Population Structure of Insect Pathogaic Bacteria in UK Soil and their Associated Nematodes

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Population structure of insect pathogenic bacteria in UK soil and their associated nematodes

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Abstract

Surveys for entomopathogenic bacteria and their associated nematode hosts were conducted locally (University of Bath campus) and across southern England. Sampling involved trialing a novel Android app. (Epicollect) to manage sample collection data. *Galleria* larvae were used to bait UK soil samples. Insects which became infected were placed on White traps to collect any emerging nematodes, from which bacteria were isolated. Bacteria were also isolated from the haemolymph of any infected larvae. Bacterial isolates were classified on the basis of 16s rDNA and *recA* gene sequences. *Serratia proteamaculans-like* strains dominated the samples, and Multilocus sequence analysis (MLSA) was developed for the characterization of these *Serratia* isolates. We determined the sequences of (350-450-bp) fragments from five housekeeping genes of 84 isolates of *Serratia proteamaculans*. MLSA was shown to be effective for distinguishing closely related strains found in the insects' haemolymph and from different nematodes. goeBURST was used to visualize the relationships between the STs, and the data showed a high level of discrimination, resolving 69 STs from the 84 isolates. In addition, the data derived from this study were represented in a phylogenetic network using the Splits Tree-network methods, to show the rate of recombination within and between the genes.

From a total of 256 infected *Galleria* 23.04% were nematode positive. The nematodes were identified based on 18S rDNA 19 isolates were close relatives of the species *Pristionchus entomophaga* and *Diplogasteriodes magnus* (Diplogastridae). A further 16 isolates were more closely related to *Steinernema glaseri* (Steinernematidae). All three nematode types were isolated from diverse habitats and soil types, but were isolated more frequently in cold seasonal conditions. The bacterial sequence data suggest that the nematode- associated strains of bacteria belong to specific clades, distinct from the free living infective strains, which hints at ecological diversity within the *S. proteamaculans* population.

Two of the *Serratia proteamaculans*-like strains had been chromosomally labeled with GFP to confirm the specifics of their association with the nematode hosts. The associated *S. proteamaculans*-like isolates isolated from Bath and Chepstow soils were examined further for their pathogenicity to *Galleria mellonella* and *Manduca sexta* larvae. *Serratia* Bath isolates, isolated from *Pristionchus* were more virulent toward both insect hosts than the *Serratia* from the Chepstow isolates associated with *Steinernema* nematodes. This suggests that host specificity may play important role in the virulence of the strain.

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Abbreviations

AP	**Ampicillin**
ºC	**Degree Celsius**
Chi-seq	**Chi-squared test**
BLAST	**Basic Local Alignment Search Tool**
Bt	***Bacillus thuringensis***
bp	**base pair**
CCs	**Clonal complexes**
Cm	**Centimetre**
Cfu	**Cell forming unit**
DNA	**deoxynucleic acid**
DLVs	**Double locus variants**
dNTPs	**deoxynucleoside triphosphate**
Df	**Degree factor**
EDTA	**ethylenediamine tetracetic acid**
EPB	**Entomopathogenic bacteria**
EPNs	**Entomopathogenic nematodes**
et al.	**And others (*et alia*)**
E.coli	***Escherichia coli***
Gfp	**green fluorescent protein**
gDNA	**genomic deoxynucleic acid**
H	**Hour**
IJ	**infective juveniles**
Kb	**kilo base**
LB	**Luria broth medium**
M	**Meter**
Mcf gene	**Makes caterpillars floppy genes**
MilliQ water	**ultrapurified water**
Min	**minute**
Ml	**Millilitre**
µl	**Microliter**
Mm	**Millimetre**
MLEE	**Multilocus enzyme electrophoresis**
MLST	**Multilocus sequence typing**
Nm	**Nanometre**
OD_{600}	**Optical density at 600 nm**
PCR	**polymerase chain reaction**
PBS	**Phosphate buffer saline**
PTC	**Peltier thermal cycle**
P	**Probability**
Rpm	**revolutions per minute**
rRNA	**ribosomal ribonucleic acid**
S	**Seconds**

SDS	**Sodium dodecyl sulphate**
SSU	**small subunit**
SLVs	**single locus variants**
ST	**Sequence type**
Sp	**Species**
TAE	**tris-acetate EDTA**
Taq	***Thermophilus aquaticus* (DNA polymerase)**
Temp	**Temperature**
Tris	**Tris(hydroxymethyl)methylamine**
TLVs	**Trible locus variants**
Tcs	**toxin complexes**
U	**unit (of enzyme activity)**
***vs*.**	***Versus***
UV	**Ultraviolet**
w/v	**Weight per volume**

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Chapter 1

Introduction

Interactions between eukaryotes and prokaryotes are widespread in nature and range from mutualistic to pathogenic. Entomopathogenic bacteria and their nematode vectors provide an excellent model system to study these interactions. Nematodes and bacteria cover the most biologically and phylogenetically diverse domains of organisms, and many soil dwelling nematodes with a bacterium have entered into mutualistic associations [1]. New entomopathogenic nematodes and their bacterial endo-symbionts are still likely to be waiting to be discovered [1].

Recently, insect pathogenic bacteria have attracted the attention of many researchers because of their importance for pest control and in transmission of disease. The application of the entomopathogenic bacteria (EPB) and the associated bacteria with their entomopathogenic nematodes (EPNs) as bio-control agents is used worldwide. Moreover, recent studies have focused on surveys for both EPB and EPN in order to increase the understanding of their roles in nature, and how they interact in the ecology of the environment. However, little is known regarding the diversity and distribution of EPNs in UK soils, or the extent to which co-adaptation has occurred between entomopathogenic bacteria and nematode vectors.

In my thesis I focused on the following aims:

1. Characterization of common entomopathogenic bacteria both associated with, and non- associated with nematodes in UK soil from different locations and habitats.

2. Discovery and characterization of novel associations between *Pristionchus* sp and *Sterinernema* sp nematodes with *S. proteamaculans-like* strains in UK soil. Understanding the virulence properties of *S. proteamaculans* towards insects.

3. Using phylogenetic analysis to understand the degree of ecological (host) specialization in the *S. proteamaculans* population.

1.1. Entomopathogenic Bacteria (EPB)

Entomopathogenic bacteria (EPB) invade and cause disease in insects. Gram-negative and Gram-positive bacteria are present in the insect gut as microflora; some of them are beneficial to the insect host and others are pathogenic. Entomopathogenic bacteria have an application in insect pest control.

There are two types of EPB:

A. Free living EPB and the well-known examples include the spore-forming Gram-positive EPB *Bacillus thuringensis* (Bt) and *Bacillus sphaericus* and Gram-negative entomopathogens include *Yersinia entomophila, Pseudomonas entomophila, Serratia entomophila* and *Serratia proteamaculans.*
B. The symbiotic EPB including Gram-negative genera *Photorhabdus* and *Xenorhabdus* are well-characterized entomopathogenic bacteria which have evolved a symbiotic relationship with nematode hosts. Those bacteria produce a wide range of virulence factors including proteases, chitinases and toxins [2].

1.1.1. Bacterial-Nematodes symbiosis

Xenorhabdus and *Photorhabdus* spp. are rod-shaped gamma-proteobacteria associated symbiotically with *Steinernematidae* and *Heterorhabditid* soil nematodes [3]. The nematode-bacterial associations are typically very specific and participate in a complex life cycle in which they parasitize and kill insect hosts in the soil. The bacterial partners produce a range of toxins and employ various mechanisms to evade the immune system and kill the insect host. This life cycle requires the nematode associated bacteria to exhibit both symbiotic and pathogenic traits. In both the *Steinernematidae* and *Heterorhabditid* nematodes, the partner bacteria are carried monoxenically in the gut of non-feeding forms of the nematodes termed infective juveniles (IJ).

The IJs seek out an insect host in the soil and enter the insect's open blood system (the hemocoel) either via natural openings (mouth, anus or spiracles) or by burrowing in through the cuticle [4]. Once the nematodes have invaded the hemolymph they un-sheath and regurgitate around 100 bacterial cells (in the case of *Photorhabdus*) [5]. As the bacteria grow in the insect host they produce toxins to kill the larvae, and potent antimicrobials to ward off potential competitors and saprophytes. Bioconversion of the insect tissue into more bacteria provides a food source for the growing and replicated nematodes [6]. Additionally, both of these EPBs produce antimicrobial compounds such as bacteriocins that shelter the insect cadaver from the growth of other bacteria, fungi or yeasts [7]. Nevertheless, certain resistant bacteria can often still be found growing in the infected host insects such as *Acinetobacter* [8]. Although they are unrelated, the life cycles of the *Steinernematidae* and *Heterorhabditid* worms appear very similar; however, detailed examination of the two model systems does reveal some differences [9]. Although the symbiotic association is essential for the reproduction and the growth of both *Photorhabdus* and *Xenorhabdus* in the environment, they can also be cultured independently as free-living forms in laboratory conditions [10] & [11].

***Photorhabdus- Heterorhabditis* system:**

Photorhabdus is a member of the Enterobacteriacae and so is closely related to the *Yersinia* and *Escherichia*, although they have the distinction of living in an obligate mutualistic association with the soil dwelling nematodes belonging to the family *Heterorhabditis*. The dual symbiotic-pathogenic life cycle of this bacterium provides a very tractable model system for studying the interactions between bacteria and eukaryotic hosts [12]. *Photorhabdus* bacteria colonize the intestines of *Heterorhabditis spp* IJs. In the IJ, the mouth and the anus are closed and the pharynx and gut are collapsed [12]. The interaction between the bacteria and the nematode appears to occur between the specific receptors on the intestinal epithelium of the nematodes and the cell surface molecules of the associated bacteria, creating species-specific colonization.

The IJs enter the insect host via the natural openings or directly through soft areas of cuticle using a tooth-like appendage [13]. An unknown “food signal” then induces the IJ to “recover” and regurgitate the *Photorhabdus*. The bacteria are able to overwhelm the insect’s innate immune system and replicate by using both proteinaceous and small-molecule toxins. In addition to insecticidal toxins they also employ more specific toxins such as type 3 secreted proteins that allow them to resist phagocytosis by the insect hemocytes [14].

Examples of well characterized protein toxins include the Toxin Complexes, or TC’s, such as Tcc, Tcb, Tca and Tcd. All show toxicity to *Manduca. sexta* when injected, although the last two also show oral toxicity to the same insect model. Other toxins include the Makes Caterpillars Floppy (Mcf1 and 2) toxins [15] [6]& [16], the PVC system (*Photorhabdus* virulence cassettes) and an RTX-like metalloprotease (PrtA) [17] & [6]. PrtA is a protein that carries the characteristic RTX repeated motifs typical of type I secreted proteins. This protease shows similarities to proteases from other Gram-negative pathogens such as *Erwinia chrysanthemi, Pseudomonas aeruginosa* and *Serratia marcescens* [18]. Several other hydrolytic enzymes such as proteases and chitinases secreted from the bacteria are believed to be used to break down the insect tissues to provide suitable nutrients for both bacteria and nematode growth and reproduction inside the insect cadaver. Moreover, the symbiotic bacteria produce antibiotics that inhibit the growth of any competing microorganisms [2]. *Photorhabdus spp* are also able to resist death by humoural aspects of innate immunity such as the Phenol-Oxidase cascade and antimicrobial peptides by extracellular polysaccharide associated factors [19]. Ciche et al [16] found that as part of the symbiotic association, *Photorhabdus* not only provides food for the worm, but is also able to invade the nematode cells and mesoderm by binding to distal INT9 gut cells. It was shown that each mature IJ carries approximately 100 cfu of bacterial cells in their guts [20]&[5].

Upon the initial invasion of the insect blood, the single IJ recovers to become a self-fertile adult hermaphrodite. The hermaphrodite can lay eggs that hatch into either males, females or more hermaphrodites. The nematodes have four larval stages (L1-L4) before adulthood, and the IJ's represent an alternative developmental stage of L2, similar to the environmentally resistant Dauer juveniles of *C. elegans*. When the insect resources run low, the bacteria invade the nematode and manipulate development to force all the nematodes to the IJ developmental stage. Finally, the IJs leave the cadaver and search for a new insect to infect [16]. Typically, a single IJ can result in the emergence of > 1000,000 IJs from a single infected insect host within 2-3 weeks. This highly efficient process makes these symbiotic EPN associations important and useful biocontrol agents against crop pest insects [21].

Three distinct species comprise the genus of *Photorhabdus : P. luminescens, P. temperate* and *P. asymbiotica* [22]. The first two are restricted to insect hosts while the *P. asymbiotica* has also been associated with human infections in the USA and Australia [23]. Genomic comparisons of *P. luminescens* strain TTO1 indicates that *P. asymbiotica* has a smaller genome than TTO1, lacking the diversity of toxin genes seen in the TT01. In addition, the human pathogenic strains have also acquired plasmids similar to the pMT1 plasmid found in *Yersinia pestis*, the causative agent of the bubonic plague [14] & [24]. Furthermore, the genomic studies of *P. luminescens and P. asymbiotica* revealed the importance of secondary metabolite synthesis in this genus, used to facilitate their infection to the host [24]. In *P. luminescens* >6% of the genome TTO1 encodes genes expected to be responsible for the production of small bioactive compounds such as stilbene (ST) and corbapenem antibiotics and an anthraquinone pigment (AQ) [12]. Furthermore, some of these novel bioactive compounds were identified in *P. asymbiotica* using a technique called Rapid Virulence Annotation. This allowed the identification of two genetic loci responsible for the production of cytotoxic cyclic peptides [25].

***Xenorhabdus-Steinernema* system:**

A second well-characterized EPN partnership is the *Xenorhabdus-Steinernema* complex. *Xenorhabdus,* like *Photorhabdus sp,* are Enterobacteriacae that have also evolved a nematode-mutualistic / insect-pathogenic lifestyle. While the bacterial genera are closely related and the life cycle they employ is convergent with that of the *Photorhabdus*, the *Xenorhabdus sp* form mutualistic associations with entomopathogenic *Steinernem*, which are genetically unrelated to the *Heterorhabditid* nematodes [26]. For example, *Xenorhabdus nematophila* is mutualistically associated with the nematode *S. carpocapsae* [27]. Again, these EPNs have great utility as biological pest control agents.

One central difference can be seen in the colonization of the different bacteria genera in these different nematodes. *Xenorhabdus spp*. are carried in a specialized region of the *Steinernema* intestine, termed the receptacle [28]. Furthermore, the transmission of *P. luminescens* to *H. bacteriophora* infective juvenile progeny also appears to be significantly different than the transmission of the *Xenorhabdus* to *S. carpocapsae*. *Photorhabdus* transmission requires bacterial colonization and invasion of parental rectal gland cells followed by the induction of egg-hatch within the mother (endotokia matricida). Conversely, no endotokia matricida is needed for IJ colonization by *Xenorhabdus* in *S. carpocapsae* [16].

Also, work published by Akhurst compared the virulence of *Xenorhabdus* and *Photorhabdus* on different insect hosts [29]. He found that some species of *Xenorhabdus* are non-pathogenic towards certain hosts. For example *X. japonica* was unable to successfully infect *Spodoptera* larvae while others could. It is possible that *X. japonica* may not have certain virulence factors present in other *Xenorhabdus* species. It is likely that different strains of *Photorhabdus* and *Xenorhabdus* all encode and deploy slightly different virulence factors in different hosts. Indeed, this is suggested by genome sequence comparisons.

Interestingly, strains of both genera produce phenotypic phase-variant cells, which arise during the prolonged culture in the laboratory [2]. Phase I is characterized by phenotypic characteristics such as the ability to produce antibiotics, exoenzymes, and are motile with peritrichous flagella able to swarm on agar [2]. Phase II cells produce few exoenzymes, have reduced crystalline inclusion proteins and pigments and are unable to support symbiosis with the host nematode. [30].

Conversely, both phases are equally virulent to insects as seen from *Galleria mellonella* studies [2]. However in *X .nematophilus,* phase II were non virulent when tested with *M. sexta,* and do not make the outer membrane protein OpnB in the stationary phase. Moreover, a mutation does not appear to be involved in the phase II formation as described by Forst et al [2]. Known virulence factors of *Xenorhabdus* include TC and other toxins in addition to secreted lipases and lecithinases [31]. *X. nematophila* also produces haemolysins, and immune suppressants that are likely to be involved in the infection process. Brillard *et al* found haemolytic activities in most *Xenorhabdus* species, including *X. nematophila* culture supernatants, which could break down both mammalian erythrocytes and insect haemocytes [32].

It has been found that entomopathogenic bacteria have a signal transduction system, induced to express certain genes as a result of infection; some regulatory systems such as Quorum sensing systems have been identified in *Xenorhabdus* and.*Photorhabdus* [33].

1.1.2. Entomopathogenic *Serratia*

Free living entomopathogenic *Serratia* spp.

Serratia are Gram-negative, oxidative negative, catalase positive, heterotrophic, facultative anaerobic bacteria belonging to the family Enterobacteriaceae [34]. *Serratia* occupies many habitats such as water, soil, plants, and animals and occasionally causes human disease within health

care settings. Insect-associated *Serratia* spp. have been found widely distributed and with a variety of insects [11]. For example, *Serratia marcescens* is a pathogen frequently found infecting insects such as the Boll weevil [34]. Little is known regarding host-interaction or virulence factors in such strains. One of the central AMP molecules used by insects against Gram-negative bacterial invaders is Cecropin. It was shown that certain strains of insect pathogenic *Serratia marcescens* are resistant to this AMP by virtue of a secreted protease that degrades the Cecropin [35]. This protease virulence factor is essential for full pathogenicity.

The nematode worm *C.elegans* was used as a model system to identify virulence factors in *S. marcescens* Db11. It was discovered that these bacteria produce proteases, lipases and chitinases as virulence factors during infection [36]. In addition, the lipopolysaccharide (LPS) component of *S. marcescens* bacterial cells was found to stimulate immune defense reactions in certain insects [37], which was a surprise as work on *Drosophila* suggests that purified LPS cannot be recognised by insect innate immunity. Bedick et al [37] show that injections of purified *Serratia marcescens* LPS into larvae of the tenebrionid beetle reduced stimulated hemocyte microaggregation reactions in the insects.

Another recognized insect pathogenic *Serratia* is *S. entomophila*. This is used as a microbial control agent in New Zealand against grass grubs. The bacteria are prepared and delivered as granular formulations allowing them to survive in a stable form in the soil for up to five months over a wide range of soil moisture conditions [38]. *Serratia entomophila* and *Serratia proteamaculans* both cause amber disease in the New Zealand grass grub *Costelytra zealandica*. When injected into the hemocoel, approximately 10^4 cells are sufficient to kill 50% of the treated insects [39] & [40].

The main virulence determinants for this disease are encoded on a 153,404-bp plasmid, called pADAP (amber disease-associated plasmid) [41]. This plasmid encodes the *sep A B C* toxin and the “anti-feeding–prophage” (*afp*) operons which are homologues of the *Photorhabdus* Toxin Complex and PVC insect toxins respectively. Interestingly, homologues of the TC toxin genes have also been found encoded in the genomes of many other bacteria cited by Jackson et al [40], such as *Yersinia pestis* C092, *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae pv. syringae* B728a (gi:28876514), *Pseudomonas fluorescens* PfO-1 (gi:48732052) and *Chromobacterium violaceum* ATCC 12472. Many of which likely interact with insects in the soil [42] & [43].

Kaska group [44] stated that several secreted enzymes have been shown to play important roles in the virulence of *Serratia* including proteinases, chitinases and lecithinases cited by Grimont [44, 45]. It should be noted that the genus *Serratia* contain several species that have also been isolated from human clinical specimens including; *Serratia ficaria*, *Serratia fonticola*, *Serratia odorifera*, *Serratia plymuthica*, *Serratia rubidaea* and *Serratia entomopa* [46]. This suggests many diverse virulence factor genes may be present and available for horizontal gene transfer in these related bacterial species.

Nematode associated *Serratia* spp,

Recently, a novel *Serratia* spp was found to be associated with nematodes. To date four *Serratia* species have been shown to be associated with nematodes [47], [48], [49] & [50]. It was found that all of the three species have a close evolutionary relationship based on molecular studies [51].

The first identified *Serratia* sp found in symbiotic association with nematodes was *Serratia nematophila* sp. *S .nematodiphila* sp. Nov. was found in the intestine of EPNs belonging to the family (Rhabditida:Rhabditidae) called *Heterorhabditidoides chongmingensis* [49]. The strain was isolated by two

methods, the first was from crushing the surface sterilized isolated nematodes, followed by identifying the isolated colonies in the nutrient bromothymol blue agar (NBTA) plates. And the second was from streaking the nematode-infected insects' haemolymph on the (NBTA) plates. Physiological characteristics and phenotypic characterization of this novel *Serratia,* such as the inability to use oxalate as a source of carbon and arginine dihydrolase activity, confirmed the discovery of this new sp of *Serratia* [49].

The second associated *Serratia* was *Serratia* sp. SCBI detected in South African soil samples with *Caenorhabditis* species KT0001 as well as other *Caenorhabditis* nematodes [52] *&* [29]. This newly discovered *Serratia* SCBI was recovered from the soil baited with *G*. *mellonella*. It was suggested that the *Serratia* are able to enter the insects along with the nematodes and overcome the immune response. This study by Abebe et al [52] suggested that the nematodes formed an IJ like stage containing the *Serratia* sp. SCBI and that the females also underwent endotokia matricida like Heterorhabditid nematodes. Despite this, they found that this association lacked specificity for several *Caenorhabditis* species they tested [52]. Based on 16S rDNA, *Serratia* sp SCBI was identified as closely related to *S. marcescens* Db11[52]. Another study confirmed that these two strains are 99% identical (32). The third *Serratia* sp was found in the cuticle of *Oscheius carolinensis* (Nematoda: Rhabditidae), able to penetrate, colonize and kill five different insect species in the laboratory. The pathogenicity of *Serratia* isolated from the nematodes was compared to that of surface sterilized nematodes. It was found that the surface sterilization reduced the nematode's ability to colonize in the insects [48].

It is clear that the *Serratia marcescens* associated with the *Caenorhabditis* species KT0001 provides the nematodes with entomopathogenic potential [48]. Comparative studies on *Serratia* sp SCBI and *Serratia marcescens* Db11 showed that while both strains have similar virulence properties and

temperature dependent responses, they do differ in the swarming activity. *Serratia* sp. SCBI has been found to swarm on media with different agar concentrations, whereas *S*. *marcescens* Db11 does not [51].

The fourth nematode associated *Serratia* was found in 2013 and was identified as a *S. marcescens* strain which was associated with a Rhabditis sp. nematode[50]. The pathogenicity of this new bacterial isolate was tested in *G. mellonella.* This strain was shown to produce virulence factors able to suppress the insect immunity. In addition it produces several proteases, such as a serralysin-like metolloprotease protein lethal to the host. This protein is significantly different from the genes found in *S. proteamaculans* 568, and it has been suggested that this protein could be used as a biological control [50].

1.1.3. Other examples of Entomopathogenic bacteria

Moraxella osloensis is a Gram-negative bacterium, associated with slug-parasitic nematodes *phasmarhabditis* (Rhabditida: Rhabditidae). These EPN complexes have been developed as a biological pest control for slugs. The nematode acts as a vector to transport the bacterium into the body cavity of the slug, which is then killed within 4-16 days post infection[53]. It was found that *Moraxella* produces a highly toxic LPS endotoxin, which has a molecular weight of 5300 KD and is heat resistant. When injected into the slug it alone can cause the death of the slug [54] .

A selective capture of transcribed sequences (SCOTS) technique was used in order to identify relevant gene expression by *M. osloensis* in the slug. A protein-disulfide isomerase and a protein kinase were characterized as important virulence factors of *M. osloensis*. In addition, the *sclB*, *vspC*, and *spp*, genes were also identified which encode structural proteins of the outer membrane. It has been suggested these proteins are important in immune evasion [55].

Perhaps the most famous and certainly the best-studied examples of entomopathogenic bacteria are members of the genus *Bacillus* such as ***B. thuringiensis*** **and** ***B. sphericus***. Many of these bacteria produce crystal and binary toxins, such as the economically important δ- endotoxins. These toxins can be divided into two families: the crystal proteins (Cry) and cytolytic (cyt) toxins, both produced during sporulation. They can have potent effects on the insect midgut [5, 56, 57]. Cry and Cyt proteins are related toxins and commonly carried on plasmids [58]. In addition, some molecular studies have indicated that many Cry proteins implicated in toxicity are often highly conserved over evolution [59]. The activity spectrum of different examples of these toxins often differs widely.

For example many of the Cyt toxins are toxic to Diptera only while the Cry toxins can be active against many different insect orders including Lepidoptera, Diptera, Hymenoptera and Coleoptera [60]. Toxin production is not restricted to sporulation as exemplified by the *B. thuringiensis* vegetative insecticidal proteins (Vips), which are produced during the vegetative growth of the bacteria. Three types of Vip have been identified; Vip1 and 2 are highly active against western corn rootworm beetle (*Diabrotica virgifera*) [61], whilst Vip3 has been shown to be highly active against several Lepidopteran pests [62]. Insecticidal toxins produced by *B. sphaericus* include the BinA, BinB and Mtx toxins, which have been shown to have potent activity against mosquito larvae [57]. While many of the toxins discussed so far are encoded on mobile plasmid replicons, there are examples of proteins encoded on the chromosomes of these *Bacillus* species that also play a role in virulence against insect hosts; for example, phospholipase C, proteases and hemolysins in the closely related *Bacillus thuringiensis* and *Bacillus cereus*. These are regulated by the common PlcR/PapR quorum sensing system and have been shown to be important virulence determinants [63].

Pseudomonas entomophila is a soil bacterium that kills insects of different orders including the important model insect *Drosophila melanogaster. P.*

entomophila is known to produce a number of virulence factors including: insecticidal toxins, proteases, hemolysins and hydrogen cyanide. In addition, several potential adhesins encoded with type I or II secretion system genes and unique secondary metabolite synthesis genes are important in infection and killing insects [64].

P. entomophila also secretes a metalloprotease called AprA that acts as a virulence factor, which is controlled by the GacS/GacA system. AprA suppresses the activity of antimicrobial peptides produced in the gut as part of the innate immune response of the insect. *P. entomophila* can also kill diverse insect orders including the Lepidopteran silkworm *Bombyx mori* and the disease vector mosquito *Anopheles gambiae*. As such, this entomopathogenic bacteria has great potential for development as a biological pest control agent in both agriculture and vector control[64].

1.2. Diversity and adaptation of Entomopathogenic nematodes

Soil provides a rich source of many insect pathogens, such as viruses, bacteria, fungi and nematodes. Judicious application of these natural enemies of insects has been deployed to help in the fight against insect crop pests [65].

Some studies suggest that there may be over 10 million different nematode species occurring in diverse environments on earth [66]. Nematodes can be found in all habitats rich in organic carbon sources. In the soil, nematodes adapted to many different food sources may be found. Some are purely saprophytic or feed on bacteria and fungi in the soil, whilst others may be specialised plant or animal parasites and predators. Many may be able to utilise diverse sources of nutrition. In the work presented here, the entomopathogenic parasites and predators of insects are of primary interest. Previous work has shown that nematodes may associate with insect

pathogenic bacteria either in obligate mutualistic relationships or potentially more short term and less stringent associations [66].

This spectrum of bacterial-nematode associations presents ideal model systems for studying host-bacterial interactions, both symbiotic and pathogenic, and also for understanding the principles of co-evolution. Interest in studying the Entomopathogenic nematodes (EPNs) has increased because of their importance as biological control agents for pests, in addition to basic research in ecology, evolution and symbiosis [67] & [68]. As discussed above, there are two important well studied EPN families. The first family is the *Steinernematidae* nematodes that associate with *Xenorhabdus* bacteria and the second family is *Heterorhabditidae* which associate with *Photorhabdus* sp. bacteria [69]. These EPN complexes are highly virulent to insects, killing their hosts rapidly, and can be cultured easily in the laboratory using model insects and the "White-trap" technique [68]. The free-living nematodes can carry symbiotic bacterial cells in their intestine at the infective juvenile (IJ) stage. When the infective juvenile enters the hemocoel of an insect host they release the bacteria [70]. In *Steinernema* species the nematodes also produce enzymes to suppress their immune response, although it is not known if *Heterorhabditis* nematodes also do this [15] & [30]. The nematodes reproduce in the dead insect for 1-3 generations, then release a new generation of IJs that contain the bacterial cells and are released from the insect body to hunt for a new insect [15]. It was found that unknown "food signals" influence the recovery of the EPNs, and that nematodes could often use non-cognate bacteria as food; but without their specific symbiont the virulence was reduced [71] & [72]. This indicates very specific but as yet undefined determinants of mutualistic specificity.

Natural entomopathogenic nematode (EPN) populations have previously been surveyed in different areas of the world, around Europe [73], Southern France [74], Portugal [75], Jordan [76] and Iran [77]. In some cases there was evidence of more specific prey-host-associations although in others it seemed

more opportunistic predation was common. In addition, the population structure of EPNs may vary with habitat. For example; *Steinernema* sp are found to be very common in Oak woodland soil samples in Southeastern Arizona [78] and California [1], although *Steinernema* sp and *Heterorhabditis* sp with their associated bacteria *Xenorhabdus* sp and *Photorhabdus* sp appear to be specialized to ecological niches other than woodland [79]. *H. bacteriophora* is abundant in southern Europe where it is known to harbor *Photorhabdus* spp [80]. There is some evidence that different strains of *H. bacteriophora* are also adapted to different habitats based on biotic and abiotic factors such as insect host, soil and salinity [79]. Climatic conditions and soil type may influence the distribution of bacteria and the associated host [81]. The activity of nematodes is also dependent on environmental conditions. Steinernematidae and Heterorhabditidae are adapted to a wide range of habitats, but their distribution is known to vary throughout the British soils according to soil type, and they are less commonly isolated in the summer [81].

The infectivity of *Heterorhabditis* sp and *Steinernema glaseri* on *Lucilia cuprina* larvae was lower in soils of high clay content than in loamy sandy soils, particularly when the moisture content was low. In sandy soils, however, both nematode species readily infected larvae over a wide range of moisture content [82]. Consistent with this, a study in Ireland noted that *Heterorhabditis* sp and *Steinernema sp* were more frequently recovered from sandy and wet soils than from clays and clay loams [83], and this study confirmed that these species were less likely to be recovered in the summer months. In contrast, the movement of the nematode *Phasmarhabditis hermaphrodita,* which is commonly used as a slug biocontrol agent, is higher in clay loam soil than in sandy loam soil [84].

Moreover, it has been found that habitat may play an important role in distributing the nematodes in the soil; some strains of *Heterorhabditis* have been isolated from calcareous soil such as *H. indica* and *H. marelatus*. Other

species are isolated from coastal regions in turf and weedy habitats such as *H. megidis* and also *H. bacteriophora* both found in acidic soil [15]. On the other hand, *Steinernematids* are frequently isolated from woodlands [85]. Within the Steinernematids, *S. feltiae* and *S. carpocapsae* were globally distributed, found in different habitats such as, forests, gardens, national parks and pastures [85]. Whereas other species found in grasslands and woodlands such as *S. feltiae* [86], and *S. kraussei* were found in coniferous forests (USA and Canada) and woodland [87].

Previous reports have found that United Kingdom soils commonly contain predominantly *Xenorhabdus*-Steinernema EPN complexes. Furthermore, a previous four loci MLST analysis demonstrated that only moderate variation occurs between the strains isolated from different UK soil samples [88]. Unfortunately, most previous surveys do not contain sufficiently large sampling regimes meaning there is insufficient data to understand population structure with any certainty. Increasing the sampling size and improving nematode (and bacteria) identification accuracy would improve this. In addition, the predominance of any potential host insect species is also not normally known for any given sampling site, meaning it is hard to interpret the reasons behind the special heterogeneity of EPNs [85] .

Identification and characterization surveys of EPN have recently increased. Moreover, some studies have revealed the successful relationship of specific bacterial strains with the EPB, such as *Xenorhabdus* sp with *Steinernema feltiae* and *S. affine* and *Photorhabdus* sp associated with *Heterorhabdus bacteriophora* [79]. EPN can provide effective biological control of some soil insect pests [89]. Some surveys focused on the adaptation of certain EPN with their environment and their associated bacterial strain such as the surveys in Southern France [79] and Spain [90]; other surveys were conducted in Middle Eastern countries in Jordan [89], Egypt and Oman[79]. In Britain, Hominick and Briscoe [81] studied the occurrence of EBNs in the British soil; they concluded that the frequency of the nematodes differed in different parts of Britain, suggesting that the occurrence of these nematodes

is affected by soil type [81]. To date, *Steinernema feltiae* and *Heterorhabditis bacteriophora* are the most abundant species isolated in continental Portugal. Out of 791 soil samples collected in continental Portugal, 53 were positive to *Steinernema* and *Heterorhabditis* [75].

Most surveys have used the “White trap” method described in 1975 [91] in order to isolate EPNs from the soil. This is an assumption free method used to isolate EPNs that typically uses *Galleria mellonella* larvae to “bait” soil samples. As such it is able to identify any novel entomopathogenic nematodes (or indeed any other pathogens) based purely on their infective ability [92] [81]. It should be noted that other methods have also been used in order to avoid sampling problems associated with for example host specificity, such as passive soil extraction methods. These methods are more appropriate for the study of population structure although identification becomes an issue [85].

In contrast to the EPNs, the model nematode *Caenorhabditis elegans* is very well characterized at a genetic and developmental level, although little is known about its natural life history [93]. EPNs are globally distributed and some studies show they may be key to regulating soil food chains [93]. A few species are now being developed as model systems in genetic and molecular studies. These include the full genome sequencing of a Heterorhabditid nematode [94] and also the Diplogastrid nematode *Pristionchus pacificus* [95]. A study on the genetic structure of natural populations of *C. elegans* revealed a low level of outcrossing and little geographical diversity in *C. elegans* due to frequent migration of these nematodes in the soil [96].

Caenorhabditis elegans and *C. briggsae* are closely related to each other; both are soil-dwelling, self-fertilizing hermaphrodites, with facultative males. Evidence is emerging that at least *C. briggsae* can act as an EPN if provided with an appropriate *Serratia* bacterial partner. It cannot be discounted that *C.*

elegans itself also intermittently engages in entomopathogenic activity by virtue of unstable associations with insect pathogenic bacteria. *C. elegans* has been studied in exceptional detail, and RNAi genetic knock-down studies have not been able to account for the biological role of around 1/3 of its genes. It is possible that much of this genetic material is relevant to cryptic bacterial interactions and life cycles involving parasitism in insects [97].

The nematode *Pristionchus pacificus* has been used as a model to study evolutionary and developmental biology, ecology and population biology. *P. pacificus* is a nematode with six chromosomes able to reproduce as hermaphrodites, similar to *C. elegans*; their life cycle is 4 days at 20°C [98]. The worm can also produce males under stress conditions. The essential differences between *Pristionchus* and *C.elegans* are in the feeding apparatus (valve-pump and grinder) and also in the ecological niches they occupy. *Pristionchus sp* have an embryonic molt, and the non-feeding stage in this genus is J1, which will molt to J2 before they hatch from the egg. In addition, *P. pacificus* has a highly developed vulva with an evolutionary modified structure, unlike *C.elegans* [95]. *P. pacificus* is distributed widely with a well-studied example being strain PS312 isolated from Pasadena (California) (see http://wormbase.org/db/gene/strain) [99]. Most nematodes belonging to the order Rhabditid are soil-dwelling bacterial feeders. *P. pacificus* is *a* cosmopolitan nematode with a specific necromenic association with scarab beetles and the Colorado potato beetle (*Leptinotarsa decemlineata*) [100] . This relatively specific association with these host beetle species and their habitats has facilitated the study of the nematode adaptation to their environment. Around 1200 *Pristionchus* were isolated from 15000 surveyed beetles in North America and Europe, which were shown to fall into 18 diverse species, with a specific biogeographic component [101]. While the caenorhabditids also show necromenic associations they are not specific, prompting the study of their population structure.

A study published in 1993 suggested that the specific entomopathogens *Heterorhabdutus* and *Steinernema* had evolved from a more general

necromenic nematodes species associated with an entomopathogenic bacteria [102]. Blaxter's nematode phylogram [103], suggests that the *Heterorhabditids* belongs in the same genetic clade as *Caenorhabditis* and *Pristionchus*, sharing many features in lifecycle and growth. Conversely, the *Steinernema* are placed in a more distant neighboring clade [104].

Nematodes cultured in the laboratory are often able to associate with bacteria other than a natural symbiont [10]. This means care must be taken in assigning any new mutualistic associations. The recent studies showing that certain nematodes can utilise pathogenic bacteria to kill insects, such as the associations of *Oscheius* sp, and *Caenorhabditis* with *Serratia* as a partner to invade and kill insects, are of particular interest [52]. These studies suggest that the *Serratia* may be changing the behaviour of the nematode host [105]. Typically *C. elegans* will avoid *Serratia* sp as many strains of these bacteria are pathogenic to the nematode. Despite this, it was shown that the *Caenorhabditis briggsae* KT0001 nematodes were specifically attracted to their associated *Serratia* sp. This suggests a level of co-evolution, and potentially specific adaptation of the nematode for the partner bacteria [52].

Behaviour of nematodes

In the case of the EPN nematodes, most of the research has focused upon the phoretic behaviour of the IJs because these are the only free-living stage that can be visualised outside of the complex internal environment of the insect cadaver. There is interest in the choices that the IJs make regarding host preference and ability to seek out prey, primarily as these are central to their role as biological pest control agents [106]. Some *Steinernema* sp and *Heterorhabditis* sp have been compared to understand their behaviour in the field such as their vertical distribution in the soil. *S. carpocapsae* can typically be isolated from the upper 1-2cm of the soil, whereas *H. bacteriophora* is distributed in the upper 8cm of the soil [107]. Others have found that *H. bacteriophora* could be isolated from much deeper in the soil, up to 35 cm

[106]. In addition, there is also variation in the emergence behaviour of the IJs of different EPN strains. For example male *S. glaseri* IJs were the first to arise from the hosts, and more reactive volatile cues from other host than the female [108]. However, this is not true for other *Steinernema* sp such as *S. feltiae* [109]. The condition of the insect cadaver also appears to play a role in influencing nematode behaviour, as it was shown that the infectivity of *H. bacteriophora* isolated in the laboratory from white traps could be up to ten times stronger than that of naturally isolated IJs [110]. Finally, the EPNs behaviour is also influenced by other abiotic factors such as temperature, humidity and soil type, which influences the nematode's hunting ability, movement and infectivity [111].

1.3. Evolutionary and phylogenetic inference

Phylogenetics is the study of evolutionary descent and relatedness [112]. Previously this was carried out using phenotypic markers, but most phylogenies are now based on the DNA sequences. These molecular data can be represented either as a phylogenetic tree [112] or else as a network if there are conflicting signals in the data [113]. Phylogenetics has played a key role in studying the biodiversity, geographic distribution, host range, ecology, behavior and coevolution of Entomopathogenic bacteria and nematodes [114].

