behavioral interaction and duration of the association. Behavioral interactions generally range from mutualistic or commensal, to exploitative. The duration of association can be transitory, indicating an opportunistic symbiosis, or obligate, indicating a definitive association. Mutualistic obligate symbionts are rarely found in the absence of either participant in the association, and both species generally have reduced fitness when the association is terminated (Blaxter 2003). Exploitative symbioses generally indicate parasitism, where there is increased fitness in one participant

with corresponding decreased fitness in the other. Predator/prey associations are an extension of parasitism, when one seeks and kills the other. Conversely, commensalisms, simple associations, and phoresy do not result in decreased fitness of either participant.

Nematodes have shared the environment with both vertebrates and non-vertebrates for over 550 million years (Blaxter 2003). Thus, the evolutionary pathways and events of the origin of exploitative interactions between nematodes and other organisms are likely to be obscured. There are more than 30 families of nematodes that are invertebrate parasites or pathogens (Blaxter 2003; Stock 2005). Parasitic species of nematodes have arisen multiple times within free-living genera of nematodes. Animal parasitism has arisen independently at least six times, at least four times in invertebrates, including two cases of entomopathogenic parasitism (Fig 1) (Poinar 1983; Dorris *et al.* 1999; Blaxter 2003).

Host associations range from phoretic to obligate, and are frequently specific, highly-evolved associations in many species of nematodes (Blaxter 2003). Phoretic associations are transitory, usually for the purpose of transport or protection, and there is no cost to either participant in the association. Phoresy is thought to be a precursory behavior to parasitism, or pre-adaptation, of free-living nematodes (Blaxter 2003; Poulin 2007). It is well known that many free-living nematodes utilize other organisms for transport and protection. They have been isolated from arthropods and mollusks (Fig 2) (Baird *et al.* 1994; Baird 1999; Blaxter 2003; Thomas *pers comm.*). Free-living nematodes are primarily

microherbivorous feeders and often use larger, more mobile invertebrates for transport through their microhabitat. Due to the patchiness of resources commonly found in terrestrial environments, hosts may transport nematodes across greater distances increasing the chances of locating suitable food sources. Nematode species associated with invertebrate hosts, regardless of the duration of the association, display adaptive behaviors that increase the chances of invertebrate interaction and aided transport. These behaviors include nictation on substrate surface and the secretion of cement-like adhesive structures. Nictation is observed when nematodes stand on the tail-end and wave their head in the air. This adaptation is thought to increase the chance of attachment by increasing potential surface area contact to an invertebrate host wandering nearby. The cement-like adhesive secreted by the nematodes aid in maintaining the contact between host and nematode during transport. These adaptive behaviors have been observed in phoretic, necromenic, and parasitic nematode associations.

Phoretic associations, in which there is no cost to the transport host, have been documented in many species of free-living nematodes (Blaxter 2003; Stock 2005; Kiontke and Sudhaus 2006). In some cases this association has been determined based on well-documented life history characteristics of the species, and in other cases it has been assumed because the nematode was isolated from a live host. However, phoretic behaviors are cryptic and difficult to distinguish from necromenic associations. Necromenic associations are driven by opportunistic behavior in which the nematodes attach to a live host, and upon

death of the host feeds on the decayed material and bacterial growth on the host cadaver. In this case the nematodes simply wait for the host to die, they do not directly cause death of the host. The general distinction between necromeny and phoresy is the lack of nematode/host separation in necromenic associations, while separation can occur at any point and nematodes do not propagate on the host carrier in phoresy. However, necromenic behavior is not well documented, and is often classified as phoretic because it is often difficult to determine a definitive cause of death for the host.

Entomopathogenic Nematodes

Entomopathogenic nematodes are classified as a special case of necromenic associations and parasite-like pathogens. These soil-dwelling nematodes have a symbiotic relationship with an enteric bacterium, which is an insect pathogen, and is thus termed entomopathogenic. Entomopathogenic nematodes have been studied extensively for their potential use as biological control since Rudolf William Glaser initially discovered them in the early 1930s (Glaser 1931; Glaser 1932; Glaser and Farrel 1935). The enteric bacterial symbiont remains extra-cellular and is responsible for the death of the insect host. The symbiotic bacteria are released from within the gut of the infective juvenile stage of the nematode, and cause death of the insect via secretion of various insecticidal toxins. A single clonal population of the associated bacteria is maintained within the carcass of the host through the bacterial secretion of antimicrobial compounds, preventing the colonization of competing bacteria,

yeasts and fungi (Bowen and Ensign 1998; Bowen *et al.* 1998; Adams *et al.* 2006). The two lineages of entomopathogenic parasitism are placed as two genera, both of which infect the larval stage of insects; one lineage giving rise to the genus *Steinernema* and its bacterial symbiont *Xenorhabdus*, and the other to the genus *Heterorhabditis* and its bacterial symbiont *Photorhabdus*.

The general life cycle of free-living nematodes includes four larval (or juvenile) stages (L1-L4), and an adult stage (Brenner 1973). Nematodes have a specialized diapausing stage known as the dauer juvenile stage. This is an alternative to the third larval stage, L3, which occurs during normal development; a hormonal cascade induces nematodes to enter the dauer stage in response to environmental cues including increased temperature, pheromones released during crowding, or decreased food concentration. The distinct morphologies associated with this stage are thicker cuticles and a more streamlined body shape, which aid in prevention of desiccation or starvation. Similar to parasitic nematodes, the infective stage of entomopathogenic nematodes is synonymous to the dauer juvenile stage of free-living nematodes, also referred to as the infective juvenile (IJ) stage. Most free-living entomopathogenic nematodes in nature exist only in the infective stage when not associated with a host organism; it is not until contact with a suitable insect host that the normal life cycle resumes (Fig 3) (Kaya and Gaugler 1993). Interestingly, infective juveniles are not all infective when presented with a susceptible host; generally less than 40% will infect a suitable host when in contact (Hominick and Reid 1990; Campbell *et al.* 1999). When the juveniles infect the insect host, the released symbiotic bacteria

multiply and proliferate providing ample food for the nematode to resume the normal life cycle within the insect cadaver. However, upon completion of three to four reproductive generations within the host, only infective juveniles emerge from the cadaver (Kaya and Gaugler 1993; Burnell and Stock 2000). Similar to the dauer stage in free-living nematodes, the typical infective juvenile morphologies protect the nematode from harsh environmental conditions prior to reaching a suitable host. Also, the association between bacterial symbiont and nematode is quite specific (Forst *et al.* 1997; Ciche and Ensign 2003). Most of the entomopathogenic nematodes can be maintained in the laboratory in culture through NGM agar seeded with the appropriate bacterial symbiont (Forst and Nealson 1996; Forst *et al.* 1997). The life cycle of the two genera of entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, are well studied and understood, while both are thought to have a broad range of insect hosts, there is some specificity within groups of insects.

Both genera of entomopathogenic nematodes exploit insect hosts through entry into the hemolymph by the infective juvenile stage, release of the bacterial symbiont from the gut of the nematode, reproduction within the host, and emergence of infective stage juveniles from the cadaver (Fig 3). The modes of entry, bacterial symbiont storage, and reproductive strategies vary between the two genera. *Steinernema* enters the insect host through the mouth, anus, or spiracles, and enters the hemocoel through the mid-gut epithelium (Kaya and Gaugler 1993; Koppenhöfer *et al.* 2007). Entry through the spiracles is the least common entry method due to various structural characteristics, such as average

diameter, tissue flaps, and contractile muscles, which may inhibit nematode entry into the tracheae (Koppenhöfer *et al.* 2007). Conversely, *Heterorhabditis* has a dorsal tooth that aids in burrowing between segments or vulnerable parts of the integument allowing direct access to the hemocoel through abrasion (Wang and Gaugler 1998). Upon entry into the insect host hemocoel, both entomopathogenic nematode genera release bacteria stored in the gut of the infective juvenile nematode. *Steinernema* carries the bacterial symbiont *Xenorhabdus* inside specialized vesicles of the intestinal tract and the bacteria are released through defecation (Bird and Akhurst 1983; Forst and Nealson 1996; Adams *et al.* 2006). *Photorhabdus* colonizes the entire intestinal tract of *Heterorhabditis* and is regurgitated through the mouth when in the host hemolymph (Ciche and Ensign 2003; Adams *et al.* 2006). Infected insects die within 24-48 hours after initial entry of the infective stage into the hemolymph of the host insect. The bacterial symbionts of both *Steinernema* and *Heterorhabditis* are virulent, requiring ten cells or less to kill the insect, with some *Photorhabdus* species requiring less than 30 cells to obtain a 50% lethal dose for some insects within 24 hours (Ciche and Ensign 2003). Hemocoelic injections of five cells of some *Xenorhabdus* species can yield up to 90-100% mortality rate in *G. mellonella* after 48 hours (Forst and Nealson 1996). Both genera of bacterial symbionts kill the insects through the production and release of insecticidal protein toxins, and maintain a monoculture within the insect cadaver through release of antimicrobial and antifungal compounds (Bowen and Ensign 1998; Bowen *et al.* 1998). The insect cadavers turn a characteristic reddish color post-

infection. The nematodes resume normal development while feeding on the rapidly reproducing bacteria. *Steinernema* are gonochoristic; the infective juveniles develop directly into sexually mature males and females. Thus, *Steinernema* requires both male and female infective juveniles to penetrate the insect host to resume sexual reproduction. *Heterorhabditis* infective juveniles develop into sexually mature hermaphrodites and after one generation reproduce through amphimixis, producing both males and females after the first generation. Therefore, *Heterorhabditis* does not require both male and female nematodes to penetrate into the insect host to reproduce. After three to four generations, the majority of offspring from both entomopathogenic nematode genera will develop into and arrest development at the infective stage, reassociate with the respective entomopathogenic bacterium in the gut of the nematode, followed by emergence from insect host cadaver (Forst and Nealson 1996). Once outside the insect host the nematodes will remain in the infective stage until subsequently infecting another insect larva.

Entomopathogenesis in the phylum nematoda has arisen independently twice, forming only two distinct lineages (Poinar 1983; Dorris *et al.* 1999; Blaxter 2003). Evolution of parasitism by nematodes can be potentially studied by examining free-living and entomopathogenic nematodes. They are an important model to investigate both because of their interesting evolutionary position within nematodes and parasitism, as well as playing an important role in regulating the ecosystem community structure through the modification of the insect community. The unique life cycle of entomopathogenic nematodes has sparked

increased interest in using them as an alternative to pesticide treatments of crops, and as part of integrated pest management programs (IPMs). They also have the potential to function as a useful model for vector-transmitted diseases in insects (Ciche and Ensign 2003).

Study Organisms

Recently, the WK Thomas Lab at the University of New Hampshire has been collaborating on a project involving the identification of a newly described, potentially entomopathogenic strain of *Caenorhabditis briggsae* from South Africa (*C. briggsae* SoAf). The strain was extracted from soil samples collected from various farms in South Africa using a common insect larval trap by Miriam Jumba at the University of the Witwatersrand in Johnanesburg, SA (Bedding and Akhurst 1975; Jumba *et al.* 2006). A monoculture of an associated bacterium was isolated from the *C. briggsae* SoAf strain. The isolated bacterium was determined to be most closely related to *Serratia marcescens* DB11 (Abebe *pers comm.*). The discovery of both a putative entomopathogenic *C. briggsae* and bacterial associate is unique. *C. briggsae* is most commonly found free-living in rich garden soil or compost heaps (Kiontke and Sudhaus 2006). Although there have been many strains of *C. briggsae* isolated from the terrestrial environments circumglobally, this is the first identified putative entomopathogenic association. Also, the genomes of both *S. marcescens* DB11 and *C. briggsae* AF16 have been fully sequenced and thus lend research to be more readily available for comparison and further investigation if the putative entomopathogenic *C.*

briggsae is demonstrated to be both entomopathogenic and taxonomically *C. briggsae*.

Caenorhabditis briggsae is commonly isolated in nature from anthropogenically-altered soil, such as compost heaps and recently disturbed organic matter. Strains are generally found in the dauer juvenile stage, because organically rich soil is generally patchy in distribution in nature and propagation outside microbe rich soil is low. Within the patches of rich soil dense populations may occur. Therefore, the production of dauer juveniles is an adaptation to the patchy landscape and/or crowding within a nutrient rich area often found in nature. Since the dauer stage is longer-lived and better adapted to resist desiccation, movement to another nutrient region is more likely at this stage. Another adaptation or escape mechanism observed in *C. briggsae* is nictation behavior. These invertebrate associations have been classified primarily as phoretic or necromenic, and members of the *elegans* group (*C. briggsae, C. elegans, C. remanei, Caenorhabditis* sp. 4 and sp. 5) have been isolated from gastropods, isopods, and arthropods with varying degrees of association ranging from phoretic to necromenic though most are unknown (Fig 2). Although most members of *Caenorhabditis* exhibit associations with invertebrates as well, a few have been isolated from vertebrates in single incidences with one species, *C. bovis*, identified as an ectoparasite of cattle. Another species was isolated from the intestine of a songbird, *C. avicola,* and identified as a possible bird parasite (reviewed by Kiontke and Sudhaus 2006). However, similar to the invertebrate associations observed in the *elegans* group, most *Caenorhabditis* species have

been found to have phoretic and possible necromenic associations with invertebrates (Fig 2) (reviewed by Kiontke and Sudhaus 2006). As previously stated, there have not been any documented isolations of any *Caenorhabditis* strains using traditional entomopathogenic collection methods and thus none are described as being entomopathogenic.

A bacterial symbiont is inherently necessary to be a successful entomopathogenic nematode. It is interesting that the bacterium isolated from the putative entomopathogenic *C. briggsae* is most closely related to the species *Serratia marcescens*, because *Serratia* is ubiquitous, inhabiting many different environments and utilizing varying life history strategies. It is implicated in nosocomial infections in humans, and is commonly found in soil. It has also been described as an insect symbiont (Jackson *et al.* 2004), an effective insect pathogen (Sikorowski and Lawrence 1998; McNeill 2000; Martin 2002; Tan *et al.* 2006), and a nematode pathogen (Schulenberg and Ewbank 2004; Zhang *et al.* 2005; Pradel *et al.* 2007). *Serratia marcescens* is a member of the family *Enterobacteriaceae*, similar to the distantly related common entomopathogenic symbionts *Photorhabdus* and *Xenorhabdus* (Spröer *et al.* 1999). Insecticidal toxins isolated from *Serratia* spp. were found to have high similarity to toxins produced by common insect pathogens, *Yersinia frederiksenii* and *Photorhabdus luminescens* (Hurst *et al.* 2000; Dodd *et al.* 2006). Therefore, it would not be unlikely to find *Serratia* species in association with nematodes in nature.

It is important to definitively determine if the putative entomopathogenic strain of *C. briggsae* is capable of being entomopathogenic, and to evaluate the

entomopathogenic characteristics of the associated bacterium, identified as *Serratia* sp. SCBI. Also, how this strain differs from other, wild-type naturally isolated, strains of *C. briggsae*, and if other strains are also capable of demonstrating entomopathogenic behavior in the presence of *Serratia* sp. SCBI.

Objectives

The overall goal of this project is to evaluate on molecular and behavioral levels the differences and similarities between other wild-type, naturally isolated strains of *C. briggsae* and the newly isolated strain from South Africa, as well as evaluating the entomopathogenic nature of the associated bacterium. It is hypothesized that this South African strain of *C. briggsae* will exhibit behavioral traits more similar to the classical entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*. Furthermore, it is possible that other strains of *C. briggsae* could show entomopathogenic behavior if the necessary insect pathogen, *Serratia* sp. SCBI, was present.

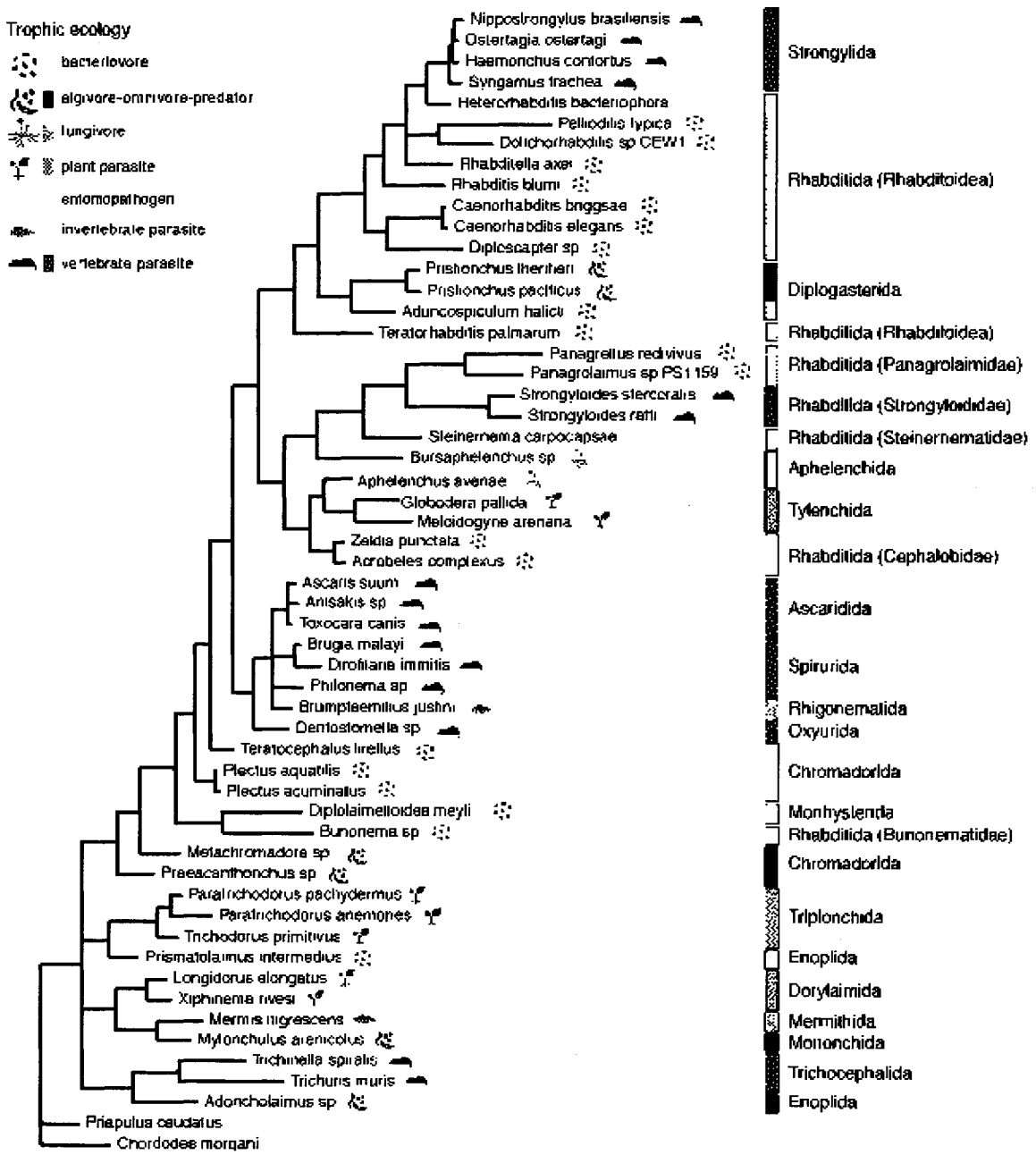
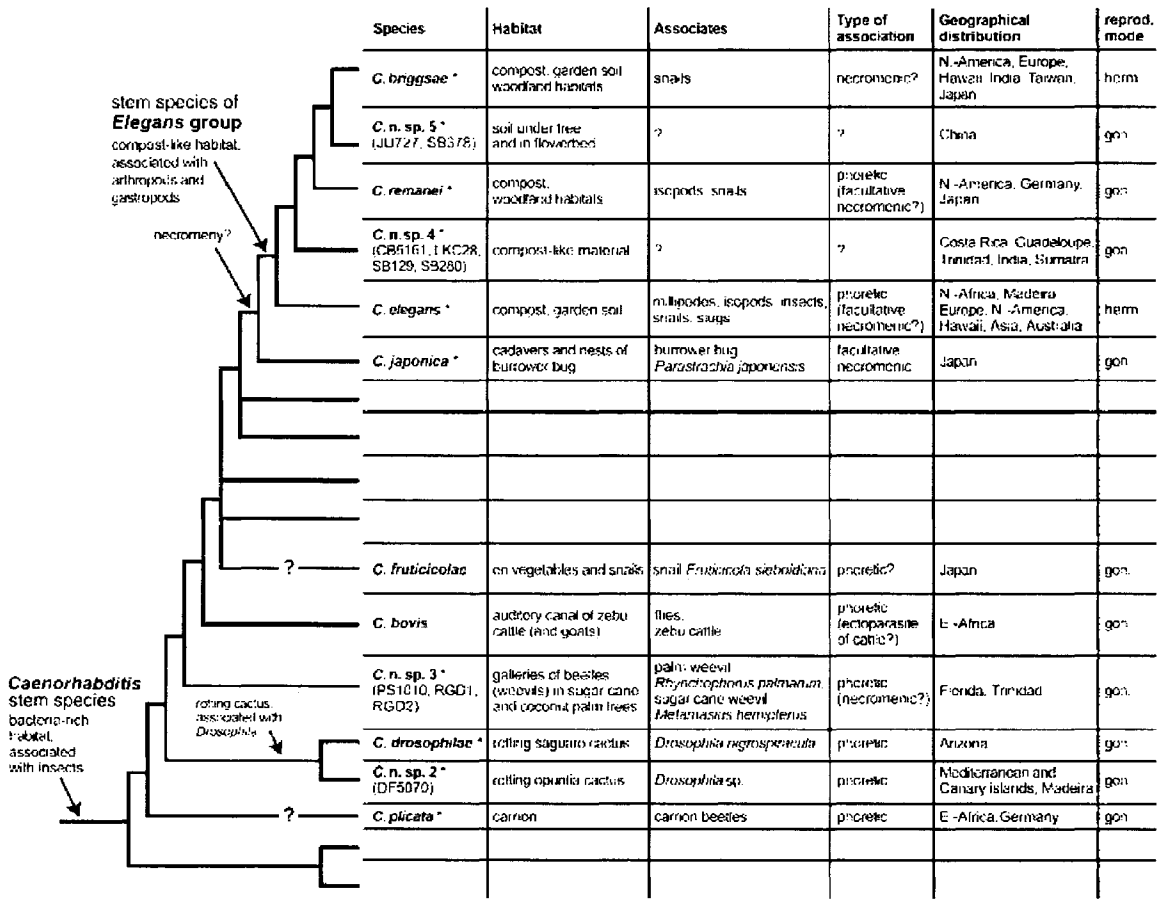