16S ribosomal DNA sequence is the most commonly used phylogenetic marker, as it is universally present in bacteria and a high level of conservation makes it possible to design PCR primers that work on a wide range of taxa [115]. However, it is often not possible to distinguish closely related species or different strains belonging to a single species using this approach. DNA hybridization techniques can also be used for the analyses of genetic relationships of closely related strains; however these can incur relatively large errors. An alternative technique was Multilocus Enzyme Electrophoresis (MLEE), which has been used as a standard method in eukaryotic population

genetics for some time [116]. This method has also been used to study the genetic diversity and structure in bacterial populations of a variety of species. MLEE allows the detection of variation within species by assaying the electrophoretic mobility of proteins on starch gels [117].

1.3.1. Multilocus sequence analysis (MLSA)

The same concept was adopted for the current methods of Multilocus sequence typing (MLST) and Mulilocus sequence analysis (MLSA) [118]&[119], except that in this case variation is determined by direct gene sequencing of several “housekeeping” genes. MLST represented an improvement on MLEE as it detects all mutations arising within an internal fragment of a gene and not just those altering the electrophoretic mobility of the final protein product. Dauga et al [120] demonstrated that the phylogenetic trees based on shared genes other than 16S DNA are more useful for distinguishing between closely related strains. Moreover, the analysis of multiple genes distributed along a chromosome as opposed to just one locus is important because it reveals evidence of horizontal gene transfer, which can occur frequently within bacterial populations [120].

MLST is now used widely for the molecular typing of different bacteria strains and species, such as *Neisseria meningitidis* [121],[122] &[9]. The allelic profile (combination of alleles at each locus) defines the sequence type (ST), which is equivalent to the Electrophoretic Type (ET) defined by MLEE data. The relatedness between two strains is then indicated by how many identical alleles they share [123]&[124]. Frequent recombination makes the interpretation of dendrograms problematic, as the different genes will not define the same patterns of relatedness between strains [125]& [126]. However, this method allows for calculating the rate of recombination in the population [127]. Whereas a point mutation results in a single base change, a recombination event may result in multiple nucleotide changes. This means it

is possible through a close examination of the sequences to determine the relative impact of these two processes on genome divergence [128].

MLST schemes have now been developed for a range of bacterial pathogens and databases are freely available (e.g. www.mlst.net & www.pubmlst.org). There are many examples of how MLST data has been used to infer recent patterns of evolution. For example, MLST has been used to demonstrate a European origin of the spirochaete *Borrelia burgdorferi*, which causes Lyme disease [129]. Another example is the characterization of the endosymbiont eubacterial genus *Wolbachia pipientis* [81, 129]. This bacterium has an important role in biological control, and is widely found within the arthropods. The MLST data reveal a high level of recombination and horizontal transmission between different host species [129].

MLST have been used widely in epidemiological research, population biology, pathogenicity studies, and in understanding the evolution of bacteria. This is a rapid technique and, as sequencing costs fall, has been adopted by many laboratories facilitated by a worldwide web-based database application.

1.3.2. eBURST

Frequent horizontal gene transfer in many bacterial lineages can make interpretation of MLST data problematic. In the absence of strong selection, a regular accumulation of variation by mutation across time means that genetic distance tends to equate to the time of divergence between alleles. As stated above each unique allele sequence, compared to the other alleles in the database, is assigned an ST-number that clearly identifies a specific lineage [123]. Patterns of evolutionary descent among closely related STs can then be inferred using the eBURST algorithm [125]. This algorithm builds an un-rooted tree based on the differences in the allelic profile; this procedure sub-divides the data into closely related clonal complexes (CCs) and predicts a founding genotype of each clone.

The program then derives the most likely evolutionary descent of all STs in the group from the founder. If the STs differ at only one locus from the founder genotype they are designated as single locus variants (SLVs) [123]. Further mutations of those SLVs can result in variation in more than one housekeeping gene in the allelic profile frame and are then classed as double locus variants (DLVs) and triple (TLVs) etc. [130] & [131]. This method provides predictions about the founding genotypes as well as giving a clearer picture of the evolutionary descent than a standard dendrogram assigned by MLST.

An alternative implementation of BURST rules has been developed, called goeBURST (global optimal eBURST), which is part of the Phyloviz platform [132]. This version differs from eBURST in that it provides the ability to connect all STs, and is based on an improved algorithm for determining the links.

An example of the acyclic graphs produced by goeBURST is shown in Figure 1.1 [130].

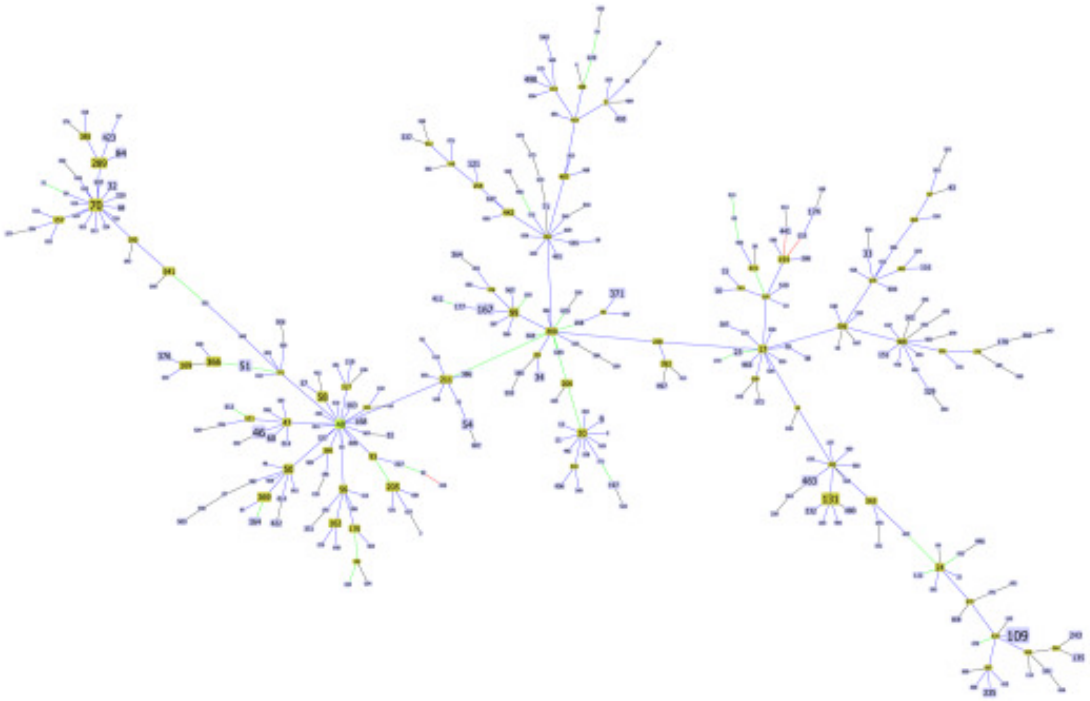


Figure 1.1. Burkholderia pseudomallei showing the Clonal Complex 48. The population snapshot represents the largest clonal complex for *Burkholderia pseudomallei*, with ST48 as the determined founder. (Adopted from Francisco et al [123])

The phylogenetic signal in molecular data is often conflicting, such that the variation within one gene might cluster strains that appear more distantly related on the basis of a second gene. The SplitsTree program is designed to illustrate the extent of such conflicts in the data [133]. The program relies on a method called “split decomposition”, and can be used to gauge the frequency of recombination events (driven by horizontal gene transfer) in the evolution of a clade [113] — as this is a common way by which conflicting phylogenetic signals can arise (Figure 1.2) [134].

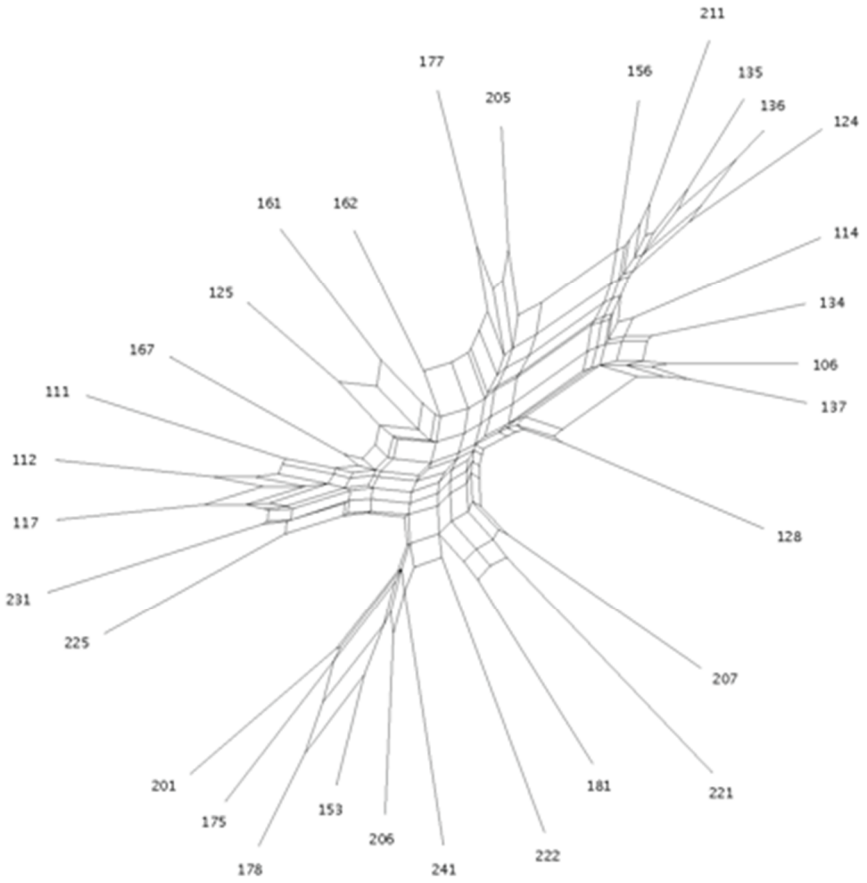


Figure 1.2. Example of a network tree generated using the SplitsTree program. Adopted from Shennan and Collard [135].

Chapter 2

Materials and Methods

2.1. General Materials

2.1.1. Bacterial strains and culture methods

E. coli strains were used in triparental mating as donor cells, as well as controls in infection experiments (Table 2.1).

Table 2.1. *Escherichia coli* strains and associated plasmids used in this study

***E. coli* strain**	**Uses**	**Notes / genotype**	**Selection**	**Source**
OP50	Strain for feeding *C. elegans* and *Serratia*	Uracil-requiring *E. coli* mutant; grows thinly on nematode growth agar, therefore nematodes can be visualised	-	Waterfield laboratory
WM3064 pURR25	Donor cells	contains aRP4 *mob* for conjugation, *pir* gene for replication R6K *ori*-based replicons **pURR25 is the plasmid** Containing miniTn7KGFP transposon	-	Waterfield laboratory
WM3064 pUX-BF13	Donor cells	contains aRP4 *mob* for conjugation, *pir* gene for replication R6K *ori*-based replicons **pUX-BF13 is the plasmid** Containing miniTn7KGFP transposase		Waterfield laboratory
pHC60	Strain for feeding *C. elegans* and *Serratia*	Tetracycline resistance marker and expressing green fluorescent protein		Waterfield laboratory

- *Photorhabdus luminescens* TT01
- *Pseudomonas fluorescens*
- *Bacillus cereus*

Bacterial culture media

Bacteria were grown on Luria Bertani (LB) agar plates at 28 °C overnight, unless otherwise stated. Stocks were stored long-term in 35% glycerol at -80 °C. Culture media used in this study are listed in Table 2.2.

Table 2.2. Bacterial culture media

Media	Use	Composition
Luria Bertani (LB) broth	Bacterial growth	10 g tryptone peptone, 5 g yeast extract, 5 g sodium chloride (pre-formulated, Sigma). 1 L dH_2O. Autoclave at 121 °C.
LB agar	Bacterial growth and isolation	10 g tryptone peptone, 5 g yeast extract, 5 g sodium chloride (pre-formulated, Sigma). 15 g Bacto agar (Difco), 1 L dH_2O. Autoclave at 121 °C

2.1.2. Nematode maintenance

Caenorhabditis elegans and the collected worms were maintained on Nematode Growth Medium (NGM) agar at room temperature, using *E. coli* OP50 as a feeding strain (200µl spread per plate) and transferred weekly to fresh plates.

Nematode Growth Medium (NGM) agar, contains:

3g NaCl, 2.5g Bactopeptone, 17g Bactoagar, dH2O to 1L. Autoclave at 121 °C for 15 min, when ~50 °C, add 1ml cholesterol, 27.6ml KH2PO4, 1ml MgSO4, 1ml CaCl2. For feeding: 200µl *E. coli* OP50 onto dry plates, incubated at 37°C overnight. For nGOT assays, add 25 ug/ml chloramphenicol. Used for maintenance and feeding (with *E. coli* OP50 bacteria).

2.1.3. Invertebrate species and maintenance

For bacterial infection threetypes of invertebrates were used as model hosts

Table 2.3. Invertebrate species used in this study

Species		Notes	Source
Manduca sexta	Order: Lepidoptera	Tobacco Hawkmoth. Insect larva used at fifth-instar stage for injection assays. Used at neonate stage for feeding assays	In-house colony, University of Bath
Galleria mellonella	Order: Lepidoptera	Greater Waxworm. Insect larva used at fifth-instar stage for injection assays and for soil baiting	Livefoods UK
Caenorhabditis elegans	Order: Rhabditida	Nematode worm. Bristol N2 strain. Used for nematode feeding assays.	Cultured in-house, University of Bath

Insect maintenance

A *Manduca sexta* colony was maintained in controlled conditions (25 °C, 80% humidity) and on an artificial diet as detailed in Table 2.4. *Galleria mellonella* larvae were purchased in batches from Livefoods Ltd and stored at 4 °C with no requirement for feeding.

Table 2.4. Details of *Manduca sexta* diet

Media	Uses	Composition
***Manduca* diet**	*M. sexta* maintenance and feeding, infection assays	Premix: 2700g wheatgerm, 1260g casein, 1080g sucrose, 540g dried active yeast, 360g Wesson's salt, 36g choline chloride (Sigma), 72g cholesterol, 36g methyl paraben, 54g sorbic acid. Stored 4 °C. Per cake: 336g premix, 1770ml dH20, 22.5g agar, 4ml corn oil, 4ml linseed oil, 8ml 4% formaldehyde, 0.2g chlorotetracycline, 0.2g Vandersant vitamins, 8g ascorbic acid [136]
Antibiotic-free *Manduca* diet	*M. sexta* feeding assays	Recipe as for complete diet (above) minus formaldehyde and chlorotetracycline.

2.1.4. Solutions used

Table 2.5. Solutions used for the nematodes

Reagent	Preparation	Source
Phosphate buffered saline (PBS)	Pre-formulated tablets dissolved 1 per 100ml as per supplier guidelines. Autoclaved at 121 °C	Sigma
Freezing solution	15 mM NaCl, 15 mM KH_2PO_4 (pH 6), 24%glycerol 110ml dH_2O autoclave, .4% Bacto agar 0.4 mM $MgSO_4$.	Sigma
Egg buffer	118mM NaCl,48mM KCL, 2mM $CaCL_2$, 2mM $MgCl_2$, 25mM pH7.3.	Sigma
Lyse buffer	5ml fresh chlorox, 1.25ml 10N NaOH ans 18.75 ml sterile water	Sigma

Table 2.6. Antibiotics used

Reagent	Preparation	Source
100 mg/ml. Kanamycin sulphate	1g of powder was dissolved in 10ml of double-distilled water.	Sigma
100 mg/ml. Tetracycline	1g of powder was dissolved in 10ml of double-distilled water.	Sigma

All the solutions were sterilized by filtration through a 0.2 µm membrane.

2.2. Methodology:

2.2.1. Sampling overview

Three separate studies were carried out involving the sampling of entemophathogenic nematodes and bacteria from the wild. In each case, approximately 50 g of soil was placed in a plastic cup, and this soil was "baited" with three *Galleria* larvae. The first was a preliminary study conducted in October 2007 where soil samples were taken from different habitats on the golf course near the University of Bath. In May-June 2008, sampling was carried out along two 15M transects on the University of Bath campus, over a five week period. The third study (February and May in 2009) was carried out on a nationwide scale and was based on two transect lines, one running east-west for 230 Km (Road distance) along the south coast of England (from Lewes to Sidmouth; E-W transect), the other running 140Km south-north (from Bath to Ludlow; S-N transect).

i. Preliminary small-scale study

Soil samples were collected in October 2007 on or near the Bath Golf course as shown in Figure 2.1. Habitat types and longitudinal-latitudinal coordinates of these samples are given in Table 2.7.

Table 2.7. Description of soil sample sites near or on the campus of the University of Bath and their coordinates, determined using the GPS device Garmin 205.

Site number	Location description	site coordinates
1	Bunker	N 51°23'2.52 W 002°19'13.38
2	Woodland	N 51°22.55.2' W 002°19.869'
3	Bunker	N 51°22.955' W 002°19.614'
4	beside the tree	N 51°22.970' W oo2°19.614'
6	Bunker	N 51°22.977' W 002°19.568'
7	compost border	N 51°22.959' W 002°19.572'
8	Woodland	N 51°22.961' W 002°19.538'
9	the rough	N 51°22.971' W 002°19.510
10	edge of the bunker	N 51°23.007' W 002°19.370'
11	middle of the bunker	N 51°23.007' W 002°19.365'
12	Woodland	N 51°23.038 W 002°19.224
13	Woodland	N 51°23.052 W 002°19.213
14	Woodland	N 51°23.042' W 002°19.223'

Figure 2.1. The location of the soil sampling sites near the Bath Golf course, using Google earth.

ii Localized survey on University of Bath campus

The second preliminary survey was carried out in May-June 2008 along two 15M transect lines on the campus of the University of Bath (location A and B in Figure 2.2). Location A was near the medical centre and Location B near Bath Cats and Dogs Home. As this study aimed only to examine spatial effects, in contrast to the preliminary study described above, all soil samples were collected from the same habitat (grassland). Samples were taken along each transect, which was marked with bamboo poles, either 0.5 M apart or 2 M apart over a period of five weeks. For each transect line, further soil samples were also collected from a site about 15 M away from the main site.

Figure 2.2. The sites of Location A and B near the University of Bath campus; location A was near the medical centre and Location B near Bath Cats and Dogs Home. Google map UK/University of Bath

The thirteen sample sites at each transect are illustrated in Figure 2.3. Seven sample sites at one end of the transect were 0.5 M apart, and a further 6 sample sites were 2 M apart. Samples were taken from all these sites for both transects, plus a separate site 15 M away, once a week for 5 weeks. At a single sample site (#9 in Figure 2.3) 3 samples were taken at each time point from different depths (0.0, 5.0, 10.0 cm). A total of 150 soil samples were taken.

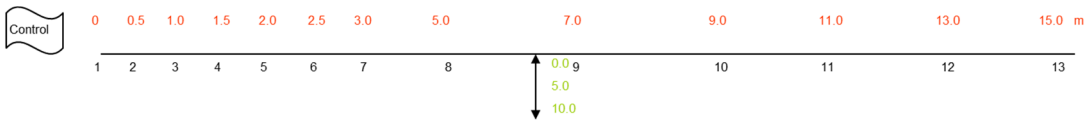


Figure 2.3. Diagram represents the transect line for the two locations A and B, showing the distances between sites numbered from 1 to 13 and different depths in site 9, with control 10 M apart from the line; the distance between location A and B was 1.5 Km.

iii) Large-scale survey in Southern England and Wales

Soil samples were collected from different habitat types along two large-scale transect-lines, one running east-west along the south coast from Lewes to Sidmouth (Transect A) and the other running south-north from Bath to Ludlow (Transect B); both samples were taken in February 2009 and May 2009. The locations of these transect lines are shown in Figure 2.4. The south coast samples (E-W) were taken from grasslands, sandy soils, woodland (the New Forest), and an island population (the Isle of Wight). The north-south transect (S-N) included mainly sheltered forested and field areas.

This survey was used to field test an experimental ecological surveillance application called Epicollect [137], designed for Android mobile devices. The system utilizes the functionality of smart phones, including the camera, SQLite database, and GPS. This allows real time monitoring of field surveys from a remote location. Soil types of each location were identified using the data available at http//www.landis.org.uk/soilscapes. Moreover, soil pH, moisture, and temperature were measured at each site.

Habitat descriptions as well as the longitudinal-latitudinal coordinates of the soil sample sites are given in the Appendix, table 1.

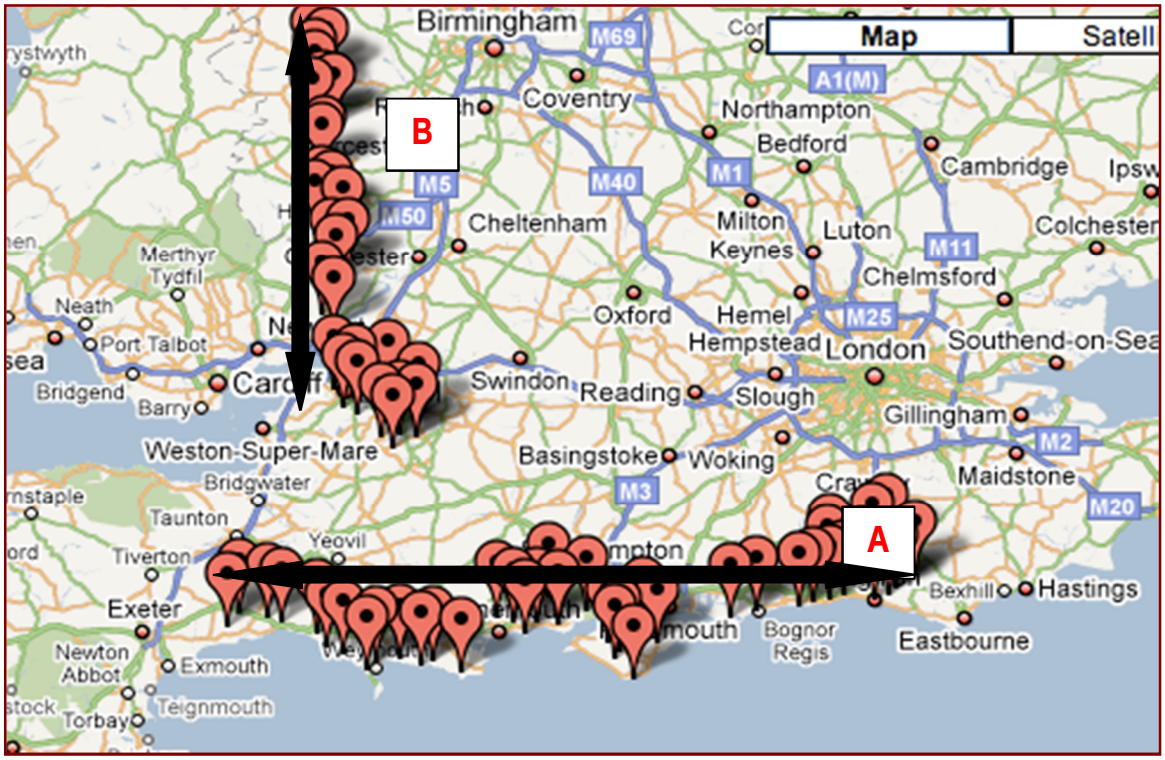


Figure 2.4. Google map of GPS of sampling sites using Epicollect application. Samples were collected in February 2009 (A) and May 2009 (B) from the two transects, http://www.spatialepidemiology.net/xenorhabdus. (Accessed on May 2009)

2.2.2. Soil "baiting" using *G. mellonella* model insects and isolation of nematodes

The soil samples were taken from a depth of 5 cm from the surface. Fifty grams of each were baited with three last instar wax moth larvae *G. mellonella* (Lepidoptera: Pyralidae) (table 2.3 page:30) placed on the surface of the soil in plastic containers. Containers were covered with a lid, and kept at room temperature (20 ± 3°C). After 5-7 days, all insects were recovered; dead larvae were placed on "White traps" [91], in order to isolate any entomopathogenic nematode (EPN) complexes from the infected insects. Briefly, infected *G.mellonella* larvae are placed on PBS-wetted strips (Oxoid) of filter paper in a Petri dish (table 2.5 page:31) . The filter paper leads down to a reservoir of the PBS, which serves to catch any EPN-nematodes leaving the insect corpse. Any emerging nematodes from the "White traps" were used to infect fresh *G. mellonella* larvae to confirm that they were the cause of death of the insect. The traps were observed over several weeks. Emerging nematodes were visualized using a Nikon Eclipse TE2000-S inverted

microscope. After one week, 2 ml of sterile water was added to each soil sample.

2.2.3. Sources of isolated bacteria

Bacteria were isolated from two sources: from crushed nematodes, and directly from the haemolymph of infected insects. Approximately one third of the nematodes extracted using white traps were crushed to extract bacteria, the others were washed in 1X PBS three times and stored at -80°C (one third), or added to a confluent plate of *Serratia* sp on nematode growth media (NGM) (one third) (section 2.1.2 page 29).

2.2.3.i Isolation of bacteria from EPN by crushing nematodes

The nematodes were washed three times in sterile PBS and surface sterilized using 1% commercial bleach for one hour (this was optimized using preliminary experiments; data not shown) [2]. In order to check the efficiency of the surface sterilization, the nematodes were washed with PBS three times and placed on LB media (Luria-Bertain Ager) (table 2.2 page 29) and the extent of bacterial growth noted (see Chapter 4 for details). Following surface sterilization, nematodes were washed three times in PBS and crushed on glass slides under sterile conditions. The nematode extract was streaked onto fresh LB agar and incubated overnight. Pure colonies were placed into a liquid medium and grown overnight (table 2.2 page:29). Bacterial stocks were maintained in 35% glycerol (final concentration) while a 1 ml aliquot of isolated uncrushed nematodes were maintained in 1 ml of freezing solution and stored at -80°C (table 2.5 page:31). Nematodes were tested for surface sterility; the nematodes were collected in a conical tube and washed three times with sterile 1X PBS, and the washed pellets were collected in a sterile 1.5ml Eppendorf tube; one percent of 20 µL bleach was added to the final washing and the nematodes were examined after 2,5,10 minutes and one hour. The nematodes were examined microscopically for survival after bleach treatment and washing. In addition, surface sterilized nematodes were also

added to LB media and incubated for 24h to check no external bacteria remained.

2.2.3.ii Isolation of bacteria from insect haemolymph

Infected *Galleria* were surface sterilized with 70% ethanol (Sigma). 100 µl sterile PBS was then injected into the larvae, and the diluted haemolymph was extracted from the larvae using a syringe (Norm-ject, Tuberkulin and 0.3x13mm of needle BD microlance-3), and transferred to a 1.5 ml microfuge tube. The haemolymph samples were then streaked out onto LB agar plates using the four-way streak method and incubated for 24h at room temperature. Single colonies were isolated and inoculated into sterile LB broth and incubated at room temperature (25°C) for 24h. Bacterial stocks were kept in 35% glycerol (final concentration) and stored at -80°C. This procedure was carried out both for insect cadavers from which nematodes had been isolated and insect cadavers from which no nematodes had been isolated (section 2.2.4).

In order to examine the normal flora of the model insect larvae *Galleria*, the haemolymph of 10 *Galleria* were streaked out onto LB plates after bleeding the insects by cutting one of their legs with sterile scissors. The plates were incubated at room temperature for 24 hours.

2.2.4. Maintenance of nematode isolates

Nematode isolates were maintained using three different methods. Firstly, nematodes were seeded onto NGM agar plates streaked with the corresponding bacteria that was found associated with them. They were incubated at 20°C to allow for ingestion and replication. Nematodes were transferred onto fresh bacterial lawns every three weeks. The nematodes were monitored for any potential contamination on occasion by using the surface sterilization, crushing and plating technique. Secondly, nematode isolates were maintained by the passage through infection cycles in *Galleria* larvae, using “White traps” to re-isolate the nematodes each time [138].

In some cases nematodes could not be maintained on the bacteria with which they were originally associated. Therefore, we also derived a third method in which nematodes were maintained on NGM agar plates seeded with a laboratory strain of *E. coli* (table 2.1 page:28). The strain selected was a tetracycline resistant *gfp* expressing strain prepared previously in our lab and therefore the NGM agar plates contained tetracycline. Furthermore, nematodes maintained on this *E. coli* strain were also used in subsequent experiments (see below).

2.2.5. Extraction of bacterial DNA

DNA was extracted from bacteria isolated from the two sources described in 2.2.4 using the DNeasy tissue kit (Qiagen, Germany) following the manufacturer's protocol. Briefly, this involved proteinase K treatment, column binding and some buffer washing steps. The genomic DNA was eluted from the column using 20µl of TE and stored at -20°C. These samples were used for PCR (described below).

2.2.6. Identification of bacterial isolates

The bacterial isolates recovered from the crushed nematodes and insect haemolymph were initially identified by PCR amplification and sequencing of the 16s rDNA gene. This was carried out using the universal 16s rDNA primers. The sequences of these primers are as follows:
Univ_16_F (5'-TGG CTC AGA ACG AAC GCT GGC GGC-3') and Univ_16_R (5'-CCC ACT GCT GCC TCC CGT AGG AGT-3').
For the large-scale survey, we also amplified the *recA* gene from the bacterial species that were isolated. This made use of the following primers *recA*-F (5'-GAG AAA CAG TTC GGC AAA GG-3') and *recA*-R (5' GTG TTG GCG TTT TTC AGG TT 3').

2.2.7. Multilocus sequence Analysis

We developed a multilocus sequence analesis (MLSA) scheme as a genotyping tool for *Serratia*. The internal fragment of ten ubiquitous genes (*recA, gmK, gyrB, glyA ,glmu, adk, AspA.pgi, rpoB* and *dnaJ*) were chosen (table 2.8) on the basis that they were physically unlinked in the genome, were presumed to be under stabilizing selection and were likely to be present in all isolates. Primers were used according to the published primers used for sequencing and amplifying Gram. negative bacteria in http://www.mlst.net/databases/default.asp.

Table 2.8. Primer sequences used in MLST analysis

Number	Gene	Nucleotide sequences	Amplicon size (bp)
1	*recA*-F	5'-GAGAAACAGTTCGGCAAAGG-3'	400
	recA-R	5'- GTGTTGGCGTTTTTCAGGTT-3'	
2	gmK-F	5'-CTGTACGACACGCAGGTTTC-3'	500
	gmK-R	5'-TGCTGATTAAAGCGTCATGC-3'	
3	gyrB-F	5'-ATGCTGTCTTCGCAGGAAGT-3'	500
	gyrB-R	5'-TCGTTCAGCAGTTTCACCAG-3'	
4	*glyA*-F	5'-TGCAATGGAAGCAAGAAGTTG-3'	360
	glyA-R	5'-GCCATGTCAACGAACAGGTA- 3'	
5	*glmu*-F	5'AAGGCGTGAATAACCGTCTG-3'	370
	glmu-R	5'- TTGGCGCCACATAGTTACA-3'	
6	*adK*-F	5'- GGCGCTGGTAAAGGTACTCA-3'	440
	adK-R	5'- TGCCGTCGATTTTACGGTAT-3'	
7	AspA-F	5'- GGTTTACGCTTCCAACCAAA-3'	400
	AspA-R	5'-TTGCCAATCACCTTGAAACA-3'	
8	*dnaJ*-F	5'- TGCCTTTAACGCCCTGTTGG-3'	514
	dnaJ-R	5'- TGACTTTCCTCACGCAACTG-3'	
9	Pgi-F	5'-GCTGGCCAAAATGAAACAGT-3'	500
	Pgi-R	5'-TTTTCTCTGCCGGAGTTTGT-3'	
10	rpoB-F	5'-CCATGGAACGGCTACAACTT-3'	500
	rpoB-R	5'-TGCTCAGCTTTTCAGCTTCA-3'	

The cross box represents the failed gene fragment amplification.

PCR reactions were prepared as shown in Table 2.9 to a final volume of 25 ml.

Table 2.9. PCR reaction

Component	Volume
2X Red PCR master mix	12.5µl
Primer-F 100pml	0.25µl
Primer-R 100pml	0.25µl
H_2O	11.0µl
Template DNA	1µl

The amplifications were performed using a Peltier thermal cycler (PTC) following the program described in table 2.10 and visualized on an agarose gel using standard methods. Under these conditions 5 out of 10 genes were reliably amplified. Amplicons were purified using Montage PCR filters according to the manufacturer's instructions and sequenced commercially by Qiagen.

The SeqMan program of the DNAstar Lasergene software was used for sequence editing. BLASTN comparison of the amplified genes sequences obtained against the NCBI nr-database was used to confirm the genus *Serratia* (insect pathogenic isolates) identification.

Table 2.10. PCR cycle details

Steps	Temperature (C°)	Time (mins)
Initial denaturation	95	2:00
Denaturation	95	00:45
Annealing	55	00:45
Extension	72	1:00
Final extension	72	10:00
Hold	4	Forever

35 cycles

2.2.8. Agarose gel electrophoresis

PCR amplification fragments and genomic DNA (gDNA) were examined by agarose gel electrophoresis in 1% TAE agarose gels (Tris-acetate-EDTA buffer). Ethidium bromide (Biorad) was added to a final concentration of 0.5µg/ml for DNA staining. A drop of 6 X loading dye was added to a 10µl sample of each PCR reaction which were then loaded onto the agarose gel. 10µl of diluted 1Kb DNA standard ladder (Qiagen) was used, and the gel was run at 100 V. The gels were run with a 1X TAE buffer for 30 minutes. The bands were visualized using a UV illuminator and the molecular size of observed band(s) for each sample was estimated by comparing its position on the gel with the DNA ladder.

2.2.9. PCR product sequencing and analyses

The PCR products were purified using Montage PCR centrifugal filter devices (Millipore) in order to remove salt, primers and unincorporated dNTPs. 100µl PCR reaction was mixed with a 300 µl TE buffer, applied on the DNA capture columns and centrifuged at 1000x for 15 minutes. The DNA fragments were eluted with a 20µl TE buffer and stored at 20°C. DNA sequencing was performed by the Qiagen sequencing service (http://www.qiagen.com/default.asp). DNA sequences were analyzed using the SeqMan program of the DNAstar Lasergene software BLASTn

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn). Comparison of the amplified genes sequences to sequences present in the NCBI nr-database was made to determine the genus with the highest similarity to the insect pathogenic isolates.

2.2.10. Phylogenetic Analysis

The sequences were aligned using ClustalW as implemented in MEGA 4.0.2 [139]. Phylogenetic trees based on 16S rDNA, *recA*, individual genes and the concatenated sequence were constructed using the neighbour-Joining method implemented in MEGA 4.0.2 [140]. Support for the trees was estimated by computing 1000 bootstrap trees.

goeBURST was used to cluster the STs as implemented in PHYLOVIZ (Phylogenetic inference and data visualization for sequence based typing methods) http://goeburst.phyloviz.net/ [125], first by loading allelic profile data and the accessory data file then running the data analysis algorithms.
The split decomposition technique was used to evaluate possible recombination for the sequences generated from the five genes, and the concatenated genes using SplitsTree4 (version (4.13.1) [113].

2.2.11. Investigating the virulence of the bacterial strains toward the model insect larvae; *Galleria. mellonella* and *Manduca. sexta*

i. Direct injection into the haemolymph.

The nematode associated *Serratia* strains isolated from Bath (B1) and Chepstow (C1) soils and an *E. coli* OP50 negative control were grown in LB broth with aeration overnight at 27°C (*Serratia*) and 37°C (*E. coli*).
Optical density (using a spectronic Unicam) was used to provide an estimate of cell number assuming an OD_{600} of 1.0 represented $1x10^9$ cells/ml. Cells were washed and diluted in sterile LB broth to give a range of cell

concentrations for injection (table 2.11). In addition to the *E. coli* negative control, cohorts injected with 10 µl of LB broth and un-injected *Galleria* were set up. *G. mellonella* were injected with 10µl / dilution (in the lamina flow cabinet) using a sterile syringe with a 0.3x13mm gauge needle. Each treatment was repeated twice.

Stock solutions of the three bacterial strains were prepared by dilution in sterile LB broth. The dilutions prepared were: $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$. Cohorts of ten *G. mellonella* larvae and three *M. sexta* larvae (table 2.2 page:30) were injected per dilution for each isolate. The larvae were placed on ice prior to injection to immobilize them and were surface sterilized with 70% ethanol. They were then incubated at 23°C and monitored daily for mortality. *Galleria* larvae were left unfed after injection, but *M. sexta* larvae were provided with an artificial diet. The median LD50 (lethal dose) and LD50 of the pathogenic bacteria *Serratia* was estimated in order to compare the virulence for each strain.

Table 2.11. Estimated bacterial cell numbers (B1, C1 and *E.coli*) in the dilutions used for insect injection experiments.

Dilution factor	number of cells per 10µl injected
$1:10^1$	695000
$1:10^2$	69500
$1:10^3$	6950
$1:10^4$	695
$1:10^5$	69.5

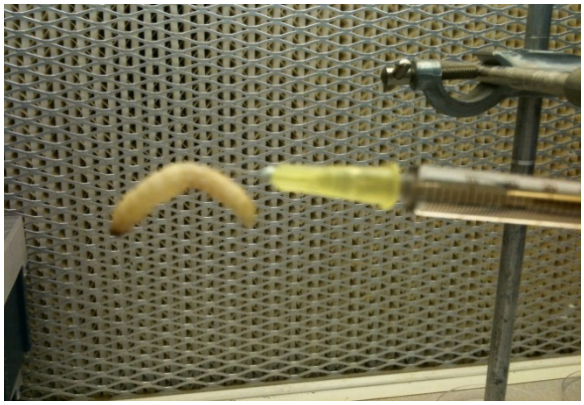


Figure 2.5.. Example of a *G. mellonella* larvae injection**.**

ii. Oral administration to *M. sexta* by feeding.

M. sexta larvae were exposed to infection by *Serratia* via the oral route by inoculation of the artificial diet, as described below.

S*erratia* and *E. coli* DH5α strains (table 2.1 page:28) were grown at 28°C for 16 h with aeration in 50ml of LB broth. Twenty ml cultures of each bacterial strain were grown overnight. The cell densities were measured using OD_{600} nm and bacterial stock solutions at equal cell densities were prepared (approximately 0.4 OD_{600} per ml). Cells were washed in 20ml of sterile PBS three times, harvesting using centrifugation at 4000 rpm 4°C for 10 min before final resuspension in 1ml of sterile PBS.

1 cm^3 disks of antibiotic free artificial diet (table 2.4 page:30) were prepared under aseptic conditions. Fifty µl of each bacterial culture sample was applied to each of these food disks and excess moisture driven off by leaving the diet disks exposed to air for 5 min in the laminar flow cabinet. Each treated food sample was placed in a plastic container, and a single 5^{th} instar *M. sexta* larvae was added and allowed to feed for 4 h. The small quantity of food ensured complete consumption of the whole bacterial dose for each larvae. After this time, the larvae were surface sterilized with 70% ethanol and transferred to fresh containers with fresh sterile food. They were returned to 28°C and monitored daily for mortality and morbidity.