Figure 1. Maximum Parsimony phylogenetic analysis of the SSU for 53 nematode taxa including representative life history characteristics. (From Blaxter *et al.* 1998)



Species	Habitat	Associates	Type of association	Geographical distribution	reprod. mode
C. briggsae *	compost, garden soil woodland habitats	snails	necromenic?	N.-America, Europe, Hawaii, India, Taiwan, Japan	herm
C. n. sp. 5 * (JU727, SB378)	soil under tree and in flowerbed	?	?	China	gon
C. remanei *	compost, woodland habitats	isopods, snails	phoretic (facultative necromenic?)	N.-America, Germany, Japan	gon
C. n. sp. 4 * (CB5161, EKC28, SB129, SB280)	compost-like material	?	?	Costa Rica, Guadeloupe, Trinidad, India, Sumatra	gon
C. elegans *	compost, garden soil	millipedes, isopods, insects, snails, slugs	phoretic (facultative necromenic?)	N.-Africa, Madeira Europe, N.-America, Hawaii, Asia, Australia	herm
C. japonica *	cadavers and nests of burrower bug	burrower bug *Parastrachia japonensis*	facultative necromenic	Japan	gon
C. fruticicolae	on vegetables and snails	snail *Euhadra sieboldiana*	phoretic?	Japan	gon
C. bovis	auditory canal of zebu cattle (and goats)	flies, zebu cattle	phoretic (ectoparasite of cattle?)	E.-Africa	gon
C. n. sp. 3 * (PS1010, RGD1, RGD2)	galleries of beetles (weevils) in sugar cane and coconut palm trees	palm weevil *Rhynchophorus palmarum*, sugar cane weevil *Metamasius hemipterus*	phoretic (necromenic?)	Florida, Trinidad	gon
C. drosophilae *	rotting saguaro cactus	*Drosophila neopsoeudoobscura*	phoretic	Arizona	gon
C. n. sp. 2 * (DF5070)	rotting opuntia cactus	*Drosophila* sp.	phoretic	Mediterranean and Canary islands, Madeira	gon
C. plicata *	carrion	carrion beetles	phoretic	E.-Africa, Germany	gon

Figure 2. *Caenorhabditis* phylogenetic analysis including life history traits, geographical location, and habitat preferences (reproductive mode herm.=hermaphroditic and gon.=gonochoristic) (From Kiontke and Sudhaus 2006)

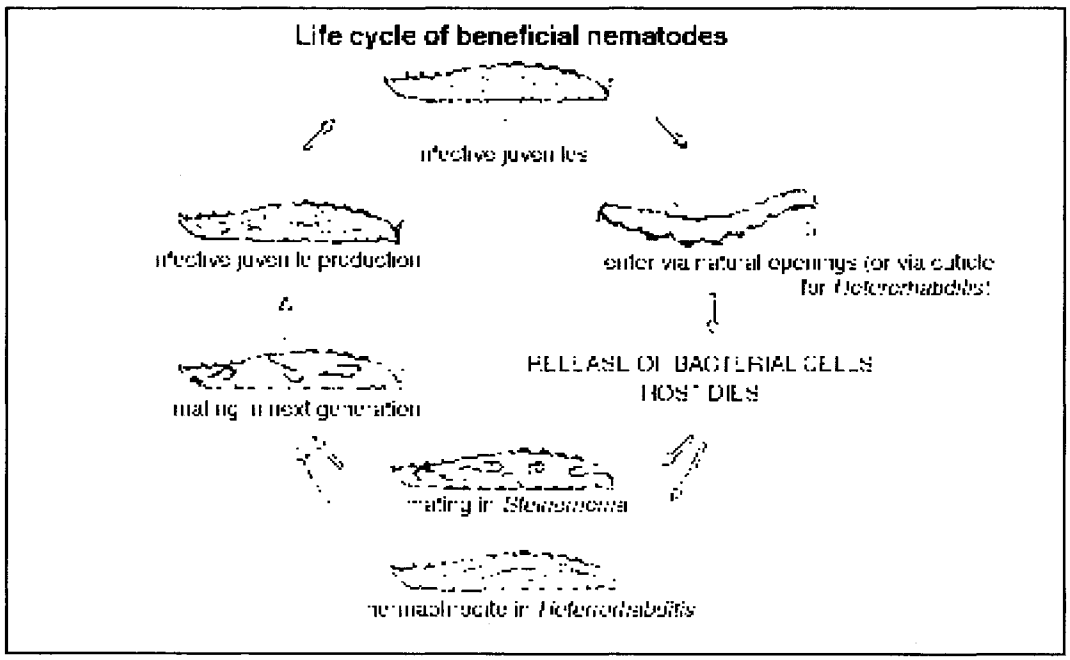


Figure 3. Generalized entomopathogenic nematode life cycle (From Gaugler 2007)

CHAPTER II

MATERIALS AND METHODS

Sample Collection

Soil samples from various farms in 3 provinces of South Africa, North West, Mpumalanga, and Kwa zulu-Natal were collected by Miriam Jumba at the University of the Witwatersrand in Johnanesburg, SA (Jumba *et al.* 2006). Samples were collected 10-20 cm below the soil surface with an area of 20 m^2 (Jumba *et al.* 2006). Entomopathogenic nematodes were extracted from the soil in the laboratory using a common insect baiting technique using late instar larval *Galleria mellonella* (Bedding and Akhurst 1975). Due to conflicting initial molecular and morphological identification, live specimens of a taxonomically unclear, putative entomopathogenic *Caenorhabditis briggsae* strain and associated bacteria were sent to the WK Thomas Lab at the University of New Hampshire for further morphological and molecular identification. [Live cultures were received in December of 2005.]

Morphological identification of the putative entomopathogenic nematode as a *Rhabditid* was completed by Dr. Eyualem Abebe at the University of New Hampshire using a compound microscope and image capturing equipment (Abebe *pers comm.*). Initial molecular identification of the putative entomopathogenic *C. briggsae* SoAf was completed using the 18S ribosomal

gene (Morris *pers comm.).* The resulting 18S rRNA sequence was compared to the GenBank database using the BLAST function at NCBI to determine the closest related sequences and species (Altschul *et al.* 1997).

The associated bacterium was isolated as a monoculture from the putative entomopathogenic *C. briggsae* on untreated LB agar plates by Dr. Eyualem Abebe. According to Jumba *et al.* (2006), the bacterium is epibiotic, primarily found on the cuticle of the nematode, although it is likely to be found in the gut as well. The relationship between nematode and bacterium is currently uncharacterized. The16S rRNA gene of the associated bacterium was subsequently sequenced and identified as *Serratia* sp. SCBI, with the closest related species being *Serratia marcescens* DB11 (Morris *pers comm.*; Abebe-Akele *pers comm.*). Stock cultures of the nematode and associated bacterium were frozen in glycerol and stored at -80°C and are maintained at the Hubbard Center for Genome Studies at the University of New Hampshire.

Nematode Culture Maintenance

Cultures of *C. briggsae* SoAf were maintained by continual infection of and emergence from late instar larval *Galleria mellonella* using the common trapping method to simulate natural conditions, and to maintain a normal life cycle. Emergent nematodes from *G. mellonella* were pooled and cultured using three different methods. One set of cultures was maintained by the infection cycle using *G. mellonella* to reflect possible natural conditions. The two other cultures were reared under normal *C. elegans* laboratory conditions using Nematode

Growth Media (NGM) agar with one set seeded with *E. coli* Op50 and the other set seeded with the isolated associated bacterium, *Serratia* sp. SCBI.

Identification of Nematode and Comparisons to Other Strains of *C. briggsae*

Upon determination of species identification of the putative entomopathogenic nematode as *C. briggsae*, further investigation and molecular comparisons between *C. briggsae* SoAf and other wild type isolates of *C. briggsae* were completed using mitochondrial, ribosomal, and microsatellite markers. Ten natural isolated reference strains of *C. briggsae* were obtained from the Caenorhabditis Genetics Center (CGC): AF16, HK104, HK105, JU725, JU726, PB800, PB826, DH1300, DR1690, and VT847 (Table 1). The strains used in this study were chosen to reflect a sample of other naturally isolated wild type strains of *C. briggsae* across geographic regions and variable substrate conditions. All strains were maintained in large populations on NGM agar seeded with *E. coli* Op50. DNA was extracted from large populations of each of the ten strains as well as the putative *C. briggsae* SoAf, using the Genomic Tip 100/G Genomic DNA Extraction Kit from QIAGEN™. The extractions were diluted to a 1:10 solution with sterile nuclease-free water and then used as template DNA for PCR amplification. Molecular comparisons were initially completed using two mitochondrial genes, cytochrome oxidase two (CO2) and NADH dehydrogenase five (ND5), and the ribosomal internal transcribed spacer region (ITS).

The mitochondrial genes, CO2 and ND5, and the ribosomal ITS region were amplified by Polymerase Chain Reaction (PCR) using AmpliTaq polymerase from New England BioLabs, using primers and corresponding amplification conditions for each reaction listed in Table 2. PCR fragments were visualized for size and banding pattern using gel electrophoresis. Samples were run with a Promega 1 kb molecular ladder on a 1.5% agarose gel in 1x TAE for ten minutes in an electrophorectic chamber filled with 1x TAE at 96 mA. Amplified fragments were purified by solid phase reversible immobilization (SPRI) magnetic bead purification using estapor™ superparamagenetic microspheres from Bangs Laboratories, Inc., or agarose gel purification with QIAquick Gel Extraction Kit from QIAGEN™. SPRI was carried out if the PCR product fragments were above 500 bp and had no secondary banding patterns. If secondary banding patterns were present or the PCR product fragment size was less than 500 bp, the appropriate band was excised from the agarose gel under an ultraviolet light lamp, and DNA was extracted and purified using QIAquick Gel Extraction Kit from QIAGEN™. Purified PCR products were prepared using Beckman sequencing chemistry, and were sequenced on a Beckman Coulter Genetic Analyzer CEQ 8000. Poor quality regions were removed manually; and a consensus was generated from the forward and reverse sequences for each strain using the software package Sequencher™. The consensus sequence of each strain for CO2 and ND5 were concatenated and then aligned using Clustal X as part of the software package Molecular Evolutionary Genetics Analysis, version 3 (MEGA3) (Kumar *et al.* 2004). The

alignments were analyzed using MEGA3 for phylogenetic analysis (Kumar *et al.* 2004). Phylogenetic trees were generated in MEGA3 using the neighbor joining, maximum parsimony, maximum evolution, and UPGMA methods. Neighbor joining and UPGMA trees were generated with a minimum of 500 bootstrap replicates and Kimura 2 substitution model (Kumar *et al.* 2004). Minimum evolution tree was generated with a minimum of 500 bootstrap replicates, with the initial tree obtained through neighbor joining method and close neighbor interchange (cni) utilizing search level 1, and using the Kimura 2 substitution model (Kumar *et al.* 2004). A maximum parsimony tree was generated with a minimum of 500 bootstrap replicates, close neighbor interchange (cni) with search level 1 and an initial tree search through random addition trees at 10 replications, and using a Kimura 2 substitution model (Kumar *et al.* 2004). Clustal X as part of MEGA3 was used to align the consensus sequences for the ribosomal ITS region of each strain (Kumar *et al.* 2004). Further phylogenetic analysis was unnecessary because all strains yielded an identical sequence. The consensus sequences of CO2, ND5, and ITS for each strain were also compared to the GenBank database using discontiguous BLAST to confirm strain and species in order to ensure a lack of contamination between strains (Altschul *et al.* 1997).

Microsatellite loci were analyzed to further investigate the relationship among the wild type isolate strains of *C. briggsae* and the putative entomopathogenic *C. briggsae* SoAf. The published genome sequence of the *C. briggsae* strain AF16 was used for the microsatellite development. The perl

script *copyByMsLoc.pl* written by Way Sung in the WK Thomas Lab at the University of New Hampshire was used to search and isolate all microsatellites found in the published strain of *C. briggsae* AF16 (Sung *pers comm.*). Information on dimer and trimer repeats was extracted from the genome, including the sequence surrounding the microsatellites. Six microsatellite loci and surrounding sequence, including three dimers and three trimers, ranging in size from 57-60 repeats for dimers and 39-70 repeats for trimers in the reference genome were chosen, respectively (Table 3). Primers were developed for each microsatellite using the open-source web program, Primer3 (Rozen and Skaletsky 2000); the optimum expected fragment size was between 500-600 bp, and a minimum distance from the microsatellite was 100 bp on both the 5' and 3' ends. Primers and corresponding PCR conditions are listed in Table 3.

All microsatellites were amplified under gradient PCR using AmpliTaq polymerase from New England BioLabs to determine optimum annealing conditions. When optimum conditions were confirmed, all strains were evaluated for all microsatellites using PCR following visualization and purification protocol previously stated. The microsatellite loci were chosen for fragment analysis based on evidence for polymorphisms via visualization of PCR amplification product. At each microsatellite locus, DNA from each strain of *C. briggsae* was amplified using fluorescent dye-labeled primers, and genotyped with fragment analysis protocol on a Beckman Coulter Genetic Analyzer CEQ 8000 using a 600bp size fragment size marker.

The fragment analysis results were binned to the nearest even integer for dimers and nearest multiple of three for trimers. All results were visually verified for appropriate bin assignments. A comparative distance matrix was generated based on the fixation index, Fst, to estimate overall allele similarity across all six loci for all strains using the population genetics analysis program Arlequin (Schneider *et al.* 2000). Using the Fst distance matrix generated, a phylogenetic tree was created with MEGA3 using the neighbor joining method with a minimum of 500 bootstrap replicates (Kumar *et al.* 2004).

Evaluating Entomopathogenic Behavior

The putative strain of *C. briggsae* SoAf was assumed to be entomopathogenic due to the manner in which it was discovered. The strain was isolated from soil samples baited with a *G. mellonella* trap, a common method employed to collect entomopathogenic nematodes (Bedding and Akhurst 1975). Experiments were carried out to confirm the entomopathogenic behavior in both the nematode and associated bacteria using *G. mellonella*. The insect larvae used for all of the following experiments were late instar *G. mellonella* from Connecticut Valley Biological Supply Company (Southampton, MA) with an average weight between 0.2 and 0.3 g. All insect larvae were kept at 25°C for 24-48 hours prior to every assay, and all experiments were completed at 25°C and in dark conditions.

Separate cultures of the putative strain of *C. briggsae* SoAf were maintained on both NGM agar plates seeded with *E. coli* Op50 and NGM agar

plates seeded with the isolated bacterial associate, *Serratia* sp. SCBI. The treatments were designated *C. briggsae* SoAf (e) for cultures that were initially bleached of the any bacterial associates and then propagated on *E. coli* OP50 and *C. briggsae* SoAf (s) for cultures maintained on the bacterial associate *Serratia* sp. SCBI. Laboratory experiments were carried out to confirm the entomopathogenic behavior of the South African strain of *C. briggsae* and the ectosymbiont *Serratia* sp. SCBI, as well as to investigate the possibility that other wild type isolate strains of *C. briggsae* and closely related *Caenorhabditis* species have the ability to exhibit entomopathogenic behavior when the appropriate bacterium is present.

Evaluation of Entomopathogenic Properties of *Serratia* sp. SCBI

The associated bacterium isolated from the entomopathogenic *C. briggsae*, was designated as *Serratia* sp. SCBI, and was previously determined to be most closely related to the common soil pathogen, *Serratia marcescens* DB11 (Morris *pers comm.*; Abebe-Akele *pers comm.*). The overall goal of this experiment was to determine if the isolated bacterial associate demonstrated entomopathogenic characteristics. Entomopathogenic properties of *Serratia* sp. SCBI was assayed through exposure of late instar insect larvae to both topical exposure and hemocoelic injection of *Serratia* sp. SCBI. If *Serratia* sp. SCBI is an effect insect pathogen, and not limited to entomopathogenic associations, then it will efficiently kill insects through both topical exposure and internal injections.

Cutaneous, or topical, exposure experiments consisted of late instar larval *G. mellonella* placed on NGM plates seeded with *Serratia* sp. SCBI (n=10), NGM plates seeded with *E. coli* OP50 (n=5), and NGM plates with no bacterial lawn present (n=5). A single *G. mellonella* was placed on each 6 cm Petri dish with one of the three treatments. Mortality was monitored at 24-hour intervals for 20 days at which time the experiment was terminated.

All injection experiments were carried out on moistened filter paper (Whatman No. 1) in 6 cm Petri dishes at 25°C in dark conditions with injections administered using a 26G 5/8 1 ml sterile syringe. Prior to the start of the experiment, both *E. coli* Op50 and *Serratia* sp. SCBI were grown in LB broth for 12-14 hours at 37°C and 20°C, respectively. The control conditions for this experiment were separated into three categories: no injection, simple puncture, and injection with control solutions (n=5 for each). For each control experiment five *G. mellonella* placed in a prepared 6cm Petri dish without any physical or chemical exposure to *Serratia* sp. SCBI: the first set of controls were undisturbed *G. mellonella*; second set was exposed to a simple penetration injection without solution; and the final set was of five *G. mellonella* that were injected with 10-20 µl of M9 buffer solution, LB broth only, or LB incubated with *E. coli* Op50 (n=5 for each injection set). The experimental injections were completed using titrations of *Serratia* sp. SCBI grown in LB broth for 12-14 hours. Three *G. mellonella* were injected with 20 µl of each *Serratia* sp. SCBI titration; the stock solution of *Serratia* sp. SCBI was diluted for the titrations using sterile LB broth. The titrations of this experiment were: full concentration, 1:10, 1:100, $1:10^3$, $1:10^4$,

$1:10^5$, $1:10^6$, $1:10^7$, $1:10^8$, $1:10^9$, and $1:10^{10}$ dilutions. Titrations were streaked on to untreated LB plates, incubated for 12-14 hours at 37°C, and colonies were counted to determine the colony forming units (cfu) for each titration. Percent mortality of *G. mellonella* was evaluated at 24-hour intervals, and was terminated after 14 days. Results were pooled into a single data set for all injection and topical exposure experiments, and the square root of percent mortality results were arcsine transformed for statistical analysis. A pairwise t-test comparing pooled experimental treatments and control treatments was used to determine if the *G. mellonella* injected with *Serratia* sp. SCBI had significantly greater mortality than the control *G. mellonella* treatments at each time interval. A regression analysis *Serratia* sp. SCBI concentration and mortality was generated to determine the strength of the relationship between bacterial concentration and mortality of the insect larvae. All statistical analyses were completed using the Sigma Plot™ and Sigma Stat™ statistical software package.

Entomopathogenic Properties of *C. briggsae*, Including *C. briggsae* SoAf

Evaluation of the entomopathogenic behavior of *C. briggsae* SoAf and other strains of *C. briggsae* as well as other closely related *Caenorhabditis*, was two fold. The first set of experiments were to determine if the nematodes were capable of reproducing within a late instar *G. mellonella*, and the second set of experiments were to determine if the nematodes were able to gain entry, and move into the insect larvae hemolymph. Detailed information on all of the strains used in this experiment is found in Table 1.

For the first set of experiments cultures of each strain were maintained on NGM plates seeded with *Serratia* sp. SCBI for two weeks prior to the start of the experiment. All cultures were maintained at 20°C until use. After two weeks, all plates were washed with M9 buffer saline solution and both nematode and bacteria were collected in 50 ml conical tubes. Ten larval *G. mellonella* were injected with a known concentration of nematodes for each strain, using a 26G 5/8 1 ml sterile syringe (Table 4). The syringe was tested prior to the injections to ensure that increased mortality of the insect or size selection of the nematode culture would not occur. Each experiment was completed in triplicate. The insect larvae were incubated in 6 cm Petri dishes with moistened filter paper (Whatman No. 1) at 25°C in the dark for 24 hours. After incubation the insect larvae were transferred to modified white traps and maintained at 25°C and in dark conditions for one month (White 1927; Kaya and Stock 1997). The white trap set-up consisted of an upside down half of a 6 cm Petri dish set inside a 15 cm Petri dish with 50 ml of sterile water surrounding the 6 cm dish. Filter paper (Whatman No. 1) was draped from the top of the upside down 6 cm Petri dish to the sterile water. The larval insect cadavers were placed on the portion of filter paper set on the 6 cm Petri dish. Emergent nematodes were expected to leave the insect cadaver and migrate to the sterile water. The sterile water was collected for each strain at two time intervals, nine and 20 days. Emergent nematodes at day nine were collected for each strain and compared using two emergence estimators. Adjusted emergence was calculated by subtracting the number of injected nematodes from the number of emerged nematodes, and the

proportional emergence was calculated by dividing the number of emerged nematodes by the number of nematodes injected into each insect larvae. A pairwise t-test comparison was completed for each emergence estimator to determine differences in emergence between the number of nematodes injected as compared to the number emerged for each strain. A single factor ANOVA was used to compare emergence between strains for both emergence estimators. A regression analysis of nematode emergence was compared to number of nematodes injected to determine if a correlation existed between the two variables.

The goal of the second set of experiments was to determine if the nematodes were capable of entering the insect hemocoel. The mode of entry would likely be through the mouth, anus, or spiracles due to the lack of a dorsal tooth like *Heterorhabditis*. It is likely the major mode of entry into the hemocoel is through the midgut epithelium after entering the digestive cavity via the mouth or the anus. The likelihood of entry into the hemocoel through the spiracles is low due to the size and structure of the opening to the tracheae (Koppenhöfer *et al.* 2007). The penetration assay followed modified procedures from Glazer and Lewis (2000) and Caroli *et al.* (1996). The penetration assay specifically measured the number of nematodes found within the insect larvae, and compared this to the total number of nematodes initially placed in the arena with the insect larvae. All experiments were carried out on 6 cm Petri dishes lined with filter paper moistened with approximately 1 ml of sterile water. Environmental conditions were maintained at 25°C, in the dark, and under moist

conditions. Appropriate moisture content was maintained to prevent nematode desiccation. Ten late instar *G. mellonella* were placed in a prepared 6 cm Petri dish, with three Petri dishes per strain of nematode (n=10 per dish). The nematodes were collected from the white trap experiment for use in this experiment; 1000 nematodes per strain were added to each prepared Petri dish (three Petri dishes per strain with 10 *G. mellonella* in each Petri dish). Control conditions were identical to the experimental conditions without the addition of nematodes. Mortality was recorded at 24, 48, 72, 96 and 168 hrs. The experiment was terminated at 168 hrs, at which point approximately 100% mortality of the insect larvae exposed to the nematodes was reached. After 168 hrs the insect larvae were collected from each Petri dish and rinsed with sterile water to remove any nematodes on the insect cuticle. Rinsed insect larvae were stored at -20°C for subsequent dissection and pepsin digestion.

To determine the number of nematodes penetrating the integument of the insect, the insect larvae were bisected longitudinally using a scalpel, and then digested in 3ml of pepsin solution at 37°C for 90 minutes with periodic vortexing every 30 minutes. The pepsin solution was made with 23 g of NaCl in 1000 ml sterile water, pH adjusted to 1.8-2.0 using concentrated HCl, and 8 g pepsin was added and mixed until all particulates were completely dissolved. The solution could be stored at 4°C for one week. After digestion, the number of nematodes within each digested insect larvae cadaver was counted and percent penetration was determined by multiplying the number of nematodes found in the cadaver by 100 and then dividing by the total number of nematodes added to the arena. The

square root of the percent penetration and percent mortality were arcsine transformed for statistical analysis. A single factor ANOVA with Dunnett's post hoc comparison was used to evaluate differences between mortality of insect larvae exposed to nematodes and the control conditions. To compare differences in percent penetration between strains, a single ANOVA with Tukey's post hoc pairwise comparison was used. A regression analysis was generated to determine if a relationship existed between percent penetration and percent mortality at 168 hrs. All statistical analyses were completed using Sigma Plot™ and Sigma Stat™ statistical software package.

Table 1. *Caenorhabditis* strains used in both molecular and behavioral assays

Strain	Species	Received at CGC	Location	Collected By	Substrate Collected On	Molecular Assay	Behavioral Assay
DH1300	*C. briggsae*	Jan-80	Unknown	Zuckerman, B	soil	x	x
DR1690	*C. briggsae*	Feb-81	Unknown	Zuckerman, B	soil	x	x
AF16	*C. briggsae*	Feb-82	Ahmedabad, India	Fodor, A	soil	x	x
HK104	*C. briggsae*	N/A	Okayama, Japan	Kagawa, H	mushroom	x	x
HK105	*C. briggsae*	Apr-98	Sendai, Japan	Kagawa, H	mushroom	x	x
JU725	*C. briggsae*	Jul-05	Yangshuo, Guangxi China	Felix, MA	mushroom compost	x	x
JU726	*C. briggsae*	Jul-05	Sanjiang, Guangxi China	Felix, MA	soil compost in cabbage field	x	x
PB800	*C. briggsae*	May-99	Dayton, OH USA	Baird, S	soil at base of mushrooms	x	x
PB826	*C. briggsae*	May-99	Hueston Woods State Park, OH USA	Hampton, RM & Baird, S	association with snail	x	x
VT847	*C. briggsae*	Apr-98	Haena, Kauai, HI USA	Ambros, V	soil	x	x
SoAf	*C. briggsae*	N/A (col. 2005)	North West, Mpumalanga and Kwa Zulu-Natal provinces South Africa	Jumba, M	grassland and strawberry cropland soil	x	x
N2	*C. elegans*	Jul-1993 (col. 1968)	Bristol, England UK	Staniland, LN	mushroom compost		x
PB4641	*C. remanei*	Aug-03	inbred derivitive of EM464	Baird, S	see EM464		x
JU727	*Caenorhabditis sp. 5*	Jul-05	Sanjiang, Guangxi China	Felix, MA	under a tree		x
CB5161	*Caenorhabditis sp. 4*	Apr-88	Trinidad	Fodor, A	sugar cane		x
EM464	*C. remanei ssp. vulgaris*	Dec-90	Brooklyn, NY USA	Baird, S	association with pill bug from soil in compost heap		x

Table 2. Amplification conditions and primers for mitochondrial and ribosomal markers

Gene / Region	Internal/External	Forward Primer (5' to 3')	Reverse Primer (5'to 3')	Reference
COII A/B[a]	internal	IF-ATTTAGATAGAAATTTAACAGTTAA	IR-CAACGATTATCAACTTCTAATAAACG	Denver *et al.* 2000
COII C[a]	internal	IF-TTTGATTCTTATATAAAATC	N/A	Denver *et al.* 2000
COII-Gly/His[a]	external	Gly-TTCAGTATGTTTGACTTCCA	His-GCTCTATATTCTCTTACACCACA	Denver *et al.* 2000
ND5-IF/Ir[b]	external	IF-AGGTGGTGTTAGAGTTTGGCTA	IR-AAATAAGGTATACCCCTTAGAATTGAT	Tucker *pers comm.*
ND5-2F/2R[c]	internal	2F-TTGGTTTATCAGTTTTTAGGGGTTA	2R-AATTACGCCCATCTTGTTGC	Tucker *pers comm.*
ITS-TW81/AB28[d]	external	TW81-GTTTCCGTAGGTGAACCTGC	AB28-ATATGCTTAAGTTCAGCGGGT	Subbotin *et al.* 2001

[a] 94°C 5min, (94°C 40sec, 55°C 30sec, 72°C 2:30min) x 35 cycles, 72°C 10min, 4°C
[b] 95°C 4min, (95°C 30sec, 51.6°C 40sec, 72°C 2:30min) x 30 cycles, 72°C 10min, 4°C
[c] 95°C 4min, (95°C 30sec, 63.7°C 40sec, 72°C 2:30min) x 30 cycles, 72°C 10min, 4°C
[d] 94°C 4min, (94°C 1min, 55°C 1:30min, 72°C 2min) x 35 cycles, 72°C 10min, 4°C

Table 3. Microsatellite locus information from reference strain AF16, amplification conditions and primers for microsatellite loci a) repeat type, length and scaffold information from the reference strain AF16 genome b) forward and reverse primer sequences and amplification protocol for each locus amplified

a)

Locus	Locus ID	Repeat Motif	Repeat Length	Start Sequence	End Sequence	Middle Sequence
1	2852	ttc	70	746507	746716	746611
2	2220	aag	39	1417236	1417352	1417294
3	NA15	tc	59	11628	11745	11686
4	4228	ag	57	184798	184911	184854
5	2454	ca	60	887318	887437	887377
6	4404	aag	45	457762	457896	457829

b)

Primer Name	Forward Primer (5' to 3')	Reverse Primer (5'to 3')
cb25.fpc2220_aag_2[a]	TTGCCTTGTTTGTTACTTGGAA	GGGGTTGACGAAGAACAAGA
cb25.fpc2454_ca_2[b]	TTTATGGCTCAAGCTAATGTTTTC	CGAGATTCTTAGATTTTGAGATTTTG
cb25.fpc2852_ttc_2[a]	GACAGATCCCCTCAGGCTATT	AGGTTGTGGATGGGAAAACA
cb25.fpc3857_ac_2[c]	GACGGTGATTTATCGGATGG	CCGTCTACATACCCCCTGAA
cb25.fpc4044_aag_2[c]	CGAATCCCTCGTTCTCTTCA	TGATTTGGTTCTGGGATGGT
cb25.fpc4228_ag_2[b]	CTTCCGCTGAAAAATGGAGA	CGCAAGTATGGGTTTGGGTA
cb25.NA_015_tc_2[b]	ATGCAGACAAGCCCTCATTT	AGCCCACTGAACCATTTTTG

[a] 95°C 5min, (95°C 40sec, 67°C 30sec, 72°C 2:30min) x 35 cycles, 72°C 10min, 4°C
[b] 95°C 5min, (95°C 40sec, 63.7°C 30sec, 72°C 2:30min) x 35 cycles, 72°C 10min, 4°C
[c] 95°C 5min, (95°C 40sec, 56.2°C 30sec, 72°C 2:30min) x 35 cycles, 72°C 10min, 4°C

Table 4. Average number of nematodes, per strain, injected into *G. mellonella* prior to transfer to white trap set-up

Strain	Avg. Injected (nematodes/50ul)
AF16	85
HK104	550
HK105	400
JU725	155
JU726	450
PB800	90
PB826	1625
DH1300	1375
DR1690	688
VT847	1250
SoAf (e)	1125
SoAf (s)	90
N2	650
PB4641	350
JU727	250
CB5161	175
EM464	350

CHAPTER III

RESULTS

The goals of this project were to taxonomically identify the putative entomopathogenic nematode through the use of molecular techniques and to evaluate the entomopathogenic behavior using classical entomopathogenic techniques, as well as evaluating entomopathogenic characteristics and taxonomic identification of the isolated associated bacterium. Additional goals included examining the relationship between the putative entomopathogenic *C. briggsae* to other wild type isolate *C. briggsae* strain using molecular tools, as well as expanding the evaluation of entomopathogenic behavior to include other wild type isolates of *C. briggsae* and closely related *Caenorhabditis*.

The initial nematode identification was accomplished by sequencing the internal transcribed spacer (ITS) one and two of the 18S ribosomal subunits by our collaborators at the University of Witwatersrand in Johannesburg, SA (Jumba *et al*. 2006). However, morphological identification conflicted with the initial molecular identification using the ribosomal ITS region due to an accidental, erroneous GenBank submission. Samples were sent to the WK Thomas Lab at the University of New Hampshire, where the nematode samples were morphologically identified as a *Rhabditid* by Dr. Eyualem Abebe, and the complete 18S ribosomal subunit was sequenced, identifying them as

Caenorhabditis briggsae (Abebe *pers comm.* and Morris *pers comm.*). The internal transcribed spacer was identical across all the wild type isolates of *C. briggsae* including the South African strain (Fig 4).

Molecular assays: Molecular Comparisons Between Strains of *C. briggsae*

To evaluate the putative entomopathogenic *C. briggsae* strain we wanted to determine the relationship between the South African strain *C. briggsae* SoAf and other wild type isolates of *C. briggsae.* Ten wild type isolate *C. briggsae* strains were used for molecular and behavioral assays in this project (Table 1). The molecular comparisons were completed using 1381 bp of two mitochondrial genes, cytochrome oxidase two (CO2) and NADH dehydrogenase five (ND5). The resulting phylogenetic tree topology was consistent across multiple tree building methods, resulting in two major clades (Fig 5). The two major clades were broken down into clade 1: including strains JU725, VT847, DH1300, DR1690, JU726, SoAf and AF16; and clade 2: including strains PB826, PB800, HK104, and HK105 (Fig 5). Pairs of *C. briggsae* strains HK104 and HK105, DH1300 and DR1690, and SoAf and AF16 were each found to be molecularly identical to the pair for mitochondrial genes CO2 and ND5 (Figs 5, 6). While it was not surprising to find strains that had identical DNA sequences for CO2 and ND5, to ensure that strain SoAf was not in fact AF16 though laboratory contamination or confusion we chose to further investigate the difference between all of the *C. briggsae* strains by analyzing microsatellite loci. Microsatellite loci were chosen because in general repetitive elements have a

greater mutation rate than other regions on the genome including the rapidly evolving mitochondrial DNA (Denver *et al.* 2000). Thus, most microsatellite loci can infer molecular differences on a fine scale. Microsatellites are a common method employed to differentiate between populations of the same or very similar species in a geographical region.

Six microsatellite loci were assayed to further investigate the similarities between SoAf and AF16 as well as the other strains (Table 5). The microsatellites chosen were intentionally large to ensure sensitivity. Three dimer and trimer repeats were assayed for each strain; they were identified as informative loci due to the highly polymorphic nature of each locus. Microsatellite fragment results were binned and overall allelic similarity was estimated using the fixation index, Fst (Table 5 and Fig 7). The results of the microsatellite fragment analysis revealed that AF16 and SoAf were not molecularly identical (Fig 7). However, after molecular analysis of HK104 and HK105, as well as DH1300 and DR1690, all were found to be molecularly identical for CO2, ND5, and six microsatellite loci (Figs 5, 6, 7).

Molecular Assays: Identification of Associated Bacterium, *Serratia* sp. SCBI

The 16S ribosomal subunit was amplified to determine the species of bacteria associated with the entomopathogenic *C. briggsae* (Morris *pers comm.)*. The associated bacterial16S sequence was determined to be most closely related to, though not identical to, *Serratia marcescens* through comparisons in the GenBank database using the BLAST function (Fig 8) (Altschul *et al.* 1997).

The phylogenetic position and unique characteristics of *Serratia* sp. SCBI are currently being investigated through genomic comparisons using the sequenced strains *Serratia marcescens* DB11 and *Serratia proteomaculans* 568 in the WK Thomas Lab at the University of New Hampshire by Feseha Abebe-Akele (Abebe-Akele *pers comm.*).

Behavioral Assays: Entomopathogenic Properties of *Serratia* sp. SCBI

The bacterial associate, *Serratia* sp. SCBI, isolated from the putative entomopathogenic nematode *C. briggsae* SoAf, was assayed for entomopathogenic properties. Late instar *G. mellonella* larvae were infected through direct hemocoelic injections with varying concentrations of *Serratia* sp. SCBI. Mortality was recorded at four time intervals: 24, 48, 72, and 96 hours post-injection. The injections ranged from 1.00 colony forming units, cfu, to 1.00 x 10^{10} cfu in LB solution. The controls for this experiment included undisturbed insect larvae, injection controls, and cutaneous exposure to *Serratia* sp. SCBI. Injection controls consisted of insect larvae that had the needle inserted without injecting a solution, and injections with buffered saline solution, sterile LB broth, and *E. coli* OP50 in LB. The control treatments and experimental treatments were pooled separately for statistical purposes. Hemocoelic injections of 1.00 x10^{6} cfu to 1.00 x10^{10} cfu of *Serratia* sp. SCBI yielded 100% insect mortality within 24 hrs of injection, while 1.00 x10^{4} and 1.00 x10^{5} cfu injections yielded 100% insect mortality within 48 hrs (Fig 9, 10). Injections of 1.00 x10^{3} cfu yielded 100% insect mortality within 72 hrs and injections of 1.00 x10^{2} cfu within 96 hrs

(Fig 9, 10). The injections with the lowest concentration of *Serratia* sp. SCBI, 1.00 x10^1 and 1.00 x10^2, reached 66% insect mortality within 72hrs (Fig 9, 10). Due to sample size, direct comparisons could not be made between injection concentration treatments. However, the mortality of *G. mellonella* in each experimental treatment, those injected with *Serratia* sp. SCBI, was significantly greater than the pooled controls at all four time intervals (Figs 9, 10 and Table 6). These results indicated that insect larvae infected with *Serratia* sp. SCBI had a significantly higher mortality rate than those uninfected, and that mortality rate was correlated with concentration of injected *Serratia* sp. SCBI. Also, insect cadavers changed from pale yellow to black post-infection.

Behavioral Assays: Entomopathogenic Properties of *C. briggsae* SoAf

In order to assess entomopathogenic behavior in the *C. briggsae* SoAf strain and other wild type isolates of the *elegans* group of *Caenorhabditis* their ability to reproduce within insect larvae with the necessary bacteria, *Serratia* sp. SCBI, and gain access into the insect needed to be evaluated. Initially, the putative entomopathogenic *C. briggsae* SoAf, wild type isolates of *C. briggsae* and other closely related *Caenorhabditis*, including *C. elegans*, were assessed for reproductive capability within larvae of *G. mellonella*. Nematodes were grown on NGM agar seeded with *Serratia* sp. SCBI for two weeks, and then harvested and injected into the insect larvae. Bacterial transfer of *Serratia* sp. SCBI from the agar plates with the nematodes occurred and insect larvae died within hours of injection. All insect larvae turned black and were dead within 24

hours. Five insect larvae were injected with each strain of *Caenorhabditis* sp. and emergence from the insect larvae carcass were collected nine days post injection (Table 7). No nematodes were recovered from insect larvae injected with *C. briggsae* DR1690 after nine days. However, emergent nematodes were also surveyed 20 days post injection and nematodes were recovered for all strains including DR1690.

For all of the strains of *Caenorhabditis* sp., except DR1690, the number of emerging nematodes from the insect larvae carcass was significantly greater than the initial injection, indicating that all strains were capable of reproducing inside late instar *G. mellonella* larvae (Fig 11 and Table 8). Comparisons were made between strains using two emergence estimations: adjusted and proportional nematode emergence. Adjusted nematode emergence was determined by subtracting the number of nematodes injected into the insect larvae from those that emerged. Proportional nematode emergence was determined by dividing the number of nematodes that emerged by those that were injected. A single factor ANOVA with Tukey's post hoc pairwise comparisons were made between each strain using both emergence estimations. In both estimations PB800 yielded the highest number of emergent nematodes and was significantly greater than all other strains, indicating it may have had a greater rate of reproduction than other strains (Fig 12 and Tables 9, 10). In the adjusted emergence estimate, PB826 was second to PB800 in reproductive capability and was significantly greater than all other strains, except PB800 (Fig 12 and Table 9). Strains AF16, HK104, JU725, VT847, CB1561, and EM464 had

comparatively moderate reproductive ability within the insect larvae (Fig 12 and Table 9), while strains HK105, JU726, DH1300, SoAf(e), SoAf(s), N2, PB4641, and JU727 demonstrated the lowest reproductive output (Fig 12 and Table 9).

The results from the proportional emergence estimates varied from the adjusted emergence estimates. While PB800 also had the greatest number of emerging nematodes, all other strains demonstrated significantly decreased ability to reproduce within insect larvae (Fig 12 and Table 10). The AF16 strain was ranked second in reproductive capability; the number of emerging nematodes was significantly greater than all other strains except PB800 (Fig 12 and Table 10). The strains JU725, SoAf(s) and CB5161 demonstrated comparatively moderate reproductive capability. The remaining strains, HK104, HK105, JU726, PB826, DH1300, SoAf(e), N2, PB4641, JU727 and EM464 showed comparatively diminished reproductive output under the proportional emergence estimate compared to PB800 (Fig 12 and Table 10). This experiment demonstrated that all strains tested were capable of reproducing within the late instar *G. mellonella*, with the exception of DR1690, as previously discussed.

When considering all strains, no correlation was found between the number nematodes injected in the late instar *G. mellonella* larvae and the number of nematodes emerging from the cadavers, no correlation was determined (adj R^2 = 0.00, p-value 0.514) (Fig 13). However, PB800 was demonstrated to be an outlier, and an additional regression analysis was completed excluding PB000 results. In the second regression analysis, the number of nematodes injected explains approximately 20% of the variation in

number of nematodes emerging from the insect larvae when the outliers were removed, PB800 (adj R^2 = 0.2 and p-value < 0.01) (Fig 13). The ability to reproduce within insect larvae was demonstrated in the white trap experiments. All strains were capable of reproducing within *G. mellonella* with the exception of DR1690, which required a much longer incubation period prior to emerging from the insect cadavers.

Another key aspect to determining if entomopathogenic behavior exists in nematodes is the ability of a nematode to gain entrance into the hemolymph of an insect. To determine if the strains were capable to invading insect larvae integument, approximately 1000 nematodes were placed in a moist arena with 10 insect larvae. The penetration, or infection rate, was determined by comparing the number of nematodes within the insect larvae compared to the total number added to the arena. Mortality of the insect larvae was recorded at 24-hour intervals for seven days, at which point approximately 100% mortality was observed and all insects color had changed to black (Fig 14). Through a single factor ANOVA and Dunnett's post hoc analysis, insect larvae mortality in all experimental treatments was significantly greater than the controls (Fig 15 and Table 11). However, regression analysis revealed that the relationship between mortality and penetration are not strongly correlated at 168 hours post exposure (adj. R^2 = 0.00, p-value = 0.366) (Fig 16).

Percent penetration ranged from 4% in strain JU726 to 27% in strain PB826, with DR1690 resulting in 0% penetration. Strains PB826, PB800, HK104, and VT847 had comparatively greater penetration rates, with overall

averages between 15-20% (Fig 17). The penetration rate of remaining strains averaged 5 to10% with the exception of DR1690, which did not penetrate the integument of the insect larvae (Fig 17). Significant differences between strains was limited, PB826 had significantly greater penetration than AF16, N2, PB4641, CB5161, and EM464 (Fig 17 and Table 12). Strain EM464 demonstrated the least penetration compared to all other strains, with the exception of DR1690 (Fig 17 and Table 12). Overall penetration across all strains tested averaged 11%, indicating approximately 11% of the nematodes placed in the arena successfully penetrated the hemolymph of the insect larvae (Fig 17). Although mixed life stages were found during the dissections and there were reproductively active individuals within the insect larvae, the majority of nematodes inside the insect larvae were dauer stage individuals (Fig 18). Thus, suggesting that gaining access into the hemolymph and reproduction within the insect larvae is possible by these nematodes.