2.2.12. Antibiotic susceptibilities

The natural levels of susceptibility of *Serratia proteamaculans*-like isolates to two different antibiotics were tested. Stock solutions of kanamycin and tetracycline (both 100 mg/ml) were prepared (table 2.6 page: 31) LB agar plates containing 100 µg/ml, 50 µg/ml, 10 µg/ml, 5µg/ml and 1 µg/ml of each antibiotic were prepared. The *Serratia* isolates were then streaked onto these plates and placed overnight at 28°C. In addition, the B1 and C1 strains were grown by serially streaking surviving colonies on a series of agar plates with increasing levels of antibiotics. Growth rates and virulence of these resistant strains were then tested to ensure they were not significantly different from the wild-type parent strain. Pathogenicity was confirmed by surface application onto *Galleria* larvae.

2.2.13. Labelling of *Serratia* isolates with *gfp* by triparental mating using mini-Tn7KSGFP

Chromosomes of the Bath *Serratia proteamaculans*-like isolate (B1) and Chepstow *Serratia proteamaculans*-like isolate (C1) were labelled using a triparental mating system [141].

Bacterial strains and plasmids

Two *E. coli* strains were used as donors in conjugations (table 2.1 page: 28):

1. The plasmid belonging to *E.coli* WM3064 pURR25 that contains the miniTn7KSGFP transposon.
2. The helper plasmid belonging to *E.coli* WM3064 pUX-BF13 that encodes the Tn7 transposase [142].

Two *Serratia proteamaculans* –like strains B1 and C1 were used as recipients.

Culturing and conjugation

We inoculated 3ml of overnight cultures of the *Serratia* B1 and C1recipient strains into 5ml of LB broth and incubated this at 28°C for 16 h. In addition, we inoculated 3ml of overnight cultures of the two donor cell strains into 5ml of LB containing 300µg/ml of diaminopimelic acid (DAP) and 100µg/ml of ampicillin (AP) and the mixture was incubated at 37°C and aerated for 16 h. WM3064 donor cells contain the RP4 *mob* for conjugation and *pir* gene for replicating R6K *ori*-based replicons; the DAP auxotroph is unable to cross-link the peptidoglycan cell wall unless DAP is added to the media. This provides good negative selection for later removal of the donor *E. coli* strains.

To perform the conjugation, 100µl of the *Serratia* recipient starter culture was transferred to 10ml of LB. In addition, 100µl of each donor strain was added to 10ml of LB with 50µl DAP and 10µl of AP. After approximately 3 h of growth, the cultures reached an OD_{600} of 0.6 nm. The recipient cells were then washed twice with 1.5ml LB, and the donor cells were washed with LB and DAP. Cells were harvested using centrifugation for 30s at 10,000 rpm. The recipient cells were resuspended in 0.5ml of LB and the donor cells were resuspended in 0.5ml of LB containing DAP. Then, 0.5ml from each of the donor and recipient cells were mixed and centrifuged for 30s at 10,000Xg. The supernatant was decanted and the remaining cells resuspended in 50µl of the washing solution. This cell mixture was then added to a dry LB DAP plate and incubated at 28°C overnight to allow mating to occur. The cells were then harvested from the plates by washing in 1.5ml of LB and centrifugated for 30s at 10.000Xg. The supernatant was decanted and the cells resuspended with 1.5 ml of LB, and the washing repeated once more. Finally, transconjugants containing min-Tn7KSGFP inserted into the chromosome were selected by plating onto LB agar containing no DAP. The cells were plated on LB containing 30µg/ml of the two antibiotics: Kanamycin and tetracycline. Expression of the gfp marker gene was confirmed after 24 h using a stereoflorescent microscope under illumination at 395nm.

2.2.14. Nematodes identification

Three of the nematodes isolated from the “White traps” were sent to Dr Patricia Stock (University of Arizona, Tucson), for further analysis. These three types were exemplified by nematodes isolated from Bath, Brighton and Chepstow soils. A molecular characterization of 39 isolates collected from different regions in the UK was also performed by the following steps. PCR amplification of the 18s rDNA gene was used to determine the genera of the nematode isolates. Genomic DNA was prepared from the frozen nematode samples, and used as a template for 18s rDNA PCR amplification. These amplicons were then sequenced.

i. DNA extraction

The method used for processing frozen samples for PCR amplification was the one described in the “DNeasy blood &tissue handbook” for purification of total DNA from animal tissues with a small modification; the nematodes were placed on autoclaved microtubes with sterile glass beads of 0.5mm, one in each tube, for disruption of the animal in order to homogenise the nematodes. The process of homogenization was done in the tissuelyser LT (Qiagen) for 2 secs at 30 oscillations.

The homogenized tissues were pipetted to a sterile microtube and digested using 20µl Proteinase K (0.8 µg/µl final concentration) and 180 µl buffer ATL; the mixture was mixed and centrifuged at 3000 rpm for 5 min, then all of the tubes were incubated at 56°C overnight for complete lyses. 410 µl of premixed buffer AL-ethanol was added per sample; after shaking, the mixture was centrifuged for 10 min at 6000 rpm and the lower part of the microtube was replaced with a clean one and 500 µl buffer AW1 was added per sample. The samples were centrifuged for 5 min at 6000 rpm and the lower part of the microtube was discarded. Then 500 µl buffer AW2 was added per sample and centrifuged for 15 min at 6000 rpm, with the samples eluted in a new microtube by adding 200 µl buffer AE per sample. Finally, the samples were incubated at room temperature for 1 min and then centrifuged for 2 min at 6000 rpm. Agarose gel electrophoresis was applied as mentioned in section 2.2.8.

ii. **Polymerase chain reaction (PCR) amplification and sequencing.**

Nematode 18S rDNA was amplified using the primers:

SSu18A (5'-AAA GATTAAGCCATGCAT G-3') forward, SSu26R (5'-CATTCTTGGCAAATGCTTTCG-3') reverse [143]. PCR products were purified and sequenced by Qiagen (Germany). The PCR technique was carried out on the 25µl reaction containing 5X Go Taq buffer, 1.25mM of $MgCl_2$, 0.25mM dNTPs, 1mM of each primer and 1µl of Go Taq polymerase containing 500 units (Promega) per reaction. The amplifications were performed using a Peltier thermal cycle (PTC). The mixture was then subjected to the following PCR conditions: 5 min at 95°C, 38 cycle including 1 min at 95°C, 1 min at 56°C, 1 min 30s at 72°C followed by 5 min at 72°C, cooling at 4° C (modified PCR condition [143]).

iii. Sequence analysis and phylogenetic relationships.

Sequences of 18S rDNA of 39 EPN were aligned using BioEdit software (DNAstar lasergene software, Madison, WI, USA). The sequences were compared with GenBank database sequences using Blastn searches using the similarity match within the sequences; then the alignments were used in the phylogenetic study using Mega4 software. The nematode trees were compared to the individual trees of the associated *Serratia* that were isolated from the crushed nematodes and with the concatenated tree, and the relatedness of each was studied.

2.2.15. Testing the stability of nematode-bacteria associations

An attempt was made to cure the Bath and Chepstow nematode isolates of their associated bacterial species by feeding them on a strain of tetracycline resistant Gfp labelled *E. coli* (table 2.1 page: 28). Mixtures of different nematode life stages were serially transferred between agar plates containing the *E. coli* food source for multiple passages (each passage took 7 days) in an attempt to "replace" any associated bacteria with *E. coli*. These experiments tested the direct ability of the *E. coli* to "outcompete" the naturally associated bacteria. In both isolates, the resident bacteria were strains of *Serratia* which were also resistant to the level of tetracycline used for selection of the *E. coli*. NGM plates containing 5 µg/ml tetracycline were streaked with the *gfp E.coli* strain in a Z shape as shown in Figure 2.6. The bacteria were grown at 37°C overnight before the addition of washed and surface sterilized nematodes. This seeding was repeated for thirty five passages on the *gfp E. coli* carrying a plasmid pHC60 (table 2.1 page: 28). Between each passage, a sample of the nematodes were surface sterilized, crushed and plated onto LB agar and onto NGM containing tetracycline plates to assess their bacterial contents. Colony morphology and the presence of the Gfp marker were used to determine the relative amounts of *E. coli* and original associated strains after overnight incubation at 28°C.

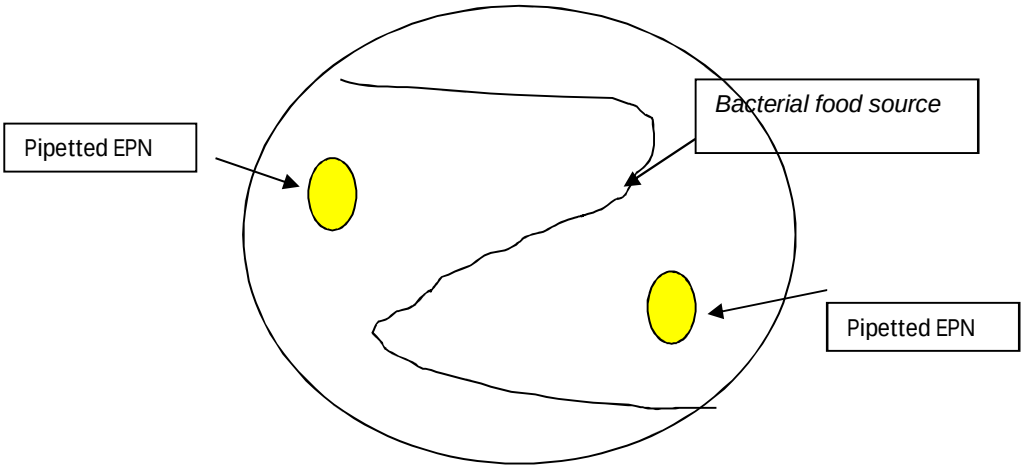


Figure 2.6. The Z-streaking method for maintaining nematodes on cholesterol NGM-Agar plates

2.2.16. Assessing the colonization of nematodes by their associated bacteria

Laser scanning confocal microscopy (LSM 510META) was used to study the colonization of the partner nematodes. Samples of the worms were washed three times with PBS and transferred onto NGM plates seeded with the labelled bacteria. They were passaged 15 times before microscopic visualization. Briefly, the worms were surface sterilized and washed (as described previously). 1% sodium azide was added to the nematode preparation to kill the worms before visualization. This was necessary as the worms rapidly moved away from the blue light used for the Gfp excitation 396 nm on the microscope. In addition, *C. elegans* (section 2.1.2 page 29) were also raised on these labelled *Serratia* strains and the fate of the bacteria was examined. Finally, nematodes were examined that had been serially passaged on the *gfp E. coli* strain in an attempt to cure them of their original bacteria.

2.2.17. Assessing insect pathogenicity of bacteria, nematodes and bacteria-nematode complexes

The relative contributions of the nematode and any associated bacteria to virulence in the insect model were investigated. To do this a soil trap technique was used. Other methods were tested, including using whetted filter paper or sand as alternative substrates [27] although autoclaved soil gave the most rapid infection times.

Soil was collected from around the University campus and baked in an oven at 200°C overnight for sterilization. When cool, 4ml of autoclaved water was added to the soil and placed in a sterile square petri dish. Ten *Galleria* were surface sterilized by immersion in 70% of alcohol for one minute [27], dried and placed on the sterile soil. Surface sterilized test nematodes or free bacteria were then added to the soil as shown in Table 2.12. To avoid

external contamination, these assays were performed in a biological safety cabinet.

The following material was added to 50g autoclaved soil + 4ml autoclaved water + 10 *Galleria* in each case. Ten replicates were set up per treatment.

Table 2.12. Soil trap method.

Treatment 1	1ml of an overnight culture ($OD600_{nm}$ was taken) of the associated *Serratia* strain diluted 1/100 in sterile PBS.
Treatment 2	1ml of washed nematodes reared on their associated *Serratia* strain (adjusted to approximately 4000 worms) in PBS
Treatment 3 control	1ml of *Caenorhabditis elegans* reared on *E.coli* OP50 (adjusted to approximately 4000 worms) in PBS.
Treatment 4	1ml of washed nematodes (adjusted to approximately 4000 worms) that had previously been fed on *gfp E. coli* (see section 5.2.5) in PBS
Treatment 5 Control	1ml of 1X PBS buffer.

* The nematode isolates used in this method were isolated from:

1. Bath, obtained from survey 1
2. Chepstow, obtained from survey 3
3. Brighton, obtained from survey 3

2.2.18. Examination of nematodes eggs for the presence of *Serratia* bacterial cells

The persistence of *Serratia* cells in the nematode worms was further investigated by the examination of the surface sterilized eggs in order to determine if bacteria could be maternally inherited and “vertically transmitted” between generations. Bath soil isolate nematodes were reared on NGM plates seeded with the nematode Gfp-labelled associated *Serratia*. Worms were harvested off the plate by washing with 1X PBS and transferred to a 50 ml sterile tube. They were washed a further three times in 30ml sterile PBS and harvested using low speed centrifugation at 1200 rpm for 3 minutes to pellet the worms, and remove the remaining external bacteria. The worm

pellet was resuspended in 15 ml of sterile PBS. The worms were then lysed by re-suspension in 6ml of lysis buffer (table 2.5 page: 31) for 10 minutes. During this time the lysis of the worms was monitored using a dissecting microscope. The lysis reaction was stopped by washing and re-suspension in egg buffer (table 2.5 page: 31). Eggs were washed three times by vortexing in egg buffer and harvested by centrifugation. After the last centrifugation, the wash buffer was removed carefully and the eggs were separated from the debris by adding 30% sucrose solution and an equal volume of egg buffer; the mixture was vortexed and again centrifuged at 1200 rpm for 5 minutes. In this solution, the eggs float to the top of the solution and may be harvested by pipetting. The eggs were transferred to a fresh 15 ml conical tube and again washed three times with the egg buffer to remove any remaining sucrose. The sterility of the eggs was tested by placing them on LB agar overnight at room temperature in the dark. The eggs were examined microscopically after hatching, and the media was examined for any bacterial growth associated with the emerging worms (htt://www.wormbook.org). In addition, sterile eggs were crushed into sterile LB broth plated out onto LB agar. DNA was prepared from any resulting bacterial colonies, and the *recA* PCR amplicon was sequenced in order to confirm if the bacteria were *Serratia* or otherwise. Sterile eggs prepared using these methods were also used to produce larvae for subsequent attraction assays discussed below.

2.2.19. Attraction assay

A method was devised to test if nematodes had a preference for the bacteria with which they were associated (the method was adapted from Zheng et al [66] and Rae et al [143]). We used this method to test if the Bath *Pristionchus* nematode would show positive phoresis toward its associated *Serratia* strain when presented with a choice of bacterial strains upon which to feed. Briefly 25µl of overnight bacterial suspension of the *Serratia* strain was placed onto an NGM agar plate 0.5 cm away from the edge of the petri dish. 25 µl of *E. coli* OP50 overnight culture was placed on the opposing side of the same plate. Either sterile eggs (see above) or approximately 50-60 nematode

individuals of different stages were placed at an equal distance between the two bacterial lawns being tested. Nematodes had previously been reared on NGM agar plates seeded with *E.coli* OP50 for several plate passages. The plates were sealed with parafilm and incubated at 20°C overnight [95]. The location of the nematodes was observed using a dissecting microscope and the number of individual worms at each bacterial lawn was counted and recorded. Three replica plates were used per assay, and the procedure was repeated three times for each tested bacterial strain. The combinations of the bacterial strains (section 2.1.1 page: 28) tested are shown below:

1. *E.coli* versus *Serratia* (B1)
2. *Photorhabdus luminescens* TT01 versus *Serratia* (B1)
3. *Pseudomonas fluorescens* versus *Serratia* (B1)
4. *Bacillus cereus* versus *Serratia* (B1)

Chapter 3

Characterization of common entomopathogenic bacteria associated and non- associated with nematodes in UK soil from different locations and habitat

3.1. Introduction

Entomopathogenic bacteria (Epb) are bacteria that can cause disease in insects and thus have potential for use as biological insect pest control agents. *Serratia* spp are entomopathogenic bacteria that belong to the family Enterobacteriaceae. This is a genus of Gram-negative bacteria, some members of which have clinical relevance [46]. *Serratia* occupies many habitats such as water, plants, animals and hospitalized human patients. Furthermore, many *Serratia* form symbiotic relations with a variety of insects; for example, the opportunistic pathogen *Serratia marcescens* was isolated from the haemolymph of the Boll weevil [34]. Molecular techniques have played a key role in studying the phylogeny, biodiversity, geographic distribution, host range, ecology, behavior and co-evolution of Entomopathogenic bacteria and nematodes [114].

To my knowledge, the work presented here represents the first biogeography study of entomopathogenic *Serratia* and their associated nematodes in UK soils. A study in 2006 [6] identified *Xenorhabdus* strains from EPN isolated from UK soil samples. These authors characterized the isolates by partial sequencing of 16S rRNA genes and four housekeeping genes. They claimed that *Xenorhabdus* are the most common entomopathogenic bacteria found in the UK soils. In addition, EPN and their symbiotic bacteria were identified via molecular tools in Southern France in 2008 [79]. These studies provided evidence concerning phylogenetic relatedness and geographic distribution of different strains belonging to the same species.

Entomopathogenic nematodes (EPN) persist in soil and are effective as biological control agents of soil insects [68]. They can be isolated by the "White trap" method, as described by Bedding and Akhurst [91]. In this method, *Galleria mellonella* are used to "bait" soil for EPNs [92].

Several surveys have studied the diversity and the distribution of EPN, and these have revealed evidence concerning habitat preferences [78, 144]. For

example, high nematode recovery from oak woodlands compared with all other isolated habitats in southern Arizona [94].

Multilocus sequence typing (MLST) is a widely used system for long-term epidemiology and micro-evolutionary studies [145, 146] . MLST is based on 5-10 housekeeping genes, and each unique allelic profile is assigned a sequence type (ST), thus defining a strain. eBURST is a clustering algorithm designed to reconstruct the likely evolutionary pathways between very closely related STs [125]. This approach has also been implemented as goeBURST, which is based on an improved algorithm compared to eBURST [125].

In this study I compared localized species distribution of entomopathogenic bacteria in soil samples, taken over a five week period from sites on or near the University of Bath campus. I also examined large-scale species distribution of entomopathogenic bacteria in soil samples taken across England and Wales.

In addition, I studied the molecular phylogeny of 84 entomopathogenic *S. proteamaculans*-like bacteria isolated from different sites by baiting with *G. mellonella*. In order to provide increased resolution of the identifying species, which was based on a single gene (*recA*), an MLST scheme was developed based on five housekeeping genes. goeBURST was used to visualize the relationships between the STs, and SplitsTree4 was used to determine the recombination within the housekeeping genes and between them. The results provided evidence of geographic and seasonal variation in the abundance of nematodes, and are consistent with the emergence of a cluster of *Serratia proteamaculans* that has co-evolved with EPNs.

3.2. Results

3.2.1. Surveying the community of insect pathogenic bacteria in UK soils.

Two surveys were carried out to identify the dominant insect pathogenic bacteria in UK soils. In each survey, soil baiting was used to isolate bacteria, as described in section 2.2.3 and 2.2.4 (page: 37 &38). Isolated colonies were then identified by amplifying and sequencing the 16s rRNA gene (section 2.2.6 page: 39). A third large-scale survey was carried out specifically on the molecular characterisation of isolates belonging to the *Serratia* genus.

i. Survey 1

14 soil samples from or near Bath golf course were baited with 3 insects each, making a total of 42 *Galleria* larvae. 30 insects died between 2-10 days (Appendix 1, table 2). 24 of the 30 insect cadavers yielded nematodes using white traps (section 2.2.2 page:36). The breakdown by habitat is given in Table 2.7. A total of 54 DNA extractions were carried out; 30 from haemolymph, and 24 from crushed nematodes. Successful amplification by PCR using the universal 16s rDNA primers were carried out in 29 of the 54 DNA samples, and 12 of the resulting amplicons were sequenced (section 2.2.10, page: 42). BLASTn was used to identify the closest hits of these sequences in Genbank (Appendix 1, figure 1). The isolates were identified as *Serratia* (n=9), *Xenorabhdus* (n=1), and *Pseudomonas* (n=2). This preliminary survey thus indicated that *Serratia* is a particularly abundant insect pathogenic bacterial taxon in UK soils. However, due to its limited scope, this survey did not provide meaningful data on spatial distribution or habitat preferences.

In order to make sure that the taxa recovered from the infected larvae were not originally present in the haemolymph of *Galleria*, we recovered bacteria from blood taken from ten healthy *Galleria* larvae, and sequenced 16S rDNA from these colonies. 3 of these colonies were identified as *Enterobacter* and the rest did not show significant BLAST hits to named taxa. No *Serratia*,

Xenorhabdus or *Pseudomonas* isolates were detected, and thus we conclude that the isolates recovered from infected larvae were associated with the infection process and were not already present in the healthy gut.

ii. Survey 2

This survey was primarily designed in order to examine to what extent bacterial communities are structured over very localized scales, and to what extent the isolates recovered from a given site are stable over the course of a few weeks. Soil samples were taken from two 15M transect lines on the campus of the University of Bath (location A and B in Figure 2.2). A total of 150 soil samples were collected from 28 sites over five weeks. As for survey 1, three insects were added to each soil sample, giving a total of 450 insects. Of these, 141 (31.3%) died within 2-10 days. Nematodes were not isolated from insect cadavers in this survey, but pure culture was grown directly from the haemolymph of 92 of the 141 insect cadavers. In 72/92 cases the 16s rRNA was successfully amplified and sequenced, thus enabling identification of the bacteria.

Seventy of the 72 isolates clustered into six taxa: *Serratia* / *Rhanella* (n = 12), *Acinetobacter* (n = 7), *Pseudomonas* (n = 11), *Enterococcus* (n=13), *Bacillus* (n = 7), *Xenorhabdus* (n = 20). The remaining isolates were identified as *Sphingobacterium* (n = 1) and *Aeromonas* (n = 1). This survey thus provided more data concerning the relative abundance of taxa. Whilst *Serratia* was found to be one of the major taxa present, the indication from survey 1 that this group dominates in the soil was not confirmed, and in this case *Xenorhabdus* was the most commonly recovered genus. Due to the small number of bacterial colonies characterized, the relative abundance of taxa is difficult to determine accurately and may even vary substantially between sites in close proximity, or from different time points. To illustrate this variation, it was noted that the relative abundance of the 6 different taxa mentioned above varied between the two transect lines, which are only ~400 M apart. *Serratia* sp and *Enterococcus* were relatively more abundant in location A than in location B, whereas *Xenorhabdus*, *Bacillus* and

Pseudomonas were relatively more abundant in location B than A (figure 3.1). However, these differences fell just outside of statistical significance (P = 0.07).

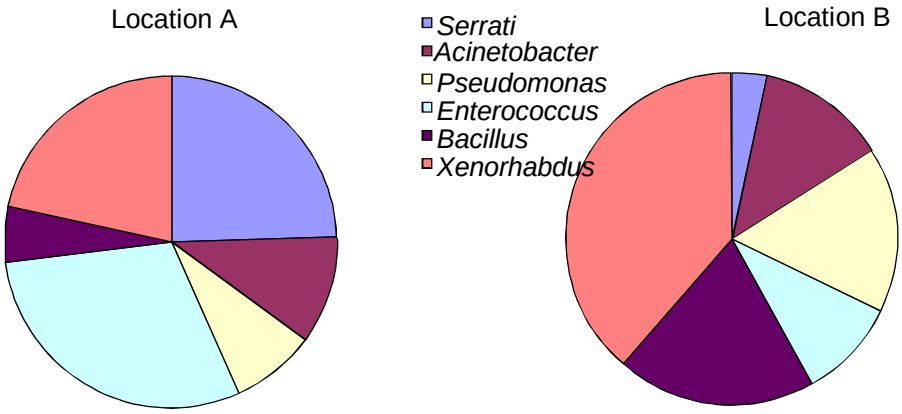


Figure 3.1. The relative abundance of the bacterial taxa is not significantly different between the two locations (Chi-Sq = 9.89, DF = 5, P-Value = 0.07).

Table 3.1 breaks down the abundance (number of isolates) of each taxon over each of the five weeks of the survey. The numbers are too small to draw conclusions regarding temporal variation in the relative frequency of each taxon when each week is considered individually. However, the five weeks over which the sampling was carried out varied with regards to the weather. Whereas weeks 1 and 5 were characterised by heavy rainfall, weeks 2, 3, and 4 were very dry. To quantify this retrospectively, I obtained weather records for Filton airfield near Bristol (http://www.martynhicks.co.uk/weather/data.php). The recorded rainfall for each of the sampling days (weeks 1 to 5) were as follows: 6mm, 0mm, 0mm, 0mm, 14mm. Pooling the data into wet weeks (1+5) and dry weeks (2+3+4) revealed significant differences in the relative frequencies of the different taxa. *Enterococcus sp* and *Acinetobacter* were relatively more likely to infect insects in rainy weeks, whereas the endospore forming bacteria *Bacillus sp* was more likely to infect insects in the dry weeks (figure 3.2).

Table 3.1. Summarized data of species identified in the two locations in the five weeks.

	Site A					Site B					Total
Weeks	1	2	3	4	5	1	2	3	4	5	
G1 *Serratia sp +Rehnella sp+Yerisinia sp*	4	4	1	0	2	1	0	0	0	0	12
G2 *Acinetobacter* sp	3	0	0	0	0	3	0	0	0	1	7
G3 *Pseudomonas* sp	3	1	0	0	1	2	3	1	0	0	11
G4 *Enterococcus* sp	4	1	2	0	3	0	0	0	1	2	13
G5 *Bacillus sp*	0	1	0	1	0	0	0	3	2	0	7
G6 *Xenorhabdus* sp	2	2	1	3	0	6	1	1	2	2	20
Total	16	9	4	4	6	12	4	4	5	5	70

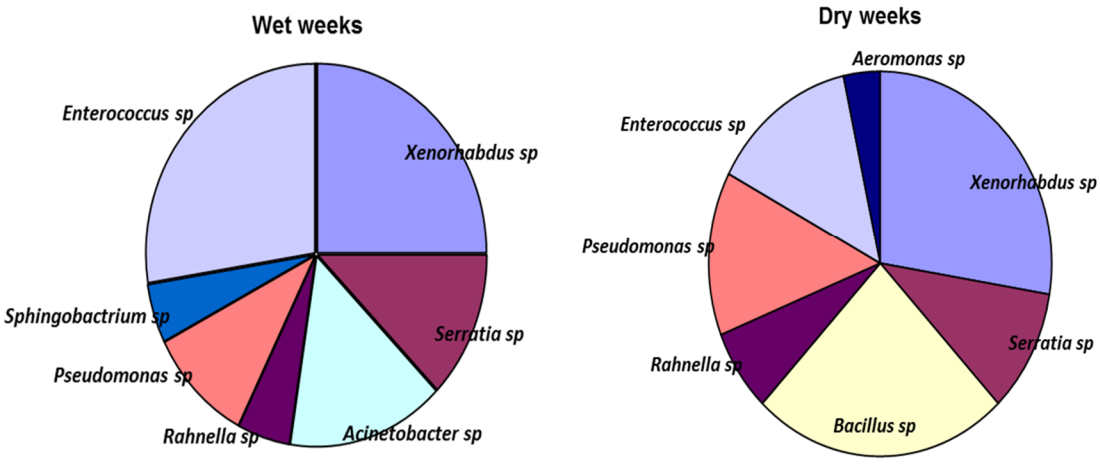


Figure 3.2. The distribution of isolated bacteria genera in wet (1+5) and dry weeks (2, 3+4). These differences are significant (Chi-Sq=18.6, DF=8, P value=0.016)

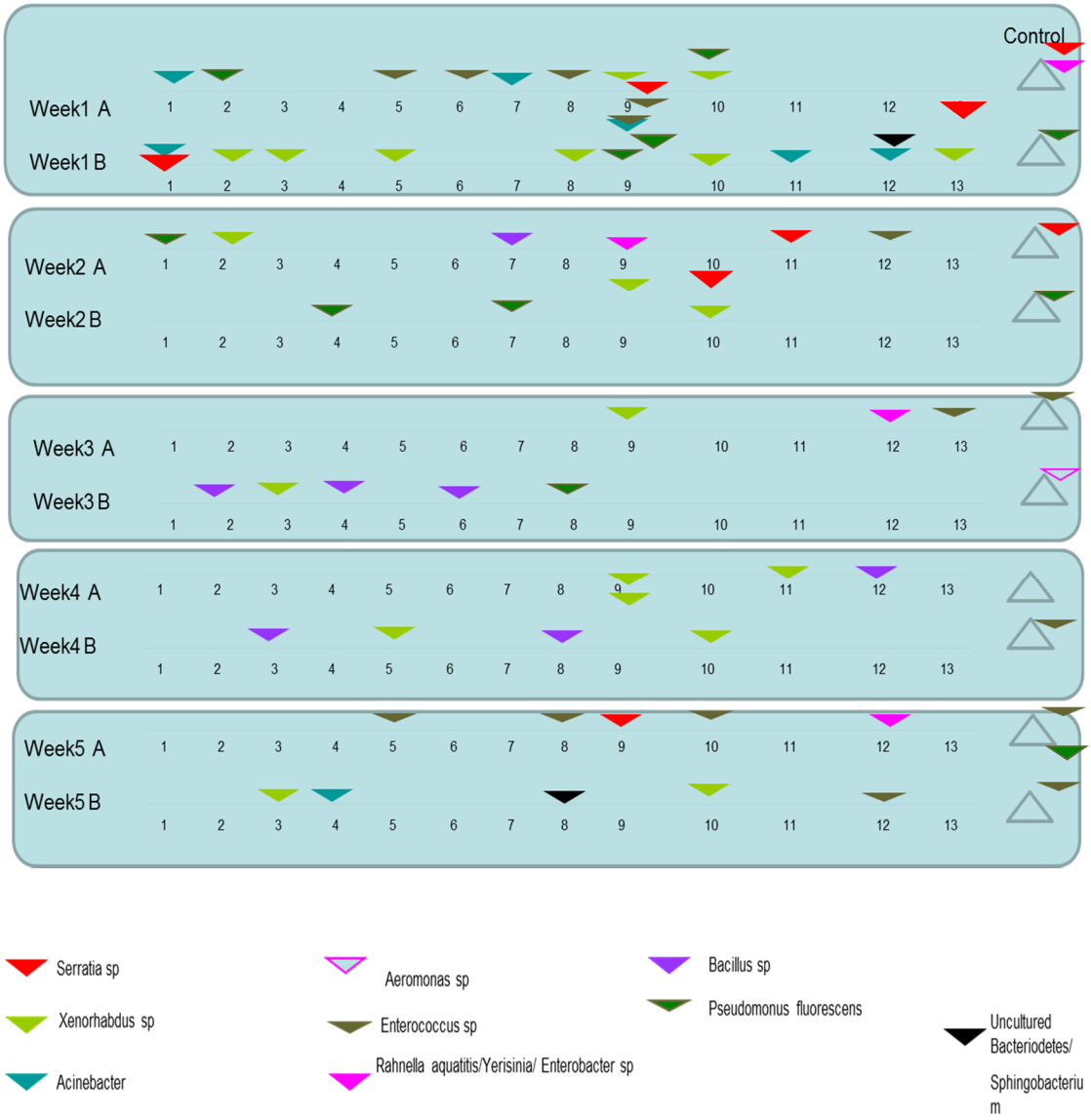


Figure 3.3 . Diagram of the putative genus isolates that are amplified with 16S rDNA primers isolated from Bath campus location A and B. The triangle represents the bacterial isolates found in the different sites along the two transects, the triangle on the side was the identified bacteria found in the soil samples collected as control.

Figure 3.3 shows identity (genus) at each position and time point for each of the 70 isolates. Spatial structuring on this scale would be evident if the same group(s) of bacteria are more likely to be recovered from neighbouring points along a given transect. There is little evidence for this from the data. Spatial stability would be evident if isolates from the same site but from different weeks were more likely to belong to the same genera than samples taken from different sites on different weeks. Again there is little evidence for this.

Although there is little evidence for either temporal or spatial structure, the following observations can be made regarding the data. *Serratia* sp, *Xenorhabdus* sp, *Acinetobacter* sp, *Enterococcus* sp, *Rehnella* sp and *Pseudomonas* sp were all recovered from soil samples from both transect lines during week 1, which showed the highest abundance and overall diversity. As noted above, *Bacillus* was only noted in the "dry" weeks 2,3 and 4. *Serratia* was abundant in location A in the first and the second week, was not recorded in weeks 3-4 but one sample was recovered in week 5. *Xenorhabdus* sp was the most commonly recorded taxon over time points and in all locations. This genus was found four times in the same location B10 in different weeks and two times in location A9 at different depths, indicating possible (but non-significant) clustering. Similarly, *Enterococcus* sp was recorded in week1A sites 5, 6, 8 and 9, and *Bacillus* sp were clustered in W3B sites 2,4, and 6.

iii. Survey 3: A UK-wide survey focusing on *Serratia*

The following conclusions were drawn from the two surveys described above. First, the methodology of soil baiting with *Galleria*, isolation from haemolymph or nematodes, and colony identification by 16s rRNA sequencing was shown to be an efficient and powerful methodological pipeline. Second, the results confirm the major insect pathogenic taxa present in UK soils, and that *Serratia* is likely to form a significant component of this community. Third, the observation that location A and B (in survey 2) showed significant differences provides evidence for spatial structuring on the level of ~400M. However, there is little evidence for spatial structuring on the scale on individual transects (~15M). Fourth, there is little evidence of stability within a site over different time points. Moreover, the data suggest that the community recovered from a single site might be largely a consequence of the prevailing weather conditions, with *Bacillus* being relatively abundant in dry conditions.

The failure to find evidence for spatial structuring within a very localized transect may have been a consequence of an insufficient sample size or the small spatial scale considered. In addition to this, the methodology used for characterising the isolates (16sr RNA) does not offer a very high level of resolution. The likelihood of finding evidence for spatial structuring would increase if: i) larger geographical scales were considered, and ii) more discriminatory markers were deployed. The use of more discriminatory markers means that it is logistically much easier to focus on single taxa, as primers for more variable protein coding genes will not work universally on all taxa.

In addition to questions relating to biogeography, an additional interesting question concerns associations with nematodes and whether the isolates for a given taxon that are recovered from nematodes are phylogenetically distinct from those isolated from haemolymph. If so, this would imply adaptation to the nematode and would raise the possibility of hitherto uncharacterized nematode / bacteria associations.

The strategy for the main survey was therefore to focus on characterizing the population of *Serratia*, both in terms of its biogeography and possible association with nematodes. The survey encompassed most of the breadth of southern England and the border country, and was carried out over two seasons. 160 soil samples were taken from 19 sites in the (E-W) transect A and (S-N) transect B described in section 2.2.1.iii, page: 35. Three *Galleria* were used in each soil sample and nematodes were recovered using white traps. Bacteria were isolated from crushed nematodes in all those cases where nematodes were isolates, and from infected haemolymph in all cases. The ecological surveillance tool Epicollect was used to record GPS and field data.

Table (1) (Appendix 1) shows the data of each sampling location submitted via Epicollect to the central database. It shows each sample location by GPS coordinate, soil pH, temperature, soil moisture and soil type which were

recorded using the smart phone. All data were synchronized using the Epicollect phone with the central database[137].

In the February 2009 survey, 114 infected insects were obtained from 160 soil samples. In the May survey 2009, 100 infected insects were obtained from 160 soil samples. A summary of the prevalence of infection and nematode recovery is given for each of the two transects in each survey in Table 3.2.

Table 3.2. Summary of the number of infected insects that produced and did not produce nematodes.

	2/09 A	2/09 B	Total	5/09 A	5/09 B	Total
Infected *Galleria*	77/300 (32%)	37/180 (20%)	114/480 (23.8%)	54/240 (22.5%)	46/180 (25%)	100/480 (20.8%)
Galleria producing nematodes	24/77 (31%)	3/37 (8.1%)	27/114 (23.7%)	8/54 (14.8%)	0	8/100 (8%)

Whereas the proportion of *Galleria* which became infected showed little variation, nematodes were significantly more commonly isolated in the February survey (pooled data 27/114; 23.7%) than in the May survey (pooled data 8/100; 8%) (chi sq. = 9.57 Df = 1, p = 0.00196). We carried out the same comparison on the basis of location, by pooling the data for the February and May samples. In transect A 32/131(24.4%) of infected insects produced nematodes, whilst the equivalent figures for location B were 3/37(8.1%). A chi sq test showed that this difference was also highly significant (chi sq = 16.080, Df = 1, p = 0.000060). Thus nematodes were more likely to be recovered in February than in May, and from transect A than from transect B.

PCR was carried out on the extracted DNA samples (section 2.2.5, page:39) using primers based on the *recA* gene sequence (section 2.2.9, page 42). We decided to focus on *Serratia sp* due to the high frequency with which this group was observed in the earlier surveys described above. *recA* was chosen as it is a widely used phylogenetic marker and provides greater resolution than 16s rRNA. The proportions of all colonies identified as *Serratia* were as follows: Feb (transect A): 61/100(66%); Feb (transect B): 12/50 (24%); May (transect A) 7/40 (17.5%); May (transect B) 10/50 (20%). A chi squared test revealed that a significantly higher proportion of bacterial colonies isolated from February was identified as *S. proteamaculans*-like sp (considering both transects; 49%) compared with the samples taken in May (18.9%) (Chi-Sq = 21.281, DF = 1, P-Value < 0.0005). Similarly, the total proportion of colonies isolated from transect A over both time points (48.6%) was significantly higher than the equivalent figure for transect B (22%) Chi-Sq = 17.573, DF = 1, P-Value < 0.0005.

In sum then, *Serratia* is predominant amongst the samples taken from February (compared to May) and transect A compared to B. To reiterate, nematodes were also more likely to be recovered in February than in May, and from transect A than from transect B. Thus, samples in which a greater number of nematodes were recovered (February/ Transect A) also showed a higher proportion of *Serratia*. Although these data in themselves do not provide evidence for a causal relationship, these observations are consistent with an association between *Serratia* and nematodes.

Pooling both transects A and B, and for both time-points, we recovered a total of 240 bacterial isolates. 201 of these isolates were recovered from insect haemolymph. Of these 201 isolates, 51 (25.3%) were identified as *S. proteamaculans*-like sp on the basis of amplification and sequencing of the *recA* gene. This is broadly consistent with the prevalence of *Serratia* observed in survey 2. The remaining 39 bacterial colonies were recovered from crushed nematodes, and 100% (35/35) of these colonies were positively identified as

Serratia on the basis of the *recA* gene. Thus, whereas 100% of colonies isolated from crushed nematodes were *S. proteamaculans*-like sp, only around a quarter of isolates from haemolymph were identified as *S. proteamaculans*-like sp ($P<0.0005$). This again hints at an association between *Serratia* and the nematodes.