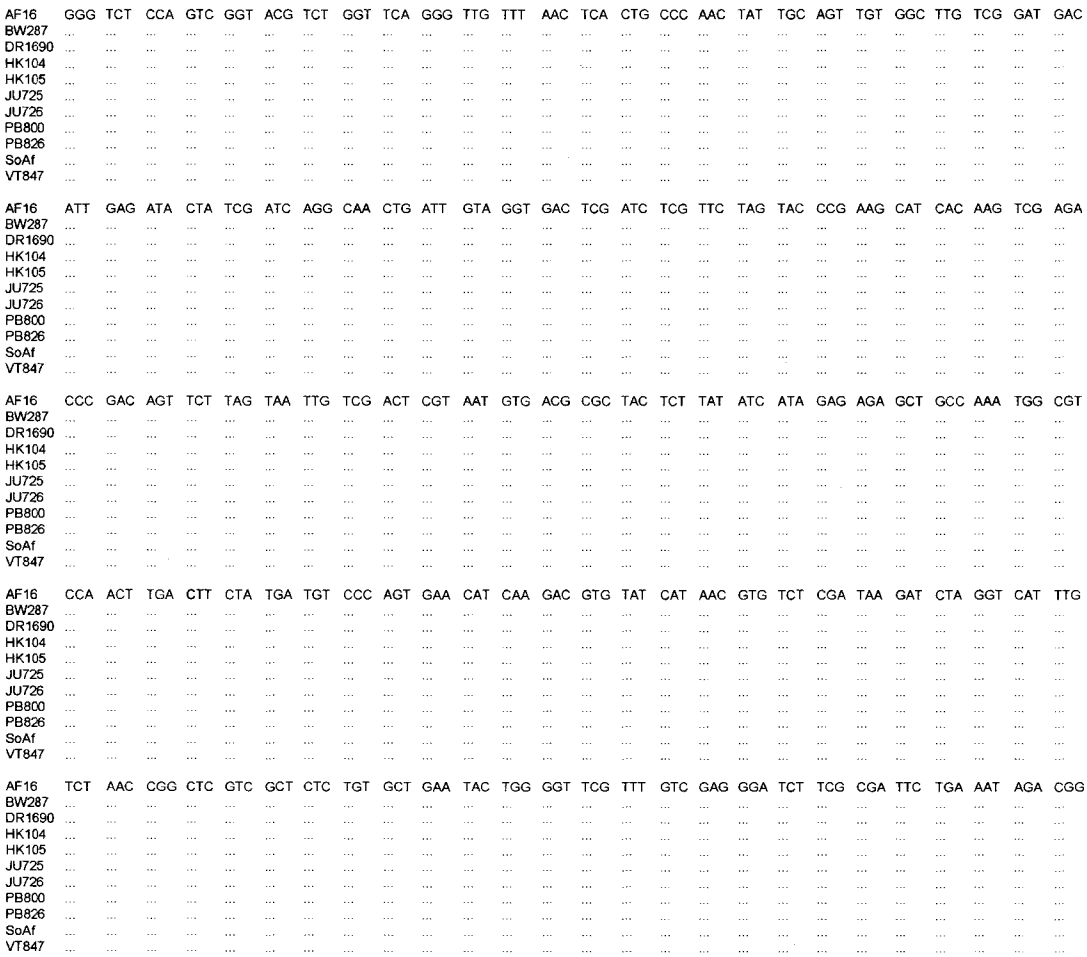


Figure 4. Alignment of 390bp of the ITS region demonstrating that all strains were molecularly identical at the ITS

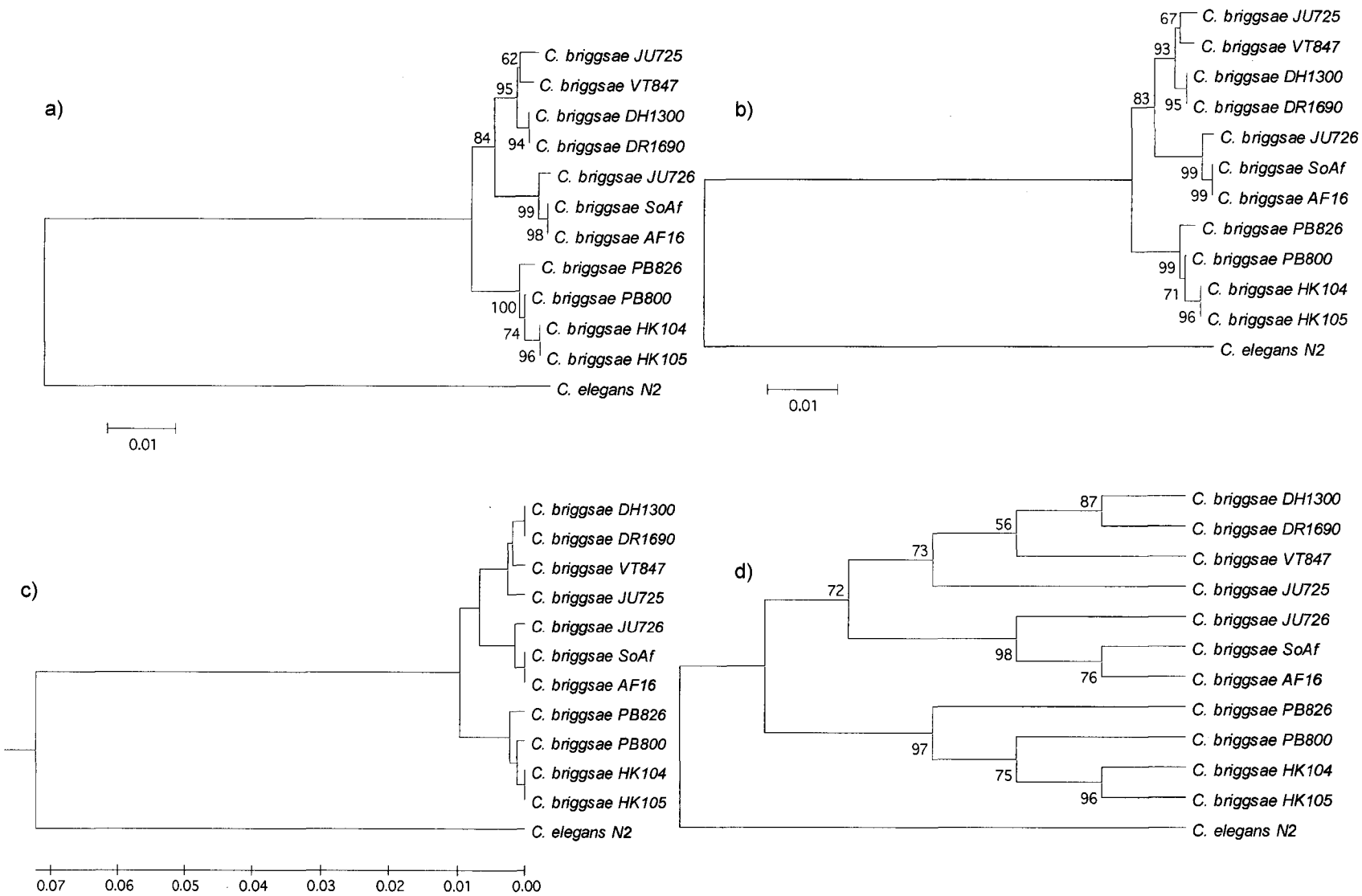


Figure 5. Phylogenetic trees of selected wild type isolates of *C. briggsae* based on 1381 bp of CO2 and ND5 and rooted with *C. elegans*
a) Neighbor-Joining Method b)Minimum Evolution c)UPGMA d) Maximum Parsimony

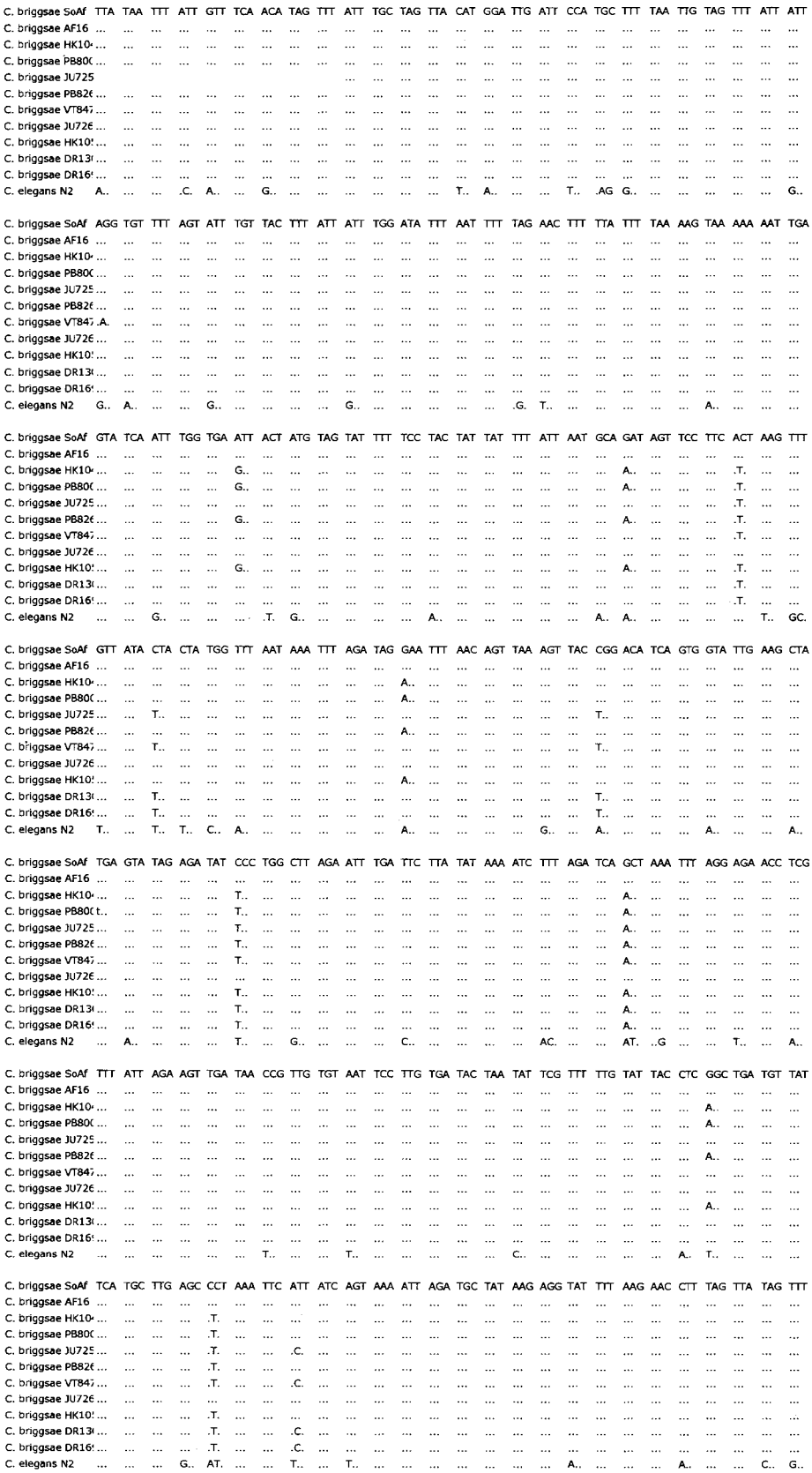


Figure 6. Alignment of concatenated sequences of CO2 and ND5 for all *C. briggsae* strains

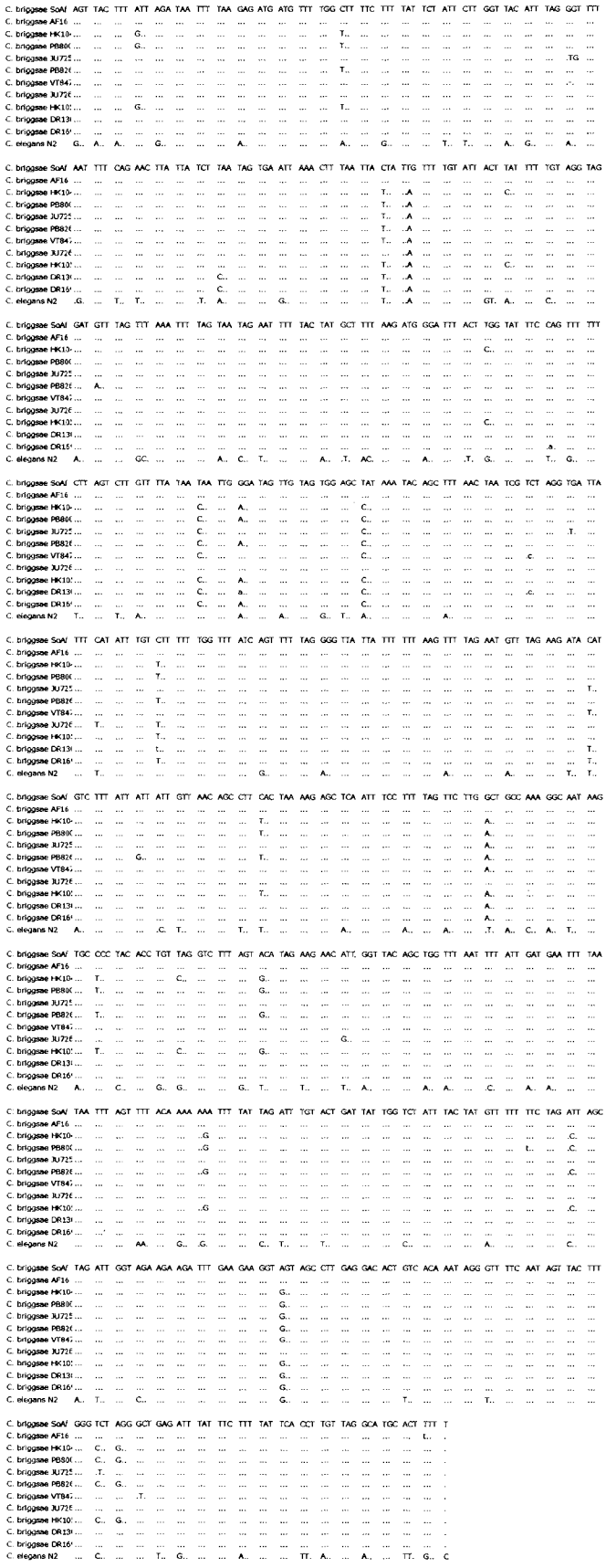


Figure 6 continued. Alignment of concatenated sequences of CO2 and ND5 for all *C. briggsae* strains

Table 5. Microsatellite loci surveyed a) loci information b) binned fragment results

a)

Locus	Locus ID	Repeat Motif
1	2852	ttc
2	2220	aag
3	NA15	tc
4	4228	ag
5	2454	ca
6	4404	aag

b)

Strain	Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6
AF16	483	487	352	408	470	490
SoAf	471	502	402	388	426	535
JU726	528	502	412	388	534	541
JU725	621	493	420	378	470	541
DH1300	450	520	412	394	450	511
DR1690	450	520	412	394	450	511
VT847	444	493	352	380	470	475
PB800	483	397	750	370	380	385
PB826	0	397	750	378	380	385
HK104	450	397	750	376	426	385
HK105	450	397	750	376	426	385

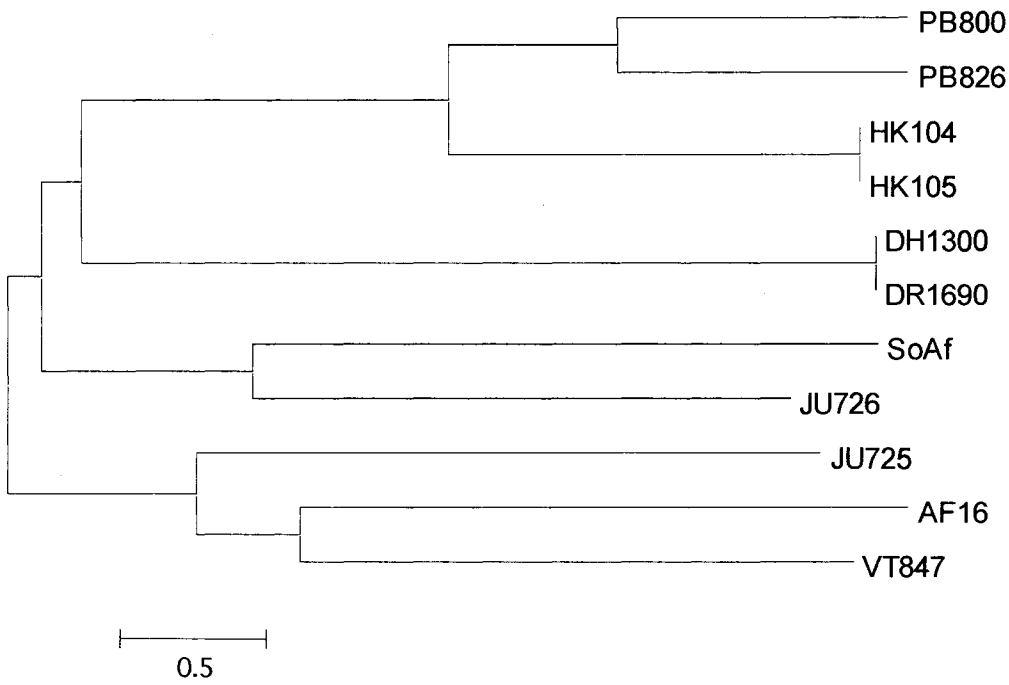


Figure 7. Neighbor-Joining phylogenetic tree of wild type isolate strains of *C. briggsae* based on overall allele similarity, Fst, across six microsatellite loci

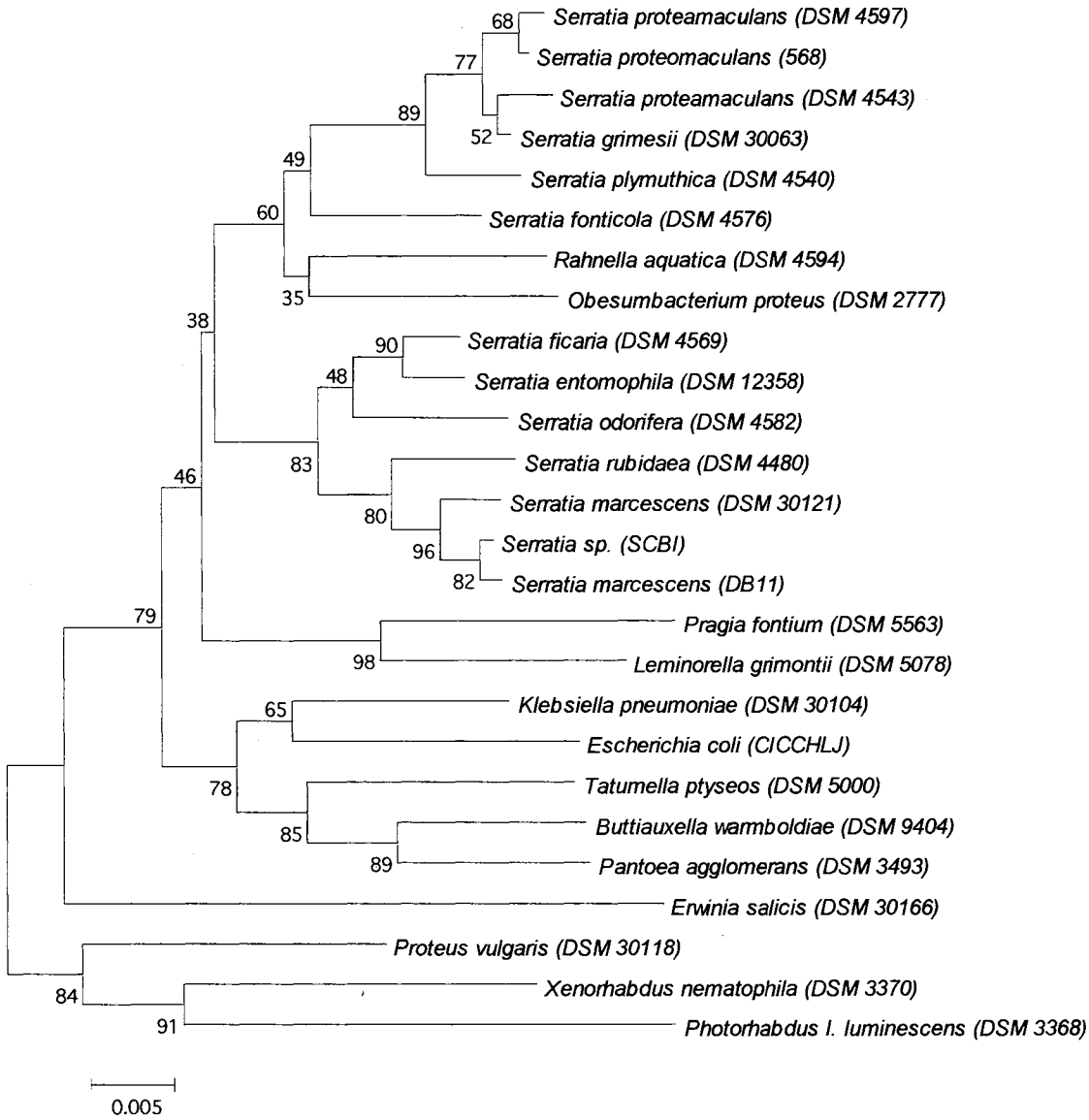


Figure 8. Neighbor-Joining phylogenetic tree based on 16S sequences of select proteobacteria including common entomopathogenic bacteria *Xenorhabdus* and *Photorhabdus* as well as recently described ectosymbiont *Serratia* sp. SCBI