A total of 90 *S. proteamaculans*-like sp isolates were identified through PCR and sequencing of the *recA* gene in the large-scale study; only 88 show a good sequence. In order to carry out a complete phylogenetic analysis (section 2.2.10, page 43), these sequences were supplemented with a further 42 *Serratia* isolates from the previous two surveys for which the *recA* gene was also successfully amplified and sequenced. 15 of these isolates derived from Bath golf course (survey 1) and 27 from the University of Bath campus (survey 2). The phylogenetic tree for all 130 isolates is given in Figure 3.4.

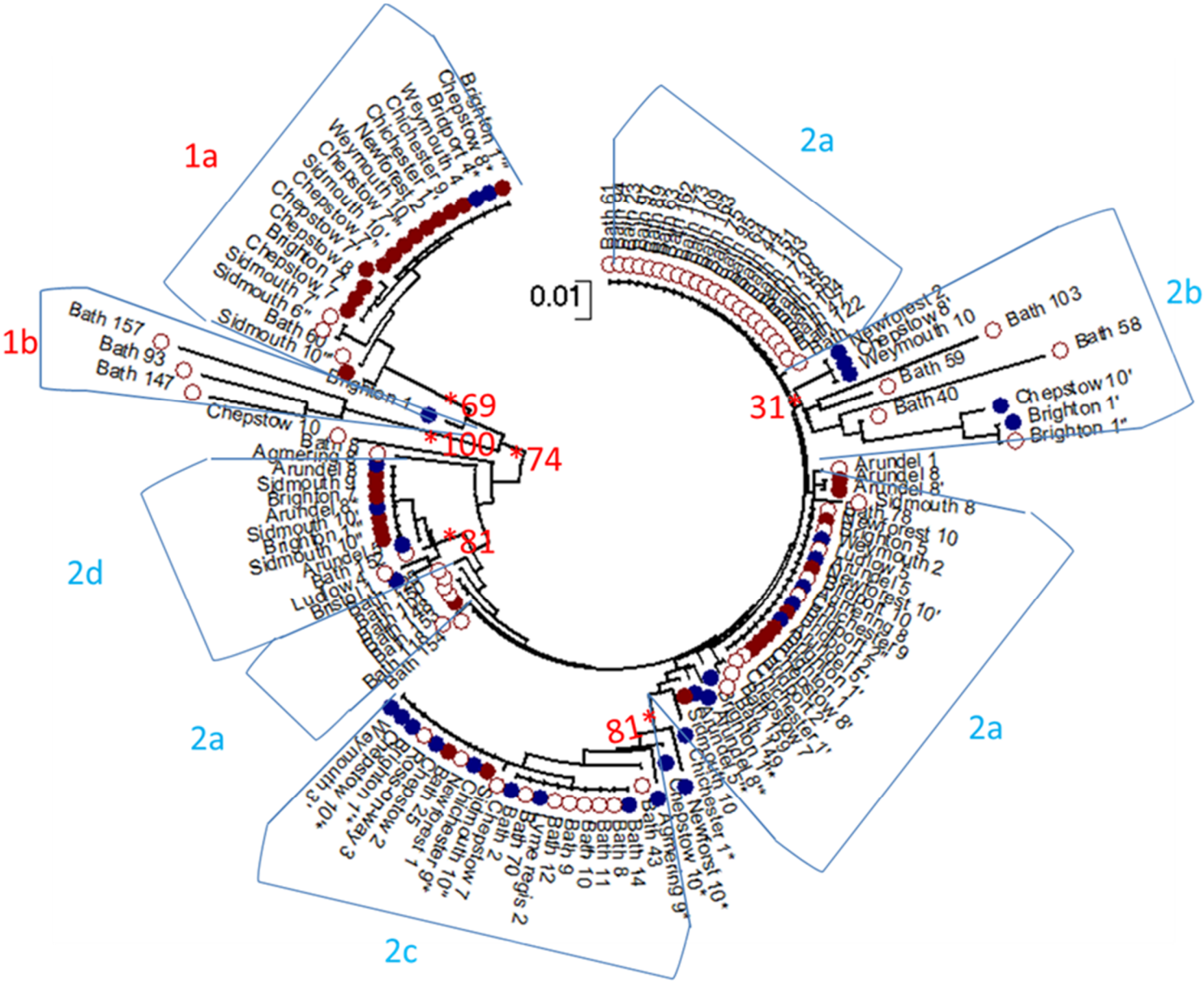


Figure 3.4. Neighbour-joining tree for 130 isolates related to *S. Proteamaculans-like sp* based on *recA* gene sequences. The associated *Serratia* were labelled as ●, *S. Proteamaculans* isolated from insect haemolymph which produced nematodes were labelled as ● Whereas S. *Proteamaculans* isolated from insects' haemolymph were labelled as ○ Bootstrap values are shown in red and the radiation tree view is shown in Appendix 1 (Figure 6.2). ',' and '"above the site name represent the number of infected *galleria* baited in the same soil sample.

The 130 *Serratia* strains isolated from this biogeographical survey are resolved into two major lineages (1 and 2) supported by a bootstrap value of 69% (Appendix 1, figure 2). Lineage 1 is subdivided into clusters 1a and 1b, whereas lineage 2 is subdivided into clusters 2a, 2b, 2c, 2d. Clusters 1a, and 2d are predominantly represented by isolates recovered from nematodes (85% and 76% respectively). In contrast, the percentage of isolates recovered directly from other nematodes in all the other clusters was much lower (overall average: 35.2%). This suggests that clusters 1a and 2d are specifically adapted to being vectored by nematodes.

Sequence diversity (π) for the whole sample and sub-samples representing the different sources of the isolates were calculated. This parameter (π) is a standard measure of sequence diversity and corresponds to the average pairwise sequence diversity (SNPs per site). Considering all 130 *Serratia* isolates, $\pi = 0.05$. For the 37 *Serratia* associated with nematode isolates $\pi = 0.07$, for the 61 haemolymph isolates $\pi = 0.04$, and for the 32 *Serratia* isolated from insect haemolymph $\pi = 0.06$. There are therefore no clear differences between the levels of sequence diversity of isolates from different sources.

We also considered geographical sub-divisions in the data. When the 52 isolates from Bath were considered, $\pi = 0.04$, when the 70 *Serratia* isolates from the north transects were considered $\pi = 0.04$, and when the 61 *Serratia* from the south were considered $\pi = 0.07$. There was therefore no obvious difference in sequence diversity according to geographical source.

Next, the phylogenetic tree was examined for evidence of geographic structuring. Whilst there is some evidence for this on a very fine scale (e.g. cluster 1b were all isolated from Bath, and 2d are predominantly from the south), in general the phylogenetic analysis does not reveal extensive geographical clustering. One interpretation of this is that there is free

migration of these bacteria or their nematode hosts between sites over evolutionary time. Alternatively, it may mean that the use of *recA* still does not provide sufficient discriminatory power to detect spatial clustering. Although this gene is more variable than 16s rRNA, it is still only a single gene. A common solution to this problem is to combine the data from multiple gene loci. Such an approach was developed for epidemiological purposes, where it is known as Multilocus Sequence Typing (MLST). For more environmental applications, where the target population is often not a well-defined species, the term Multilocus Sequence Analysis (MLSA) is used.

3.2.2. Development and use of an MLSA scheme for *Serratia proteamaculans*

We identified five suitable housekeeping genes based on comparisons with schemes for other species, and through initial studies comparing numerous candidate genes and primer sets (section 2.2.7, page 40). PCR and sequencing of these 5 MLSA genes (*adk*, *recA*, *glmU*, *glyA* and *dnaJ*) was carried out on 84 isolates previously identified as *Serratia proteamaculans*-like strains using the *recA* gene. These isolates came from all three surveys described above (Appendix 2, table 1) for a full list of strain sources, and were selected to be representative both geographically and in terms of the time of year at which they were sampled. The locations of the five genes on the genome are illustrated in Figure 3.5.

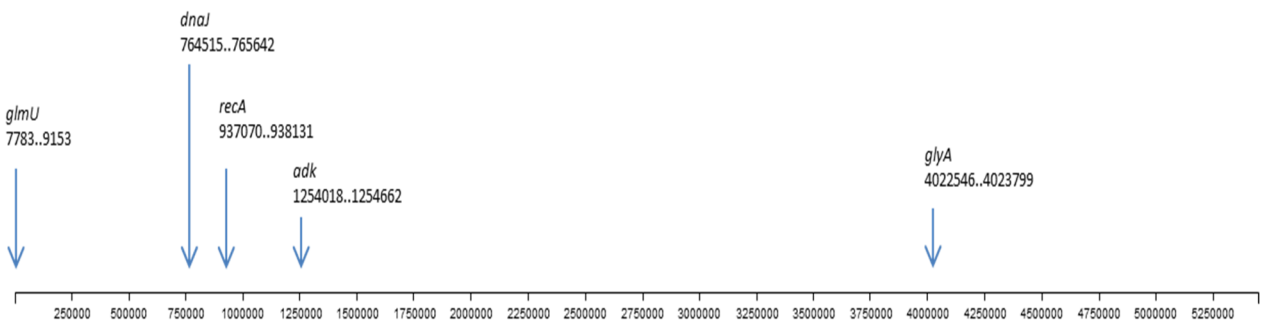
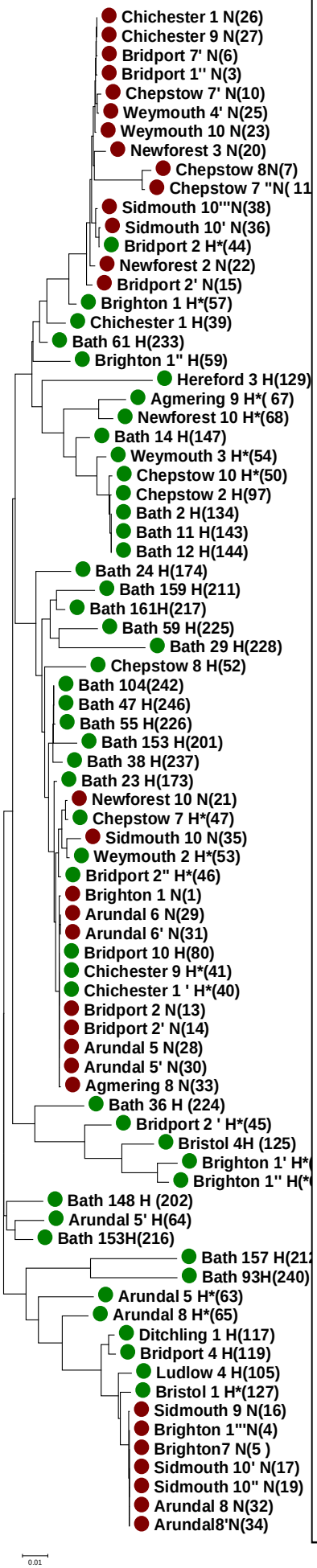


Figure 3.5. *Serratia proteamaculans* 568 chromosome (5448853 bp circular DNA)

The sequence diversity (π) for *recA*, *adk*, *glyA*, *glmU* and *dnaJ* genes respectively is, π = (0.08, 0.03, 0.03, 0.04 and 0.06). Although *recA* is the most diverse gene, these diversity levels are sufficiently similar such that no one gene will dominate the signal in the concatenated tree. The overall level of sequence diversity for the concatenated data is π = 0.05.

The concatenated sequences of the five genes were used to construct a neighbour-joining tree (Figure 3.6). This tree reveals two tight clusters of isolates that were i) recovered from the South Coast (eg. Angmering, Arundel, Bridport, Chichester, Brighton, New Forest, Sidmouth), and ii) recovered from nematodes. These clusters are labelled as clade 1 and clade 5 in Figure 3.6. Clade 2 and 4 are predominantly from haemolymph, whilst clade 3 is a mixture of isolates from nematodes and haemolymph. Isolates from Bath, Bristol, Chepstow and Hereford tend to be diverse and are scattered throughout the tree. However, there is little evidence for geographical structuring on a finer scale, that is, within the south coast isolates. As noted earlier, the south coast isolates were more commonly recovered from nematodes than directly recovered from haemolymph (green dot). In sum, these data support the impression from *recA* sequences and indicate that isolates from the south coast resolve into distinct clusters, and that these clusters tend to be nematode associated. Isolates from other regions are more phylogenetically scattered and are more typically recovered directly from haemolymph.



Origin	Isolated from	adk	dnaJ	glmu	RecA	glyA	ST
South UK	Crushed nematodes	1	6	4	2	10	19
South UK	Crushed nematodes	1	6	4	2	10	19
South UK	Crushed nematodes	1	6	4	5	10	6
South UK	Crushed nematodes	1	6	2	2	10	3
North UK	Crushed nematodes	4	6	7	5	10	9
South UK	Crushed nematodes	1	6	12	2	10	18
South UK	Crushed nematodes	1	6	4	10	10	17
South UK	Crushed nematodes	5	6	10	2	12	14
North UK	Crushed nematodes	1	6	5	6	3	7
North UK	Crushed nematodes	1	6	8	2	14	10
South UK	Crushed nematodes	8	6	11	15	12	25
South UK	Crushed nematodes	6	6	14	2	12	24
South UK	Haemolymph	6	6	11	2	12	27
South UK	Crushed nematodes	1	12	4	9	10	16
South UK	Crushed nematodes	1	17	9	8	10	12
South UK	Haemolymph	6	6	11	20	12	35
South UK	Haemolymph	9	14	15	16	25	26
North UK	Haemolymph	1	6	42	31	5	66
South UK	Haemolymph	14	29	11	21	12	36
North UK	Haemolymph	23	16	30	1	22	51
South UK	Haemolymph	15	6	24	25	7	42
South UK	Haemolymph	18	6	25	26	8	43
North UK	Haemolymph	24	3	31	30	7	53
South UK	Haemolymph	11	30	17	18	7	34
North UK	Haemolymph	11	3	17	18	7	31
North UK	Haemolymph	19	3	26	18	7	45
North UK	Haemolymph	24	3	26	30	7	52
North UK	Haemolymph	24	3	26	30	7	52
North UK	Haemolymph	24	3	26	30	7	52
North UK	Haemolymph	1	18	32	18	5	55
North UK	Haemolymph	26	28	34	33	5	58
North UK	Haemolymph	29	7	37	31	5	61
North UK	Haemolymph	31	23	39	37	17	63
North UK	Haemolymph	1	4	41	38	17	65
North UK	Haemolymph	12	21	18	19	27	32
North UK	Haemolymph	1	1	32	31	5	69
North UK	Haemolymph	1	1	32	31	5	69
North UK	Haemolymph	1	31	32	31	5	56
North UK	Haemolymph	1	1	43	31	5	67
North UK	Haemolymph	1	1	31	31	19	54
South UK	Crushed nematodes	6	8	11	1	10	15
North UK	Haemolymph	6	8	11	1	12	30
South UK	Crushed nematodes	7	14	13	14	12	23
South UK	Haemolymph	13	9	19	1	18	33
South UK	Haemolymph	1	22	4	1	12	29
South UK	Crushed nematodes	1	11	1	1	6	2
South UK	Crushed nematodes	1	1	1	12	6	21
South UK	Crushed nematodes	1	1	1	12	6	21
South UK	Haemolymph	1	1	1	1	6	11
South UK	Haemolymph	1	1	1	1	6	11
South UK	Haemolymph	1	1	1	1	6	11
South UK	Crushed nematodes	1	1	1	1	6	11
South UK	Crushed nematodes	1	1	1	1	6	11
South UK	Crushed nematodes	1	1	1	11	6	20
South UK	Crushed nematodes	1	1	1	1	6	11
South UK	Crushed nematodes	1	1	1	13	6	22
North UK	Haemolymph	30	19	38	36	23	62
South UK	Haemolymph	10	26	16	17	28	28
North UK	Haemolymph	22	25	29	28	11	49
South UK	Haemolymph	14	13	20	22	9	37
South UK	Haemolymph	15	13	20	22	9	38
North UK	Haemolymph	25	15	33	32	24	57
South UK	Haemolymph	2	2	22	24	21	40
North UK	Haemolymph	28	20	36	35	5	60
North UK	Haemolymph	27	27	35	34	16	59
North UK	Haemolymph	32	4	44	29	5	68
South UK	Haemolymph	16	6	21	23	26	63
South UK	Haemolymph	17	2	23	3	20	41
South UK	Haemolymph	2	2	3	1	4	47
South UK	Haemolymph	21	10	28	1	2	48
North UK	Haemolymph	20	2	27	27	29	46
North UK	Haemolymph	21	10	28	29	2	50
South UK	Crushed nematodes	2	2	3	3	2	13
South UK	Crushed nematodes	2	2	3	3	4	4
South UK	Crushed nematodes	2	2	3	3	4	5
South UK	Crushed nematodes	2	2	3	3	4	4
South UK	Crushed nematodes	2	2	3	3	4	4
South UK	Crushed nematodes	2	2	3	3	4	4
South UK	Crushed nematodes	2	2	3	3	4	4

Clade 1 *Serratia* associated with nematodes with one exception

Clade 2

Serratia isolated from insect haemolymph

Clade 3 Mixed *Serratia* isolates associated and non-associated with nematodes

Clade 4

Diverse *Serratia* haemolymph isolates

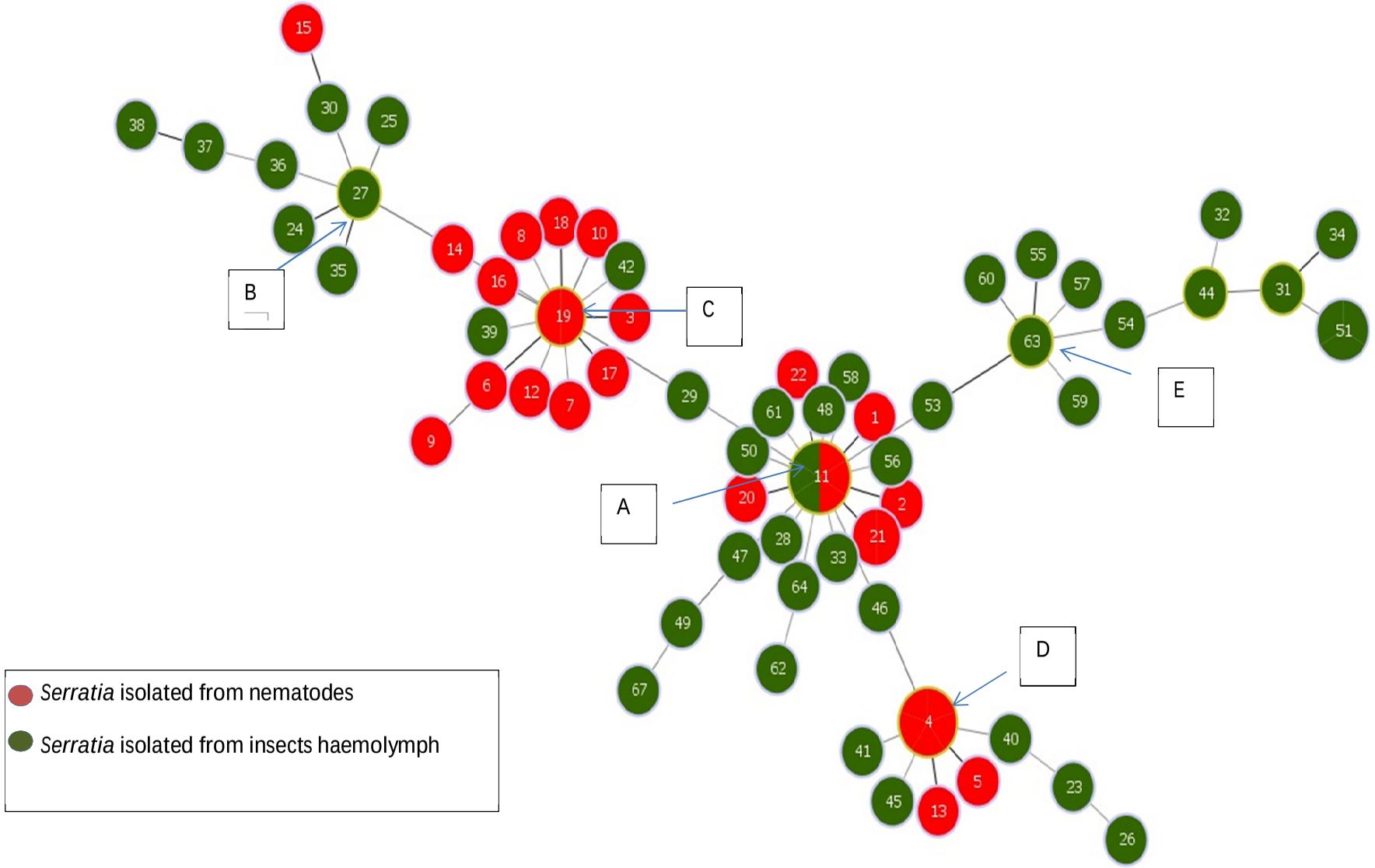
Clade5/ Associated *Serratia* isolates

Figure 3.6. Concatenated tree of the 5 housekeeping genes of 84 *Serratia* samples; ST and MLST allelic profiles of five housekeeping genes are shown for each isolate. The bootstrapped tree is shown in Appendix 2, figure 1. ‘,’’ and ‘’’above the site name represent the number of infected galleria baited in the same soil sample.

3.2.3. Clustering of allelic profiles using goe-BURST

In addition to constructing trees based on concatenated gene sequences, an additional means of visualizing MLSA data is clustering based on allelic profiles. A widely used method for this is BURST, which identifies likely founders of clusters (clonal complexes) and links near neighbours in a parsimonious fashion. goeBURST is a recent implementation of the BURST algorithm. It differs from the eBURST implementation primarily by employing a globally optimised (go) approach which corrects for the rare occasions when eBURST infers links inconsistent with BURST rules. Further, it allows the connection of all STs regardless of their divergence, although STs sharing only 1 or 2 alleles may be linked in many different ways, thus such links have low statistical robustness. Sixty-eight different STs were identified from the 84 isolates and goeBURST (PHYLOViZ) was used to determine the relatedness between the isolates based on the allelic profiles rather than the sequences (figure 3.7).

Figure 3.7. Population snapshot of 84 *S. proteamaculans*-like isolates showing major and minor clonal complexes. The primary founder of CCs is indicating by arrow A. Arrows B, C,E and D indicate the subgroup founders



The size of the circle is related to the numbers of isolates within each ST. Only ST 11 was found in both haemolymph and nematode isolates, although most STs were only found once. goeBURST works by dividing the MLSA dataset into clusters of related isolates (clonal complexes), and then identifying the most likely "founder" of each complex on the basis that it defines the highest number of single locus variants (SLV; STs that only differ at one of the seven loci). Five "founder" STs were identified in the data; STs 27, 11, 4, 19 and 63, and these define clonal complexes CC27 (B), CC11(A), CC4(D), CC19 (C) and CC63 (E). CCs 11, 4, 19 (A, D and C respectively) tend to be associated with nematodes, and these correspond to the phylogenetic clusters discussed above. In contrast, CC63 (E) consists entirely of isolates recovered from haemolymph, and CC 27 (B) contains only 1 isolate (ST) from nematodes. This analysis therefore supports the preference inference from the phylogenetic tree that the different clusters defined by MLSA in the *Serratia proteamaculans* population differ in the degree to which they are associated (and hence probably adapted to) nematodes.

3.2.4. Phylogenetic consistency and Recombination

In addition to constructing phylogenetic trees based on the concatenated sequences of all five genes, it is also possible to reconstruct trees based on each gene in turn. A comparison of these trees then makes it possible to gauge the possible role of recombination in the evolution of these strains. Homologous recombination is a common phenomenon in bacteria, resulting in the replacement of one allele with another from a related strain or species. If this process is very common then individual gene trees will not reflect strain phylogeny, but will simply reflect the phylogeny of the individual gene. In this case, gene trees will show differences in topology. It is also possible that topological inconsistencies may reflect a paucity of informative sites in the data, thus making the tree reconstructions statistically unreliable. In this case, the bootstrap scores on the trees are likely to be low. It is, of course, also possible that a combination of both these confounding effects may be operating to make the trees inconsistent.

The individual gene trees are shown in Figures 3.8 - 3.12. In general the bootstrap scores tend to be very low (Appendix 2, figures 2-6), and numerous topological inconsistencies are evident. For example, the strains 240 and 212 are closely related on the basis of gene *recA*, but distantly related on the basis of gene *glmU*. These topological inconsistencies are thus consistent with, but not proof of, frequent recombination. In order to examine this possibility further, the data were analysed using Splits Tree (version 4.13.1). First we tested the data for each gene separately. The Splits figures showed little evidence of recombination for all of the genes (Appendix 2, figure 7) and this was confirmed using the phi test, which gave a non-significant result (p)>0.05) for each of the five genes (*adk* p= 0.495, *dnaJ* p=0.238, *glyA* p= 0.376, *glmU* p=0.785 and *recA* p=0.891). However, extensive evidence for recombination was noted when the concatenated data for all five genes were analyzed (P<0.001). This pattern suggests that intergenic recombination (between genes) has been quite common, but intragenic recombination (within genes) has been rare. A simple interpretation of this is that the size of the recombination events is considerably larger than the sequenced MLST alleles.

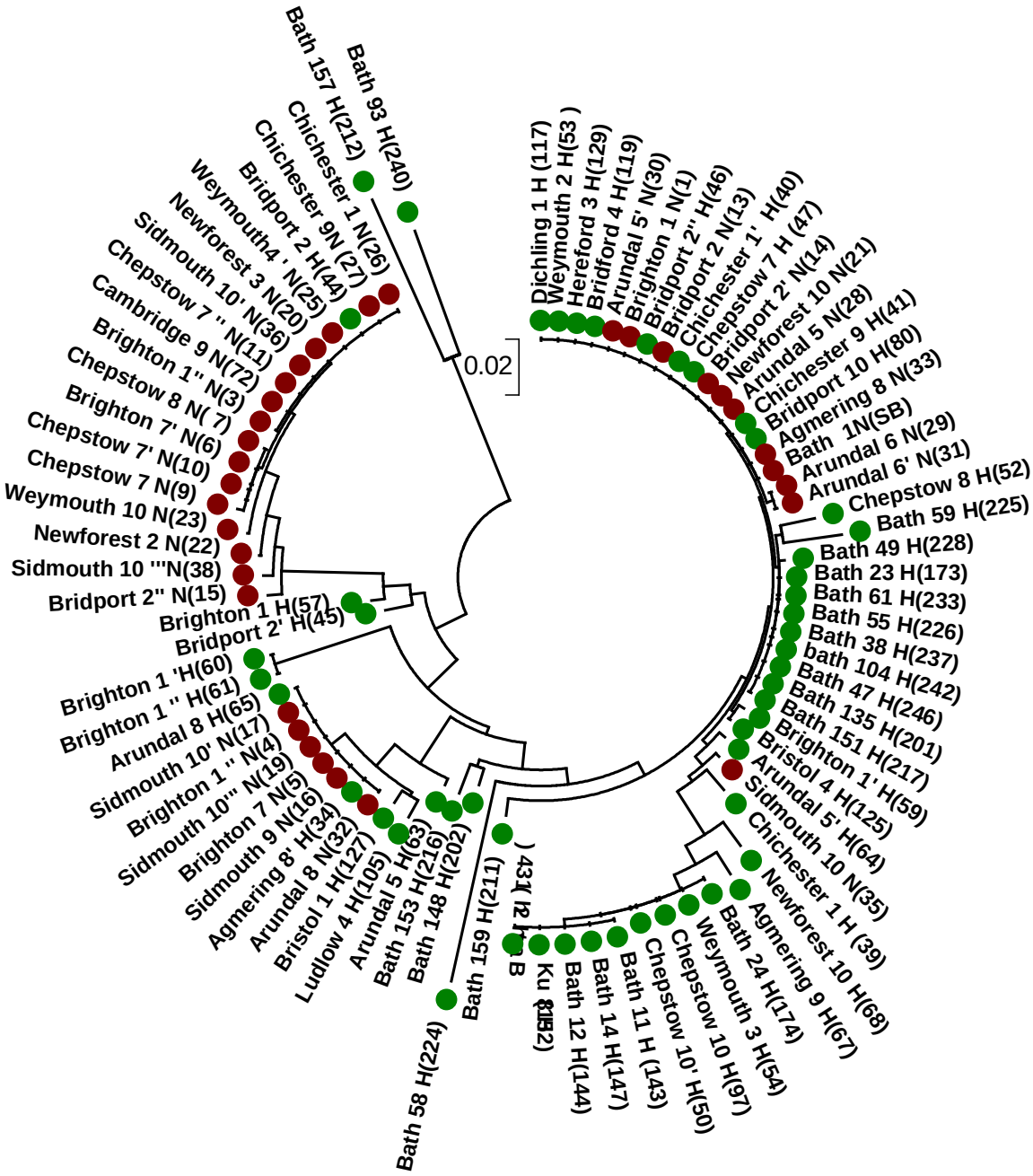


Figure 3.8 . A.Neighbour-joining tree based on *recA* gene sequence of 84 strains belonging to *S. proteamaculans*-like strains. ‘,’’ and ‘’’above the site name represent the number of infected *galleria* baited in the same soil sample.

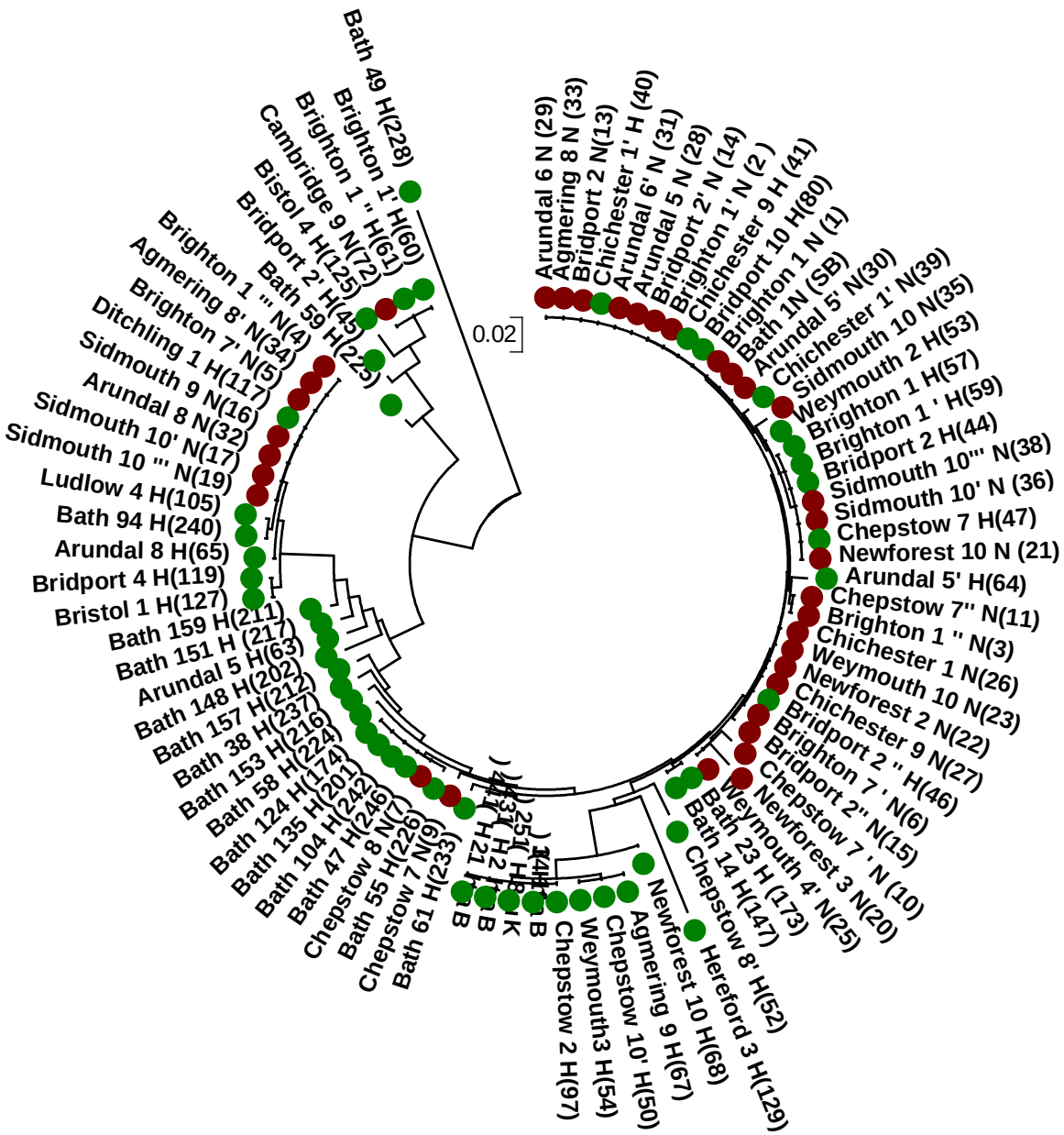


Figure 3.9 . Neighbour-joining tree based on *glmU* gene sequence of 84 strains belonging to *S. Proteamaculans*-like strains. ‘,’’ and ‘’’above the site name represent the number of infected *galleria* baited in the same soil sample.

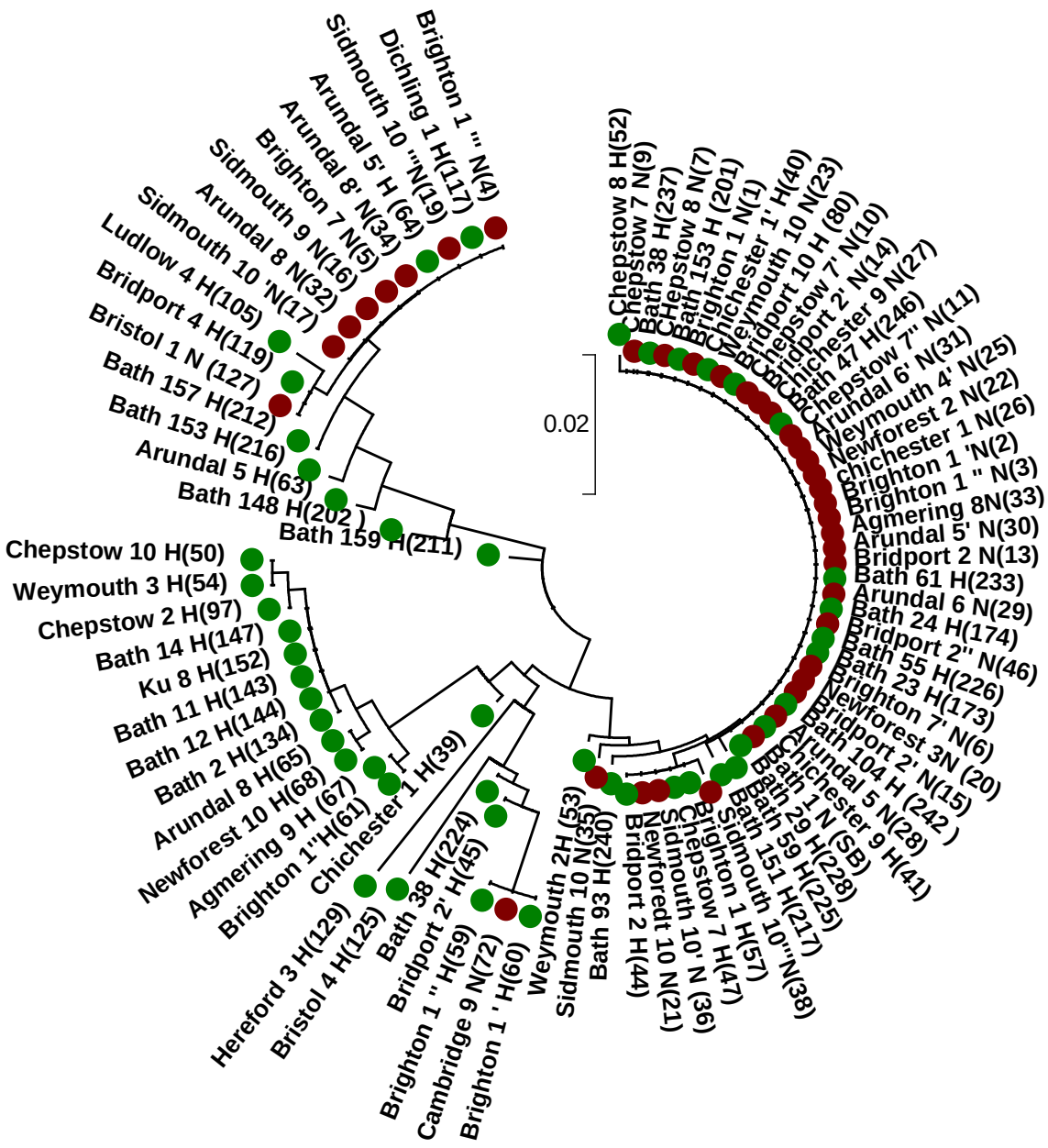


Figure 3.10. Neighbour-joining tree based on *adK* gene sequence of 84 strains belonging to *S. proteamaculans*-like strains. ‘,’’ and ‘’’above the site name represent the number of infected *galleria* baited in the same soil sample.