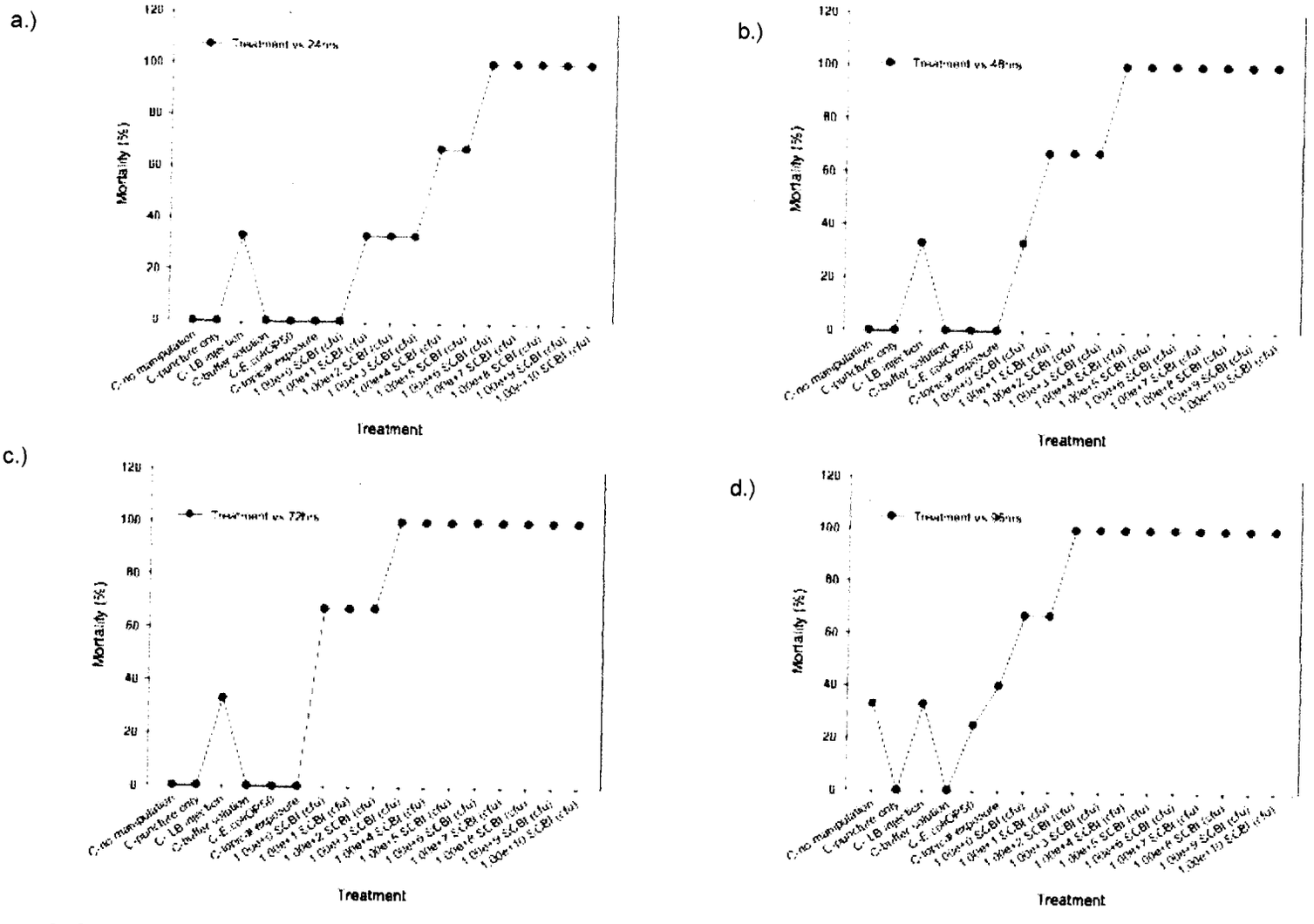


Figure 9. Comparing mortality of late instar *G. mellonella* larvae and concentration of hemocoelic *Serratia* sp. SCBI injections at a) 24 hours b) 48 hours c) 72 hours and d) 96 hours. At all time intervals there is significantly less mortality in control *G. mellonella* (C) compared to the SCBI injected *G. mellonella* (T)

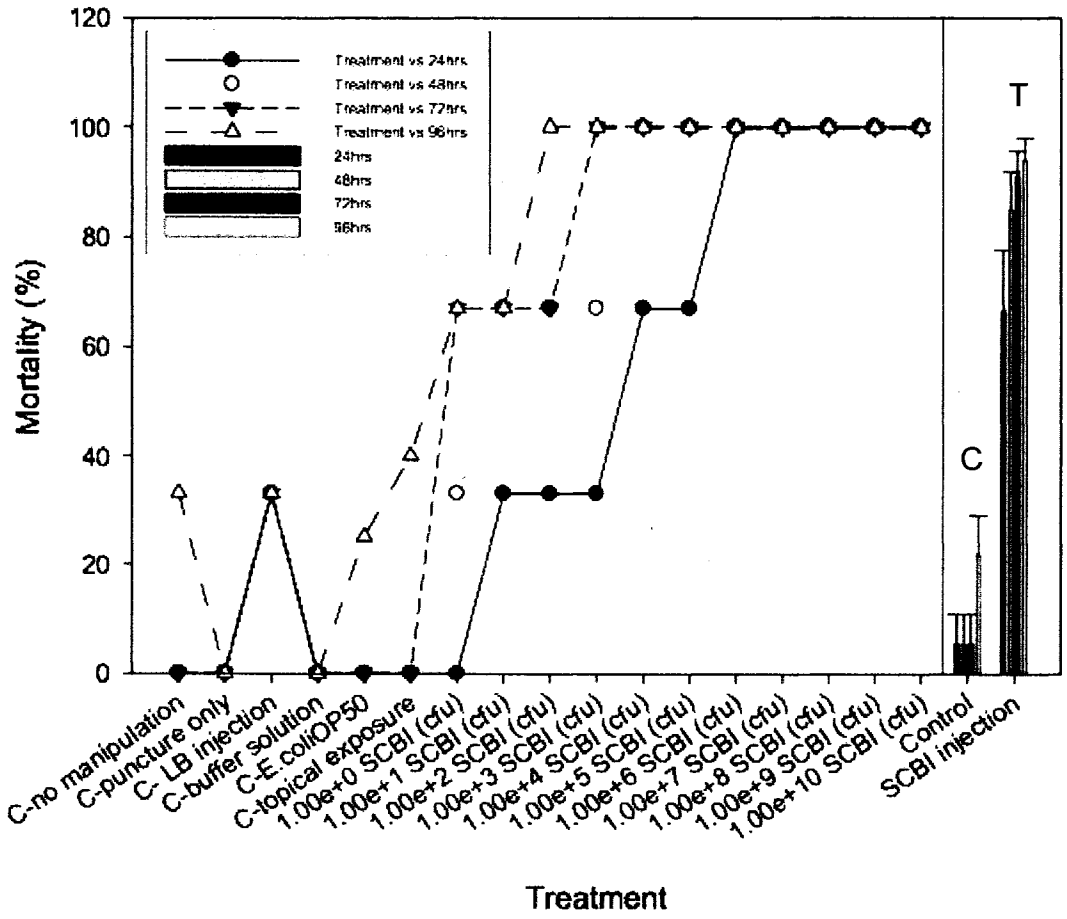


Figure 10. Summary of *G. mellonella* mortality at 24-hour intervals from 24 to 96 hours injected with varying concentrations of *Serratia* sp. SCBI. At all time intervals *G. mellonella* mortality was greater in the injected treatments (T) compared to the control treatments (C)

Table 6. Summary of *G. mellonella* mortality at 24-hour intervals from 24 to 96 hours comparing pooled injected concentrations of *Serratia* sp. SCBI and controls. Using a T-test statistic, all time intervals *G. mellonella* mortality was greater in the injected treatments compared to the control treatments

Dependent Variable: 24hrs					
Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	5.5	13.472	5.5
SCBI injection	11	0	66.636	36.607	11.037
Difference	-61.136				
t = -3.900 with 15 degrees of freedom. (P = 0.001)					

Dependent Variable: 48hrs					
Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	5.5	13.472	5.5
SCBI injection	11	0	84.909	22.915	6.909
Difference	-79.409				
t = -7.722 with 15 degrees of freedom. (P = <0.001)					

Dependent Variable: 72hrs					
Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	5.5	13.472	5.5
SCBI injection	11	0	91	15.414	4.648
Difference	-85.5				
t = -11.387 with 15 degrees of freedom. (P = <0.001)					

Dependent Variable: 96hrs					
Group Name	N	Missing	Mean	Std Dev	SEM
Control	6	0	21.833	17.566	7.171
SCBI injection	11	0	94	13.349	4.025
Difference	-72.167				
t = -9.551 with 15 degrees of freedom. (P = <0.001)					

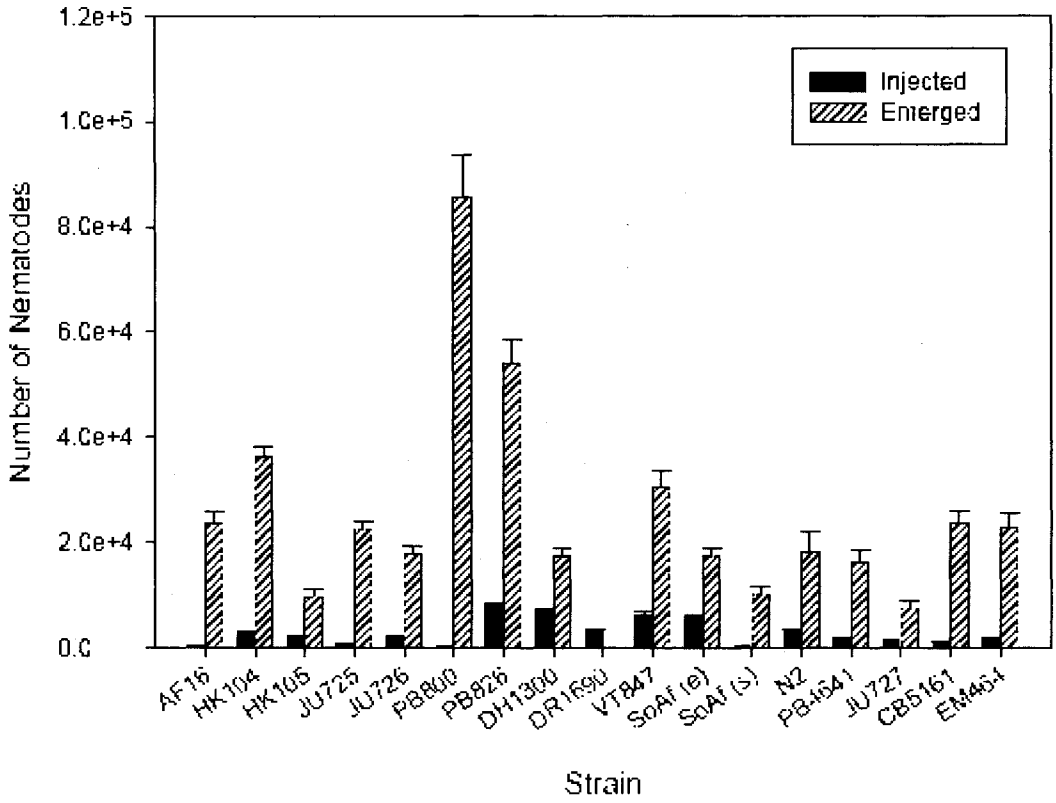


Figure 11. The number of injected nematodes into the late instar *G. mellonella* larvae were significantly less than the number of nematodes that emerged from the insect larvae cadavers in every strain, except DR1690

Table 7. Pairwise comparisons using T-test, the number of nematodes injected was significantly less than the number of nematodes that emerged from the insect larvae cadavers

Group	N	Missing	Median	25%	75%
Emerged	51	0	20000	12500	27500
Injected	51	0	2000	893.75	3687.5

T = 3752.500 n(small)= 51 n(big)= 51 (P = <0.001)

Table 8. Number of nematodes injected into and emerged from five *G. mellonella* for each strain in triplicate

Strain	Injected	Emerged	Adjusted Emergence	Proportional Emergence
AF16	350	20000	19650	57.14
AF16	425	23000	22575	54.12
AF16	500	27500	27000	55.00
HK104	2500	32500	30000	13.00
HK104	2750	36000	33250	13.09
HK104	3000	40000	37000	13.33
HK105	1750	7500	5750	4.29
HK105	2000	9500	7500	4.75
HK105	2250	12500	10250	5.56
JU725	600	20000	19400	33.33
JU725	775	25000	24225	32.26
JU725	950	22500	21550	23.68
JU726	2000	15000	13000	7.50
JU726	2250	18500	16250	8.22
JU726	2500	20000	17500	8.00
PB800	400	72500	72100	181.25
PB800	450	84500	84050	187.78
PB800	500	100000	99500	200.00
PB826	7500	47500	40000	6.33
PB826	8125	52000	43875	6.40
PB826	8750	62500	53750	7.14
DH1300	6250	15000	8750	2.40
DH1300	6875	17000	10125	2.47
DH1300	7500	20000	12500	2.67
DR1690	3125	0	0	0.00
DR1690	3437.5	0	0	0.00
DR1690	3750	0	0	0.00
VT847	5000	25000	20000	5.00
VT847	6250	31500	25250	5.04
VT847	7500	35000	27500	4.67
SoAf (e)	5000	15000	10000	3.00
SoAf (e)	5625	17500	11875	3.11
SoAf (e)	6250	20000	13750	3.20
SoAf (s)	350	7500	7150	21.43
SoAf (s)	450	10500	10050	23.33
SoAf (s)	550	12500	11950	22.73
N2	3000	12500	9500	4.17
N2	3250	17000	13750	5.23
N2	3500	25000	21500	7.14
PB4641	1500	12500	11000	8.33
PB4641	1750	16000	14250	9.14
PB4641	2000	20000	18000	10.00
JU727	1000	5000	4000	5.00
JU727	1250	7500	6250	6.00
JU727	1500	10000	8500	6.67
CB5161	750	20000	19250	26.67
CB5161	875	27500	26625	31.43
CB5161	1000	23000	22000	23.00
EM464	1500	17500	16000	11.67
EM464	1750	23000	21250	13.14
EM464	2000	27500	25500	13.75

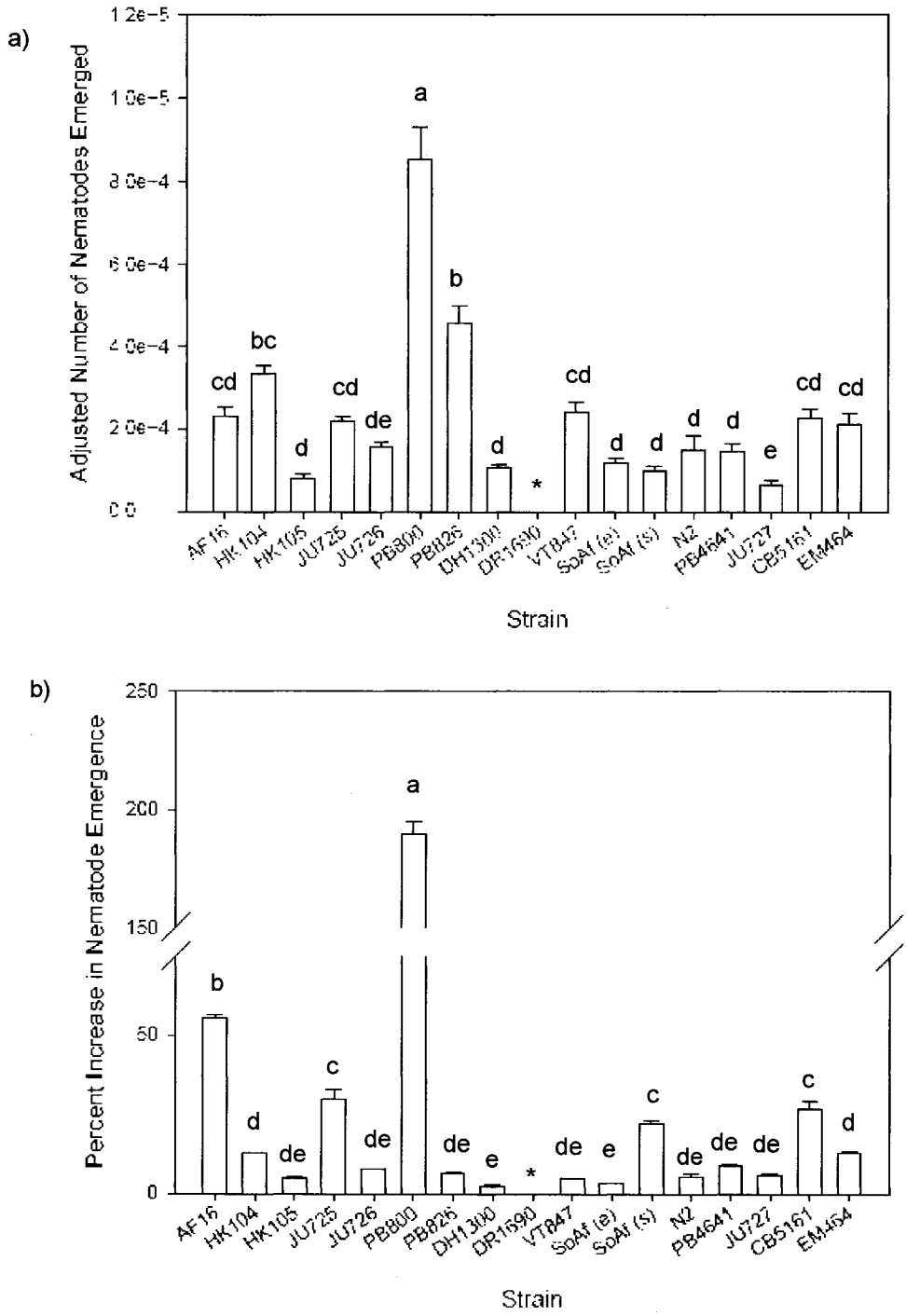


Figure 12. Direct comparisons between each strain using two emergence estimators: a) adjusted emergence and b) proportional increase emergence (* denotes zero emergence)

Table 9. Significant results for adjusted emergence estimator results using a) single factor ANOVA and b) Tukey's post hoc comparisons

a)

Source of Variation	DF	SS	MS	F	P
Between Groups	16	18512294485	1157018405	49.175	<0.001
Residual	34	799972500	23528602.94		
Total	50	19312266985			

b)

Strain Comparisons	Diff of Means	p	q	P	P<0.050
PB800 vs. DR1690	85216.667	17	30.429	<0.001	Yes
PB800 vs. JU727	78966.667	17	28.197	<0.001	Yes
PB800 vs. HK105	77383.333	17	27.632	<0.001	Yes
PB800 vs. SoAf (s)	75500	17	26.959	<0.001	Yes
PB800 vs. DH1300	74758.333	17	26.695	<0.001	Yes
PB800 vs. SoAf (e)	73341.667	17	26.189	<0.001	Yes
PB800 vs. PB4641	70800	17	25.281	<0.001	Yes
PB800 vs. N2	70300	17	25.103	<0.001	Yes
PB800 vs. JU726	69633.333	17	24.865	<0.001	Yes
PB800 vs. EM464	64300	17	22.96	<0.001	Yes
PB800 vs. JU725	63491.667	17	22.671	<0.001	Yes
PB800 vs. CB5161	62591.667	17	22.35	<0.001	Yes
PB800 vs. AF16	62141.667	17	22.189	<0.001	Yes
PB800 vs. VT847	60966.667	17	21.77	<0.001	Yes
PB800 vs. HK104	51800	17	18.497	<0.001	Yes
PB800 vs. PB826	39341.667	17	14.048	<0.001	Yes
PB826 vs. DR1690	45875	17	16.381	<0.001	Yes
PB826 vs. JU727	39625	17	14.149	<0.001	Yes
PB826 vs. HK105	38041.667	17	13.584	<0.001	Yes
PB826 vs. SoAf (s)	36158.333	17	12.911	<0.001	Yes
PB826 vs. DH1300	35416.667	17	12.646	<0.001	Yes
PB826 vs. SoAf (e)	34000	17	12.141	<0.001	Yes
PB826 vs. PB4641	31458.333	17	11.233	<0.001	Yes
PB826 vs. N2	30958.333	17	11.055	<0.001	Yes
PB826 vs. JU726	30291.667	17	10.816	<0.001	Yes
PB826 vs. EM464	24958.333	17	8.912	<0.001	Yes
PB826 vs. JU725	24150	17	8.623	<0.001	Yes
PB826 vs. CB5161	23250	17	8.302	<0.001	Yes
PB826 vs. AF16	22800	17	8.141	<0.001	Yes
PB826 vs. VT847	21625	17	7.722	<0.001	Yes
HK104 vs. DR1690	33416.667	17	11.932	<0.001	Yes
HK104 vs. JU727	27166.667	17	9.701	<0.001	Yes
HK104 vs. HK105	25583.333	17	9.135	<0.001	Yes
HK104 vs. SoAf (s)	23700	17	8.463	<0.001	Yes
HK104 vs. DH1300	22958.333	17	8.198	<0.001	Yes
HK104 vs. SoAf (e)	21541.667	17	7.692	<0.001	Yes
HK104 vs. PB4641	19000	17	6.784	0.003	Yes
HK104 vs. N2	18500	17	6.606	0.004	Yes
HK104 vs. JU726	17833.333	17	6.368	0.007	Yes
VT847 vs. DR1690	24250	17	8.659	<0.001	Yes
VT847 vs. JU727	18000	17	6.427	0.006	Yes
VT847 vs. HK105	16416.667	17	5.862	0.018	Yes
AF16 vs. DR1690	23075	17	8.24	<0.001	Yes
AF16 vs. JU727	16825	17	6.008	0.014	Yes
AF16 vs. HK105	15241.667	17	5.442	0.038	Yes
CB5161 vs. DR1690	22625	17	8.079	<0.001	Yes
CB5161 vs. JU727	16375	17	5.847	0.018	Yes
CB5161 vs. HK105	14791.667	17	5.282	0.049	Yes
JU725 vs. DR1690	21725	17	7.758	<0.001	Yes
JU725 vs. JU727	15475	17	5.526	0.033	Yes
EM464 vs. DR1690	20916.667	17	7.469	<0.001	Yes
JU726 vs. DR1690	15583.333	17	5.564	0.03	Yes
N2 vs. DR1690	14916.667	17	5.326	0.046	Yes

Table 10. Significant results for proportional emergence estimator results using a) single factor ANOVA and b) Tukey's post hoc comparisons

a)

Source of Variation	DF	SS	MS	F	P
Between Groups	16	97126.944	6070.434	709.477	<0.001
Residual	34	290.911	8.556		
Total	50	97417.856			

b)