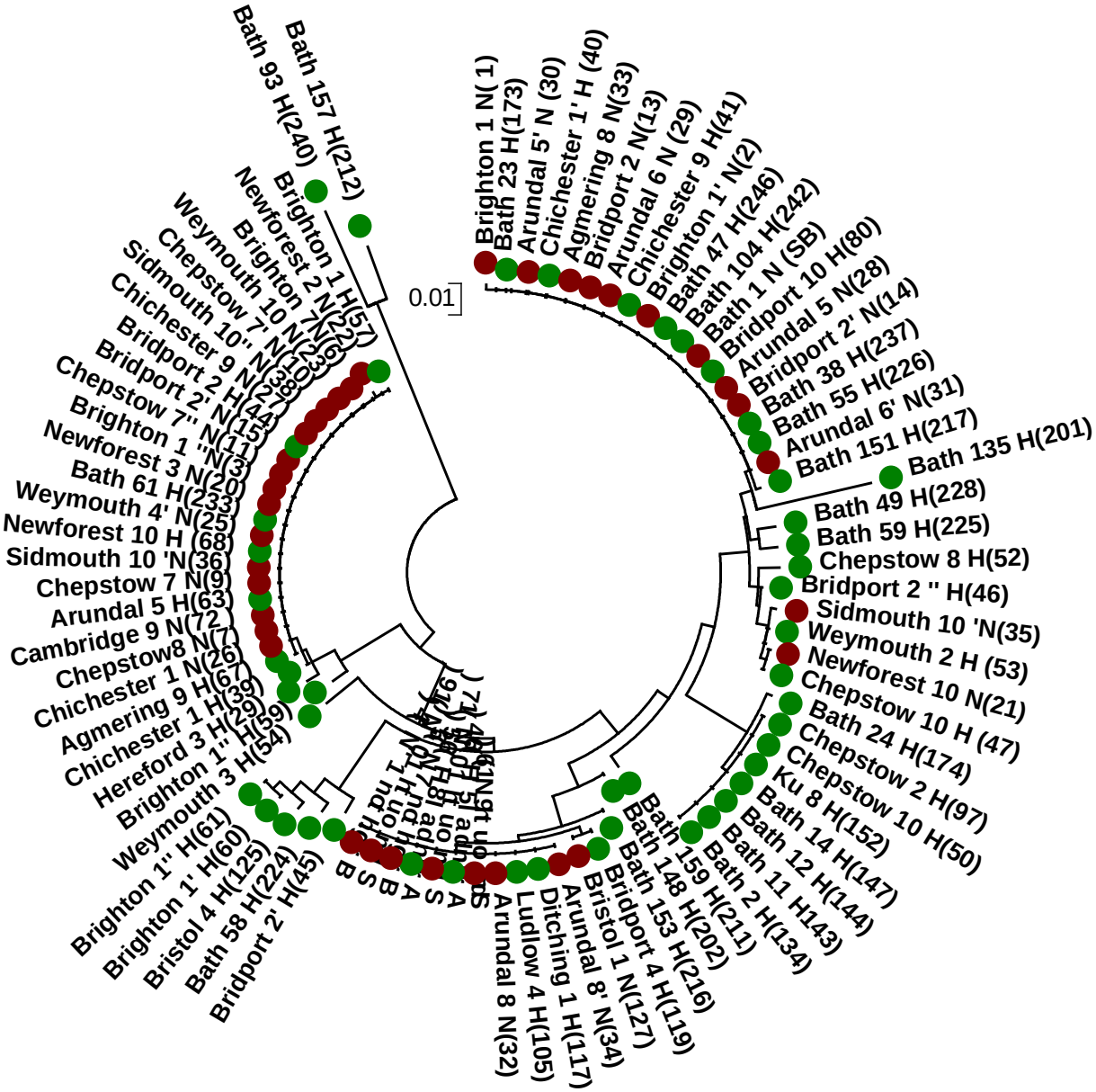


Figure 3.11. Neighbour-joining tree based on *dnaJ* gene sequence of 84 strains belonging to *S. proteamaculans*-like strain. ‘,’’ and ‘’’above the site name represent the number of infected *galleria* baited in the same soil sample.

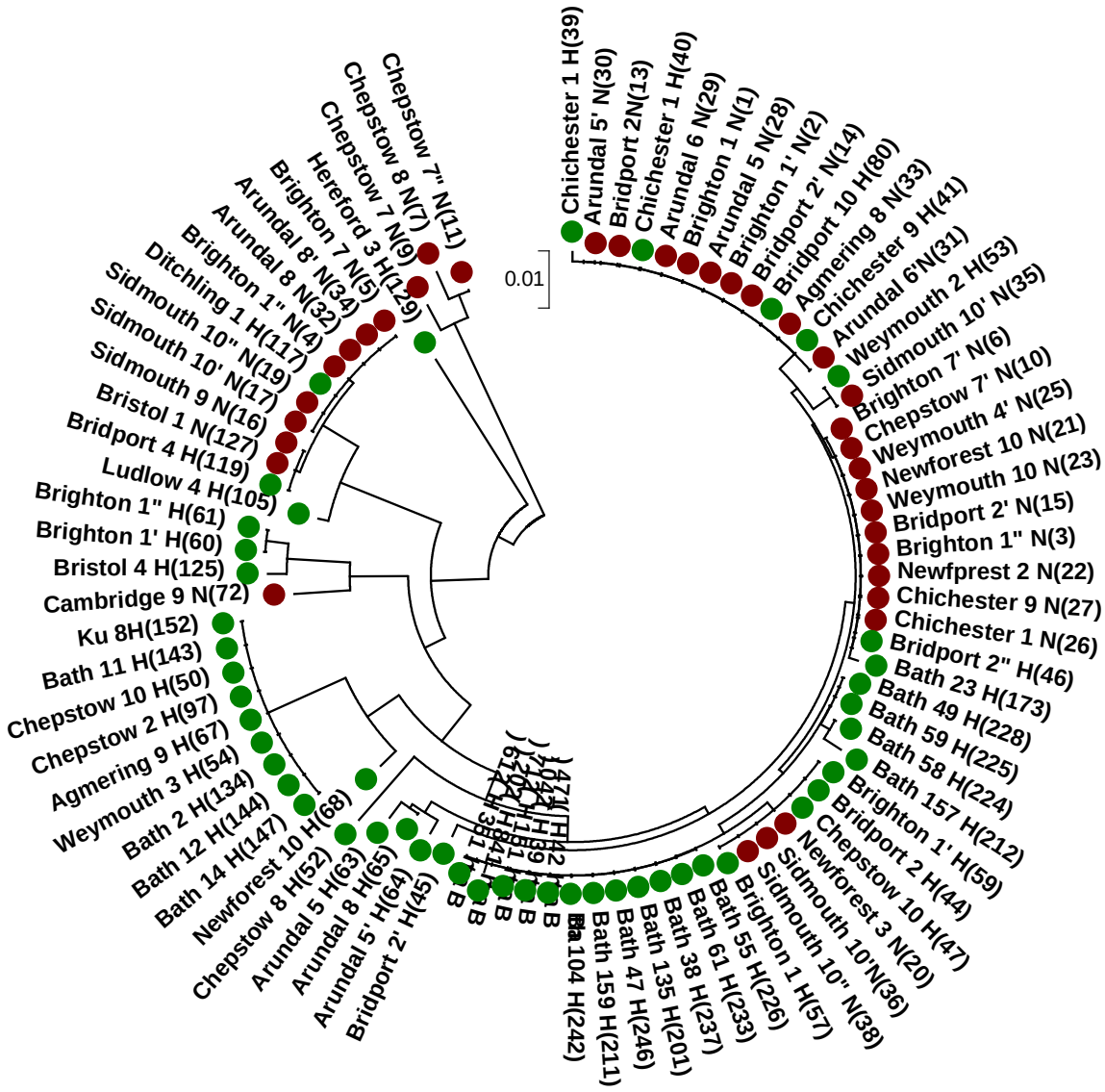


Figure 3.12. Neighbour-joining tree based on *glyA* gene sequence of 84 strains belonging to *S. proteamaculans*-like strain. ‘,’’ and ‘’’above the site name represent the number of infected *galleria* baited in the same soil sample.

3.3. Discussion:

This study aimed to survey insect pathogenic bacteria isolated from both insect haemolymph and crushed nematodes from different sites across the UK soil by molecular genotyping techniques. Three separate studies were carried out:

Preliminary small-scale study

Conducted in Bath Golf Course where soil samples were taken from three different habitats. This provided proof that the methodology was appropriate, and the insect baiting method enriches for insect pathogenic bacteria [91]. The results indicated that EPB are abundant in the soil and indicated the presence of EPN associated with some strains of *Serratia*. 16s rRNA sequencing of the isolates recovered from the infected *Galleria* revealed that they all belonged to *Enterobacteriaceae*, namely, *S. proteamaculans*, *Xenorhabdus sp.* and *Pseudomonas sp.*

Localized survey on University of Bath campus in two locations.

We recovered a total of 173 isolates from 141 dead insects, sampled from two locations on the University of Bath campus (A and B). Seventy-two of these isolates were identified on the basis of 16s rDNA sequence, and these fell into six main taxa, which were present in both locations, as well as more minor taxa which were present in either one or the other. The differences between the two locations in terms of the frequency of different taxa were not significant. The data reveal significant differences over different time points, which appear to coincide with weather patterns. Specifically, Bacillus sp are relatively more abundant in dry conditions.

Group 1 contains *Serratia, Rahnella, Yerisinia* and *Enterobacter.* *Serratia* and *Yerisinia* sp commonly occurred free living in soil; some *Serratia* sp are pathogenic and can be isolated from patients, while *Rahnella* is commonly found in plant rhizospheres. Many strains have been isolated from roots [147] in the soil and water samples [148]. They can be isolated also from the intestinal tracts of snails and slugs. **Group 2 contains isolates related to**

Acinetobacter; most frequently saprophytic, this organism occurs in soil and contributes to mineralization. They are an important source of infection in human skin and in the human respiratory tract (debilitated patients in hospital). **Group 3 are *Pseudomonas* sp**, a free-living bacterium usually found in soil and water. However, it occurs frequently on the surfaces of plants and rarely on the surfaces of animals. *Pseudomonads entomophile* were isolated from many natural niches and some species were known as an emerging opportunistic pathogen of clinical application. Several different epidemiological studies show that antibiotic resistance is rising in clinical isolates [149]. *Pseudomonas aeruginosa* has a predilection for growth in moist environments, which is probably a reflection of its natural existence in soil and water (http://textbookofbacteriology.net/pseudomonas.html)**.**

Group 4 *Enterococcus* sp, two species are commonly found in the intestines of humans as commensal organisms: *E. faecalis* and *E. faecium*. **Group 5 are *Bacillus* sp,** they are endospore forming bacteria and, due to the resistance of their endospores to environmental stress, most aerobic spore formers are ubiquitous and can be isolated from a wide variety of sources (Textbook of bacteriology). Finally, **group 6 are the *Xenorhabdus* sp** belonging to the family *Enterobacteriaceae*, and they are a symbiotic pathogen of insects and vectored by the entomopathogenic nematode. Groups 4 and 5 are most diverged as these are Gram-positive bacteria, whilst the others are Gram-negative gamma -proteobacteria.

There were differences in the frequency with which the various genera were observed in the two locations, although these differences were not significant. The Gram-positive bacteria *Enterococcus* sp and the Gram-negative bacteria *Serratia* sp were more commonly isolated from location A than location B. In contrast, *Bacillus* sp, which is Gram-positive spore forming bacteria, and *Xenorhabdus* are more common in location B than location A (Figure 3.1). Habitat type may play an important role in mixing different types of bacteria. Volume of the sample, a mixture of abiotic factors (moisture, soil pH and nutrient content) and biotic factors (nematodes, other bacteria) might influence microbial composition, and may contribute to the distribution and the characterization of these bacteria.

We investigated the repeatability of the results over time, sampling from the same locations every week for five weeks. This revealed some temporal variation. For example, *Bacillus* sp was commonly recovered in location B in weeks 2, 3 and 4, but was not recovered in week 1 and 5 in this location. This may have been related to rainfall and soil moisture as weeks 2, 3 and 4 were dry and week 1 and 5 were wet. Further, we found that the total number of infected *G.mellonella* increases on rainy days, which may reflect the increased growth of the enteropathogenic bacteria in wet conditions. This finding is supported by the previous investigation done by Iovieno P& Bååth E [150], who studied the effects of drying on bacterial growth rates in soil and on the respiration rates of those bacteria; they reported that the rate of bacterial growth was lower in the dry soil than in the moist soils and the respiration rates increased within 1 h to a level>10 times higher than that in the moist soil. This paper focuses on free-living bacteria but for the bacteria that are vectored by nematodes it is possible that the effect will be different. Our results suggested that *Enterococcus* sp and *Serratia* sp are dependent on wet conditions for their growth and survival. *Serratia* sp have the ability to infect insects because some of them are vectored by nematodes, whereas the endospore forming bacteria *Bacillus* sp were found in dry conditions as the spore resists the dry conditions. Other factors such as soil quality, climate and the vegetation type in the soil may influence the bacteria community structure, and these can be investigated in the larger-scale survey.

Large-scale survey in Southern England and Wales

160 soil samples were collected from ninety sites across two transect-lines: A. E-W & B. S-N (figure 2.4). These two lines represent a range of different habitat types, soil types and environmental conditions. In this field work we used for the first time a new mobile-phone based sample-recording protocol system called Epicollect (figure 3.13).

Figure 3.13. Screenshot of soil survey using the Epicollect system, (Adopted from Aanensen et al. (2009) [137]

This system employs software written for the Android operating system, which supports the analysis of epidemiological data [137]. As described in the methods section, soil temperature, pH, moisture and images of each sample area were taken and recorded on the mobile phone, which also recorded the GPS location of the site. This information was instantly transmitted by the handset to the central web database allowing the project coordinator to monitor the sample collection. This study represented the first successful trial of the Epicollect system; currently there are 700 registered projects using this application.

In the large-scale survey we recovered a total of 201 isolates from dead insects. Thirty-five of the bacterial isolates were recovered directly from crushed EPN that were isolated from infected insects using “White-traps”. Unlike the small-scale soil collection survey from the University of Bath, we observed only one *Xenorhabdus* isolate. It is possible that this difference reflects seasonal variation as the Bath study was carried out in summer, whilst the one survey of the large scale study (February) was carried out during very cold weather. The most predominant bacterial species isolated in the large-scale survey was a *S. proteamaculans*-like species. Sequencing of the *recA* gene revealed that this species accounted for 51/90 (25.3%) of the strains isolated directly from the insect haemolymph, and 35/35 (100%) of the

strains isolated from the crushed nematodes. Interestingly, a far higher proportion of *Serratia* isolates was observed from the samples collected in February (49%) than in May (18.9%).This difference might be explained by a close association between *S. proteamaculans*-like strains and the nematodes. Griffin *et al*., in 1991 carried out a survey of *Steinernema* nematodes from fields, grassland and woodland in the Republic of Ireland. They noted a higher prevalence of nematodes in January and February than in May and June; It is possible that this difference is due to temperature and/or soil moisture [19].

Similarly, a higher proportion of *Serratia* isolates were observed from samples taken along the south transect (Brighton, Arundel, Angmering, Chichester, Newforest, Weymouth, Bridport and Sidmouth) than on the north transect. This is likely to be explained by soil type: the south samples were taken from grasslands, sandy soils and woodland whilst the south-north transect included mainly sheltered field areas. Again, it is possible that the high proportion of *Serratia* from the south transect may be due to increased nematode activity rather than differences in the bacterial communities *per se*. In support of this, it has been suggested that changes in the soil moisture do not always lead to a change in the bacteria community [151]. A possible confounding factor is that the soil samples were taken from different depths, as the community diversity has been shown to be maximal at approximately 5 cm below the surface (http://www.ehow.com/list_7665226_bacteria-soil-different-depths.html).

However, it is unclear how this can explain why *Serratia* isolates are more common in February, and more common in the south transect. A previous survey detected that Heterorhabditis became inactive in low soil temperatures. 12°C is the lower limit for this type of nematode development [82]. By contrast, Hominick and Briscoe [81] recovered species from steinernematids that occur in different habitats and soil types and that also adapted to cooler temperatures and demonstrated that these nematodes are distributed unevenly in Britain soil [81].

We did not note any differences in the distribution of bacterial taxa associated with differences in pH, although this is thought to be an important factor impacting on the distribution of the soil bacteria [152]. In the present case it might be similar or different results of what they found because they studied the free living soil bacteria whereas we aimed to study the insect pathogenic bacteria.

An evolutionary tree based on *recA* sequences was constructed by the neighbour-joining method; this gene has been widely used as a phylogenetic marker [153]. We built a Phylogenetic tree of 130 *Serratia* isolates collected from all *Serratia* isolates in the four surveys based on *recA*; the sequence data for *recA* was generated from 42 *Serratia* isolates isolated from crushed nematodes and 90 isolates recovered from the haemolymph of infected larvae. There was little evidence that isolates recovered from the same geographical area are more likely to be related, and it is striking that the members of a single nematode associated cluster were isolated from different geographical locations. This suggests that rates of migration have been sufficient to erode any biogeographical structure, at least at the resolution afforded by a single gene.

Entomopathogenic Nematodes

Gouge and Snyder [10] determined that several free-living bacteria could be associated with a range of nematode hosts, through casual colonization of the cuticle rather than intimate symbiosis [10]. These bacteria (e.g. *Vibrio*, *Pseudomonas*, *Serratia*) can be found on many different types of nematodes. However, such relationships may be distinct from well described obligate nematode/ bacteria symbioses such as Steinernematidae and Heterorhabditidae with *Xenorhabdus* spp and *Photorhabdus* spp. Both of these EPN nematodes inoculate insect haemolymph with their bacteria which will multiply and produce toxins and antimicrobial compounds to suppress the growth of competing pathogens [10], indicating a long-term co-evolutionary relationship. The nature of the relationship between *S. proteamaculans* and

the nematode hosts will be more closely characterized in the following chapters.

Adams et al [36] demonstrated that EPNs are nearly ubiquitous, but that *heterorhabditids* are mainly isolated from sandy coastal soils, and that other taxa are more common in calcareous soils. Adams and his group also found that other types of EPN are broadly distributed in turf and weedy habitats and tropical forests. The results presented here agree with previous surveys which found that some EPNs tend to be most common in woodlands, grassland and some in forest habitats [78] & [144]. They recovered entomopathogenic nematodes from different habitats in California; it has been found that *Steinernema sp* are most commonly recovered from acid soil that contain high organic matter— some species were also recovered from sandy loamy soil [78]. However, another study recovered EPNs mostly from clay loams and they demonstrated that they are most effective in the porous soils with low organic-matter content [154]. A further study recovered aggregation of *Steinernema sp* in grassland soil [155].

Finally, the genotypic characterization of *Serratia* strains isolated from crushed nematodes suggests that there may be distinct genotypes, which are more likely to be associated with nematodes as shown in clusters 1a and 2d (figure 3.4). This figure illustrates the diversity of *S. proteamaculans*-like isolates. The two clusters 1a and 2d are predominantly associated with nematodes (85% and 76% respectively) and form distinct phylogenetic clusters. Sequence diversity (π) for the whole sample (130) was measured and we concluded that there is not a huge difference in the (π) value from the haemolymph and nematode isolates; not only did we not find differences in the level in sequence diversity regarding the sources but also there were no differences in the sequence diversity based on the geographical source.

There was little evidence of clustering based on geographic source, but it was thought that this may reflect the limited genetic resolution in the data. To address this, a more powerful Multilocus Sequence Analysis scheme was developed. This scheme utilizes five unlinked housekeeping genes; *adK*.

recA, *glmu*, *glyA* and *dnaJ,* and the data showed a high level of discrimination, resolving 69 STs from the 84 isolates.
This work shows that: i) the MLST primers work reliably for *Serratia proteamaculans* isolated from the insect's haemolymph and from the crushed nematodes; (ii) there is considerable allelic diversity in the five housekeeping genes and among *Serratia* isolates, with recombination occurring between the MLSA genes. (iii) MLSA has sufficient resolution to distinguish closely related strains.

The concatenated sequences of the five genes clustered the 84 isolates into 5 lineages. These lineages vary in the degree to which they were recovered from crushed nematodes or insect haemolymph, and this impression is borne out by the goeBURST analysis [156]. This analysis defined five clonal complexes, whereas isolates corresponding to CCs 19, 11, 4 tended to be more typically associated with nematode isolates corresponding to CCs 27 and 63, which were almost exclusively recovered from haemolymph (figure 3.7). A lack of geographical structure was also evident from these data, thus this study lends further support to the conclusion on the use of *recA* that host associated ecology is more important than geography in driving and maintaining sequence divergence within this bacterial population.

In summary, the simplest interpretation of these observations is that sub-clusters of *S. proteamculans* adapted to nematodes have emerged, and these are more common on the South coast (E-W) than in other regions covered by these surveys. The broad biogeographical patterns observed are therefore more likely to reflect adaptation than migration. One possibility is that these clusters are adapted to nematodes, which themselves are adapted to the habitat and conditions prevalent in the south.

The rate of recombination was calculated using the phi test, as implemented in SplitsTree4. This revealed extensive recombination in the concatenated dataset, but limited recombination when each gene was considered separately. The simplest interpretation of this is that recombination affects regions that are considerably longer than the sequenced alleles. This can explain why intergenic recombination (that is, between genes) is much more common than intragenic recombination (within genes).

Chapter 4

Characterization of novel entomopathogenic strains of *Serratia* and their association with a nematode host

4.1. Introduction

4.1.1. Pathogenicity of *Serratia*

Serratia species are ubiquitous in nature, found in water, soil, animals (including man) and on the surfaces of plants [115]. Bucher *et al* [116] stated that the genus *Serratia* belongs to the Enterobacteriaceae, and some of them are considered important insect pathogens including *S. marcescens, S liquefaciens* and *S. proteamaculans,* cited by Grimont in 2006 [115] as causing lethal septicemia through invasion of the insect haemolymph. It has been shown that different insect hosts are highly susceptible to infection by these pathogens. For example in *G. mellonella*, only 40 cells of *S. marcescens, S. proteamaculans and S. liquefaciens* are lethal to the larvae by injection into the haemolymph [117]. A similarly low dose of only 10-50 cells will kill the grasshopper *Melanoplus sanguinipes* [118].

It is important to understand the reasons why different entomopathogenic bacteria cause different degrees of virulence to the host in an evolutionary biology study. The common bioassay used to evaluate the pathogenicity and virulence of a pathogen is determining the dose needed to kill 50% of an experimental cohort [157].

Bacterial pathologists usually use dose-response bioassays to evaluate the dose LD50 or concentration LC50 needed to kill 50% of the population. Moreover, the time taken to cause the death of 50% of the population also can be measured, giving what is known as an LT50. This method was devised by Trevan [158] & [159]

Serratia entomophila and *Serratia proteamaculans* are the causal agents of Amber disease in the New Zealand grass grub *Costelytra zealandica*; this insect feeds on the young roots of grassland causing severe losses in the grass yield [160]. The disease was first prominent in 1981, and the mature insects' larvae stop feeding as a result of the infection. The signs of infection include clearance of the larval mid gut, causing it to turn pale yellow, followed

by death [161] Other related Coleoptera do not show any susceptibility to *S. entomophila*, suggesting that *S. entomophila* is highly host specific [38].

The first step in the infection is the attachment of the bacteria to the host gut cells, facilitated by the interaction of specific proteins which have ability to adhere with the specific receptors of the host. Many species belonging to Enterobacteriaceae have Fimbriae or pili which are protein appendages located on the cell surface. These proteins are often found to be associated with the host's specificity, pathogenicity and virulence. Other non-fimbrial adhesins have also been reported [162] & [163]. Pili and fimbriae are also often important to allow bacteria to survive and persist in different abiotic environments.

Another important determination of the pathogenicity is bacteria toxins. Kaska et al [44] (as cited by Grimont [45]) stated that several secreted enzymes have been shown to play important roles in the virulence of *Serratia,* including proteinases invading insects' haemocytes, and chitinases injecting toxins into larvae hemolymph and lecithinases. It was found that Phospholipases, proteases and chitinases depress the insect immune system and are involved in the virulence of bacteria. Toxin proteins have been identified as the major cause of the symptoms of amber disease. A 115-kb plasmid pADAP was identified in *S. entomophila* which is important to this infection [7]. This plasmid encodes the *sepA, sepB, and sepC* and anti-feeding–prophage (*afp*) operons which are homologues of the *Photorhabdus* Toxin Complex and PVC insect toxins respectively. It was found that *sepA*, *sepB*, and *sepC* together are essential virulence genes on pADAP.

Many *P. luminescens* strains have been shown to produce Toxin-Complex proteins with strong insecticidal activity against insects from different orders including the Coleoptera, Dictyoptera, Hymenoptera, and Lepidoptera. The Toxin Complex proteins of *P. luminescens* are homologous to S. *entomophila*

sepA, sepB and sepC and were first identified as the tca, tcb, tcc, and tcd complexes. These toxin proteins are now known to be widespread in many species of pathogen and have been shown to be active against a range of hosts, including humans in the case of the *Yersinia pestis* TCs [164].

4.1.2. Evolution of the nematodes

Nematodes are multicellular organisms, and many are parasitic on humans, animals or crop plants. Moreover, those parasites of insect pests are important because of their use as biological control agents [68].

The figure below demonstrates the evolution of the nematodes, starting from the free living and ending in two ways depending on their uses of pathogenic bacteria, which will make the nematodes more or less harmful to their host [165].

Some nematodes use symbiotic bacteria to facilitate their pathogenicity such as entomopathogenic nematodes.

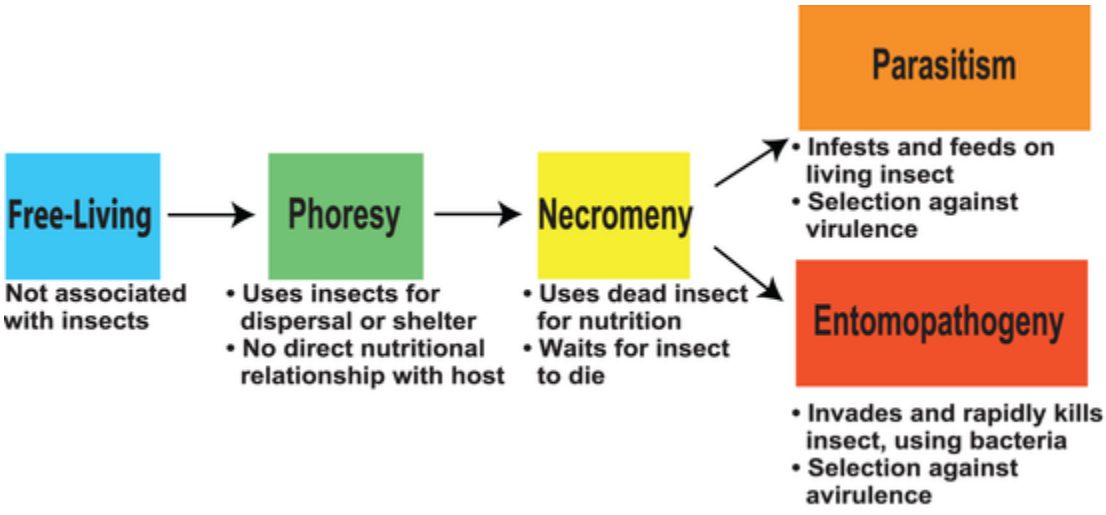


Figure 4.1. The evolution of the nematodes associated with insects. adopted from Dillmon et al [165]

Two abundant types of entomopathogenic nematodes are the *Steinernema* and *Heterorhabditis* nematodes, which are highly infective to insects. These nematodes are symbiotic with *Xenorhabdus* and *Photorhabdus* respectively, which are potent Gram-negative entomopathogens (insect pathogens) [68]. The symbiotic relationships between the nematode hosts and specific bacterial strains are normally very strict. However, there have been several reports in which bacteria from the genus *Serratia* have been found to be associated with either *Steinernema* spp or *Heterorhabditis* spp [2]. Many species of *Serratia* are considered to be opportunistic or facultative pathogens to insects including *S. marcesens*, *S. entomohila* and *S. proteamaculans*.

Other nematodes such as *Oscheius chongmingensis* and *Caenorhabditis briggsae* have been identified as insect pathogenic and both are seen to be associated with poorly defined insect pathogenic *Serratia* sp. In both cases, after the infection is finished, the dauer juveniles emerging from the insect cadaver were found to be associated with their vectored pathogen, suggesting a classic EPN-like relationship had become established [165].

The term “entomopathogenic nematode” was developed to distinguish between parasitic nematodes. Entomopathogenic nematodes have specific characteristics. The association between the nematodes and the pathogenic bacteria should facilitate the pathogenicity and the death of the infected insect. This should be clearly distinguished from other types of association, in which the bacteria are more readily lost from the nematode after an infection[165].

Pristionchus sp and *Caenorhabditis elegans,* are two nematodes species that are widely studied as model organisms because they are easily cultured in the laboratory, feeding on *Escherichia coli* OP50 [95] & [143] and because they have other desirable characteristics, such as the ease of performing genetic crosses and their highly specified developmental processes. While in the wild they are found as free-living soil-dwelling nematodes[166], little is known about their natural history.

The life cycle of *Pristionchus sp* is quite short; they can complete their growth within 4 days at 20°C, starting from egg to hermaphrodite (J1 to J4), which means no sex partner is developed. However, males can spontaneously occur and are named residual males [95].

In this chapter:

1. I examined the virulence of the novel nematode-associated *Serratia proteamaculans*-like strains isolated from Bath (B1) and Chepstow (C1) soils, toward *Galleria. mellonella* and *Manduca. sexta.* And antibiotic susceptibilities of these strains to allow the construction of *gfp*-tagged derivatives for use in subsequent work.

2. I described the discovery of a novel association partnership between two different species of nematodes, *Pristionchus* sp. and *Steinernema* sp, and the normally free-living *Serratia proteamaculans*-like sp found in UK soils. While this partnership was seen in soil samples from many sites in the UK, the focus was placed on a nematode-bacterial pair isolated from soil in Bath, Chepstow and Brighton as a model system.

4.2. Results

4.2.1. Phenotypes test for two strains of nematodes associated *S. proreamaculans-like isolates*

Virulence bioassay of B1 and C1 toward the model insect larvae; *Galleria. mellonella* and *Manduca. sexta*

i. *Galleria* Injection experiments.

To quantify the essential virulence of each bacteria isolate we performed a bioassay assessing the LD (lethal dose) and LT50 (lethal time), an estimate of the dose and time required to kill 50% of the hosts. This is considered a standard measure of virulence for *Serratia spp.*

100 *Galleria* larvae were injected for each dilution of *Serratia* and this was repeated twice (figure 4.2) as described in section 2.2.11, page: 43. The average number of dead insects per replicate is shown in Table 4.1 and graphically represented in Figure 4.3 and 4.4.

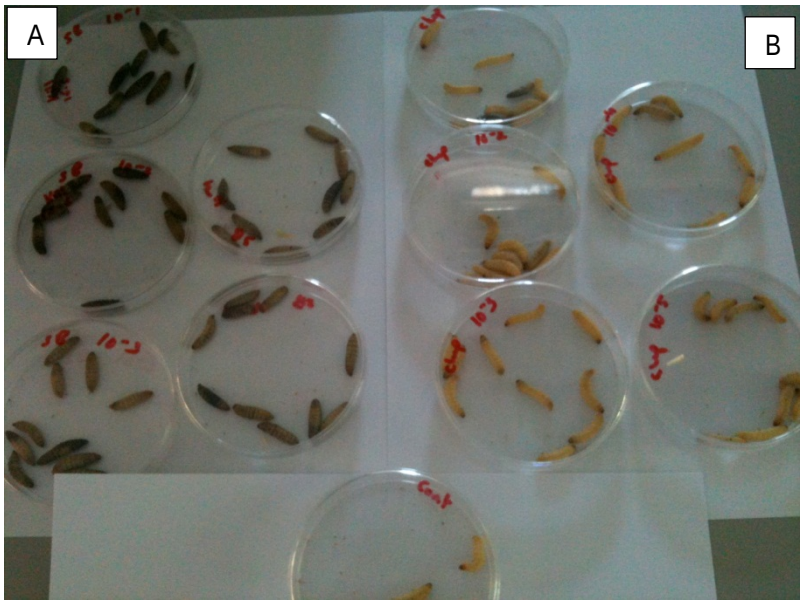


Figure 4.2. A. Infected insects injected by nematodes associated with *S. proteamaculans* B1. B. Infected insects injected by nematodes associated with *S. proteamaculans* C1.The image represents the injection of *Serratia* isolates dilution 3 after 72 h.

Table 4.1. Average number of dead insects injected by *Serratia* sp after 24 and 72 h. The number in brackets refers to the dilution factor and correlates with Figures 4.3 and 4.4.

Number of cells Injected (dilution)	Average number of dead *Galleria* injected by B1 strain		Average number of dead *Galleria* injected by C1 strain	
	24 h	72 h	24 h	72 h
695000 (1)	95	100	22.5	97.5
69500 (2)	67.5	100	12.5	75
6950 (3)	0	100	0	0
695 (4)	0	100	0	0
69 (5)	0	100	0	0

For *Galleria* injected with 69500 of *S. proteamaculans* B1 cells, 67.5% of the insects died after 24 h. However, when only 6950 cells were injected none died at 24 h, but 100% were killed by 72h. We note 100% mortality by 72 h even for the lowest dose of only 69 cells (table 4.1). This was not true of the Chepstow isolate (C1) as at 6950 cells or less we observed no killing of the larvae at either 24 or 72 h. Even at the highest dose of 695000 cells, C1 only killed 22.5% of the larvae at 24h compared to 95% by the B1 strain. None of the *E. coli* or LB injected negative control insects died. The lethal dose of Bath isolates and Chepstow isolates was measured to estimate the number of bacterial cells required to kill 50% of the hosts; for B1 the LD_{50}= 6.6, and for C1 the LD_{50}= 7.64 24h after the injection (Appendix 3, figure 1).

This indicated that B1 was more virulent than C1. A high LD_{50} is considered as less virulent. Whereas, when injecting the insects with B1 the insects die far more rapidly after 24h, LT50= 12.27, while for C1 LT50= -3.5 (Appendix 3, figure 2). The results are not significant because we took the reading after two time points only.

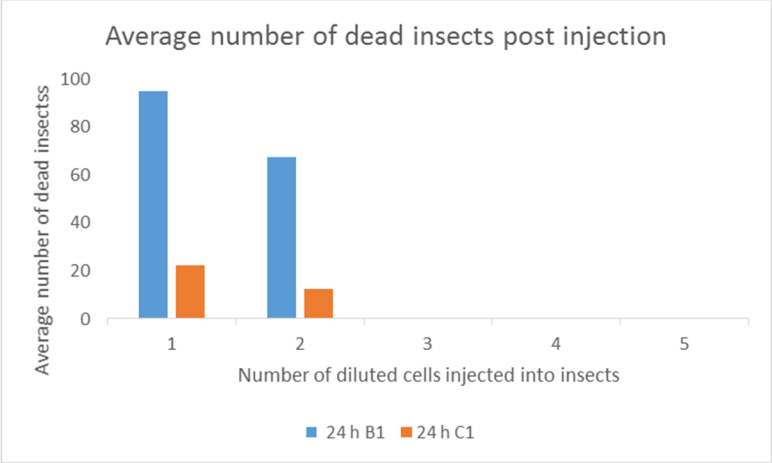


Figure 4.3. The average number of dead insects from two replicates of 100 *Galleria* each after 24 hours post injection with *Serratia proteamaculans* B1 and C1 strains.

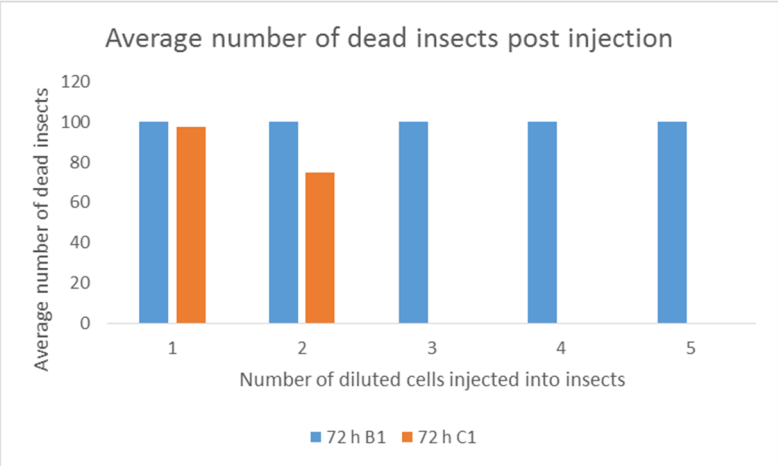


Figure 4.4. The average number of dead insects from two replicates of 100 *Galleria* each after 72 hours post injection with *Serratia proteamaculans* B1 and C1 strains.

M. sexta injection experiments.

A preliminary experiment was performed in which three 5th instar *M. sexta* larvae were injected for each dilution of *Serratia* (table 4.1) and this was repeated twice. For *M. sexta* all of the insects were killed within 24h for both the B1 and C1 isolates compared with the control insects (figure 4.5).

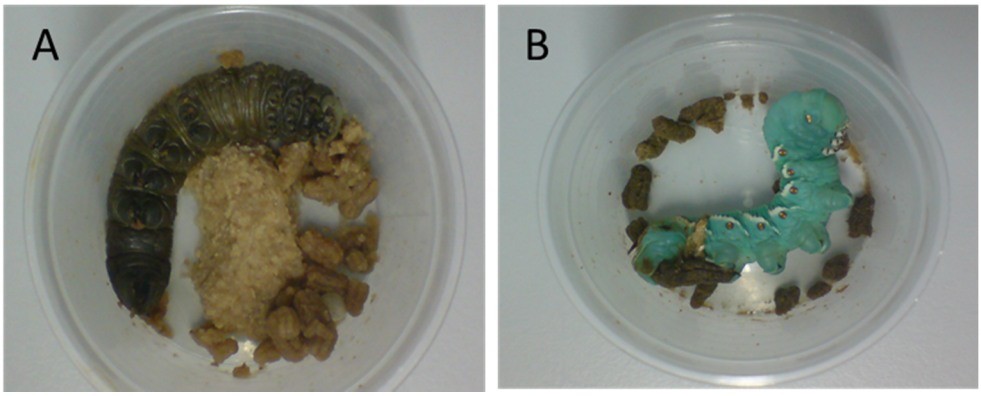


Figure 4.5. Pathogenicity of *Serratia proteamaculans*-like strain (B1) on *M. sexta*, (A). injected insects with 69 c.f.u lowest concentration of cells, (B). control insects injected with LB broth**.**

ii. *M. sexta* feeding experiments.

Six 5th instar *M. sexta* larvae were fed on either the *Serratia* B1 strain or *E. coli* overnight culture for 4 h before they were transferred onto fresh food. The insects were weighed before feeding and after 4 days. No mortality or morbidity was observed in the treated insects, implying that the *Serratia* B1 strain was not able to infect orally. In addition, there was no obvious reduction in weight gain (figure 4.6). Both isolates are not orally toxic to *M.sexta.*

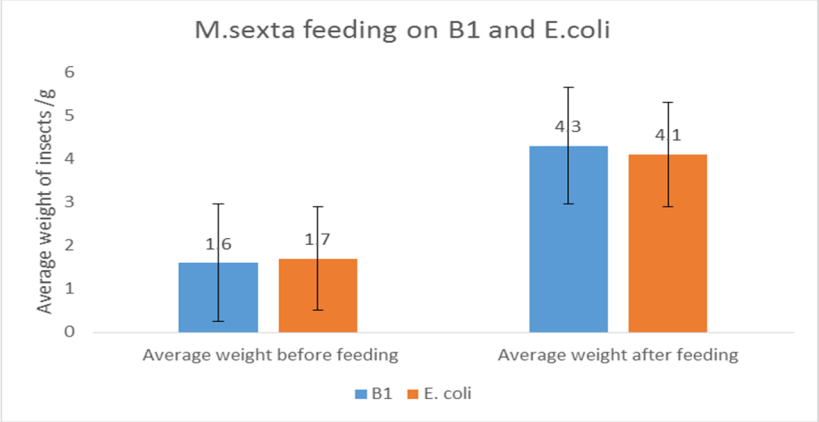


Figure 4.6. The average weights (g) of *M. sexta* before and after 4 days post oral exposure to *Serratia proteamaculans* (B1) and *E. coli* OP50. A total of 12 insects were fed each bacterial strain. Error bars represent the standard error. The numbers above the bars show the mean weight in grams of the cohorts.

4.2.2. Antibiotic resistance B1 and C1

The susceptibility of two of our nematode associated *Serratia proteamaculans*-like strain isolates was tested by streaking them out onto agar plates containing different concentrations of two antibiotics, kanamycin and tetracycline (table 4.3) (section 2.2.12, page:46).

Table 4.3. *Serratia proteamaculans-like* strain antimicrobial sensitivity on LB agar plates. "+" indicates a normal level of growth,"-" indicates no growth

A. Concentration of kanamycin or tetracycline					
Serratia	1µg/ml	5µg/ml	10µg/ml	50µg/ml	100µg/ml
Bath strain (B1)	+	+	-	-	-
Chepstow strain (C1)	+	+	-	-	-

These observations indicate that the two *Serratia* isolates are relatively sensitive to both antibiotics. Typically, they were unable to grow at 10 µg/ml or above. B1 and C1 parental strains were used for chromosomal *gfp*-labelling (see below).

4.2.3. Determination of the location of associated bacteria within the nematode

Identification of nematode associated bacterial genera using *recA* gene sequence

Nematode associated bacteria were identified as *S. proteamaculans*-like species based on *recA* gene sequence as discussed before.
From the Bath samples, 80% of the dead insects produced nematodes in the White traps. From the (E-W) samples, 24.4% of infected insects produced nematodes, whilst the equivalent figure for (S-N) was 8.1% (A chi sq test showed that this difference was significant (chi sq = 16.080, df = 1, p = 0.000060).

We selected three independent nematode isolates and characterized them in greater detail. These nematodes were isolated from infected insects from soils taken from Bath, Brighton and Chepstow. These worms were sent to Dr Patricia Stock at the University of Arizona who typed them using light and electron microscopy and also molecular diagnostics, using the sequence of the 18S rRNA gene as a "Barcode sequence". The Bath and Brighton isolates were identified as *Pristionchus sp,* whereas the Chepstow nematodes were identified as *Steinernema* sp (chapter 5). We were not sent the sequence of the samples, hence why we had to sequence the samples again as mentioned in chapter5 .

We assign the strain names B1, C1 and B2 to the *Serratia* isolated from the nematodes from Bath, Chepstow, and Brighton respectively. Conversely, we named the associated nematodes as NB1, NC1 and NB2 respectively.

With the aim of testing if bacteria were associated with the surface of the nematode isolates or whether they were being carried internally (in the gut lumen or in the tissues) we tested a range of surface sterilization regimes on

strains NB1 and NC1 (section 2.2.3.i, page: 37). We examined how long the worms could survive exposure to 1% bleach [2] & [167] (table 4.4).
Note, nematodes sterilized in this way were also used for the phoresis-attraction assays (section 4.2.9). In these experiments, after exposure to the bleach, nematodes were also crushed into sterile media and plated onto LB agar to assess the survival of any associated bacteria.

Table 4.4: Effect of exposure to 1% bleach upon nematode survival.

Exposure time to 1% bleach	Nematode condition	Bacterial growth from active nematodes	Bacterial growth from crushed nematodes
2 minutes	Nematodes are active	Bacterial growth on the agar plates	Bacteria growing
5 minutes	No survival	Bacterial growth on the agar plates	Bacteria growing
10 minutes	No survival	No bacterial growth	Bacteria growing
1 hour	No survival	No bacterial growth	Bacteria growing

The nematode strains are killed by this 1% within 10 minutes, but we observed bacteria growing on the media after crushing the dead nematodes.

4.2.4. Comparing the virulence of the *Serratia* bacteria alone with that of the nematode isolates that had been fed on their cognate *Serratia* strains

Crushing experiments showed that 37 of the 59 nematodes collected from the previous surveys contained *Serratia proteamaculans* like bacterial strains. We therefore decided to investigate the role of these associated bacteria in the virulence of the nematodes upon *Galleria* infection in sand/soil – trap experiments (section 2.2.17, page 51). Two model nematodes and their bacteria were used in these experiments (NB1-B1 and NC1-C1). We tested the insect infectivity of nematodes that had been fed upon their cognate bacteria, the bacteria alone and also nematodes that had been passaged on laboratory *E. coli*. (section 2.2.15, page: 50). Combinations of *C. elegans* nematodes and *E. coli* were used as negative controls.

The impact of using autoclaved soil in place of the sand substrate in these infection experiments was subsequently tested as the composition of the substrate has been shown to affect nematode activity in other parasitic nematode isolates [84].

In these experiments, washed worms (40 nematodes in 10µl) were added to the substrate or to 1ml of diluted overnight culture of bacteria. We used 100 *Galleria* per test chamber and performed three replicates per treatment. The number of dead insects was counted after three days; averages from these experiments are graphed in Figure 4.7.

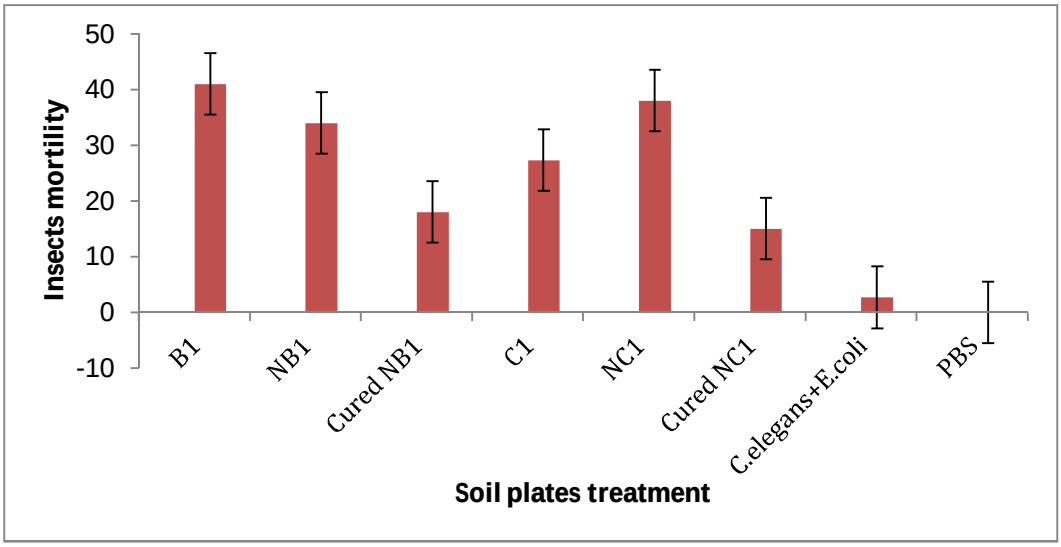


Figure 4.7. The pathogenicity of nematode and bacterial isolates in soil-trap baiting experiments. The graphs show the mean percentages of dead *Galleria* after three days of exposure to the treatments. These are the means of 3 replicates of 100 larvae per replicate (n=300). Error bars represent one standard error.

When the 3 replicates are combined we can see that the NC1 nematodes that had been raised on their cognate *Serratia* strain killed more larvae than when the *Serratia* bacteria were added to the soil alone. The bacterial numbers added cannot be directly compared between these two treatments as the number of *Serratia* cells per nematode was not known. Nevertheless, significantly more bacteria would have been added in the case of the bacterial culture treatment. Conversely, in the case of the NB1 isolates we saw that their associated bacteria were able to kill more *Galleria* when added to the soil alone than when the *Serratia* fed *Pristionchus* isolates were added. The

isolated *Serratia* B1 were seen to infect more insects than when associated with the cognate nematode host. As expected, the PBS control and the *C.elegans* (reared on *E.coli* OP50) showed little or no insect mortality over 10 days.

In the case of cured NB1 and NC1 nematodes the results show that virulence towards the test insects was less than for the complexes of NB1 and NC1; this was not surprising because in this experiment the cured nematodes had been treated by passaging *E.coli* 35 times in an attempt to eliminate the original associated bacteria. The results in section (4.2.5) below indicate that passaging the Gfp-labelled *E.coli* was ineffective in getting rid of *Serratia*— their number was reduced but they were not eliminated.

We confirmed that the nematodes and bacteria were both present during the lethal infection by placing infected larvae on white traps and collecting emerging nematodes. We confirmed these nematodes were still associated with the *Serratia* by surface sterilization and crushing. We did not determine the exact contribution of the nematode and bacteria to the killing process, as we were not able to cure the nematodes of their associated bacteria. However the bacteria were shown to be highly pathogenic independently of the nematode. This would require further work.

In initial experiments autoclaved sand was used as a substrate, into which *Galleria* larvae were added as “bait”. Either nematodes or bacteria were added and insect mortality was measured. The results showed the average number of insects that became infected when exposed directly to the B1 strain culture was slightly higher than when the test insects were exposed to NB1 nematodes that had been fed on the B1 strain. This was consistent with the observations from the soil-trap experiments. The results also suggest that the infectivity of the NC1 nematode isolate was higher than that of the NB1 isolate. The difference between the two methods was the time it took for the infection to occur, being slightly longer in the sand trap than in the soil trap.

4.2.5. Testing the stability of the association between the Bath nematode (NB1) isolate and the *Serratia* strain (B1)

The stability of the association between B1 bacteria with the NB1 nematodes isolate was assessed by prolonged feeding of NB1 on *E. coli* with the aim of "displacing" the *Serratia* (section 2.2.15, page 50). The results were observed and recorded in Table 4.5.

Table 4.5 : Determination of the presence or absence of *Serratia* B1 in *E. coli* passaged NB1

Type of colony in the 10 plates after 10 passage	Both *Serratia* and *E.coli* Colonies were observed
Type of colony in the 10 plates after 20 passage	The majority of colonies were *E. coli* ,few *Serratia*
Type of colony in the 10 plates after 30 passage	Both *Serratia* and *E. coli* Colonies were observed

Despite extensive passage on an *E. coli* food source we were not able to eliminate the *Serratia* B1 strain from the NB1 nematode population. Figure 4.8 shows examples of plates from crushing experiments of passage 15 exhibiting both Gfp labelled *E. coli* and the orange pigmented B1 *Serratia* colonies. It should be noted that the experimental procedure used did not assess whether all nematodes retained *Serratia* cells or just a proportion of them in the population.

Nevertheless, the persistence of the *Serratia* in the nematodes confirms that the virulence of *E. coli* passaged nematodes could not be reliably assessed as the presence of the associated B1 *Serratia* could not be ruled out. Furthermore, given the number of passages used, these results suggest that there is a very tight association between the B1 *Serratia* bacteria and the NB1 nematode host.

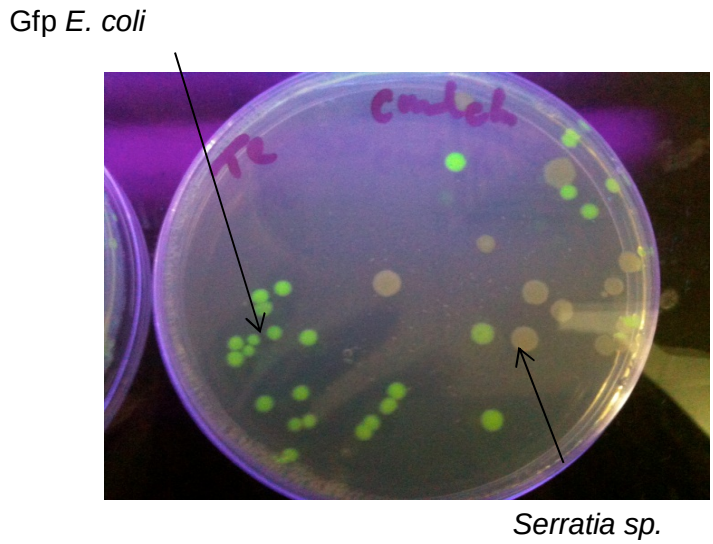


Figure 4.8. An example showing the presence of both *E. coli* and *Serratia* colonies on NGM plates from crushed NB1 after *E. coli* passage 15.

4.2.6. Microscopic examination of the colonization of NB1 nematodes by *Serratia* strain B1

The mini-Tn*7* transposon system is a suitable tool for site-specific labelling of Gram negative pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Photorhabdus luminescens* [168]. We successfully used the pURR25(mini-Tn7KSGFP) and pUX-BF13(Tn7 transposase) plasmids in a triparental mating strategy to deliver the mini-Tn7 transposon, which expresses the fluorescent Gfp protein from the *E. coli lac* derived promoter. In other Proteobacterial genomes the Tn7KSGFP usually inserts into an *att* site located downstream of the highly conserved *glmS* genes. This is assumed to be a neutral site that should not affect the rest of the genome. Further work would be required in future, to determine the exact integration site of the transposon in our strains

The triparental mating system described above was used to label the B1 and C1 nematode associated *Serratia* strains with a *gfp* marker gene; both were successfully labelled using this method (section 2.2.13, page:46). The *gfp* gene (Tn7KSGFP) was inserted into the chromosome of these strains to

ensure stability during *in vivo* insect and nematode infections. Figure 4.9 illustrates the green fluorescence of the labelled strain B1 as observed under the inverted fluorescence microscope.

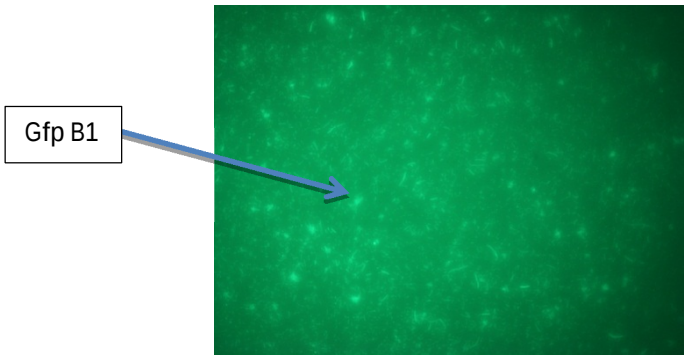


Figure 4.9 An example image of Gfp expression from the labelled Bath *Serratia proteamaculans*-like strain B1

In order to determine the physical location of the *Serratia* B1 cells inside the NB1 nematodes we labelled strain B1 with a Gfp reporter gene. Gfp labelled *E. coli* was used as a "non-symbiont" control strain and also *C. elegans* as an alternative nematode "host". The nematodes were passaged (15 times) on NGM plates seeded with these bacterial strains, washed, immobilised using 1% sodium azide and analysed using laser scanning confocal microscopy to visualize the location of the bacteria in the worm. We assessed four combinations of feeding types as summarized in Table 4.6.

Table 4.6. Nematode host and bacterial strain combinations.

Food	*C. elegans*	Bath *Pristionchus* nematodes NB1
E. coli Gfp	Figure 4.11	Figure 4.13
Serratia Gfp	Figure 4.10	Figure 4.12

The control of *gfp E. coli* fed *C. elegans* confirmed as expected that the few intact bacterial cells seen were restricted to the gut of the worm, with the majority presumably having been crushed by the worm's "grinder" and subsequently digested (figure 4.11). More intact *E. coli* cells were typically seen in the gut of the *Pristionchus* nematodes, possibly because this genus lacks a "grinder" (figure 4.13). Interestingly, the *Serratia* Gfp cells could be

seen throughout the mesoderm tissues of the *C. elegans*, and were not restricted to the gut (figure 4.10). This suggests that this strain of bacteria has the ability to cross the gut and invade the tissues. It should be noted that no obvious deleterious effects were seen on *C. elegans* fed on *Serratia* B1, suggesting this tissue invasion is not associated with any pathogenic effects. Finally, when *Serratia* Gfp bacteria were fed to the NB1 nematode it was seen that the bacteria invaded the tissue surrounding the pharynx almost immediately after ingestion (figure 4.12). It is important to note that the confocal microscopic examination verified that the bacteria were indeed within the nematode tissues using Z-stack 3D reconstruction (Appendix 4, CD1)

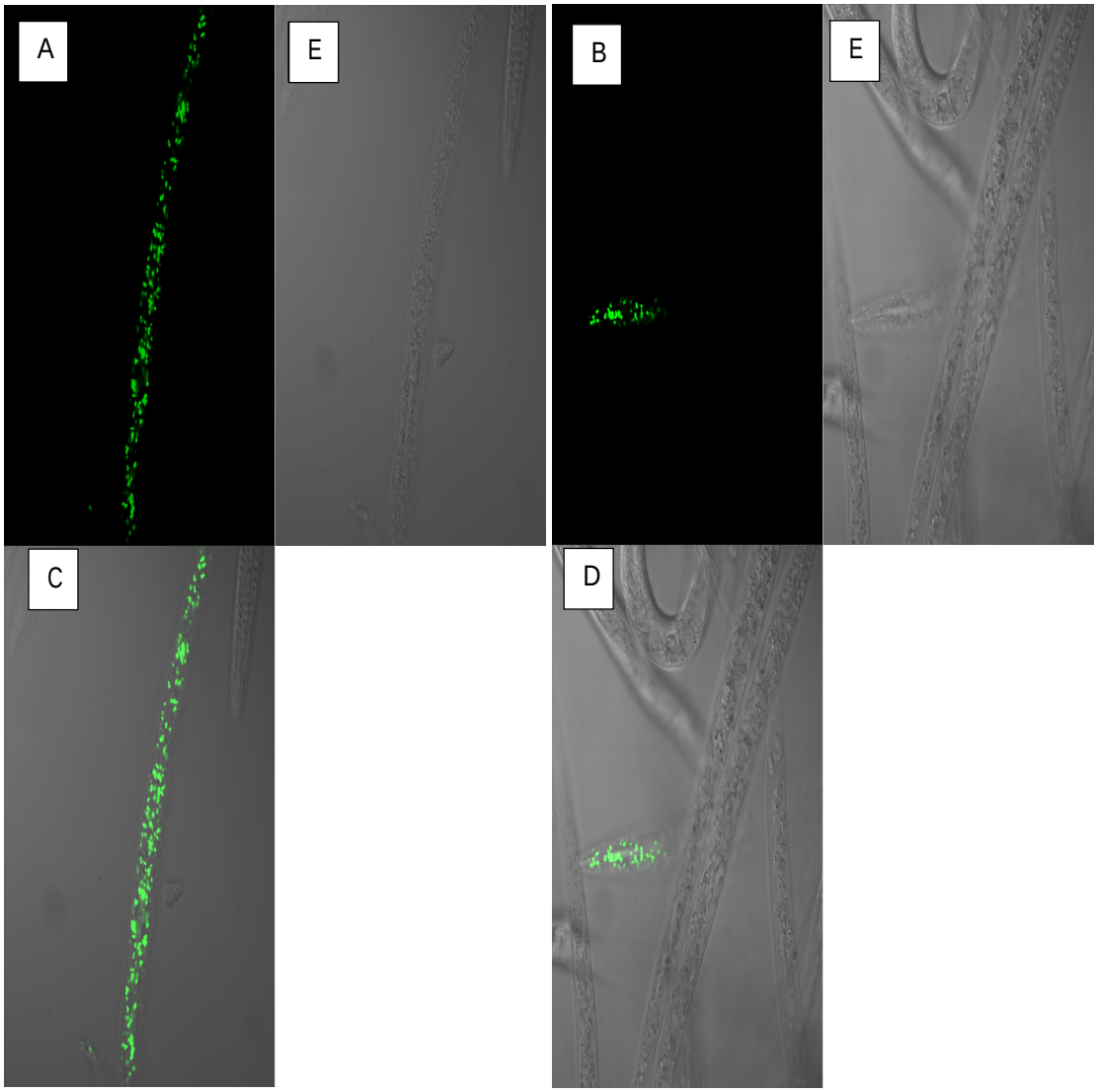


Figure 4.10. Example images of two individual *C. elegans* nematodes which had been fed on Gfp labelled *Serratia*. A and B represent illumination under the Gfp excitation wavelength of 396 nm, C and D show an overlay with bright field illumination and E in both shows the bright field illumination

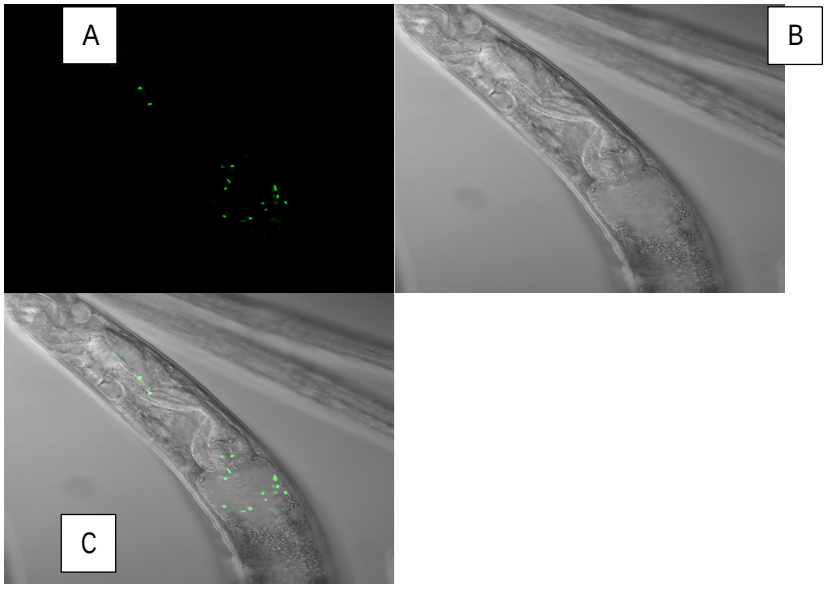


Figure 4.11. An example image of a *C. elegans* nematode which had been fed on Gfp labelled *E. coli*. A represents illumination under the Gfp excitation wavelength of 396 nm, B shows the bright field illumination, while C shows an overlay with bright field illumination

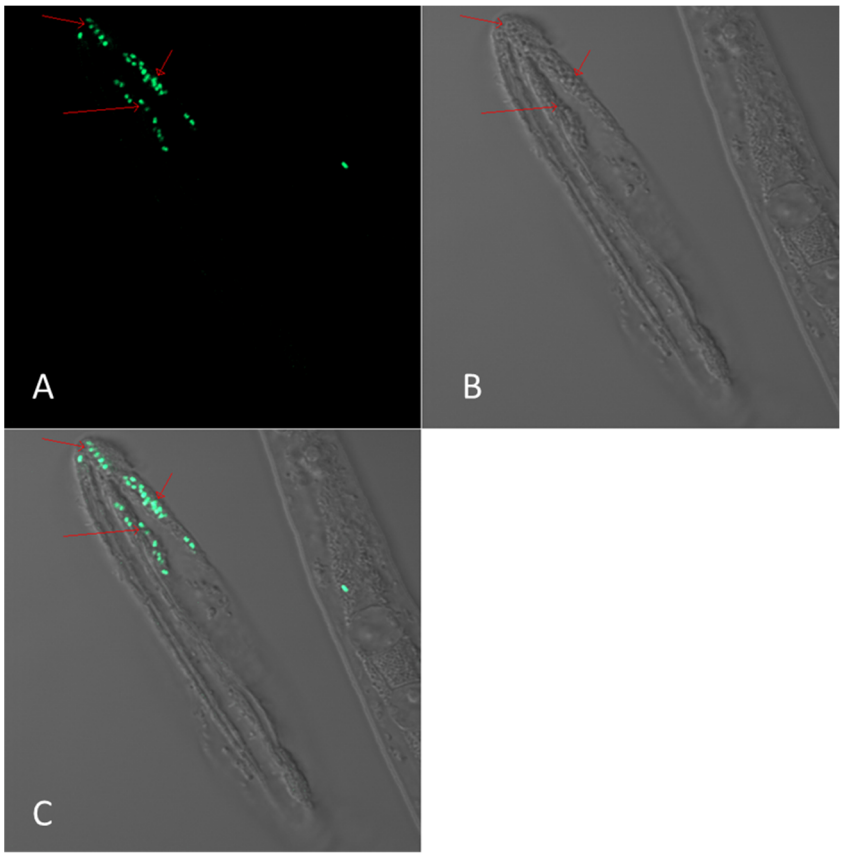


Figure 4.12: An example image of a NB1 *Pristionchus* nematode which had been fed on Gfp labelled *Serratia*. A represents illumination under the Gfp excitation wavelength of 396 nm, B shows the brightfield illumination and C shows an overlay of the two. Red arrows denote the location of the bacteria invading the tissues inside the mouth.

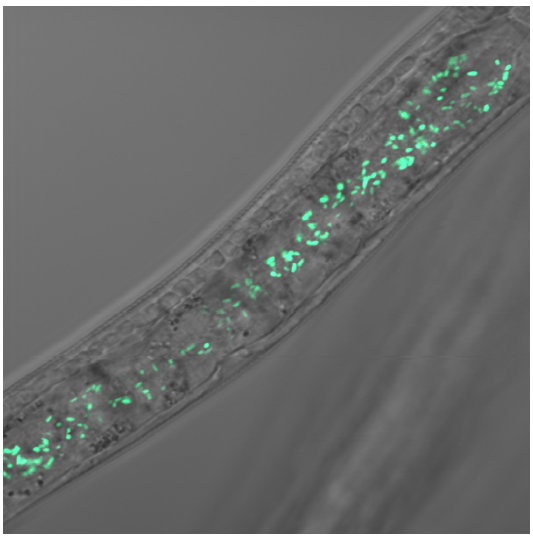


Figure 4.13. An example image of a NB1 *Pristionchus* nematode which had been fed on Gfp labelled *E. coli.* The image shows an overlay illumination under the Gfp excitation wavelength of 396 nm and bright field.

4.2.7. Can the *Serratia* B1 bacteria be found in the eggs of the NB1 nematode?

In preliminary experiments, eggs from the NB1 nematode were isolated from worms that had been grown on NGM plates seeded with the associated *Serratia* strain (section 2.2.18, page: 52). Worms and eggs were washed from the plates with PBS and then treated with bleach to kill any free bacteria or surface associated bacteria and also to release eggs from adults. Eggs were then washed several times and resuspended in egg buffer (figure 4.14). The eggs were then crushed (without counting how many there were) in sterile media before plating out onto NGM agar plates and incubating at room temperature in the dark. As a control, we also added washed and surface sterilized eggs directly onto the plates to control for any external bacteria that had not been killed. After 4 days of incubation colonies were observed. Six

colonies were found on plates; these colonies were picked and their *recA* genes were amplified using PCR (Figure 4.15). These amplicons were sequenced to identify the bacterial species. BLASTN analysis of the *recA* amplicon sequences identified colony 3 as *Serratia proteamaculans*, 4 and 6 as *Serratia liquefaciens* (ATCC 27592) and *Serratia marcescens* (WW4) respectively and band 1 as *Enterococcus* (Appendix 5). No colonies grew on the non-crushed egg control plates confirming that surface sterilization was complete.

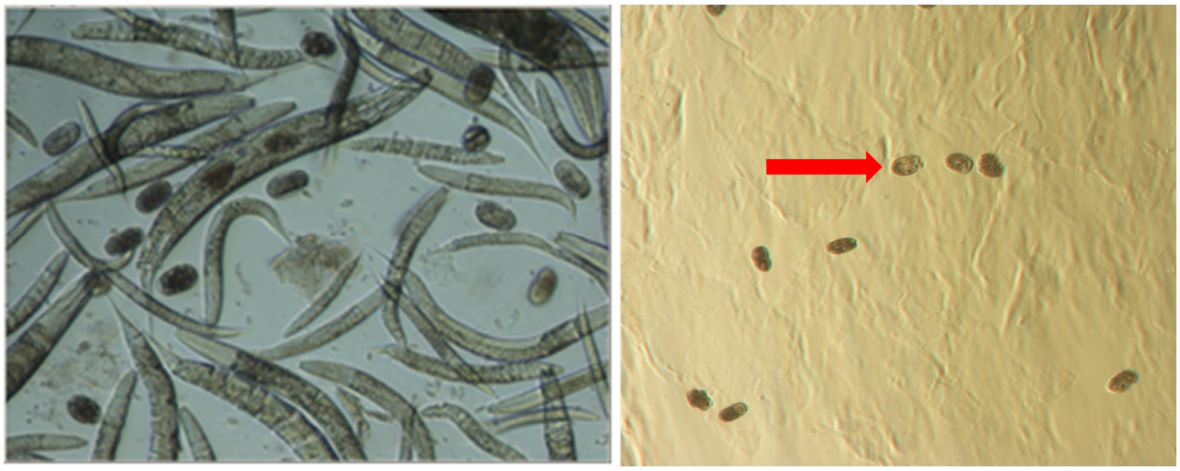


Figure 4.14. Isolation of the NB1 eggs, (red arrow) taken using a Nikon SMZ1500 inverted microscope.

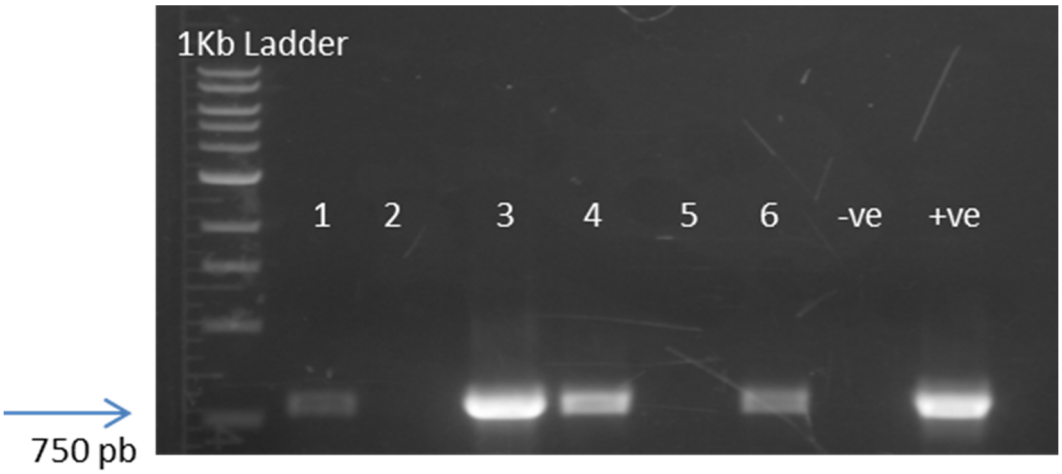


Figure 4.15. Gel electrophoresis of the PCR amplicons of the *recA* gene from the six colonies isolated from surface sterilized and crushed eggs. *S. Proteamaculans*-like species was used as positive control.

4.2.8. Nematode bacterial phoresis assays

In order to determine if the NB1 nematodes showed a preference for feeding on their associated *Serratia* B1 strain we performed phoresis "choice" assays (section 2.2.19, page: 53).

Briefly, different bacterial strains were seeded onto two sides of an NGM plate, and 50-60 individual NB1 nematodes at different stages were added in the centre of the plate equidistant from the bacterial lawns. In addition, in separate experiments sterile eggs were added to the centre of the plates in place of worms.

The preliminary experiments using complete NGM agar were inconclusive as nematodes became distributed randomly across the plate, possibly because the plate represented a "homogenous" food source. Therefore, the experiments were repeated using NGM plates without the addition of peptone. In addition, the nematodes were starved for one week before their addition to the plate. These low nutrient plates were also used for the sterile egg-hatching experiments. These experimental conditions led to clear directional phoretic movement of the nematodes and allowed the numbers of nematodes choosing the different bacterial lawns to be microscopically counted. The results from the hatching eggs experiments are presented in Table 4.7.

We do not present the results of phoresis experiments using adult and juvenile worms as the worms appeared to release their associated *Serratia* when grown on the plates, confounding interpretation. The population of nematodes used in these studies were a mixture of ages so we cannot comment on which specific stage was releasing bacteria.

Table 4.7: Hatching nematode bacterial phoresis results. The numbers of hatched worms counted within the given bacterial lawn are shown.

Observation after	*E.coli* versus *Serratia*	*Photorhabdus luminescens* TT01 versus *Serratia*	*Pseudomonas fluorescens* versus *Serratia*	*Bacillus cereus* versus *Serratia*
24 hrs	4 toward *Serratia* 2 toward *E. coli*	5 toward *Serratia* 2 toward *Photorhabdus*	4 toward *Serratia* 0 toward *Pseudomonas*	5 toward *Serratia* 0 toward *Bacillus*
48hrs	32 toward *Serratia* 20 toward *E.coli*	30 toward *Serratia* 2 toward *photorhabdus*	20 toward *Serratia* 4 toward *Pseudomonas*	25 toward *Serratia* A few dead worms in *Bacillus* lawn

These experiments illustrate that up to 48 hours the NB1 nematode preferred lawns of *Serratia* B1 bacteria over those of *Photorhabdus, Bacillus* or *Pseudomonus*. Interestingly, there was no obvious bias in nematode phoresis when presented with a choice between *Serratia* and *E. coil* lawns.
After three days, nematodes were distributed across the plates, even in pathogenic bacterial lawns, where some worms were seen to be dead.

This finding might not confirm that the worms prefer *Serratia*, they may randomly move and only those that got to *Serratia* survived.

4.4. Discussion

Pathogenicity of *Serratia* to insect hosts

The *Serratia* strains that have been investigated in this project were all isolated from the insect soil baiting experiments discussed in the previous chapter. In some cases, the bacterial strains were associated with nematodes and in others they appeared to be “free living”. In this chapter the pathogenicity of two of the nematode associated strains, B1 and C1, was compared when they were injected into insects without their nematode partners. We found that both strains were pathogenic for *Galleria* and *Manduca*, but that the B1 strain was significantly more virulent than the C1 strain.

These findings are consistent with previous work by Abebe [52]. In their study, they isolated a strain designated *Serratia* sp. SCBI that was found associated with *Caenorhabditis briggsae* KT0001 isolated by insect baiting studies using South African soil. This strain was also highly pathogenic when injected into *Galleria* larvae, even in the absence of its nematode host [52]. Furthermore, other studies have confirmed that some *Serratia* sp such as *S. proteamaculans* and *S. marcescens* are lethal to insects by injection, with only up to 50 cells able to kill the grasshopper *Melanoplus sanguinipes* as well as *G. mellonella* [169].

Interestingly, even though both of our strains were able to infect *Galleria* when added directly to autoclaved soil, we note that they were not able to infect *Manduca* larvae orally, even when fed in high doses. This suggests that these strains are able to invade the insect haemolymph either directly through openings such as the spiracles or by vector transmission by the nematode host. It is interesting to note that the highly virulent obligate nematode symbionts *Photorhabdus* and *Xenorhabdus* are also unable to infect *Manduca* via the oral route. It is possible that exposure to the very high pH of the insect gut is acting as a barrier to the establishment of infection. It is not clear why the B1 and C1 strains show a difference in virulence; however, as they belong

to different phylogenetic groups (chapter 4) we may speculate that their genomes encode different virulence factors. It is interesting to note that the bacterial-nematode complex of *Steinernema/Serratia* NC1-C1 is highly virulent to *Galleria* compared with the *Pristionchus/Serratia* NB1-B1 complex (figure 4.8), suggesting the nematode species are determining the actual virulence level. We note that the lethal dose (LD50) of Bath isolates was less than LD50 of Chepstow isolates, indicating that the B1 isolate is more virulent than the C1 when injected alone.

Antibiotic resistance of nematode associated *Serratia*

It has been found that many soil bacteria (up to 40%) are resistant to artificial antibiotics, often mediated by plasmid encoded determinants[170]. *Serratia marcescens* has been known to be the cause of many hospital epidemics. It causes several diseases as a secondary infection. It has been found that *S. marcescens* has the ability to develop resistance to many β-lactam antibiotics by virtue of chromosomally encoded, inducible Amp^c β-lactamases or by the acquisition of plasmid-encoded extended-spectrum β-lactamases (ESBLs) [171].

Antibiotic resistance is an increasing clinical problem. The origin of these resistance genes is poorly understood. Previously it has been suggested that horizontal gene transfer responsible for the spread of such genes is more likely to occur during the association of bacteria with invertebrates in the environment. It was therefore decided that the antibiotic resistance profiles of our *Serratia proteamaculans*-like isolates should be examined to determine if there was any correlation between nematode association and resistance. However, the two genetically distinct strains that were examined in this study showed no strong natural resistance to the two antibiotics tested.

Insect pathogenic nematode distribution

Two different families of insect pathogenic nematodes belonging to the *Pristionchus* and *Steinernema* genera were recovered from our UK soil sampling as confirmed in chapter 5. Previously, Adams et al [15] concluded

that EPNs are nearly ubiquitous, and that heterorhabditids are mainly isolated from sandy coastal soils with some taxa being more common in calcareous soils; whereas, other types of EPN are distributed in turf, weedy habitats and tropical forests. Consistent with this, the present study showed that 80%, 24.4% and 8.1% of infected insects from soils from Bath, Southern England (E-W) and Southern Wales (N-S) respectively successfully produced nematodes.

Initial molecular diagnostics with 18S rDNA barcoding were performed by Patricia Stock (University of Arizona) on three of our model nematode isolates (NB1, NB2 and NC1). This suggested that the nematodes we isolated from Bath and Brighton soils belong to the genus *Pristionchus,* a known insect-associated nematode previously found in UK soils. On the other hand, the nematode isolated from the Chepstow soil sample was seen to be a close relative of the genus *Steinernema,* and which therefore belongs to the family Rhabditidae. In the next chapter, I describe in more detail the molecular characterisation of these different nematodes.

Nematode associated bacterial strains

In all cases when the different *Pristionchus* nematode isolates were crushed it was found that they were associated with strains of bacteria belonging to the genus *Serratia*. Sequence analysis of the recA amplicon of these strains indicated that they were all close relatives of *Serratia proteamaculans* sp. Recently, strains of *Serratia marscens* have also been found associated with the nematode *Caenorhabditis briggsae* isolated from South African soil [52]. While no reports exist that specifically link *Pristionchus* with a bacterial symbiont, it has previously been shown to interact successfully with different species of pathogenic bacteria which can accumulate within the nematode gut and on the nematode cuticle [143]. Furthermore, *Pristionchus* is commonly used as a model organism in the laboratory to study development as they can be easily cultured by feeding on *Escherichia coli* OP50 in a manner similar to *C. elegans* [98]. We believe it is significant that we were able to isolate

Pristionchus-Serratia complexes from soil widely distributed across our sampling transects. We are unaware of any previous studies that have uncovered such a ubiquitous distribution of these highly insect pathogenic nematode complexes in the UK. This suggests that either (i) these nematode complexes are recently evolved or (ii) they have been simply discarded in previous sampling studies. Interestingly, despite being designated a member of the Steinernematidae, the NC1 isolate was shown also to contain a *Serratia proteamaculans* sp. strain, rather than the obligate symbiont *Xenorhabdus* normally associated with these entomopathogens. It is possible that these nematodes were associated with both types of bacteria but that the *Serratia* was able to out-compete the *Xenorhabdus* in the time scales used in these experiments. We cannot comment on the long term ecological stability of such an association. Alternatively, this situation may represent a genuine case of replacement of the normal *Xenorhabdus* symbiont by a more successful *Serratia* strain. Repeated sampling of these soils to determine the natural frequency of such associations would be required to address this issue.