Strain Comparisons	Diff of Means	p	q	P	P<0.050
PB800 vs. DR1690	189.676	17	112.314	<0.001	Yes
PB800 vs. DH1300	187.163	17	110.825	<0.001	Yes
PB800 vs. SoAf (e)	186.572	17	110.476	<0.001	Yes
PB800 vs. HK105	184.812	17	109.434	<0.001	Yes
PB800 vs. VT847	184.774	17	109.411	<0.001	Yes
PB800 vs. N2	184.162	17	109.049	<0.001	Yes
PB800 vs. JU727	183.787	17	108.827	<0.001	Yes
PB800 vs. PB826	183.051	17	108.39	<0.001	Yes
PB800 vs. JU726	181.769	17	107.631	<0.001	Yes
PB800 vs. PB4641	180.517	17	106.89	<0.001	Yes
PB800 vs. EM464	176.823	17	104.703	<0.001	Yes
PB800 vs. HK104	176.535	17	104.532	<0.001	Yes
PB800 vs. SoAf (s)	167.18	17	98.993	<0.001	Yes
PB800 vs. CB5161	162.644	17	96.307	<0.001	Yes
PB800 vs. JU725	159.917	17	94.693	<0.001	Yes
PB800 vs. AF16	134.256	17	79.497	<0.001	Yes
AF16 vs. DR1690	55.42	17	32.816	<0.001	Yes
AF16 vs. DH1300	52.907	17	31.328	<0.001	Yes
AF16 vs. SoAf (e)	52.316	17	30.978	<0.001	Yes
AF16 vs. HK105	50.556	17	29.936	<0.001	Yes
AF16 vs. VT847	50.518	17	29.913	<0.001	Yes
AF16 vs. N2	49.907	17	29.551	<0.001	Yes
AF16 vs. JU727	49.531	17	29.329	<0.001	Yes
AF16 vs. PB826	48.795	17	28.893	<0.001	Yes
AF16 vs. JU726	47.513	17	28.134	<0.001	Yes
AF16 vs. PB4641	46.261	17	27.393	<0.001	Yes
AF16 vs. EM464	42.567	17	25.205	<0.001	Yes
AF16 vs. HK104	42.279	17	25.035	<0.001	Yes
AF16 vs. SoAf (s)	32.924	17	19.495	<0.001	Yes
AF16 vs. CB5161	28.388	17	16.81	<0.001	Yes
AF16 vs. JU725	25.662	17	15.195	<0.001	Yes
JU725 vs. DR1690	29.759	17	17.621	<0.001	Yes
JU725 vs. DH1300	27.245	17	16.133	<0.001	Yes
JU725 vs. SoAf (e)	26.655	17	15.783	<0.001	Yes
JU725 vs. HK105	24.895	17	14.741	<0.001	Yes
JU725 vs. VT847	24.856	17	14.718	<0.001	Yes
JU725 vs. N2	24.245	17	14.356	<0.001	Yes
JU725 vs. JU727	23.87	17	14.134	<0.001	Yes
JU725 vs. PB826	23.133	17	13.698	<0.001	Yes
JU725 vs. JU726	21.851	17	12.939	<0.001	Yes
JU725 vs. PB4641	20.6	17	12.198	<0.001	Yes
JU725 vs. EM464	16.905	17	10.01	<0.001	Yes
JU725 vs. HK104	16.617	17	9.84	<0.001	Yes
CB5161 vs. DR1690	27.032	17	16.006	<0.001	Yes
CB5161 vs. DH1300	24.519	17	14.518	<0.001	Yes
CB5161 vs. SoAf (e)	23.928	17	14.169	<0.001	Yes
CB5161 vs. HK105	22.168	17	13.126	<0.001	Yes
CB5161 vs. VT847	22.13	17	13.104	<0.001	Yes
CB5161 vs. N2	21.518	17	12.742	<0.001	Yes
CB5161 vs. JU727	21.143	17	12.519	<0.001	Yes
CB5161 vs. PB826	20.406	17	12.083	<0.001	Yes
CB5161 vs. JU726	19.124	17	11.324	<0.001	Yes
CB5161 vs. PB4641	17.873	17	10.583	<0.001	Yes
CB5161 vs. EM464	14.179	17	8.396	<0.001	Yes
CB5161 vs. HK104	13.89	17	8.225	<0.001	Yes
SoAf (s) vs. DR1690	22.496	17	13.321	<0.001	Yes
SoAf (s) vs. DH1300	19.983	17	11.833	<0.001	Yes
SoAf (s) vs. SoAf (e)	19.393	17	11.483	<0.001	Yes
SoAf (s) vs. HK105	17.633	17	10.441	<0.001	Yes
SoAf (s) vs. VT847	17.594	17	10.418	<0.001	Yes
SoAf (s) vs. N2	16.983	17	10.056	<0.001	Yes
SoAf (s) vs. JU727	16.608	17	9.834	<0.001	Yes
SoAf (s) vs. PB826	15.871	17	9.398	<0.001	Yes
SoAf (s) vs. JU726	14.589	17	8.639	<0.001	Yes
SoAf (s) vs. PB4641	13.338	17	7.898	<0.001	Yes
SoAf (s) vs. EM464	9.643	17	5.71	0.023	Yes
SoAf (s) vs. HK104	9.355	17	5.539	0.032	Yes
HK104 vs. DR1690	13.141	17	7.781	<0.001	Yes
HK104 vs. DH1300	10.628	17	6.293	0.008	Yes
HK104 vs. SoAf (e)	10.038	17	5.944	0.015	Yes
EM464 vs. DR1690	12.853	17	7.611	<0.001	Yes
EM464 vs. DH1300	10.34	17	6.123	0.011	Yes
EM464 vs. SoAf (e)	9.749	17	5.773	0.021	Yes
PB4641 vs. DR1690	9.159	17	5.423	0.039	Yes

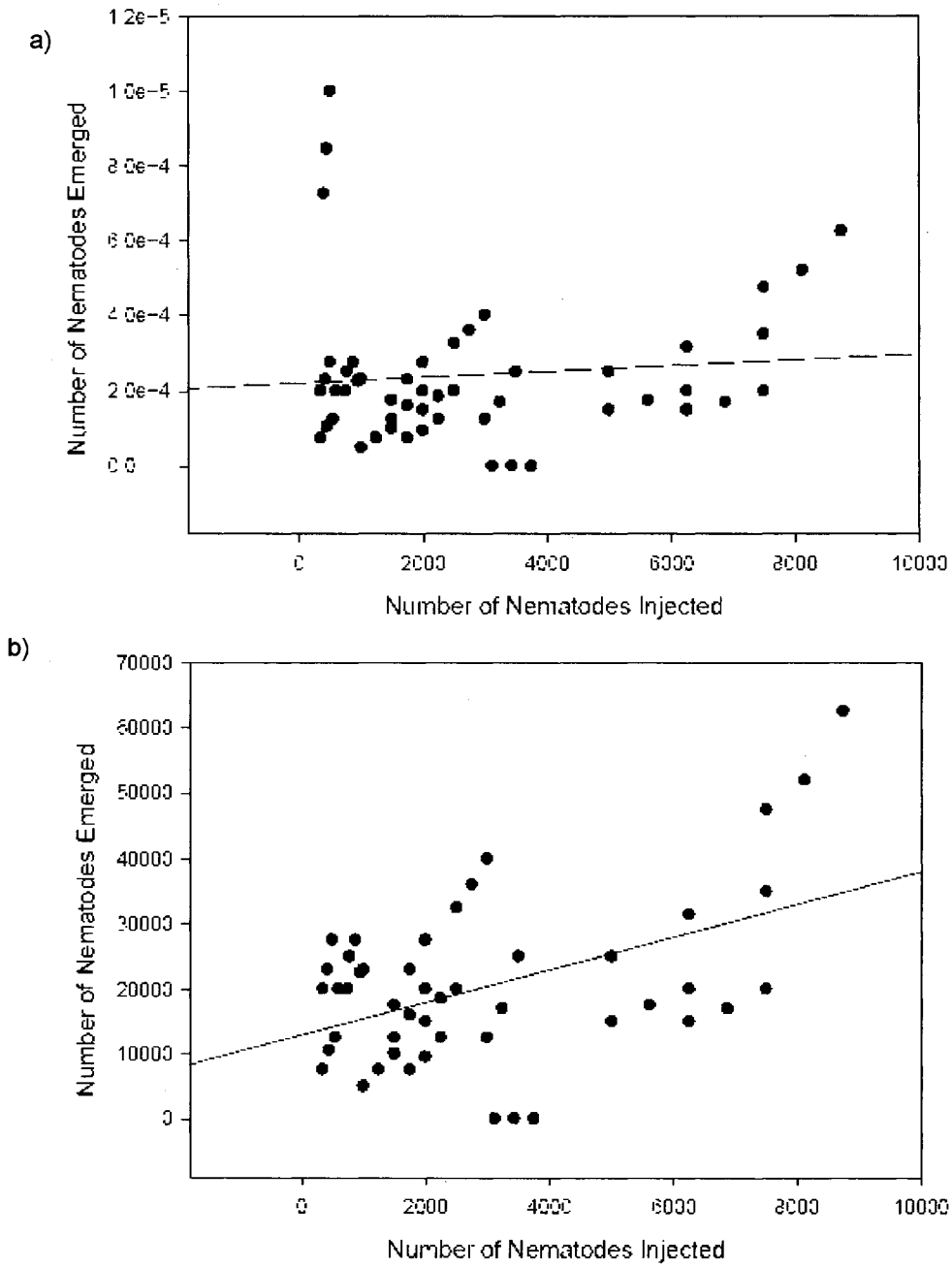


Figure 13. Correlations between number of nematodes injects compared to the number that emerged from the insect larvae cadavers a) all strain data included (adj R^2 = 0.01 p-value = 0.5) b) all strain data included except PB800 (adj R^2 = 0.2 p-value <0.05)

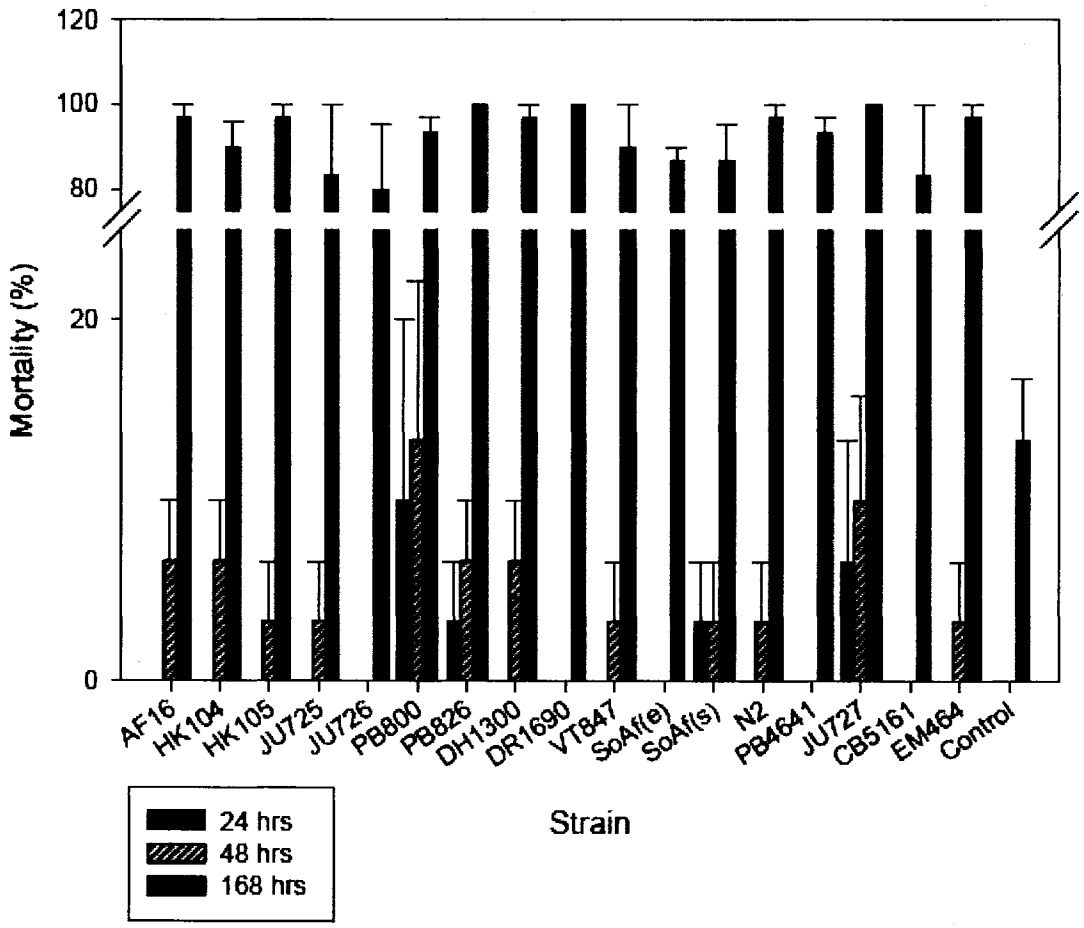


Figure 14. Late instar *G. mellonella* larvae mortality at 24, 48 and 168 hours post exposure to strains of nematodes previously emerged from *G. mellonella* cadavers

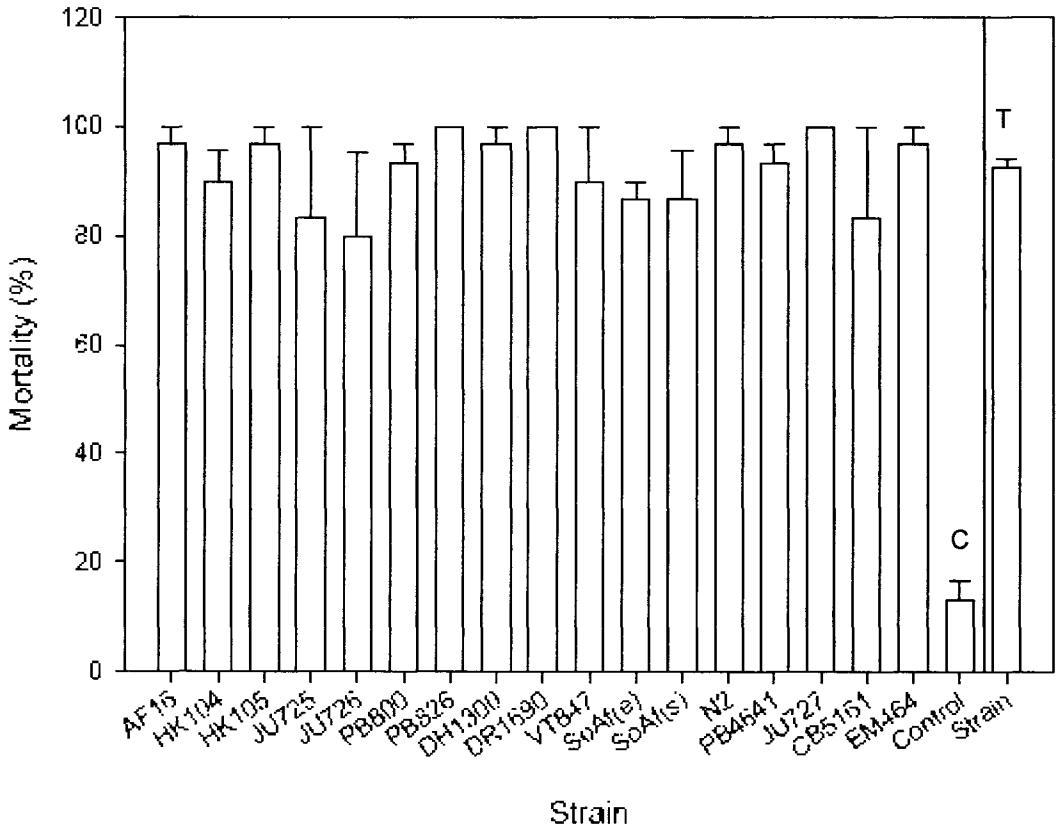


Figure 15. The mortality in control treatments (C), or unexposed late instar *G. mellonella*, was significantly lower than in experimental treatments (T) late instar *G. mellonella* larvae mortality at168 hours post exposure to strains of nematodes emerged from previous *G. mellonella* cadavers

Table 11. Significant results for comparing mortality at 168 hours between experimental and control treatments using a) single factor ANOVA and b) Dunnett's post hoc comparisons

a)

Source of Variation	DF	SS	MS	F	P
Between Groups	17	3.68	0.216	3.625	<0.001
Residual	36	2.15	0.0597		
Total	53	5.83			

b)

Comparison	Diff of Means	q'	P<0.050
Control vs. JU727	1.202	6.023	Yes
Control vs. PB826	1.202	6.023	Yes
Control vs. DR1690	1.202	6.023	Yes
Control vs. N2	1.094	5.486	Yes
Control vs. EM464	1.094	5.486	Yes
Control vs. AF16	1.094	5.486	Yes
Control vs. HK105	1.094	5.486	Yes
Control vs. DH1300	1.094	5.486	Yes
Control vs. VT847	1.009	5.055	Yes
Control vs. PB800	0.987	4.948	Yes
Control vs. PB4641	0.987	4.948	Yes
Control vs. JU725	0.94	4.711	Yes
Control vs. CB5161	0.94	4.711	Yes
Control vs. HK104	0.94	4.711	Yes
Control vs. SoAf(s)	0.901	4.517	Yes
Control vs. JU726	0.833	4.173	Yes
Control vs. SoAf(e)	0.833	4.173	Yes

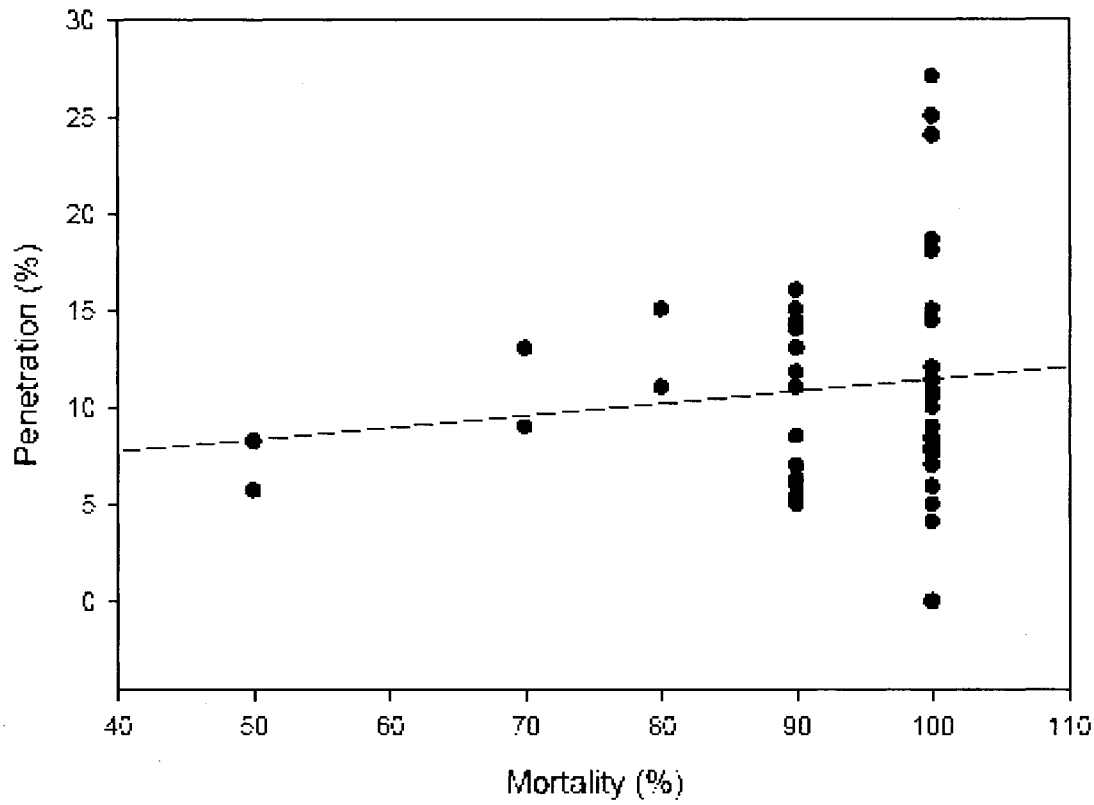


Figure 16. No significant correlation was found between percent mortality of late instar *G. mellonella* larvae at 168 hrs and percent penetration, or nematode load (adj R^2 = 0.00 and p-value = 0.366)

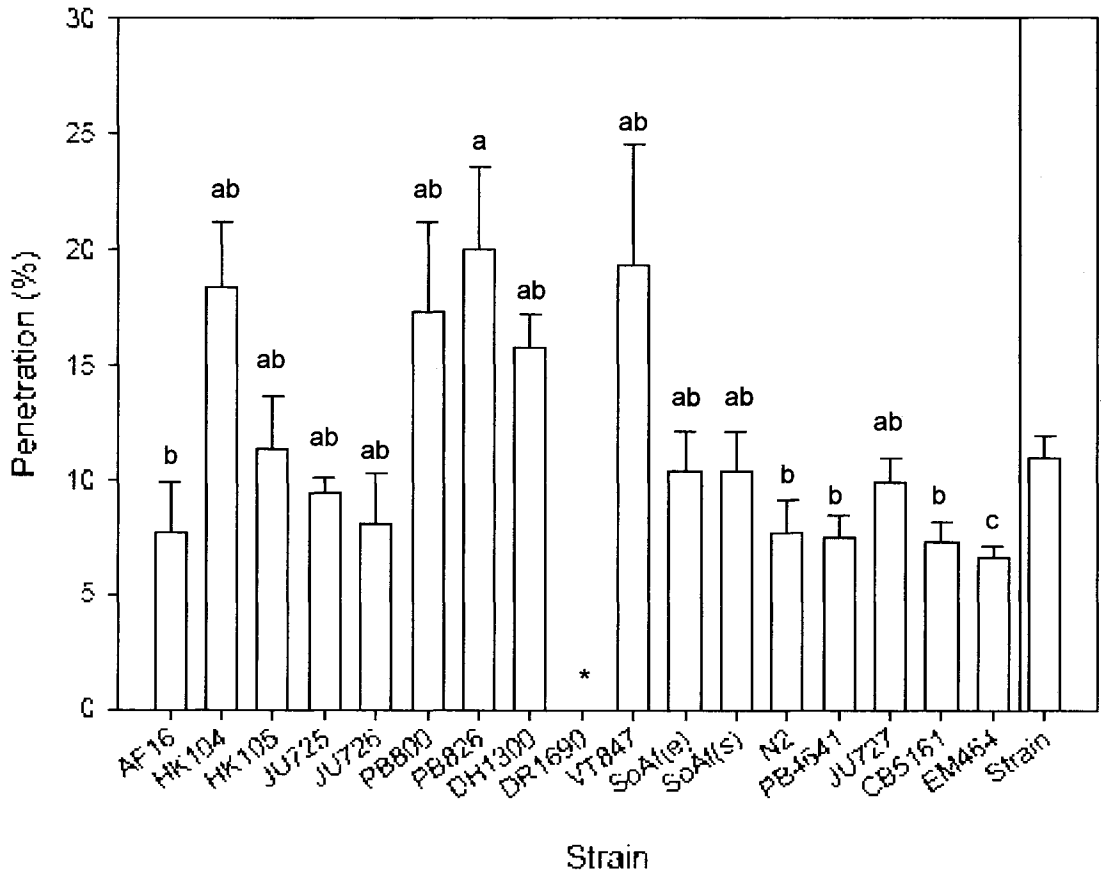


Figure 17. Penetration rate into late instar *G. mellonella* larvae168 hours post exposure to strains of nematodes emerged from previous *G. mellonella* cadavers. Average penetration rate was 11% and ranged from 4% - 27% (* denotes strains with a penetration rate of zero)

Table 12. Significant results for penetration rates between strains using a) single factor ANOVA and b) Tukey's post hoc comparisons

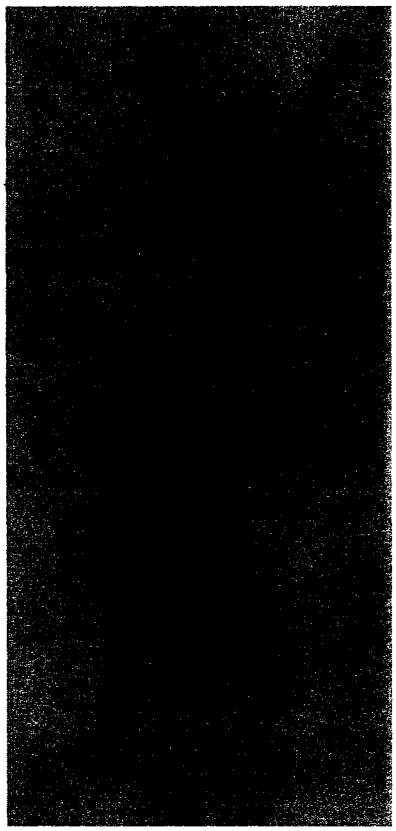
a)

Source of Variation	DF	SS	MS	F	P
Between Groups	16	0.556	0.0348	9.959	<0.001
Residual	34	0.119	0.00349		
Total	50	0.675			

b)

Strain Comparisons	Diff of Means	p	q	P	P<0.050
PB826 vs. DR1690	0.461	17	13.508	<0.001	Yes
PB826 vs. EM464	0.202	17	5.92	0.016	Yes
PB826 vs. CB5161	0.188	17	5.512	0.033	Yes
PB826 vs. PB4641	0.186	17	5.451	0.037	Yes
PB826 vs. AF16	0.185	17	5.429	0.038	Yes
PB826 vs. N2	0.183	17	5.358	0.044	Yes
VT847 vs. DR1690	0.447	17	13.098	<0.001	Yes
VT847 vs. EM464	0.188	17	5.51	0.033	Yes
HK104 vs. DR1690	0.44	17	12.911	<0.001	Yes
HK104 vs. EM464	0.182	17	5.323	0.046	Yes
PB800 vs. DR1690	0.425	17	12.469	<0.001	Yes
DH1300 vs. DR1690	0.408	17	11.965	<0.001	Yes
HK105 vs. DR1690	0.34	17	9.96	<0.001	Yes
SoAf(s) vs. DR1690	0.325	17	9.525	<0.001	Yes
SoAf(e) vs. DR1690	0.325	17	9.525	<0.001	Yes
JU727 vs. DR1690	0.319	17	9.346	<0.001	Yes
JU725 vs. DR1690	0.311	17	9.106	<0.001	Yes
JU726 vs. DR1690	0.282	17	8.258	<0.001	Yes
N2 vs. DR1690	0.278	17	8.15	<0.001	Yes
AF16 vs. DR1690	0.276	17	8.079	<0.001	Yes
PB4641 vs. DR1690	0.275	17	8.057	<0.001	Yes
CB5161 vs. DR1690	0.273	17	7.996	<0.001	Yes
EM464 vs. DR1690	0.259	17	7.588	<0.001	Yes

a)



b)

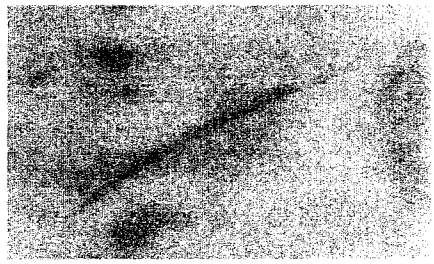


Figure 18. Pictures of *Caenorhabditis* sp. 5 strain JU727 isolated from infected late instar *Galleria mellonella* larvae from the penetration assay experiment a) adult, eggs present b) juvenile stage

CHAPTER IV

DISCUSSION, CONCLUSION, AND FUTURE INVESTIGATON

Discussion

The overall goals of this project were to 1) taxonomically identify the putative entomopathogenic *Caenorhabditis* from South Africa through the use of molecular markers, 2) employ the same and additional molecular markers to compare the putative entomopathogenic *C. briggsae* SoAf to other wild type isolate strains of *C. briggsae*, 3) to taxonomically identify and 4) evaluate the effectiveness of the isolated bacterium *Serratia* sp. SCBI, and 5) to evaluate putative entomopathogenic behavior in *C. briggsae* SoAf and other closely related taxa. Ribosomal, mitochondrial and nuclear markers were used to evaluate molecular similarity between wild type isolates of *C. briggsae* and *C. briggsae* SoAf. *C. briggsae* SoAf and members of the *elegans* group of *Caenorhabditis* were evaluated for potential entomopathogenic characteristics using the isolated associated bacterium *Serratia* sp. SCBI.

Goal 1: Taxonomic Identification of the Putative Entomopathogenic Nematode

It was hypothesized that the putative entomopathogenic *C. briggsae* SoAf was in fact most similar to other wild type *C. briggsae* in contrast to the previous identification as *Pseudodiplogasteriodes n.* sp. (Jumba *et al.* 2006). The

previous identification was the result of close sequence similarity to an accession in GenBank of *Panagrolaimus* sp. strain BW287 that was contaminated with *C. briggsae*, thus explaining the high level of sequence similarity. Evidence to support this hypothesis was obtained using ribosomal and mitochondrial markers. Through sequencing the ITS region and subsequent alignments with other *C. briggsae*, it was determined that the putative entomopathogenic *C. briggsae* SoAf was identical to the other wild type isolates of *C. briggsae* (Fig 4). The ITS region was also sequenced for *Panagrolaimus* sp. strain BW287, the strain was received from the Caenorhabditis Genetics Center, independently to confirm contamination hypothesis and was also subsequently identified at *C. briggsae*.

Goal 2: Molecular Comparisons of Isolates of *C. briggsae*, Including *C. briggsae* SoAf

Mitochondrial genes cytochrome oxidase two (CO2) and NADH dehydrogenase (ND5) were analyzed to further evaluate the relationship between wild type isolates of *C. briggsae* and *C. briggsae* SoAf. The resulting topology of the phylogenetic tree using concatenated mitochondrial sequences was consistent with previous studies of wild type isolates of *C. briggsae* (Thomas and Wilson 1991; Graustein *et al.* 2003; Cutter *et al.* 2006; Howe and Denver 2007) (Fig 5). As originally proposed by Cutter *et al.* (2006), the results imply a possible correlation between latitude of collection and molecular phylogeny with strains in clade one (AF16, SoAf, JU726, VT847, JU725) isolated from latitudes

ranging from 20° 57' N to 24° 46' N and clade two (HK104, HK105, PB800, PB826) ranging from 34° 40' N to 39° 53' N, with the possible exception of strains DR1690 and DH1300 because collection information is unknown (Fig 5). Cutter *et al.* (2006) suggested that the differences between temperate and tropical could potentially be explained through a recent colonization and expansion of *C. briggsae* within the temperate regions from a small founding population. However, additional strain collection and analysis is necessary to demonstrate more support for this hypothesis.

This study also revealed three pairs of strains with identical sequences within the pair for mitochondrial sequences, strains AF16 and SoAf, HK104 and HK105, and DH1300 and DR1690 were identical. Similarity between haplotypes across geographic locations was not unexpected. This is a common finding in natural isolates of *C. elegans* and *C. briggsae*, where low global phylogeographic structure, and high local genetic diversity has been documented (Graustein *et al.* 2003; Sivasunder and Hey 2003; Haber *et al.* 2005; Barrière and Félix 2005; reviewed in Barrière and Félix 2006). As a result, there was a high degree of similarity of natural isolate strains of *C. elegans* from varying geographic locations (Denver *et al.* 2003; Cutter 2006). It is hypothesized that the reduction in variation overall is likely the result of low rate of out-crossing, lack of production of males due to the reproductive strategy of hermaphroditic selfing, and observed increased frequency and length of life-span spent in dauer J3 stage. This suggests there is a lower reproductive rate in wild populations compared to laboratory conditions, which would reduce overall global diversity

(reviewed in Barrière and Félix 2006). It is presumed that, hermaphroditic selfing and low dispersal are likely driving increased local genetic diversity among geographically close populations. Haplotype similarity among natural isolates of *C. briggsae* across geographic regions has been noted in previous studies; high sequence similarity between strains from France and Iceland, as well as similarities between strains HK104 and HK105 were observed by Cutter *et al.* (2006). The discovery that similar mitochondrial haplotypes occurred across geographic regions was not unexpected, but to determine whether the resulting similarity between *C. briggsae* SoAf and AF16 was the consequence of contamination or confusion, six microsatellite loci were used to analyze the relationship between all *C. briggsae* wild-type strains assayed.

The microsatellite loci surveyed were sufficiently large to ensure sensitivity for fine-scale molecular comparisons between the *C. briggsae* strains without a bias toward overestimating differences resulting from extreme rapidly evolving loci. The results of the microsatellite survey indicated allele differences at all six loci between strain AF16 and SoAf (Fig 7 and Table 5). While having identical mitochondrial sequences, differences between AF16 and SoAf at all six nuclear markers indicate a disconnect between maternally inherited mitochondrial DNA and bi-parentally inherited nuclear DNA. In a completely hermaphroditic selfing clonal population, similar phylogenetic patterns would be observed when comparing both mitochondrial and nuclear markers. These results suggest that there may be a higher incidence of males than previously thought, and influencing the phylogenetics of *C. briggsae*. Also, the microsatellite assay failed

to differentiate between mitochondrial identical pairs HK104 and HK105 as well as DR1690 and DH1300. Collection records for strains DH1300 and DR1690 from the *Caenorhabditis* Genetics Center indicated that DH1300, previously known as *C. briggsae* BO, is a subclone of DR1690, previously *C. briggsae* Zuckerman; both strains were noted to have been in culture for a long period of time and were likely to have accumulated mutations. Thus, a high degree of similarity between the two strains would be expected, and consequently were utilized as controls to ensure that appropriate loci were chosen for this analysis, as they have been in separate cultures since 1980 (Fodor *et al.* 1993). The use of sibling strains with a common ancestor originating 25 years ago in this assay (DR1690 and DH1300), ensures that the resulting alleles observed in SoAf did not arise post-isolation from nature. Also, strains HK104 and HK105 remained molecularly identical across all microsatellite loci. The strains HK104 and HK105 were collected in different locations in Japan, suggesting a possible single colonization event occurred and all strains share the same genotype in Japan, or could represent a common genotype among natural isolates. However, more extensive sampling would be required to provide sufficient support for that hypothesis. Also, population genetic diversity trends in natural isolate strains of *C. briggsae* could be similar to those determined in *C. elegans*, and not yet observed due to a small sample size and relatively few over all collections.

However, the goal of this aspect of the project was to taxonomically identify the putative entomopathogenic strain of *C. briggsae* and to examine the relationship between wild type isolates of *C. briggsae* and the putative

entomopathogenic *C. briggsae.* The results definitively determined the South African strain to indeed be *C. briggsae* and share the same mitochondrial haplotype as the sequenced strain, AF16. The life history of the putative entomopathogenic *C. briggsae* is interesting and may provide additional information and insight on the associations between invertebrates and nematodes in the *elegans* group of *Caenorhabditis.* The putative entomopathogenic *C. briggsae* was assumed to be entomopathogenic because of the manner in which it was collected. Therefore, a need to qualify the entomopathogenic properties of the South African isolate, evaluate potential entomopathogenic properties in other wild type isolates of *C. briggsae* and other *Caenorhabditis*, as well as identifying and evaluating the associated bacterium was necessary.

Goal 3: Taxonomic Identification of the Associated Bacterium

Serriatia marcescens strain DB11 was identified as the sequence most closely related to the associated bacterium through sequencing the rRNA gene, 16S (Fig 8); the sequences were not identical and the bacterium was subsequently named *Serratia* sp. SCBI (Morris *pers comm.*; Abeb-Akele *pers comm.*). As a result, F. Abebe-Akele in the WK Thomas Lab at the University of New Hampshire is completing further investigation comparing the completed genome sequences of *S. marcescens* DB11 and *S. proteomaculans* 568 to the isolated *Serratia* sp. SCBI, sharing greatest genomic similarity to *S. marcescens* DB11 (Abebe-Akele *pers comm.*).

The bacterial species complex *Serratia marcescens* is cosmopolitan with diverse life histories, strains have been identified as free-living soil microbes, causative agents in nosocomial infections in humans, an insect symbiont (Jackson *et al.* 2004), insect pathogens (Sikorowski and Lawrence 1998; McNeill 2000; Martin 2002; Tan *et al.* 2006), and in some cases a nematode pathogen (Schulenberg and Ewbank 2004; Zhang *et al.* 2005; Pradel *et al.* 2007), among many other lifestyles. Therefore, it is not surprising that the putative bacterial symbiont in this system is likely a member of the *Serratia* species complex because of the diversity of life history traits and ubiquitous nature. The probability of an opportunistic association is high if two species are commonly found in the same region and the cost of association is low and benefits are high (Poulin 2007). Therefore, since the bacterium is commonly found in the soil an association with the putative entomopathogenic *C. briggsae* would not be unexpected.

The associations between nematode and bacterium in classical entomopathogenic complexes is both highly specific and highly evolved; the bacterium provides the nematode with a reliable food source through the release of virulence factors killing the insect and antimicrobial and antifungal compounds to prevent growth of other bacteria, while the nematode acts as a vector for the transmission of the bacterium (Sicard *et al.* 2004; Sicard *et al.* 2005; Sicard *et al.* 2006). The two common genera identified with entomopathogenic nematodes are *Xenorhabdus* and *Photorhabdus*, associated with *Steinernema* and *Heterorhabditis*, respectively. Although distantly related, similar to both

entomopathogenic symbionts *Photorhabdus* and *Xenorhabdus, Serratia marcescens* is a member of the family *Enterobacteriaceae* (Spröer *et al.* 1999). Insecticidal toxins isolated from *Serratia* spp. were found to have high similarity to toxins produced by common insect pathogens, *Yersinia frederiksenii* and *Photorhabdus luminescens* (Hurst *et al.* 2000; Dodd *et al.* 2006).

In contrast to the variety of life history traits observed in the *Serratia* species complex, nearly all species of *Xenorhabdus* and *Photorhabdus* are entomopathogenic, with relatively few clinical strains, which are primarily saprophytic (Forst *et al.* 1997). The effectiveness and virulence of each entomopathogenic bacterium is dependent upon host specificity, as well as the host immune response (Li *et al.* 2007). Both classical entomopathogenic bacteria *Photorhabdus* and *Xenorhabdus* have been well studied and well documented insect pathogens (Ciche and Ensign 2003; Forst and Nealson 1996; Forst *et al.*1997). This study demonstrates similarities in pathogenicity observed in common entomopathogenic bacteria and the recently isolated *Serratia* sp. SCBI.

Goal 4: Entomopathogenic Properties of the Bacterium

While virulence varies within the genus *Photorhabdus*, it is commonly reported that LD_{50} in late instar *G. mellonella* larvae is reached with hemocoelic injections of 30 cells or less of *Photorhabdus* (Ciche and Ensign 2003). Initial hemocoelic injections of five cells of *Xenorhabdus* species have been documented to kill *G. mellonella* with 90-100% mortality within 48 hours (Forst

and Nealson 1996). Similar analysis of *Serratia* sp. SCBI demonstrated 100% mortality of late instar *G. mellonella* within 24 hours post-injection of 10^6 colony forming units (cfu) *Serratia* sp. SCBI and 10^4 cfu *Serratia* sp. SCBI within 48 hours (Figs 9, 10). While 100% mortality was reached with injections of 10^3 cfu *Serratia* sp. SCBI within 96 hours (Figs 9, 10). Mortality of insect larvae injected with concentrations of *Serratia* sp. SCBI was significantly greater than all control manipulations, including topical exposure (Figs 9, 10). While the number of cells necessary to yield nearly 100% mortality within 48 hours post injection of late instar *G. mellonella* larvae was greater for *Serratia* sp. SCBI than both commonly reported values for *Xenorhabdus* and *Photorhabdus*, this experiment sufficiently demonstrated that *Serratia* sp. SCBI was effective at killing insect larvae when injected directly into the hemocoel. However, insects infected by the classical entomopathogenic bacterium generally turn red, the insect infected by *Serratia* sp. SCBI turned black. Host response to the bacterial infection from *Serriatia* sp. SCBI is likely different from infections by *Photorhabdus* and *Xenorhabdus* as evident by the disparity of color change in the insect cadavers. This change in color is likely the result of differences in toxins and other antimicrobial and antifungal compounds released by the invading bacterium coupled with a different host immune response trigger.

The possibility that *Serratia* sp. SCBI a suitable entomopathogenic symbiont is evident through the insect pathogenicity studies. However, it is important to note that this study does not definitively demonstrate that *Serratia* sp. SCBI is the entomopathogenic bacterial symbiont of *C. briggsae* SoAf.

While *Serratia* sp. SCBI was isolated from *C. briggsae* SoAf in a monoculture suggesting an association between the two, selection for easily culturable bacterium may have occurred and other bacterium are likely present. However unlikely, it is possible that other bacteria may play a greater role in the entomopathogenic interactions and may in fact be the causative agent in mortality of the insects. Attempts to culture additional bacterial species from *C. briggsae* SoAf collected from the wild may aid in elucidating the relationship between *C. briggsae* SoAf and *Serratia* sp. SCBI.

Goal 5: Entomopathogenic Properties of the Nematodes

While *C. briggsae* SoAf was isolated using common entomopathogenic trapping methods, it was important to definitively demonstrate capability of being entomopathogenic. Because entomopathogenic behavior had not be documented in any *Caenorhabditis*, determining if other wild type isolates of *C. briggsae* and other closely related taxa were capable of becoming entomopathogenic when the appropriate bacteria was present.

In the soil, nematodes and insects are constantly interacting and have likely coevolved (Blaxter 2003). Considering this fact, there are very few entomopathogenic nematodes; this is likely due to the many adaptations to prevent infection in insect larvae. Entomopathogenic nematodes must overcome many obstacles when trying to gain entry into the insect larvae hemocoel and subsequently the hemolymph, it is not merely attaching to the cuticle of the insect larvae and then simply entering through the anus or mouth or abrasion through

the integument. The first obstacle is simply finding insect larvae. Entomopathogenic nematodes exhibit both ambush and seeker foraging behavior including both standing and nictation behavior (Campbell *et al.* 1999; Lewis *et al.* 2006). Once in contact with a potential host additional obstacles to entry persist. Many insect larvae have adapted behaviors to prevent nematode invasions, such as meticulous and aggressive grooming to remove nematodes attached to the insect cuticle through brushing with legs, rubbing with an abrasive raster located on the ventral end of the abdomen, and scraping and chewing with mandibles (Koppenhöfer *et al.* 2007). The aforementioned only represent possible external inhibitors to infection. Internal inhibitors, such as host immune response in the form of encapsulation and melanization also affect the success of infection by entomopathogenic nematodes. Also, reductions in secondary invasions by nematodes entering an already infected host is reduced and is thought to be a result of chemical cues released by the infected host in response to pathenogenesis (Glazer 1997). However, it has been demonstrated that entomopathogenic nematodes generally show a preference to infect previously infected insect hosts compared to uninfected hosts because an uninfected host has a comparatively better immune system and thus there is a greater chance for mortality of the invading nematode (Grewal *et al.* 1993; Campbell *et al.* 1999).

The effectiveness of an entomopathogenic nematode is governed by the specificity and duration of the association between the bacteria and the nematode, the bacteria and the insect host as well as ability of the nematode to gain entry into the insect and transmit bacteria to the hemolymph. Thus, for the

putative entomopathogenic *C. briggsae* SoAf to be a successful entomopathogen it must be able to gain access into the insect larvae and to reproduce within the insect larvae on *Serratia* sp. SCBI.

Reproductive capability within the insect larvae

All strains of *C. briggsae* tested were capable of reproducing on associated bacterium *Serratia* sp. SCBI both when seeded on NGM agar and injected along with the nematodes into late instar *G. mellonella* larvae. For the reproductive assays, all strains were reared on NGM seeded with *Serratia* sp. SCBI and washed prior to injection into *G. mellonella* larvae to ensure sufficient supply of bacteria for nematode growth. White traps were used to collect emerging nematodes (White 1927; Kaya and Stock 1997). For all strains except DR1690, the number of nematodes injected into the insect larvae was significantly less than number of emerged nematodes from the insect cadavers after nine days (Fig 11). However, emergence for all strains, including DR1690, was observed at day 20. While the goal of this experiment was to simply determine if any or all of the strains tested were capable of reproducing within the insect larvae, interestingly we found variability in reproductive capabilities between some of the strains.

The greatest reproductive output was seen in *C. briggsae* PB800 in both adjusted and proportional emergence estimates (Fig 12). As previously mentioned, strain DR1690 demonstrated deficient capabilities of reproducing within the insect larvae. Because the goal of this experiment was not to directly

compare strains, insect larvae were not injected with the same number of nematodes across strains and the total number of bacteria cells injected along with nematodes was not controlled for each strain injection. Injections of each strain were of mixed populations and the lack of a strong correlation between the number of nematodes injected, and the number of nematodes emerging overall strains indicates that the number of nematodes injected is not the only factor in determining emergence (Fig 13). It is possible that variability in host immunity and total bacterial cells available as a food source at the time of injection may play a greater role in nematode reproduction within the insect larvae.

Ability to enter insect larvae hemocoel

To assess penetration capability, emergent nematodes from the white traps previously used to assess reproductive capability within the insect larvae in the presence of the bacterial associate *Serratia* sp. SCBI were collected (White 1927; Kaya and Stock 1997). As previously stated, to determine entomopathogenic capability, it was important to assess both the reproductive ability of the strains within the insect larva and the ability to get inside the insect larvae. Nematodes grown inside the insect larvae were used for the penetration assay to simulate continuation of the behavior in the wild.

Mortality rate of penetration assays for both *Steinernema* and *Heterorhabditis* after 48 hours yields approximately 95% mortality in late instar *G. mellonella* larvae (Caroli *et al.* 1996; Ricci *et al.* 1996). However, the penetration assays in this study only yielded near 100% mortality of *G. mellonella* at 168

hours post exposure, with mortality rates between zero and 20% at 48 hours post exposure (Figs 14, 15). Mortality at 168 hours was significantly greater than control insect larvae indicating a strong causative impact (Fig 15). The mortality at 48 hours post exposure is quite diminished compared to traditional entomopathogenic nematodes. However, as previously demonstrated, more bacterial cells are required to kill the insect larvae than traditional entomopathogenic bacteria. Since the bacterial load per nematode is unknown, it is possible that the number of bacterial cells carried is such that a greater number of nematodes are needed to effectively kill the insect larvae in this system compared to other entomopathogenic systems. A lack of correlation between number of nematodes penetrating the insect larvae and the mortality at 168 hours indicate that the number of nematodes penetrating has little impact on mortality rate (Fig 16). However, this aspect of the study should be completed as a time trial to evaluate the impact of the percent penetration on mortality as exposure time increases (Fig 16). Also, to determine average bacterial load the transmission efficiency should be calculated to determine how many bacterial cells are entering the insect hemolymph per nematode penetration.

The average penetration rate overall strains was approximately 11% at 168 hours post exposure, compared to 40% in *Steinernema* and 5% in *Heterorhabditis* (Fig 17) (Caroli *et al.* 1996; Ricci *et al.* 1996). The degree of penetration varies greatly between the common entomopathogenic nematodes and is thought to be the result of variation in the primary mode of reproduction. *Heterorhabditis* infective juveniles develop into hermaphrodites and thus require

the penetration of a single individual to infect and reproduce inside the insect larvae; although after the first generation sexual reproduction resumes. However, *Steinernema* infective juveniles become amphimictic and consequently require at least one male and one female to penetrate for reproduction. *C. briggsae* is primarily hermaphroditic with a small occurrence of males. Therefore, presumably only a few nematodes penetrating are necessary for reproduction. However, as previously discussed the bacterial load carried by each nematode is unknown and plays a major role in insect mortality and nematode reproduction. This experiment has demonstrated that all strains were capable of penetrating into the insect larvae with strains PB800, PB826, DH1300, HK104 and VT847 having comparably elevated penetration rates to the other strains and DR1690 demonstrating deficiency in penetration (Fig 17). The overall conclusions of the entomopathogenic assays was that all strains were capable or both reproducing and penetrating the insect larvae, with PB800 demonstrating elevated capabilities and DR1690 demonstrating deficiency.

Conclusion

The goals of this experiment were to taxonomically identify both the putative entomopathogenic *Caenorhabditis* and associated bacterium, as well as evaluate entomopathogenic characteristics for both and to expand the experiments to include other closely related *Caenorhabditis.* Through taxonomic identification using molecular tools, as well as completing molecular comparisons to wild-type isolates of *C. briggsae*, the South African strain was definitively

identified as a new isolate of *Caenorhabditis briggsae*. The associated bacterium was identified as a member of the *Serratia* group, denoted *Serratia* sp. SCBI. As previously stated further, comparisons using full genomes of closely related taxa as well as to common entomopathogenic taxa is being undertaken by F. Abebe-Akele.

The studies in this experiment included other wild-type isolates of *C. briggsae* strains in the molecular assays, and were expanded to include comparisons to other closely related *Caenorhabditis* in the behavioral assays. The South African strain of *C. briggsae* was expected to show entomopathogenic characteristics based on the manner in which it was collected. However, the experiments in this study demonstrated entomopathogenic capability in all of the strains tested, with the exception *C. briggsae* DR1690. All strains were capable of penetrating, reproducing, and emerging from insect larvae.

These experiments merely demonstrate the capacity for exhibiting entomopathogenic behavior in members of the *elegans* group of *Caenorhabditis*; they do not infer that this behavior occurs frequently in the wild for any or all of the strains tested in this survey. However, considering the South African strain was in fact collected from within a dead insect larva with an associated bacterium it is possible that this behavior could be common. *Caenorhabditis briggsae* strains have been found to have both phoretic and necromenic associations with invertebrates. It is not surprising to find organisms that already have the adaptations to enable phoretic and necromenic associations that could potentially lead to entomopathogenic behavior, especially in *C. briggsae* where the ecology

is not well described or known. Phoresy and necromeny are pre-parasitic associations, and serve as predisposition behaviors in the evolution from free-living to becoming parasitic. However, it is a concern that entomopathogenic behavior has not been documented before in *C. briggsae* or any other *Caenorhabditis* species, and many strains have been collected in nature. Most *C. elegans* are collected from anthropogenically-altered soil, such as compost, suggesting that there are sufficient bacteria present in the decaying organic matter to support bacteriovorous nematode populations. The nematodes tested in this study exhibited normal life cycles when grown on both *E. coli* OP50 and *Serratia* sp. SCBI, where the insect host was not necessary for nematode population growth. The association between *C. briggsae* SoAf and *Serratia* sp. SCBI is likely to be opportunistic, not obligate.

Also, the nematode behavior demonstrated in this experiment could be driven by the presence of the bacterium, not simply the predisposition of the nematode. The association between the bacterium *Serratia* sp. SCBI and *C. briggsae* is undefined; it is likely an opportunistic symbiosis as the nematode can survive without the bacterial counterpart. These experiments merely provide a starting point for other experiments on this system, and opens the door for future analysis of the evolution of parasitism in free-living nematodes, or more specifically the evolution of entomopathogenic nematodes. There remains much to investigate within this system; we have only brushed the surface.

Future Investigations

The opportunities for further investigation in this system are abundant. Additional investigation is crucial in all facets of this work to increase the understanding and implications of an entomopathogenic *Caenorhabditis briggsae*, from determination of nematode behavior in nature, the type and duration of the association between the bacterium and the nematode, insect host specificity, and to expand the behavioral assays to include additional taxa of free-living nematodes. Within the laboratory, transmission efficiency estimates would yield the bacterium load per nematode, and would aid in elucidating the penetration assay results. Controlling both bacterium and nematode injections across all strains may provide clearer results in the reproductive assays. While genomic comparisons between *Serratia* sp. SCBI other *Serratia* are currently being carried out, molecular comparisons of identified virulence factors between *Serratia* sp. SCBI and other entomopathogenic bacteria may aid in elucidating the relationship between the bacterium and nematode. It would be interesting to compare genes involved in forming the association with the symbiotic bacterium in common entomopathogenic nematodes to the *Caenorhabditis briggsae* AF16 genome, which is already available would also be interesting.

Also, further exploration within the region of the initial South African isolate of *C. briggsae* would prove useful for both population genetic analysis to compare to other *C. briggsae* population studies, and to determine the frequency at which they are found, as well as observation of common stage, prevalence of males and other life-history traits. Collection and dissection of insect larvae

cadavers in nature to look for evidence of parasitism by *C. briggsae* would support the possibility of true entomopathogenic behavior. The discovery of a new isolate of *C. briggsae* alone is interesting, discovery of a potentially entomopathogenic *Caenorhabditis briggsae* strain and demonstrating entomopathogenic capabilities within the *Caenorhabditis* group elicit a whole new set of questions about *Caenorhabditis* ecology and the evolution of entomopathogenesis.

REFERENCES

Adams, B. J., Fodor, A., Koppenhofer, H. S., Stackebrandt, E., Stock, S. P., & Klein, M. G. (2006). Biodiversity and systematics of nematode-bacterium entomopathogens. *Biological Control, 38*(1), 4-21.

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B., & de Rosa, R. (2000). The new animal phylogeny: Reliability and implications. *Proceedings of the National Academy of Sciences of the United States of America, 97*(9), 4453-4456.

Aguinaldo, A. M. A., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A., et al. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature, 387*(6632), 489-493.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402.

Baird, S. E. (1999). Natural and experimental associations of *Caenorhabditis remanei* with *Trachelipus rathkii* and other terrestrial isopods. *Nematology, 1*, 471-475.

Baird, S. E., Fitch, D. H. A., & Emmons, S. W. (1994). *Caenorhabditis vulgaris* Sp-N (Nematoda, Rhabditidae) - A necromenic associate of pill bugs and snails. *Nematologica, 40*(1), 1-11.

Barriere, A., & Felix, M. A. (2005). High local genetic diversity and low outcrossing rate in *Caenorhabditis elegans* natural populations. *Current Biology, 15*(13), 1176-1184.

Barriere, A., & Felix, M. A. (2006). Natural variation and population genetics of *Caenorhabditis elegans*. In D. H. A. Fitch (Ed.), *WormBook*: The *C. elegans* Research Community.

Bedding, R. A., & Akhurst, R. J. (1975). Simple technique for detection of insect parasitic Rhabditid nematodes in soil. *Nematologica, 21*(1), 109-110.

Bird, A. F., & Akhurst, R. J. (1983). The nature of the intestinal vesicle in nematodes of the Family Steinernematidae. *International Journal for Parasitology, 13*(6), 599-606.

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 Namtoda. *Nature, 392, 71-75.*

Blaxter, M. L. (2003). Nematoda: Genes, genomes and the evolution of parasitism. *Advances in Parasitology, Vol 54, 54*, 101-195.

Bowen, D., Rocheleau, T. A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R., et al. (1998). Insecticidal toxins from the bacterium *Photorhabdus luminescens. Science, 280*(5372), 2129-2132.

Bowen, D. J., & Ensign, J. C. (1998). Purification and characterization of a high-molecular-weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens. Applied and Environmental Microbiology, 64*(8), 3029-3035.

Brenner, S. (1973). Genetics of behavior. *British Medical Bulletin, 29*(3), 269-271.

Burnell, A. M., & Stock, S. P. (2000). *Heterorhabditis, Steinernema* and their bacterial symbionts - lethal pathogens of insects. *Nematology, 2*, 31-42.

Campbell, J. F., Koppenhofer, A. M., Kaya, H. K., & Chinnasri, B. (1999). Are there temporarily non-infectious dauer stages in entomopathogenic nematode populations: a test of the phased infectivity hypothesis. *Parasitology, 118*, 499-508.

Caroli, L., Glazer, I., & Gaugler, R. (1996). Entomopathogenic nematode infectivity assay: Comparison of penetration rate into different hosts. *Biocontrol Science and Technology, 6*(2), 227-233.

Ciche, T. A. & Ensign, J. C. (2003). For the insect pathogen *Photorhabdus luminescens*, Which end of a nematode is out? *Applied and Environmental Microbiology, 69 (4), 1890-1897.*

Ciche, T. A., Darby, C., Ehlers, R. U., Forst, S., & Goodrich-Blair, H. (2006). Dangerous liaisons: The symbiosis of entomopathogenic nematodes and bacteria. *Biological Control, 38*(1), 22-46.

Cutter, A. D. (2006). Nucleotide polymorphism and linkage disequilibrium in wild populations of the partial selfer *Caenorhabditis elegans. Genetics, 172*(1), 171-184.

Cutter, A. D., Felix, M. A., Barriere, A., & Charlesworth, D. (2006). Patterns of nucleotide polymorphism distinguish temperate and tropical wild isolates of *Caenorhabditis briggsae. Genetics, 173*(4), 2021-2031.

De Ley, P. (2006). A quick tour of nematode diversity and the backbone of nematode phylogeny. In D. H. A. Fitch (Ed.), *WormBook*: The *C. elegans* Research Community.

Denver, D. R., Morris, K., Lynch, M., Vassilieva, L. L., & Thomas, W. K. (2000). High direct estimate of the mutation rate in the mitochondrial genome of *Caenorhabditis elegans*. *Science, 289*(5488), 2342-2344.

Denver, D. R., Morris, K., & Thomas, W. K. (2003). Phylogenetics in *Caenorhabditis elegans*: An analysis of divergence and outcrossing. *Molecular Biology and Evolution, 20*(3), 393-400.

Divergence. (2006). Plant parasitic nematodes, animal parasitic nematodes, and human parasitic nematodes. Retrieved May 2007, from http://divergence.com/Investors/index.html

Dodd, S. J., Hurst, M. R. H., Glare, T. R., O'Callaghan, M., & Ronson, C. W. (2006). Occurrence of sep insecticidal toxin complex genes in *Serratia spp.* and *Yersinia frederiksenii*. *Applied and Environmental Microbiology, 72*(10), 6584-6592.

Dorris, M., De Ley, P., & Blaxter, M. L. (1999). Molecular analysis of nematode diversity and the evolution of parasitism. *Parasitology Today, 15*(5), 188-193.

Fitch, D. H. A. (2005). Introduction to nematode evolution. In L. R. Girard (Ed.), *WormBook*: The *C. elegans* Research Community.

Fitch, D. H. A., & Thomas, W. K. (1997). Evolution. In D. L. Riddle, T. Blumenthal, B. J. Meyer & J. R. Priess (Eds.), *C. elegans II* (pp. 815-850). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Fodor, A., Riddle, D. L., Nelson, F. K., & Golden, J. W. (1983). Comparison of a new wild-type *Caenorhabditis briggsae* with laboratory strains of *Caenorhabditis briggsae* and *C. elegans*. *Nematologica, 29*(2), 203-217.

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

Forst, S., & Nealson, K. (1996). Molecular biology of the symbiotic pathogenic bacteria *Xenorhabdus spp* and *Photorhabdus spp. Microbiological Reviews, 60*(1), 21-43.

Gaugler, R. (2007). *Biological Control: A guide to natrual enemies in north america*. Retrieved May 2007, from http://www.nysaes.cornell.edu/ent/biocontrol/pathogens/nematodes.html.

Glaser, R. W. (1931). The cultivation of a nematode parasite of an insect. *Science, 73*, 614.

Glaser, R. W. (1932). Studies on *Neoaplectana glaseri*, a nematode parasite of the Japanese beetle *Popillia japonica*. *NJ Dept of Agricultures, Circ. No. 211*.

Glaser, R. W., & Farrel, C. C. (1935). Field experiments with the Japanese beetle and its nematode parasite. *Journal of the New York Entomological Society, 43*, 345-371.

Glazer, I. (1997). Effects of infected insects on secondary invasion of steinernematid entomopathogenic nematodes. *Parasitology, 114*, 597-604.

Glazer, I., & Lewis, E. E. (2000). Bioassays for entomopathogenic nematodes. In A. Navon & K. R. S. Ascher (Eds.), *Bioassays of entomopathogenic microbes and nematodes* (pp. 229-247). New York, NY: CABI Publishing.

Graustein, A., Gaspar, J. M., Walters, J. R., & Palopoli, M. F. (2002). Levels of DNA polymorphism vary with mating system in the nematode genus *Caenorhabditis*. *Genetics, 161*(1), 99-107.

Grewal, P. S., Selvan, S., Lewis, E. E., & Gaugler, R. (1993). Male insect-Parasitic nematodes - Colonizing sex. *Experientia, 49*(6-7), 605-608.

Haber, M., Schungel, M., Putz, A., Muller, S., Hasert, B., & Schulenburg, H. (2005). Evolutionary history of *Caenorhabditis elegans* inferred from microsatellites: Evidence for spatial and temporal genetic differentiation and the occurrence of outbreeding. *Molecular Biology and Evolution, 22*(1), 160-173.

Hausdorf, B. (2000). Early evolution of the bilateria. *Systematic Biology, 49*(1), 130-142.

Hominck, W. M., & Reid, A. P. (1990). Perspectives on entomopathogenic nematology. In R. Gaugler & H. Kaya (Eds.), *Entomopathogenic Nematodes in Biological Control* (pp. 327-345). Boca Raton, FL: CRC Press.

Howe, D. K., & Denver, D. R. (2007). Evolutionary impact of a *Caenorhabditis briggsae* mitochondrial genome deletion. *in prep*.

Hurst, M. R. H., Glare, T. R., Jackson, T. A., & Ronson, C. W. (2000). Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *Journal of Bacteriology, 182*(18), 5127-5138.

Jackson, T. A., McNeill, M. R., & Madurappulige, D. (2004). Use of *Serratia spp.* bacteria for monitoring behaviour of parasitoids. *International Journal of Pest Management, 50*(3), 173-176.

Jumba, M., Vincent, G. M., Thomas, W. K., & Abebe, E. (2006). Molecular and morphological characterization of *Pseudodiplogasteroides n.* sp. (Nematoda: Pseudodiplogasteroides) associated with a bacteral ectosymbiont. *in prep*.

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 L. A. Lacey (Ed.), *Manual of Techniques in Insect Pathology* (pp. 281-324). Boston, MA: Academic Press.

Kiontke, K., & Sudhaus, W. (2006). Ecology of *Caenorhabditis* species. In D. H. A. Fitch (Ed.), *WormBook*: The C. elegans Research Community.

Koppenhofer, A. M., Grewal, P. S., & Fuzy, E. M. (2007). Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. *Journal of Invertebrate Pathology, 94*(3), 184-195.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics, 5*, 150-163.

Lewis, E. E., Campbell, J., Griffin, C., Kaya, H., & Peters, A. (2006). Behavioral ecology of entomopathogenic nematodes. *Biological Control, 38*(1), 66-79.

Li, X. Y., Cowles, R. S., Cowles, E. A., Gaugler, R., & Cox-Foster, D. L. (2007). Relationship between the successful infection by entomopathogenic nematodes and the host immune response. *International Journal for Parasitology, 37*(3-4), 365-374.

Longhorn, S. J., Foster, P. G., & Vogler, A. P. (2007). The nematode-arthropod clade revisited: phylogenomic analyses from ribosomal protein genes misled by shared evolutionary biases. *Cladistics, 23*(2), 130-144.

Martin, P. A. W. (2002). An in vitro inhibition test that predicts toxicity of bacterial pathogen combinations in the Colorado potato beetle. *Biocontrol Science and Technology, 12*(5), 643-647.

McNeill, M. R. (2000). Effect of container type on suitability of the pathogen *Serratia marcescens - Microctonus hyperodae* (Hym., Braconidae) association to indicate parasitoid oviposition attempts. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie, 124*(2), 93-98.

Philippe, H., Lartillot, N., & Brinkmann, H. (2005). Multigene analyses of bilaterian animals corroborate the monophyly of Ecdysozoa, Lophotrochozoa, and Protostomia. *Molecular Biology and Evolution, 22*(5), 1246-1253.

Poinar, J., G. O. (1983). *The natural history of nematodes.* Englewood Cliffs, NJ: Prentice-Hall, Inc.

Poulin, R. (2007). *Evolutionary ecology of parasites* (second ed.). Princeton, NJ: Princeton University Press.

Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C. I., & Ewbank, J. J. (2007). Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America, 104*(7), 2295-2300.

Ricci, M., Glazer, I., Campbell, J. F., & Gaugler, R. (1996). Comparison of bioassays to measure virulence of different entomopathogenic nematodes. *Biocontrol Science and Technology, 6*(2), 235-245.

Rogozin, I. B., Wolf, Y. I., Carmel, L., & Koonin, E. V. (2007). Ecdysozoan clade rejected by genome-wide analysis of rare amino acid replacements. *Molecular Biology and Evolution, 24*(4), 1080-1090.

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (pp. 365-386). Totowa, NJ: Humana Press.

Schneider, S., Roessli, D., & Excoffier, L. (2000). Arlequin: A software for population genetics data analysis Ver.2000.

Schulenburg, H., & Ewbank, J. J. (2004). Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens. Bmc Evolutionary Biology, 4*, 1-9.

Sicard, M., Ferdy, J. B., Pages, S., Le Brun, N., Godelle, B., Boemare, N., et al. (2004). When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). *Journal of Evolutionary Biology, 17*(5), 985-993.

Sicard, M., Hinsinger, J., Le Brun, N., Pages, S., Boemare, N., & Moulia, C. (2006). Interspecific competition between entomopathogenic nematodes (*Steinernema*) is modified by their bacterial symbionts (*Xenorhabdus*). *Bmc Evolutionary Biology, 6*, 1-9.

Sicard, M., Tabart, J., Boemare, N. E., Thaler, O., & Moulia, C. (2005). Effect of phenotypic variation in *Xenorhabdus nematophila* on its mutualistic relationship with the entomopathogenic nematode *Steinernema carpocapsae*. *Parasitology, 131*, 687-694.

Sidow, A., & Thomas, W. K. (1994). A molecular evolutionary framework for eukaryotic model organisms. *Current Biology, 4*(7), 596-603.

Sikorowski, P. P., & Lawrence, A. M. (1998). Transmission of *Serratia marcescens* (Enterobacteriaceae) in adult *Heliothis virescens* (Lepidoptera : Noctuidae) laboratory colonies. *Biological Control, 12*(1), 50-55.

Sivasundar, A., & Hey, J. (2003). Population genetics of *Caenorhabditis elegans*: The paradox of low polymorphism in a widespread species. *Genetics, 163*(1), 147-157.

Sproer, C., Mendrock, U., Swiderski, J., Lang, E., & Stackebrandt, E. (1999). The phylogenetic position of *Serratia, Buttiauxella* and some other genera of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology, 49*, 1433-1438.

Stock, S. P. (2005). Insect-parasitic nematodes: From lab curiosities to model organisms. *Journal of Invertebrate Pathology, 89*(1), 57-66.

Subbotin, S. A., Vierstraete, A., De Ley, P., Rowe, J., Waeyenberge, L., Moens, M., et al. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution, 21*(1), 1-16.

Tan, B. L., Jackson, T. A., & Hurst, M. R. H. (2006). Virulence of *Serratia* strains against *Costelytra zealandica*. *Applied and Environmental Microbiology, 72*(9), 6417-6418.

Thomas, W. K., & Wilson, A. C. (1991). Mode and tempo of molecular evolution in the nematode *Caenorhabditis*: Cytochrome oxidase-II and calmodulin sequences. *Genetics, 128*(2), 269-279.

Wang, Y., & Gaugler, R. (1998). Host and penetration site location by entomopathogenic nematodes against Japanese beetle larvae. *Journal of Invertebrate Pathology, 72*(3), 313-318.

White, G. F. (1927). Scientific apparatus and laboratory methods: A method for obtaining infective nematode larvae from cultures. *Science, 66*(1709), 302-303.

Wolf, Y. I., Rogozin, I. B., & Koonin, E. V. (2004). Coelomata and not Ecdysozoa: Evidence from genome-wide phylogenetic analysis. *Genome Research, 14*(1), 29-36.

Zhang, Y., Lu, H., & Bargmann, C. I. (2005). Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature, 438*(7065), 179-184.