Benefit of the *Serratia* to the Nematode worm?

Soil contains millions of bacterial cells of different species and often a very large diversity of nematodes. Consequently, a large range of interactions and outcomes can occur. For example, some bacterial species may serve as food for certain nematodes while others may be pathogenic or toxic to certain nematodes, including *C. elegans* and *Pristionchus.* It has been found that *C. elegans* can detect the presence of certain bacterial pathogens, or at least the negative impact of pathogenic activity, such that they may enter a lawn of pathogenic bacteria but will later exit and remain near the edge of the lawn, displaying avoidance behaviour [105]. Examples include the avoidance of *Serratia marcescens*, which has been traced to the detection of a natural product, Serrawettin W2, produced by the bacteria [105].

These observations suggest that the NB1 nematodes are not necessarily being attracted to the *Serratia* B1 bacterial lawn; rather they seem to be avoiding the bacterial lawns of the three potential pathogenic strains. Indeed, this is consistent with previous reports by Rae [143] that show that

Pristionchus nematodes do not seem to prefer a particular food source, but will avoid certain *Bacillus* strains.

It is interesting to note that the novel model *Pristionchus* isolates investigated here could readily feed and reproduce on these nematode associated *Serratia* strains. This is despite the very high level of virulence exhibited by these bacteria to insect hosts. When the nematodes were presented with a choice between lawns of their cognate *Serratia* strains and *E. coli*, *Photorhabdus luminescens, Bacillus cereus or Pseudomonas fluorescens* the worms avoided the potential pathogens but were happy to feed on the *E. coli* or *Serratia*. This suggests they were not attracted to the *Serratia per se*, but did avoid the other pathogens.

The biological and ecological interaction between Entomopathogenic nematodes and symbiotic bacteria has only been studied in any detail in the well characterised *Heterorhabditidae* and *Steinernematidae* EPN complexes. This is in part because of the obligate relationship of the symbiotic partnerships between these hosts and their bacteria. It has proved difficult to clarify non-obligate symbiotic relationships between nematodes and bacteria because their often bactivorous diet means their guts usually contain different bacteria, confounding the identification of any more stable ecological associations. The discovery of any other such symbiotic entomopathogenic complexes would be important ecologically and possibly economically and would clearly warrant further research. The unexpected findings of this project suggest the existence of a novel stable ecological association between *Pristionchus* nematodes and specific strains of *Serratia proteamaculans*, a bacterium which had previously only been described as a free living insect pathogen [172]. *Pristionchus* nematodes have previously been associated with a “necromonic” life-cycle only [143]. That is, they persist on the surface of insects such as scarab beetles for dispersal, and ingest the insect tissue upon its death. They have not previously been observed to exhibit direct invasive insect pathogenicity as we demonstrate here. We propose that the acquisition of a symbiotic relationship with the potent insect pathogenic

Serratia proteamaculans strains has facilitated the ability of these *Pristionchus* strains to actively kill insect hosts. In support of this the soil trap assays described above revealed an increase in virulence when either of the NB1 and NC1 nematodes were fed upon the *Serratia* rather than *E. coli*. It should be noted that both NC1 and NB1 were able to infect *Galleria* larvae more rapidly when placed in autoclaved soil as opposed to the traditional sand-trap assays used for *Heterorhabditis* EPN studies. This preference for different substrates has been noted before in studies of other entomopathogenic nematodes. It was not possible to assess the insect pathogenicity of the NB1 and NC1 isolates in a total absence of their associated *Serratia* strains however, as our attempts to completely cure them failed. Despite extensive repeated passaging of these nematodes on *E. coli* OP50 on NGM agar, we always observed low levels of *Serratia* present (in at least some members of the nematode population) when we subsequently crushed the worms. This indicates that *Serratia* sp bacteria must possess a very effective persistence mechanism. Further attempts to cure the nematodes would require the testing of a large panel of antimicrobial agents and/ or conditions not possible in the time frame of these studies.

How and where does the *Serratia* persist in *Pristionchus*?

One essential difference in the physiology of members of the *Caenorhabditis* and *Pristionchus* genera is that the *Caenorhabditis* nematodes possess a grinder in the terminal bulb of the pharynx, which is used for physically lysing the food bacteria [143]; this grinder is absent in *Pristionchus* worms. This suggests that more live bacteria are likely to reach the gut of a *Pristionchus* worm, affording more potential for bacterial persistence and survival. Indeed, this was seen in our confocal microscopy studies that followed the fate of ingested Gfp labelled *E. coli* in these two worms. Few intact Gfp labelled bacteria could be seen post-grinder in *C. elegans* while in the *Pristionchus* the bacterial cells could be seen right along the gut length (Figure 4.12& 4.14). More striking was the ability of the Gfp labelled *Serratia* strains to invade the tissues surrounding the gut in both *C. elegans* and the *Pristionchus*. In the case of the *C. elegans* the bacteria could be seen throughout the mesoderm of the nematode, while in the *Pristionchus* the bacteria seemed to immediately

invade the tissues surrounding the pharynx. It was not clear if this tissue invasion represented intracellular invasion in these experiments and further studies would be required to assess this. Nevertheless, this mechanism does provide an explanation for the observed *Serratia* persistence in the nematodes despite repeated passage on an *E. coli* food source. Our preliminary egg-crushing experiments also suggested that the *Serratia* could be vertically "inherited" by transmission in the eggs. Unfortunately, the numbers observed were low and we were not able to repeat these experiments as an incubator failure killed our stock of nematodes. Re-isolation of the *Pristionchus*-*Serratia* complex from soil taken from the same GPS co-ordinates would be required to confirm this.

In summary, we have shown certain strains of *Serratia proteamaculans* are tightly associated with *Pristionchus* and *Steinernema* nematodes. The visualization of non-pathogenic tissue invasion of the nematode by Gfp labelled bacteria illustrates this clearly in *Pristionchus* nematodes. Interestingly, the bacteria were also capable of infecting *Galleria* larvae as free living cells not associated with the nematode. The benefit to the bacteria may therefore be through a provision of a more environmentally stable niche (inside the worm). The benefit to the nematode is likely to be that the bacterial association allows the nematode to switch from a necromonic life style to an actively predatory one. Overall, the bacterial / nematode association is not an obligate one as exemplified by the better studied EPNs, although there are clear signs of genetic co-adaptation. The "food choice" experiments revealed that while the nematodes were able to grow and reproduce on the *Serratia*, they were not preferentially attracted to them given a choice of *E. coli*. The suggestion that the bacteria may be able to be vertically inherited in the egg is particularly intriguing although requires further study.

Chapter 5

The relationship between nematodes isolates and the molecular phylogeny of their associated *Serratia* strains

5.1. Introduction

Entomopathogenic nematodes (EPN) are commonly dispersed throughout the world and some of them have a wide range of insect hosts [79]. Identification and characterization surveys of EPN have recently increased. Moreover, some studies have revealed the successful relationship of specific bacterial strains with the EPB, such as *Xenorhabdus* sp with *Steinernema feltiae* and *S. affine* and *Photorhabdus* sp associated with *Heterorhabdus bacteriophora* [79]. EPN can provide effective biological control of some soil insect pests [89]. In Britain, Hominick and Briscoe [81] studied the occurrence of Rhabditida (Steinernematidae and Heterorhabditidae) in the British soil; they concluded that the frequency of the nematodes differed in different parts in Britain, suggesting that the occurrence of these nematodes is affected by soil type [81].

The genus *Steinernema glaseri* are normally associated with bacteria of the genera *Xenorhabdus* [5]. Nematodes of this genus are found in almost all terrestrial habitats with vegetation [79]. The first effort to use sequencing data for *Steinernema* phylogenetic analysis was based on examining the partial 18S gene of rDNA [173].

Other common insect associated nematodes are *Pristionchus entomophagus* and *Pristionchus pacificus*; the first is a free living nematodes belonging to Diplogastridae; and is highly diverse in dispersal and has been isolated from different regions [174] & [95]. Meanwhile, *P. pacificus* is a parasite of the oriental beetle *Examala orientalis* [172]. The genome size of *P. pacificus* is substantially larger than that of *C. elegans,* approximately169 Mb in size. *Pristionchus* is believed to have diverged from the common *C. elegans* ancestor millions of years ago [172].

In 2011 Sommer and Rödelsperger [174] suggested that the horizontal gene transfer of a “cellulase” gene from insect host to nematode in *P. pacificus* may have been responsible for a parasitic life style being adopted.

Recently, a newly discovered EPN called *Heterorhabditidoides chongmingensis* was found in Shanghai, associated symbiotically with *Serratia nematodiphila* sp. These nematodes are closely related to *Heterorhabditis*, another common and highly studied EPN belonging to the Rhabditidae family (based on 18S rDNA phylogeny [175].

In the present study, I provide results of the molecular characterization of an EPN that I found associated with *Serratia proteamaculans*-like strains, conducted in UK soil surveys based on 18S rDNA phylogenetic analysis; the resulting fragments of 39 samples were sequenced directly and identified. This chapter aimed to:

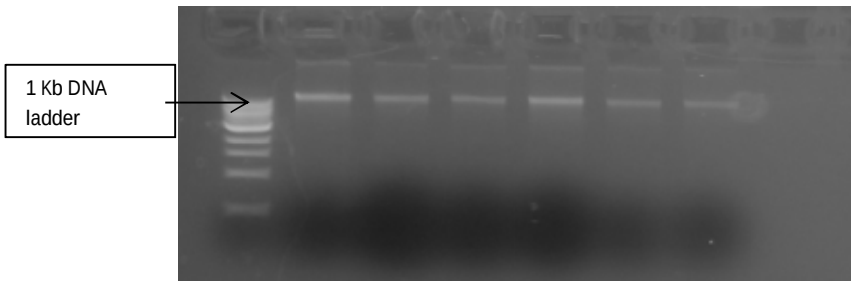
1. determine a molecular phylogeny of 39 insect pathogenic nematode isolates.
2. correlate the nematode phylogeny to the phylogeny of associated *Serratia proteamaculans like*-bacterial strains identified previously with five housekeeping genes.
3. determine if there is any correlation between the nematode genotype and different habitats and soil type from which it was isolated.

The soil type includes factors such as temperature, moisture content and pH (Appendix 1, table 1).

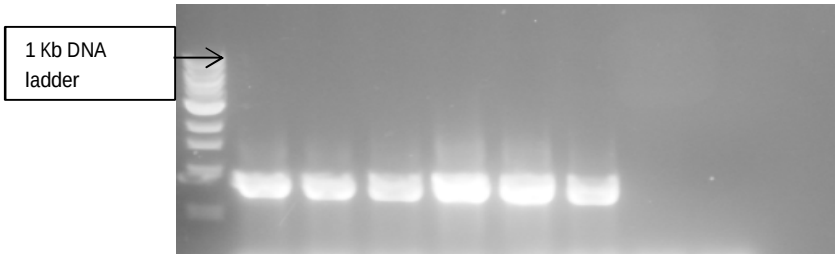
5.2. Results

5.2.1.i. Nematode genomic DNA (gDNA) and PCR amplifications

Nematodes gDNA was prepared (section 2.2.14, page: 48), from 39 frozen nematode samples that were collected by “White traps” from the infected insect cadavers of our baiting experiments. All 39 nematodes were shown to be associated with *Serratia proteamaculans*-like bacterial strains.



A.



B.

Figure 5.1. A. Representative example of genomic DNA analysis using gel electrophoresis prepared from -80 ‘C frozen. B. PCR amplifying bands using specific SSu18A & SSu26R primer pairs to amplify approximately 1000pb of the 18S rDNA gene using 1 Kb DNA ladder.

5.2.1.ii. PCR amplification and BLASTN comparisons

An approximate 1 kb fragment of the 18S rDNA was PCR sequenced and used as a molecular marker to identify the genus of the thirty nine nematode isolates (figure 5.1). BLASTN alignment was used to determine the genus of the isolates. Thirteen isolates were most similar to *Pristionchus entomophaga* (98-100% identity) and six to *Diplogasteroides magnus* (99-100% identity), both of which are Diplogastridae. Sixteen isolates most closely matched *Steinernema glaseri* (Rhabditida: Steinernematidae) (98-100% identity) and four samples were discarded from the study because of bad sequence reads. Examples of BLASTN comparisons for those three groups are shown in Figures 5.2-5.4. NB1, NB2 and NC1 described in the previous chapter were identified in this chapter to confirm the result sent by the University of Arizona, and the results confirmed that NB1 and NB2 belonged to *Pristionchus entomophaga*, and NC1 belonged to *Steinernema glaseri.*

Score	Expect	Identities	Gaps
1584 bits(824)	0.0	837/841(99%)	3/841(0%)

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Query  1    AATAATTGACTTGACGATATATTCCTTAAATGGATAACTGTGGTAATTCTAGAGCTAATA  60
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Sbjct  74   AATAATTGACTTGACGATATATTCCTTAAATGGATAACTGTGGTAATTCTAGAGCTAATA  133
Query  61   CATGAATTTAAGCAGAGTGTCTTTG---GCGCTCTGTTCATTTATTAGACCAAAACCAAG  117
            ||||||||||||||||||||||| |   ||||||||||||||||||||||||||||||||
Sbjct  134  CATGAATTTAAGCAGAGTGTCTTGGTTCGCGCTCTGTTCATTTATTAGACCAAAACCAAG  193
Query  118  CGGCCTCGGTCGTTTATTTCGGTGACTCGTAGTAATTTCGTTGATCACATGATCTTGTAT  177
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  CGGCCTCGGTCGTTTATTTCGGTGACTCGTAGTAATTTCGTTGATCACATGATCTTGTAT  253
Query  178  CGGTGACACGTCTATCAAGTTTCTGCCCTATCAACTTTTGATGGTTCTTTATTGGAGAAC  237
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  CGGTGACACGTCTATCAAGTTTCTGCCCTATCAACTTTTGATGGTTCTTTATTGGAGAAC  313
Query  238  CATGGTTATAACGGGTAACGGAGAATTAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAAC  297
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  314  CATGGTTATAACGGGTAACGGAGAATTAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAAC  373

Query  298  GGCTACCACATCCAAGGAAGGCAGCAGGCGCGAAAATTACCCACTCTCAGTGCGAGGAGG  357

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Sbjct  374  GGCTACCACATCCAAGGAAGGCAGCAGGCGCGAAAATTACCCACTCTCAGTGCGAGGAGG  433

Query  358  TAGTGACGAAAAATAACAAGGCTGATCGCTTTGCGAGCAGCTATTGGAATGGGTACAATT  417

             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

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Query  418  TAAACCCTTTAACGAGGACCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTC  477

             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  494  TAAACCCTTTAACGAGGACCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTC  553

Query  478  CAGCTCTCAAAATGTACTTAACCATTGTTGCGGTTAAAAAGCTCGTAGTTGGATCTCTGC  537

             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

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Query  538  AACGCGAAGTGGTTCGTTCATTGAACGATTACTTCTTCGCGTTGCTCTCTTTTGTCGGTT  597

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Sbjct  614  AACGCGAAGTGGTTCGTTCATTGAACGATTACTTCTTCGCGTTGCTCTCTTTTGTCGGTT  673

Query  598  TTTGGCAGTGTTCCTCACGGAGTGCTGTCGTGACTGACGAGTTTACTTTGAATTAATTAG  657

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Sbjct  674  TTTGGCAGTGTTCCTCACGGAGTGCTGTCGTGACTGACGAGTTTACTTTGAATTAATTAG  733

Query  658  AGTGCTTAAAACAGGCGTTTCGCTTGAATAGTCTAGCATGGAATAATGGAATAGGACTTC  717

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Query  718  GGTTCGATTTTATTGGTTTTATGGATCGAAGTAATGATTAGTAGGAATAAACGGGGGCAT  777

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Query  778  TCGTATTGTTACGTTAGAGGTGAAATTCTGGGACCGTAGCAAGACGATCGACTGCGAAAG  837

             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

Figure 5.2. Example of the blast results of *Steinernema glaseri***,** isolate SteiGla 18S small subunit ribosomal RNA gene, partial sequence. Sequence ID: gb|AY284682.1|

Score	Expect	Identities	Gaps	Strand	Frame
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Features:

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Sbjct  122  CACCAAATCTCGATCCTCTGGATCCGAGAGCACTTGTTAGACCAAGACCATTCCGGGCAA  181

Query  121  CCGGGTTTTGGTGACTCTGAATAATTTCGCTGATCGCACGGTCTCGTACCGGCGACGTAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  CCGGGTTTTGGTGACTCTGAATAATTTCGCTGATCGCACGGTCTCGTACCGGCGACGTAT  241

Query  181  CGTTCAAGTATCTGCTTTATCAACTTTCGATGGAAGTCTATATGGCTACCATGGTTATGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  CGTTCAAGTATCTGCTTTATCAACTTTCGATGGAAGTCTATATGGCTACCATGGTTATGA  301

Query  241  CGGATAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAACGGCTACCACAT  300
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Sbjct  302  CGGATAACGGAGAATAAGGGTTCGACTCCGGAGAGGGAGCCTGAGAAACGGCTACCACAT  361

Query  301  CCAAGGAAGGCAGCAGGCGCGTAAATTACCCACTCTCAATTCGAGGAGGTAGTAACTATC  360
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Sbjct  422  AATAACGAGACAGATCTCTTTGAGGCCTGTTATCGGAATGGGTACAATTTAAACCCTTTA  481

Query  421  ACGAGGATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA  480
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Sbjct  482  ACGAGGATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTCAAA  541

Query  481  GTGTATATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGAGTCTTTGGACGCG  540
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            |||||||||||||||||||||||||||||||||||||||||||| |||||||| |||||
Sbjct  842  GAGGTGAAATTCTTGGACCGCAGCGGGACGTCCTATTGCGAAAGCATTTGCCAA-GAATG  900
```

Figure 5.3. Example of the blast results of *Diplogasteroides magnus* strain RS1983 18S ribosomal RNA gene, partial sequence. Sequence ID: gb|JQ005869.1|

Score	Expect	Identities	Gaps	Strand	Frame
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Features:

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Sbjct  137  AATGGTAGTGCGCACTTATTAGTTTAAGGCCGATTGGGGCAACCCTCTTGGTGACTCTGA  196

Query  61   ATAATTTTGCGGATCGCATGGTCTTGTACCGGCGACGTACTGGTCGAGCGGGTGCCCTAT  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  ATAATTTTGCGGATCGCATGGTCTTGTACCGGCGACGTACTGGTCGAGCGGGTGCCCTAT  256

Query  121  CAACTATTGATGGAAGTCTATGTGTCTTCCATGGTTGTAACGGGTAACGGAGAATAAGGG  180
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Sbjct  497  AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCCAACGTGTATATCGTCATTGCTGCG  556

Query  421  GTTAAAAAGCTCGTAGTTGGATCTAAGTTCATGACTGTAGTTCTCCATGTGAGATACTGC  480
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Sbjct  557  GTTAAAAAGCTCGTAGTTGGATCTAAGTTCATGACTGTAGTTCTCCATGTGAGATACTGC  616

Query  481  TAGTCTGGACTGTTTCGCCGGTTTTCCGTAGCTTCGGCTGCGGTGACTGGTGTTGTAACT  540
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Sbjct  617  TAGTCTGGACTGTTTCGCCGGTTTTCCGTAGCTTCGGCTGCGGTGACTGGTGTTGTAACT  676

Query  541  TTGATTAAATCAATGTGATTAAAACAGGCGTTTGCTTGAATGCTTTATCATGGAATAATA  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  677  TTGATTAAATCAATGTGATTAAAACAGGCGTTTGCTTGAATGCTTTATCATGGAATAATA  736

Query  601  GAATATGA  608
           ||||||||
Sbjct  737  GAATATGA  744
```

Figure 5.4. Example of the blast results of *Pristionchus entomophagus* isolate 1349 18S small subunit ribosomal RNA gene, partial sequence. Sequence ID: gb|FJ040441.1|

5.2.2. Phylogenetic analysis and geographical dependence

The 18s rDNA sequences were compared to one another using Mega 4 to construct a molecular phylogeny. Figure 5.5 illustrates how the phylogeny of these nematodes can be sub-divided into three distinct clades. Clade 1 is represented by the thirteen *Pristionchus entomophagus*–like isolates which were mainly isolated from soils taken from the south (E-W) UK sampling transect. Clade 2 is represented by the six *Diplogasteriodes magnus***-**like isolates which were mainly isolated from soils taken from the (S-N) UK sampling transect. The sixteen *Steinernema glaseri*-like isolates were found mainly from the south (E-W) transect soil samples. We note that members of clades 1 and 3 were isolated from samples collected from either May or February, while the clade 2 nematodes (*Diplogasteriodes magnus* like) were only seen in samples collected in February.

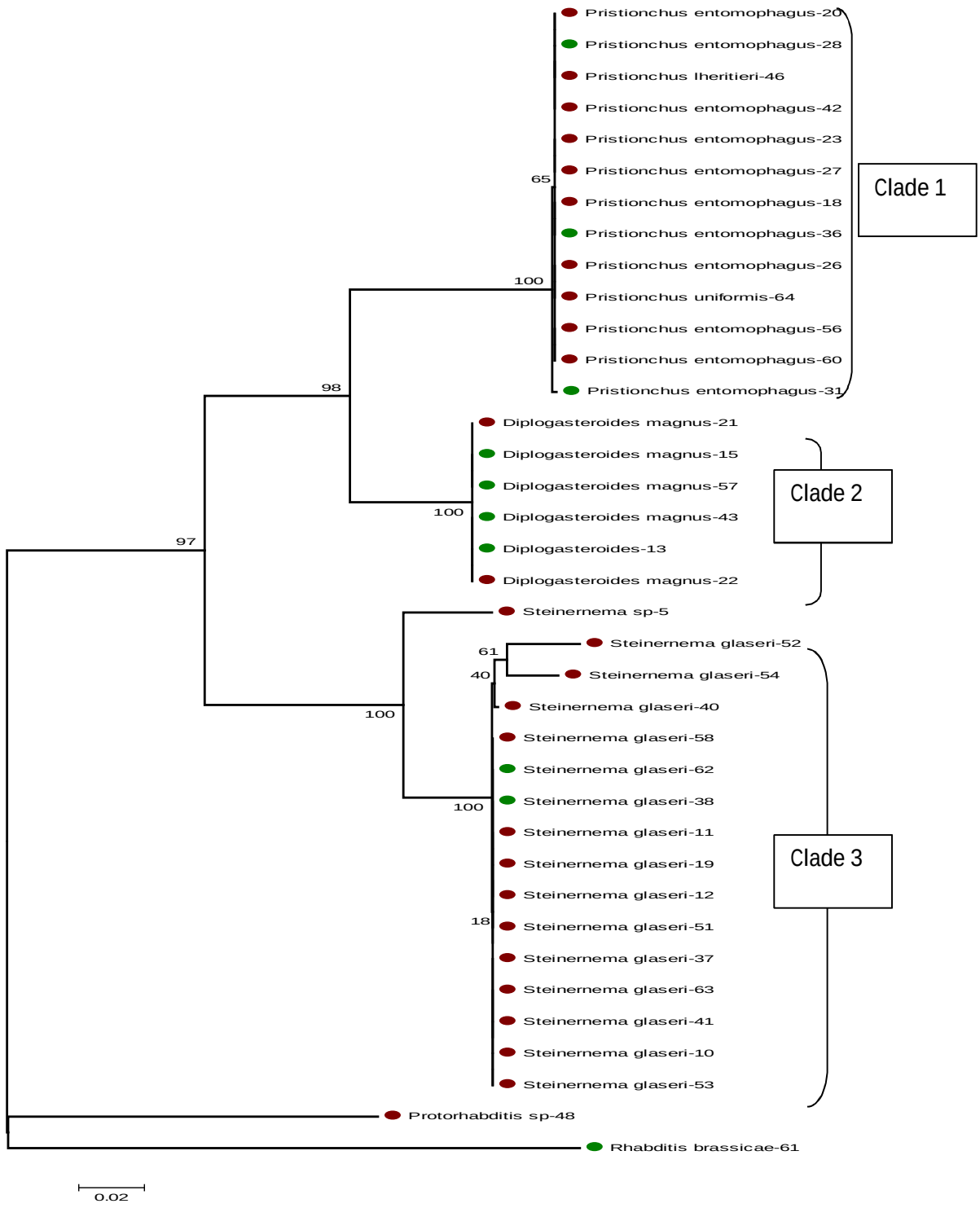
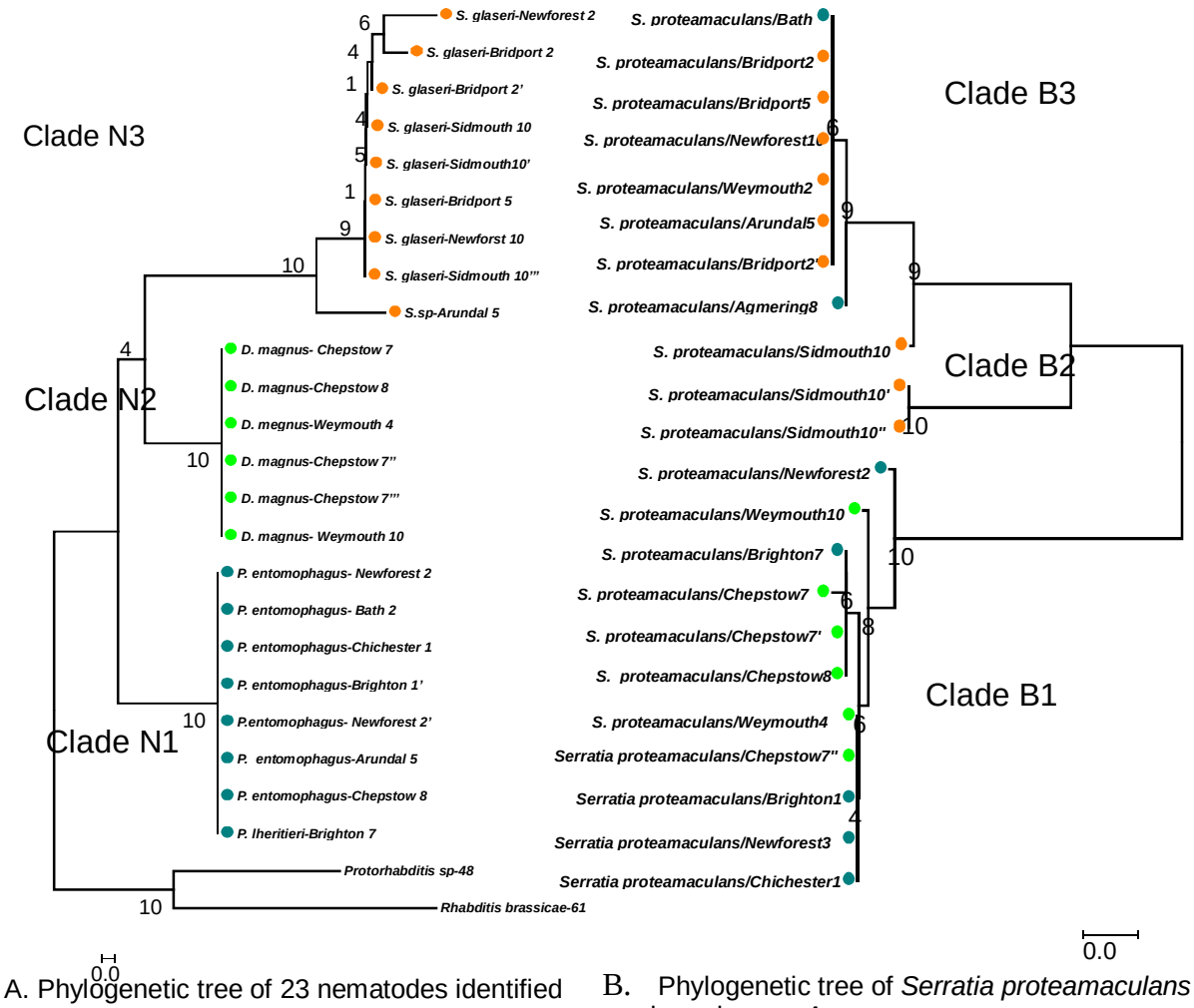


Figure 5.5. Phylogenetic relationship of the three types of associated nematodes *Pristionchus entomophagus, Diplogasteriodes magnus* and *Steinernema glaseri* based on 18S rDNA. Bootstrap values are shown as anodes, the coloured circles indicate the isolated region either from the South (E-W) or North (S-N) UK transect line. The bar represents 2% sequence divergence**.**

5.2.3. A correlation between the phylogenetic trees of the nematodes and their associated *Serratia* strains

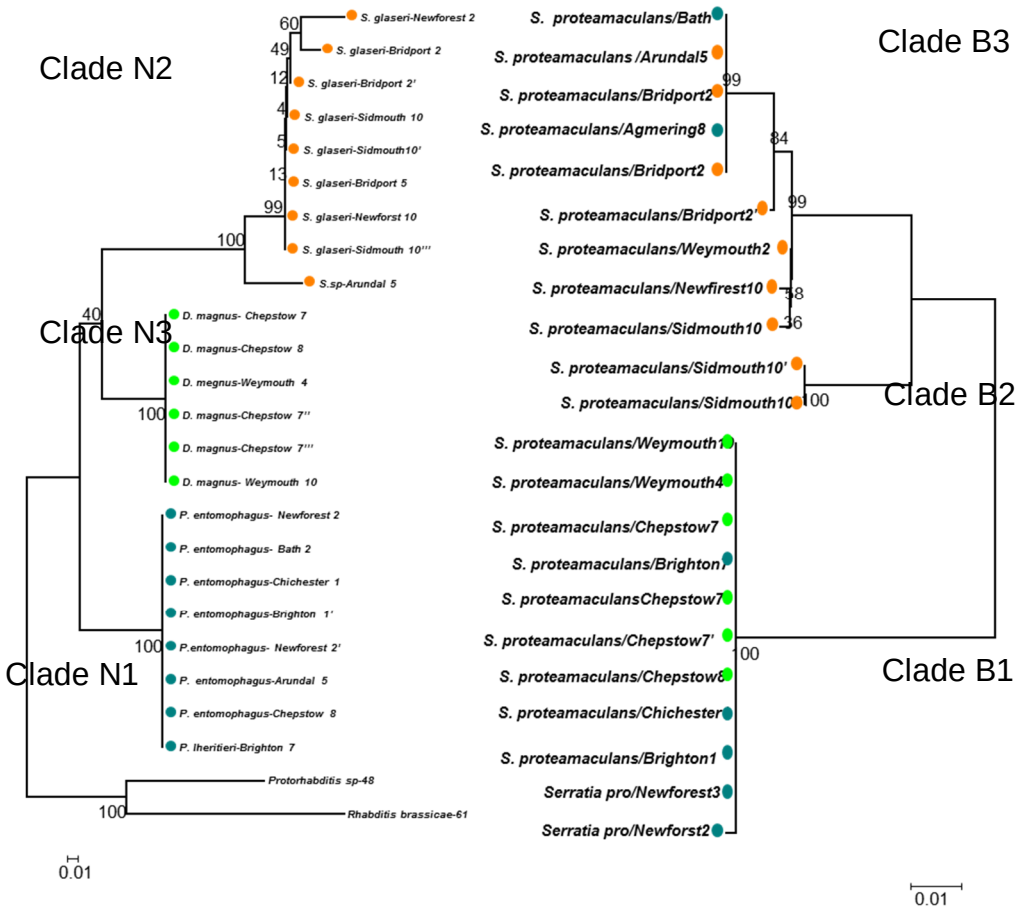
We selected twenty three isolates of nematodes belonging to the 3 clades described above and did a direct comparison of the phylogenetic relationship between the nematodes and the bacterial strains they are carrying. This should provide evidence of any co-speciation of the bacteria with their associated hosts. We did this using the 5 MLSA gene sequences (*recA, dnaj, glmu, adk* and *glyA*) independently and also as a concatenated sequence. A comparison of the trees can be seen in Figures 5.6-5.11.



A. Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Phylogenetic tree of *Serratia proteamaculans* based on *recA* gene.

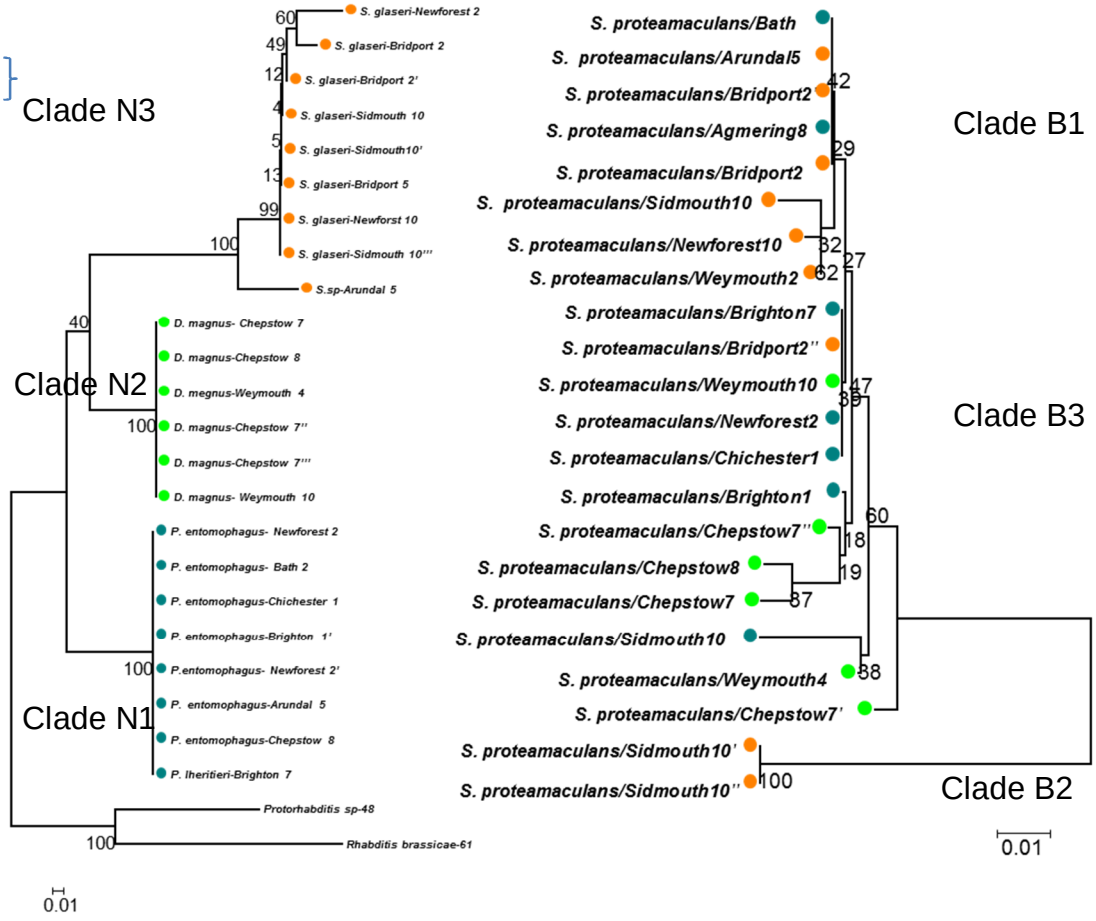
Figure 5.6. Comparative phylogenetic trees. (A).The phylogeny of the host nematodes based on 18S rRNA. (B).The phylogeny of the *S. proteamaculans* strains based on *recA* gene. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.



A. Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Phylogenetic tree of *Serratia proteamaculans* based on *dnaj* gene

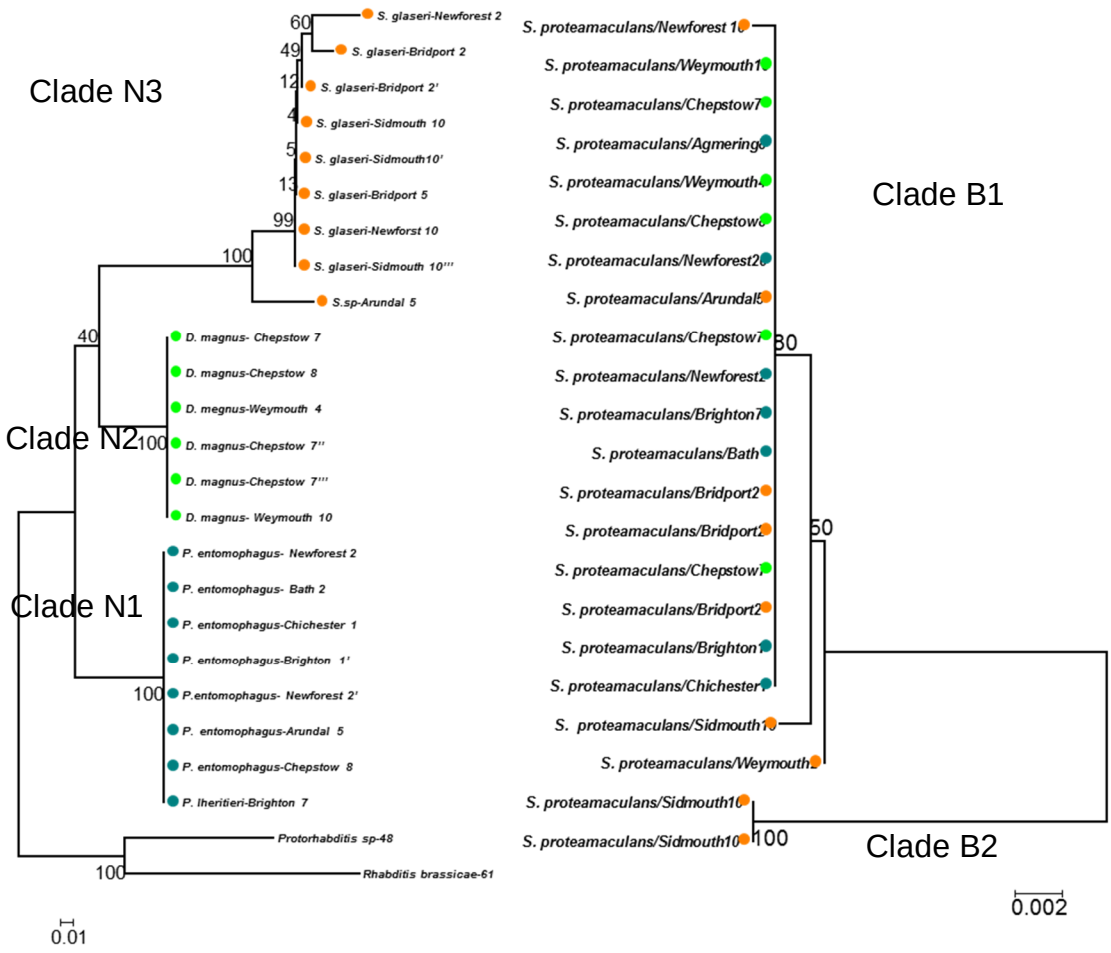
Figure 5.7. Comparative phylogenetic trees. (A).The phylogeny of the host nematodes based on 18S rRNA. (B). The phylogeny of the *S.proteamaculans-like* strains based on *dnaj* gene. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.



A. Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Phylogenetic tree of *Serratia proteamaculans* based on *glmU* gene

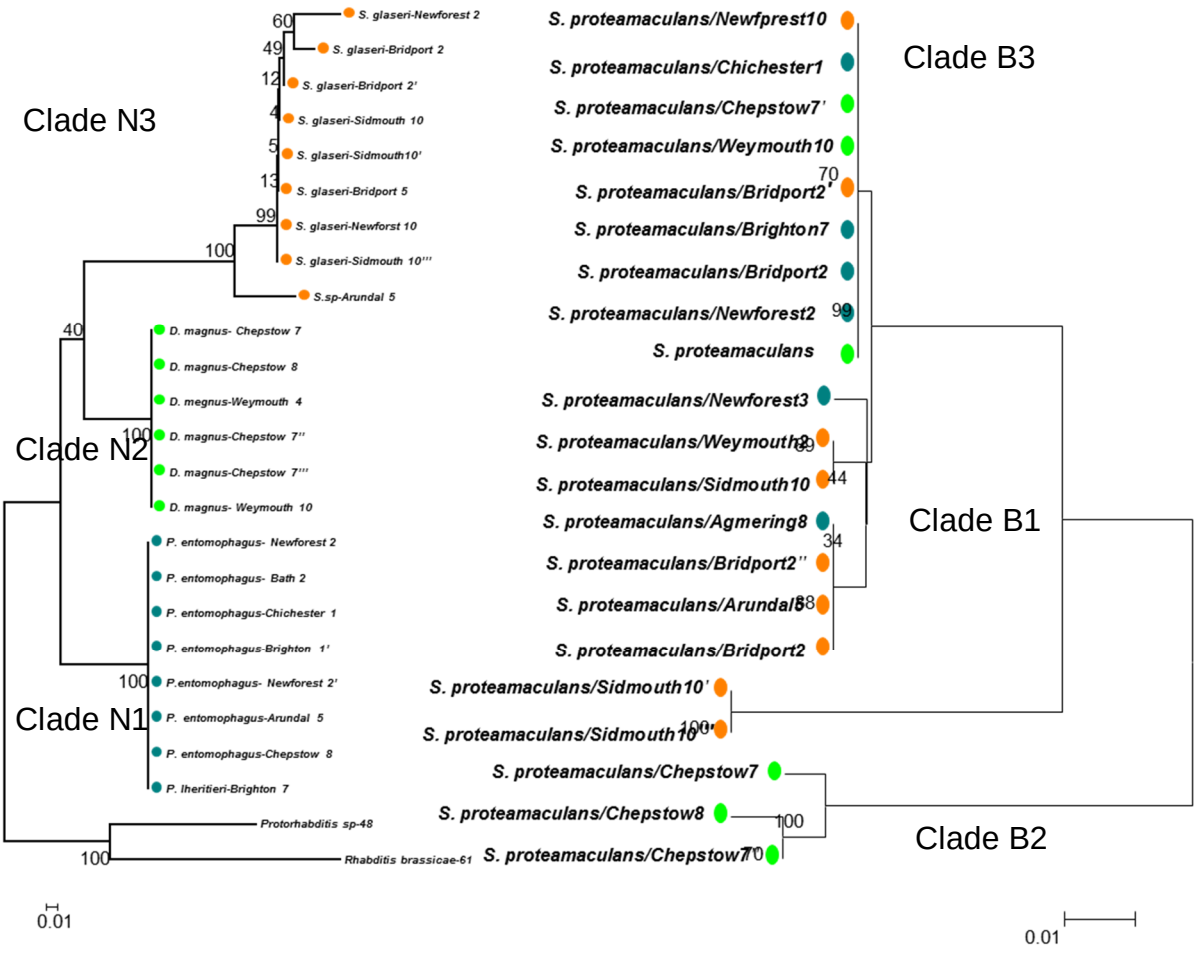
Figure 5.8. Comparative phylogenetic trees. (A).The phylogeny of the host nematodes based on 18S rRNA. (B).The phylogeny of the *S.proteamaculans-like* strains based on *glmU* gene. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.



A, Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Phylogenetic tree of *Serratia proteamaculans* based on *adk* gene

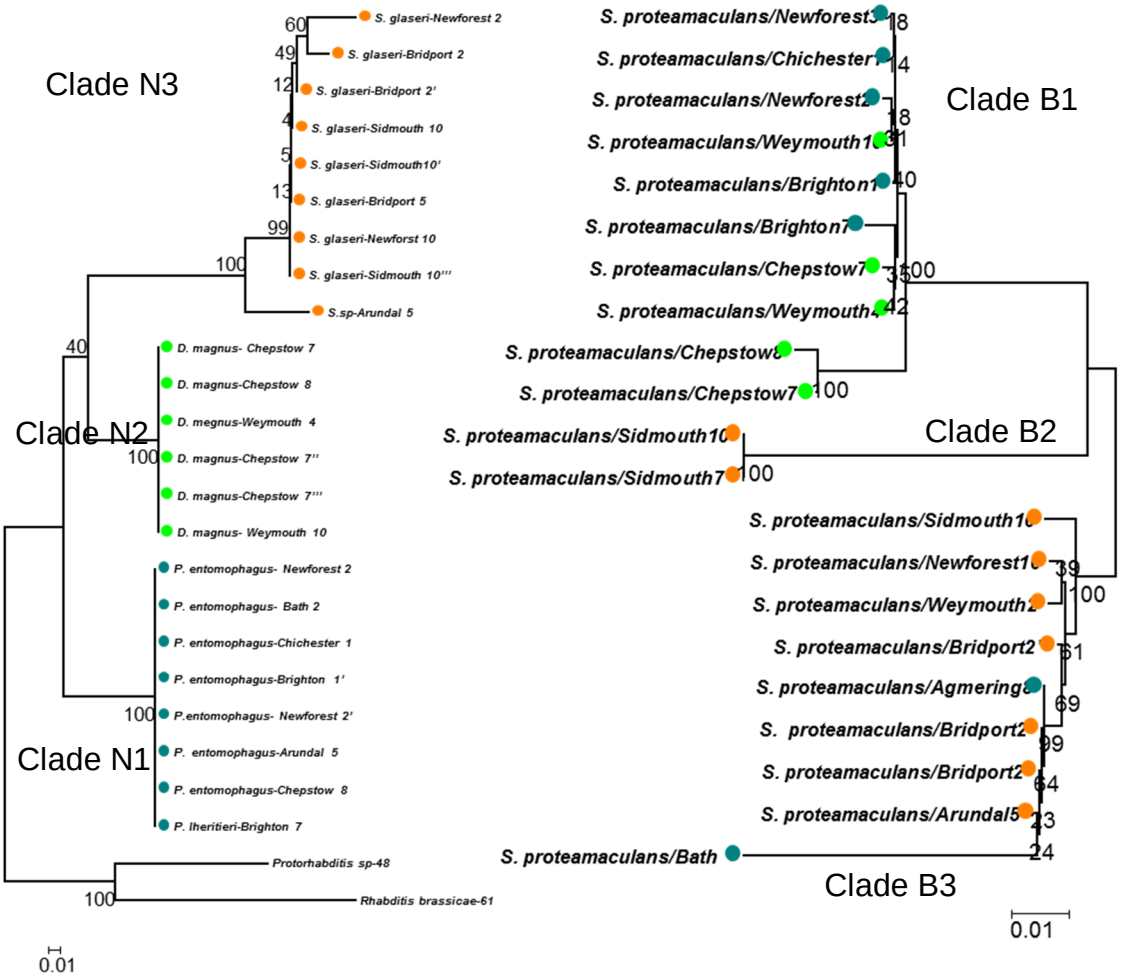
Figure 5.9. Comparative phylogenetic trees. (A).The phylogeny of the host nematodes based on 18S rRNA. (B).The phylogeny of the *S. proteamaculans-like* strains based on *adk* gene. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.



A. Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Phylogenetic tree of *Serratia proteamaculans* based on *glyA* gene

Figure 5.10. Comparative phylogenetic trees. (A).The phylogeny of the host nematodes based on 18S rRNA. (B). The phylogeny of the *S. proteamaculans-like* strains based on *glyA* gene. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.



A. Phylogenetic tree of 23 nematodes identified based on 18S rRNA

B. Concatenated tree of S. *proteamaculans* based on the five genes

Figure 5.11. Comparative phylogenetic trees. (A). The phylogeny of the host nematodes based on 18S rRNA. (B). The phylogeny of the *Serratia proteamaculans* strains based on the five genes. The coloured dots next to the bacterial strains indicate the clade to which their host nematode belongs. Blue is clade 1, green is clade 2 and orange is clade 3. The bar represents 1% sequence divergence in A and B trees. The neighbour-joining phylogenetic tree indicates the number of occurrences (%) of the branching order in 100 bootstrapped trees.

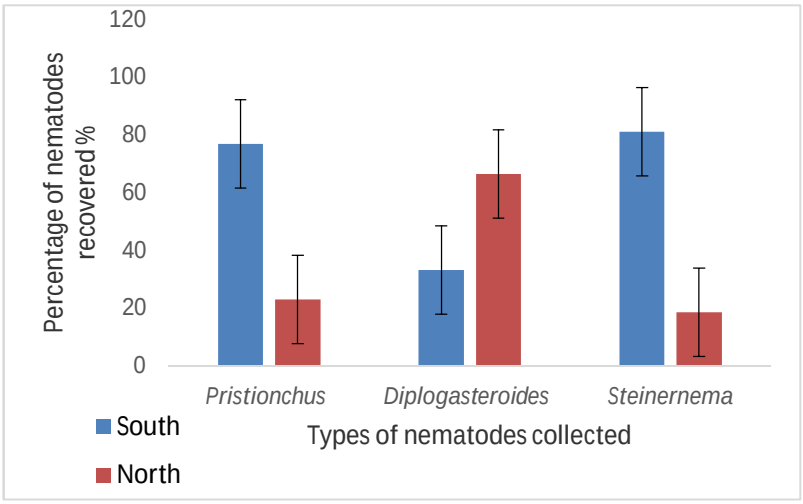
Three of the genes, *recA, dnaJ* and *glmU*, were seen to reflect a phylogeny that correlated with that of the nematodes from which they were isolated. That is bacteria clustering in any one clade were isolated from the same nematode type. The same correlation was seen when we produced a bacterial phylogeny based upon the full MLSA five gene concatenations. Conversely, two of the genes, *adk* and *glyA*, did not show such a clear correlation (figures 5.9 & 5.10). We propose that the phylogeny from the concatenated sequences reflects the most likely relationship between the bacteria and their corresponding nematodes.

For example, two strains of *Serratia* isolated from Chepstow *Diplogasteriodes magnus* nematodes were grouped in B3 using the *glyA* phylogenies; whereas, we find them in B1 in the other four trees.

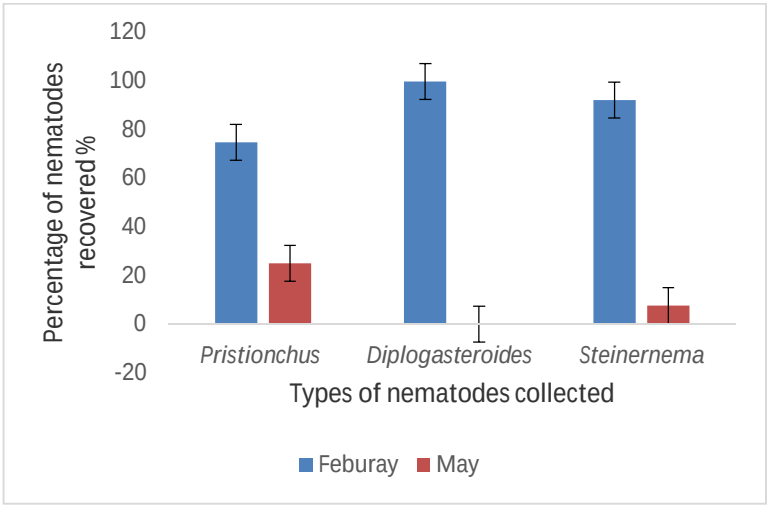
Serratia strains isolated from nematodes in clades N1 and N2 (both belonging to the Diplogastridae family) can be seen to belong to the same bacterial clade B1. Conversely, *Serratia* strains isolated from nematodes grouped into clade N3 can be seen to form a distinct bacterial clade B3. There are exceptions to this correlation, for example in the concatenated MLSA bacterial phylogeny two isolates that cluster with bacteria from *Steinernema* nematodes can be seen even though they were isolated from *Pristionchus* -like nematodes themselves.

5.2.4. Correlating nematode species with habitat

The south (E-W) transect line yielded soil samples that contained the highest numbers of entomopathogenic nematodes. Within this transect, the woodland samples were the most productive followed by the grassland samples. Figure 5.12 shows the types of each nematode recovered from different transects at different time points.



A.



B.

Figure 5.12. Percentage of nematodes recovered A. From two transect lines(E-W) and (N-S). B. In two different time point conditions (February) and (May) n=214 infected *Galleria*.

There was a significant association between sampling date and frequency of recovery of nematodes from infected insects in the baiting experiments, with more nematodes overall being recovered from the February sampling. As shown in chapter 4, nematodes were recovered from 23.7% of insects in soil collected in February compared to only 8% for May. This difference was shown to be significant using Chi-squared analysis (Chi.seq= 9.58, DF=1, PV=0.002). In addition, a significant association was seen between nematode type and the site of sampling. Whereas 24.4% of insects produced nematodes from the soil from southern England, only 8.1% produced nematodes from the northern soil. Again this was statistically significant using Chi-squared analysis (Chi.seq= 16.1, DF=1, PV=0.000).

Generally, the Diplogasteriod family nematodes, which include the *Diplogasteriodes* sp and *Pristionchus* sp were the most ubiquitous nematodes recovered, followed by *Steinernema.*

An attempt was made to correlate the soil sample type with the type of nematode species recovered in our two transects (Appendix 6). For example, ten *Pristionchus* sp isolates were recovered from samples taken along the south transect including Brighton, Angmering, New Forest, Chichester and Bridport, and three isolates from Bristol, Bath and Chepstow. As can be seen from appendix 3, the habitats where the nematodes were recovered are diverse, ranging from woodland to grassland. *Pristionchus* sp were recovered mainly from slightly acid loamy and clayed soil. *Diplogasteriodes sp* were recovered from Chepstow and Weymouth soils taken from grassland habitats. These isolates were found in slightly acid and base rich soil.

In the case of *Steinernema* sp, we recovered thirteen isolates from Sidmouth, New Forest, Weymouth, Bridport, Angmering and Arundel and three from Chepstow and Bath soils. Habitats varied from grassland, woodland and sandy regions. The soil types were mainly slightly acidic sandy, loamy and clay soils. Interestingly, two nematode isolates, one *Pristionchus* and one *Steinernema*, were collected from shallow lime-rich soils over chalk or limestone from Bath.

5.4. Discussion

A phylogenetic analysis of nematodes using small-subunit ribosomal RNA has been undertaken, in an attempt to understand nematode evolution [70] & [142].

Determination of nematode 18S rDNA sequence allowed us to identify entomopathogenic nematodes captured from UK soil using soil baiting and "White trap" techniques. The phylogenetic tree constructed using this 18S rDNA data shows that 19 isolates are close relatives of the species *Pristionchus entomophaga* and *Diplogasteriodes magnus,* which belong to the same taxonomic group (Diplogastridae). A further 16 isolates are more closely related to *Steinernema glaseri* (Steinernematidae).

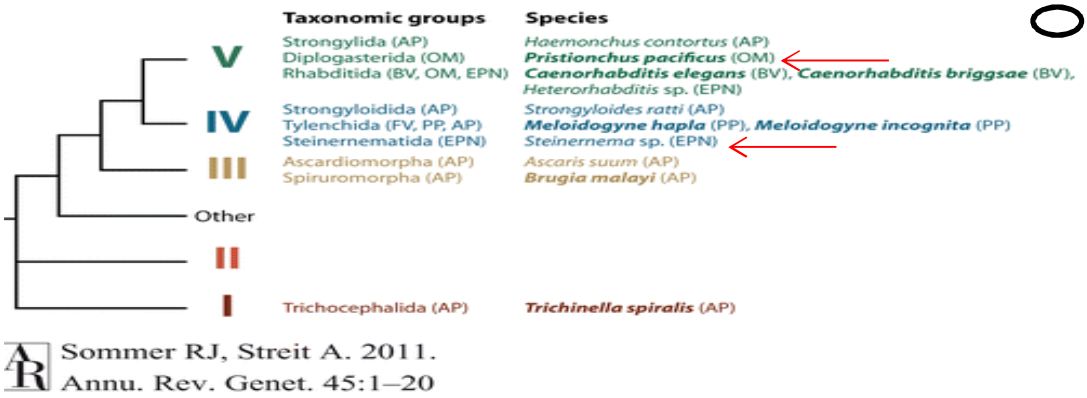


Figure 5.14. Phylogenetic relationship of some of the nematodes published in 2011 [143]. Our identified species were shown by red arrows.

Figure 5.14 illustrates the phylogenetic grouping which distinguishes the five major nematode clades in the classification system of Sommer, R.J. and A. Streit [143]. In clade V, free-living *Pristionchus* nematodes are characterized as OM, or omnivores, and can be seen to belong to the family Diplogastrida. In Clade IV, entomopathogenic nematodes *Steinernema* sp are present, which normally live in symbiotic association with *Xenorhabdus* spp. bacteria. In the present study, specific strains of *Serratia* were found to be associated

with nematode groups. It can be confirmed that these bacteria were not derived from the *Galleria* bait themselves as “sterile control killed” larvae were at no time found to contain *Serratia* cells. Nevertheless, the correlation of specific *Serratia* genotypes with specific nematode types does support an evolved relationship between specific strains of bacteria and nematode host. In addition, the ability of the *Serratia* to support efficient nematode growth and reproduction and the microscopic tissue invasion observations shown in chapter 5 further argue for a specific association. Interestingly, there seemed to be little genetic population structure in the *Serratia* that appeared to infect insects as a free living form, independent of subsequent nematode recovery or association (chapter 4).

To my knowledge, this is the first report of an apparently evolved association between *Serratia* and *Diplogastridae* nematodes such as *Pristionchus*. It is also of note that the *Serratia* associated with the different nematode species show specific phylogenic relationships. That is, it is possible to divide them into three clades based on the species of nematode from which they were recovered. Clade B3 and B2 were mainly recovered from *Steinernema* sp; clade B1 includes bacteria isolated from either *Pristionchus* sp or *Diplogasteriodes* sp. These results appeared in the three genes (*recA, dnaJ* and *glmU*); whereas, the isolates in the two genes (*adk* and *glyA*) did not match with them (figures 5.9 & 5.10). Therefore, the concatenated tree is regarded here as the phylogeny that is most likely to be correct.

The discovery of an association between certain *Serratia* strains and members of the *Diplogastridae* nematode group is novel although perhaps not too surprising. Conversely, the repeated isolation of *Steinernema* nematodes associated with *Serratia* rather than a more usual *Xenorhabdus* strain is harder to explain. It should be noted that most of these isolates were recovered in soil collected in the month of February.

Recently, other types of nematodes have been found to use pathogenic bacteria to parasitize insects, such as *Oscheius* sp and *Caenorhabditis briggsae*. These nematodes have been found to be associated with *Serratia* sp [57].

A novel association was found symbiotically with EPN *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae) and *Serratia* from the investigation of Zhang et al [141]; this also agrees with the results of the present investigation which need further analysis to test if the *Pristionchus* found in different regions in the UK are newly associated with nematodes or not.

A recent report by Abebe et al [33] revealed that some *Caenorhabditis* species such as *C. briggsae* can be transformed into active insect parasites by association with a specific strain of *Serratia marcescens* (*Serratia* sp. SCBI). In this form, the nematode-bacterial complex adopts an entomopathogenic life cycle typical of the better characterized *Steinernema* and *Heterorhabditis* EPNs. This is consistent with observations presented here for the *Diplogastridae* group nematodes (*Pristionchus* and *Diplogasteriodes* types) and the *Serratia proteamaculans* strains isolated in this study. Indeed, *Caenorhabditis* species fall in the same taxonomic clade V as our *Diplogastridae* isolates, suggesting such associations with *Serratia* species may be a relatively ancient, if not obligatory association.

It should be noted that soil from the woodland habitats yielded the greatest diversity of associated *Pristionchus* sp; this finding is in agreement with studies from other surveys [1] & [94] which found that EPN had the greatest diversity in woodlands and forest habitats; whereas grassland yielded the greatest diversity of *Steinernema.* A previous study recovered *Steinrnema* spp distributed in aggregation form in grassland soil [105].

In this study a greater number of Diplogasteriods nematodes was identified than nematodes belonging to the *Sterinernema.* It may be speculated that this result is related to the distribution of preferred insect host types and also soil type. For example, the *Heterorhabditis* EPNs are known to prefer sandy soil types rather than organic-rich woodland soils, and as a result are more readily identified in coastal soils. The comparison between the efficiency of insect infection in sterilized soil compared to sand confirms a preference in the case of the NB1 isolate.

In summary, specific strains of *Serratia proteamaculans* have been identified that show a preference for association with different species of nematode in UK soils. These *S. proteamaculans* strains are associated with three species of nematodes, *Pristionchus entomophaga, Diplogasteriodes magnus,* and *Steinernema glaseri* isolated from UK soil, the habitats where the nematodes were recovered are diverse, ranging from woodland to grassland. Unlike the better studied EPN complexes, these bacteria are also capable of free-living survival in the soil and infection of insect hosts, confirming this is not an obligate symbiotic association. Nevertheless, the observation that specific genetic sub-populations are repeatedly associated with certain species of nematode does suggest host adaptation and that these associations are common in nature [11] & [144]. The *Serratia* associated with the different nematode species show specific phylogenic relationships, dividing them into three clades based on the species of nematode from which they were recovered. The long term stability of any such association in the environment remains to be determined, although laboratory tests do suggest mechanisms for long term persistence.

Chapter 6

General discussion and future perspectives

This thesis describes the development of a novel MLST scheme [121], to type 80 entomopathogenic *Serratia proteamaculans* isolates recovered from soil samples from several locations in the UK. goeBURST [130] supported by the PHYLOViZ program has also been used to cluster related STs, whilst SplitsTree4 was used to check for evidence of recombination. Although there was little evidence of geographical structuring within the *S. proteamaculans* population, the MLSA data resolved distinct clusters, some of which appeared to be associated with three types of nematodes on the basis of the 18s rDNA gene (*Pristionchus entomophagus, Diplogasteriodes magnus* and *Steinernema glaseri*). The work also confirms that climatic factors, such as temperature and rainfall, have a large impact on the recovery rates of both bacteria and nematodes from a given site. For example, a far higher proportion of the entomopathogenic bacteria recovered from the soil traps corresponded to *Serratia* when the samples were collected in February (49%) than in May (18.9%)

Whilst there was limited evidence for recombination in *S. proteamaculans*, when data for single gene loci was considered, there was evidence for extensive recombination when the concatenated data for all 5 genes was subjected to phylogenetic analysis. This suggests that intra-genic (within gene) recombination is rare, whilst inter-genic (between gene) recombination is common. The most likely explanation is that recombination events affect regions considerably larger than the genes in question, hence recombination breakpoints tend to fall between the sequenced genes rather than within them.

The present study also confirms the previous study of Stock et al [78] in that grassland and woodland habitats yielded the greatest diversity of EPN. Torr et al [176] showed adaptation to different habitats in two *Steinernema* species. In the present work S. *glaseri* was isolated from a wide range of habitats, even at very low temperatures (7-10^{o}C), which contrasts with the work of Kung [111] suggesting that these nematodes were more frequently recovered at higher temperatures [111]. Emelianoff et al [79] argued that the adaptation of nematodes to different environments is also dependent on associated bacteria, thus it would be interesting to further study to what extent the different strain *S. proteamaculans* affects nematode distribution. *Pristionchus pacificus* is known to avoid *Serratia marcescens*, and Rae et al noted 23 different bacterial strains associated with *Pristionchus* (gut and cuticle) recovered from beetles [143]. The current work not only demonstrates that *Serratia proteamaculans*-like strains are associated with *Pristionchus,* but that particular bacterial variants may be associated with different types of nematode. Other examples of highly specific associations between bacteria and nematodes have recently been reported, such as that between *Caenorhabditis briggsae* and *Serratia* sp SCBI [47].

In this study we did not confirm 100% that this association between *S. proteamaculans*-like strains and the two types of nematodes *Pristionchus entomophagus,* and *Steinernema glaseri* are real EPN complexes; this will require further study to confirm that this association increased the insects' pathogenicity, as described by Dillma et al [165], to have full characteristics of the EPN complexes.

Thus, the overall suggestion from the current work is that extensive variation exists within single named species of entomopathogenic bacteria, and that this variation is more readily explained by ecological adaptation to different nematode hosts and environmental conditions, than by geographical variation.

The main conclusions of this thesis are therefore as follows:

1. *Serratia proteamaculans*-like bacteria are a major insect pathogenic bacteria widely distributed in UK soil.
2. MLSA data suggest a lack of geographical structure, but that certain clades are adapted to specific nematode hosts.
3. Some *Serratia proteamaculans* strains (e.g. those isolated from Bath and Chepstow) are highly insect pathogenic by injection; both of the isolates can directly infect *Galleria* when free living in the soil.
4. *Serratia* proteamaculans is associated with three types of nematodes *Pristionchus entomophagus, Diplogasteriodes magnus* and *Steinernema glaseri*), particularly in cold temperatures.
5. Certain lineages of these *Serratia proteamaculans-like* bacteria are found associated with the nematodes from a range of sites.
6. Gfp labelled *Serratia* strains invade the tissues surrounding the gut in both *C. elegans* and the *Pristionchus*. In the *Pristionchus* the Gfp bacteria seemed to immediately invade the tissues surrounding the pharynx.

Future perspectives:

1. Different insect hosts and other invertebrates could be examined to elucidate the extent and specificity of the host range of the EPN complex (e.g. beetles, Drosophila, woodlice and spiders).

2. The possibility that *Serratia* produces antimicrobial compounds could be examined. These bacteria may also act as an environmental reservoir of antibiotic resistance genes.

3. The mechanisms of pathogenicity and virulence towards insects employed by the *Serratia proteamaculans*-like bacteria are unknown. Studying the migration of *gfp Serratia* within the host insect, and when in contact with insect cells *in vitro* would be rewarding in this context. Recent work has shown that other bacterial insect pathogens interact both directly and indirectly with *Drosophila* hemocytes [60].

4. The effect of environmental variables on communities of entomopathogenic nematodes and their associated bacteria in soils is very poorly understood. In this context, it would be very interesting to study insect pathogenic bacteria and nematodes isolated from geographic regions with extremely high soil temperature (Kuwait would be a good example), and to compare this with the UK data. Does high temperature lead to closer or looser associations between nematodes and bacteria? Is the diversity of nematodes and/or bacteria greater or less than in lower temperature soils? Is pathogenicity towards insect hosts greater or lesser (etc). It would be logical to extend this to look at other environmental variables, such as soil water content, salinity, soil type (etc).

5. Are *Serratia. proteamaculans*-like bacteria found elsewhere in the world, and are they associated with nematodes (and if so, which ones)?

6. It would be particularly interesting to determine whether the *Serratia. proteamaculans*-like bacteria are able to form long term functional relationships with laboratory model nematode species such as *C. elegans,* which would allow an experimental genetic investigation of the proposed mutualism, in relation to the genetics of both the nematode host and the associated bacteria.

7. Further study is required to confirm that the association between *S. proteamaculans*-like strains and the two types of nematodes *Pristionchus entomophagus,* and *Steinernema glaseri* are EPN complexes, in order to achieve the definition of EPN complexes as described by Dillman et al [165].

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Chapter 7

Appendixes

Appendix 1.

Table 1. Large-scale survey in (E-W) Southern England and (S-N) Wales data including: Soil samples sites, Date, soil PH, soil temperature, soil moisture, Habitat descriptions, long-lat coordinates for each soil sample sites.

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
1	Bri 1	Ditchling 1	16/02/2009 09/May/2009	6	8°C 18°C	5	Woodland	-
2	Bri 2	Ditchling 2	16/02/2009 09/May/2009	6	8°C 18°C	6	Grass land	-
3	Bri 3	Brighton beach	17/02/2009 09/May/2009	6.3	10°C 18°C	5	No vegetation	-
4	Bri 4	Brighton park	17/02/2009 09/May/2009	7	10°C 18°C	2	Beside tree	Lat :50.8317393.644984 Log: -0.132822999041748047 Alt: 207.0
5	Bri 5	Brighton park	17/02/2009 09/May/2009	7	10°C 18°C	4	tree root	Lat :50.8317393.644984 Log: -0.132822999041748047 Alt: 207.0
6	Bri 6	Brighton (beside tree)	17/02/2009 09/May/2009	7	10°C 18°C	2	grass land	Lat: 50.85746169090271 Log: -0.10410189628601074 Alt: 88.0
7	Bri 7	Lewes (beside lake)	17/02/2009 09/May/2009	7	7°C 18°C	3	Woodland	Lat: 50.87523937225342 Lon 0.015063285827636719 Alt: 51:0
8	Bri 8	Lewes (beside lake)	17/02/2009 09/May/2009	6.3	7°C 18°C	3	Woodland beside tree	Lat: 50.877331495285034 Lon: 0.006802082061767578 Alt: 64.0
9	Bri 9	Lewes	17/02/2009 09/May/2009	6	9°C 18°C	3	beside lake	Lat: 50.877315402030945 Lon: 0.006775259971618652 Alt: 52.0
10	Bri 10	Lewes	17/02/2009 09/May/2009	7	10°C 18°C	4	Wood, beside tree root	Lat: 50.87724030017853 Log: 0.007188320159912209 Alt: 65.0
11	Ang 1	Angmering	17/02/2009 10/May/2009	7	8°c 18°C	5	Beside tree	Lat: 50.83918511867523 Lon: -0.3071558475494385 Alt: 50.0
12	Ang 2	Angmering	17/02/2009 10/May/2009	6	9°C 17°C	7	Farm soil far from Ang1 36meter	Lat: 50.831342339515686 Lon: -0.47624766682662964 Alt: 63.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
13	Ang 3	Angmering	17/02/2009 10/May/2009	7	9°C 17°C	4	Woodland	Lat:50.83125650882721 Lon: -0.4746477813720703 Alt: 57.0
14	Ang 4	Angmering	17/02/2009 10/May/2009	6	9°C 17°C	3	Woodland	Lat: 50.83124041557312 Lon: -0.4775887727737427 Alt: 59.0
15	Ang 5	Angmering	17/02/2009 10/May/2009	7	10°c 17°C	6	Grass land	Lat: 50.83103120326996 Lon: -0.4778248071670532 Alt: 60.0
16	Ang 6	Angmering	17/02/2009 10/May/2009	7	8°C 17°C	5	Woodland	Lat: 50.82757115364075 Lon: -0.4869818687438965 Alt: 54.0
17	Ang 7	Angmering	17/02/2009 10/May/2009	7	9°C 17°C	2	Tree root	Lat: 50.82773208618164 Lon: -0.48696577548980713 Alt: 51.0
18	Ang 8	Angmering	17/02/2009 10/May/2009	7	9°C 17°C	3		Lat: 50.82128405570984 Lon: -0.48508286476135154 Alt: 58.0
19	Ang 9	Angmering	17/02/2009 10/May/2009	7	9°C 17°C	3	Play ground School grass land	Lat: 50.8210426568985 Lon: -0.48524496172759 Alt: 52.0
20	Ang 10	Angmering	17/02/2009 10/May/2009	6	9°C 17°C	3	Play ground School grass land	Lat: 50.82044184207916 Lon: -0.4851311445236206 Alt: 55.0
21	Aru 1	Arundel	17/02/20091 0/May/2009	7	9°C 17°C	3	Woodland	Lat: 50.82044184207916 Lon: -0.48515260219573975 Alt: 56.0
22	Aru 2	Arundel	17/02/2009 10/May/2009	7	9°C 17°C	5.5	Grass land	Lat: 50.85395336151123 Lon: -0.5506521463394165 Lon: 45.0
23	Aru 3	Arundel	17/02/2009 10/May/2009	7	9°C 17°C	7	grass land 100 meter from Aru2	Lat: 50.85377097129822 Lon: -0.5495363473892212 Alt: 47.0
24	Aru 4	Arundel	17/02/2009 10/May/2009	7	9°C 17°C	7.5	Grass land 100 meter from Aru3	lat: 50.85379779338837 Lon: -0.5488765239715576 Alt: 44.0
25	Aru 5	Arundel	17/02/2009 10/May/2009	7	8°C 17°C	7	Grass land	Lat: 50.85344910621643 Lon: -0.5498743057250977 Alt: 46.0
26	Aru 6	Arundel	17/02/2009 10/May/2009	7	10°c 17°C	6	Woodland	Lat: 50.85348665714264 Lon: -0.5498367547988892 Alt: 51.0
27	Aru 7	Arundel	18/02/2009 10/May/2009	7	10°c 18°C	7	Grass land	Lat: 50.856823325157166 Lon: -0.5784344673156738 Alt: 83.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
28	Aru 8	Arundel	18/02/2009 10/May/2009	7	10°c 17°C	8	Woodland	Lat: 50.85619032382965 Lon: -.05783754587173462 Alt: 84.0
29	Aru 9	Arundel	18/02/2009 10/May/2009	7	8°C 17°C	7	Forest soil	Lat: 50.8561635017395 Lon: -0.5783593654632568 Alt: 80.0
30	Aru 10	Arundel	18/02/2009 10/May/2009	7	8°C 17°C	6.5		Lat: 50.85446298122406 Lon: -0.5658817291259766 Alt: 80.0
31	Chi 1	6 mile to Chichester (Arundel road)	18/ 02/2009 11/May/2009	7	10°C 17°C	3	Grass land	Lat: 50. 3555348110198975 Lon: -0.6501996517181396 Alt: 73.0
32	Chi 2	Chichester	18/ 02/2009 11/May/2009	7	10°C 17°C	4	No vegetation	Lat: 50.853551030159 Lon: -0.6810826063156128 Alt: 75.0
33	Chi 3	Chichester	18/ 02/2009 11/May/2009	6.8	8°C 17°C	4	grass land	Lat: 50.83846628665924 Lon: -0.7747131586074829 Alt: 40.0
34	Chi 4	Chichester	18/ 02/2009 11/May/2009	6.9	10°C 17°C	3	Wood, beside tree root	Lat: 50.83895444869995 Lon: -0.7747131586074829 Alt: 63.0
35	Chi 5	Chichester	18/ 02/2009 11/May/2009	7	10°C 17°C	5	grass land	Lat: 50.839099287986755 Lon: -0.7745307683944702 Alt: 60.0
36	Chi 6	Chichester	18/ 02/2009 11/May/2009	7	9°C 17°C	2	Woodland	Lat: 50.83826243877411 Lon: -0.774809718132019 Alt: 73.0
37	Chi 7	Chichester (Bosham)	18/ 02/2009 11/May/2009	7	10°C 17°C	3	Grass land	Lat: 50.83942115306854 Lon: -0.8519446849822998 Alt: 54.0
38	Chi 8	Chichester (Bosham)	18/ 02/2009 11/May/2009	6	10°C 17°C	4	no vegetation	Lat: 50.83924949169159 Lon: -0.85193932056427 Alt: 50.0
39	Chi 9	Chichester	18/ 02/2009 11/May/2009	7	10°C 17°C	3	Woodland	Lat: 50.83699107170105 Lon: -0.851815938949585 Alt: 52.0
40	Chi 10	Chichester	18/ 02/2009 11/May/2009	7	10°C 17°C	3	Grass land	Lat: 50.82044184207916 Lon: -0.48515260219573975 Alt: 56.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
41	Isle1	Isle of wight Haven street forest	18/ 02/2009 11/May/2009	7	10°C 17°C	6	woodland (forest)	Lat: 50.761985778808594 Lon: -1.1694002151489258 Alt: -148.0
42	Isle2	Isle of wight Garlic farm	18/ 02/2009 11/May/2009	7	10°C 17°C	6	Farm soil	Lat: 50.67959368228912 Lon: -1.2151157855987549 Alt: 117.0
43	Isle3	Isle of wight Garlic farm	18/ 02/2009 11/May/2009	7	10°C 17°C	6	Grass land	Lat: 50.679582953453064 Lon: -1.2148261070251465 Alt: 88.0
44	Isle4	Isle of wight Ventnor	18/ 02/2009 11/May/2009	7	10°C 17°C	4	Beside tree	Lat: 50.6796258687973 Lon: -1.214761734008789 Alt: 88.0
45	Isle5	Isle of wight Ventnor	18/ 02/2009 11/May/2009	7	10°C 17°C	3	No vegetation	Lat: 50.59310317039999 Lan: -1.2019729614257813 Alt: 54.0
46	Isle6	Isle of wight New port	18/ 02/2009 11/May/2009	7	10.1°C 17°C	3	No vegetation	Lat: 50.61896502971649 Lon: -1.22064561653137207 Alt: 160.0
47	Isle7	Isle of wight New port	18/ 02/2009 11/May/2009	7	8°C 17°C	3	grass land	Lat: 50.6985408006770325 Lon: -1.2898153066635132 Alt:69.0
48	Isle8	Isle of wight New port	18/ 02/2009 11/May/2009	7	8°C 17°C	3	beside tree	Lat: 50.69871246814728 Lon: -1.2899225950241089 Alt: 66.0
49	Isle9	Isle of wight Yarmouth	18/ 02/2009 11/May/2009	7	10°C 17°C	2	grass land	Lat: 50.69890558719635 Lon: -1.2899547815322876 Alt: 89.0
50	Isle10	Isle of wight Yarmouth	18/ 02/2009 11/May/2009	6	8°C 17°C	3	grass land	Lat: 50.70415198802948 Lon: -1.4995747804641724 Alt: 40.0
51	New 1	Minstead	19/02/2009 12/May/2009	7	9°C 18°C	6	Forest soil	Lat: 50.70418417453766 Lon: -1.499934196472168 Alt: 55.0
52	New 2	Minstead	19/02/2009 12/May/2009	6.6	9°C	4	Forest soil/ 50 meter from before	Lat: 50.87170958518982 Lon: -1.5713292360305786 Alt:99.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
53	New 3	Minstead	19/02/2009 12/May/2009	6.6	10°C 18°C	3	Forest soil/ 50 meter from before	Lat:50.8714896440506 Lon: -1.571270227432251 Alt: 72.0
54	New 4	Minstead	19/02/2009 12/May/2009	6.9	9°C 18°C	4	Forest soil/ 50 meter from before	Lat:50.87125360965729 Lon:-1.5716028213500977 Alt: 78.0
55	New 5	Minstead	19/02/2009 12/May/2009	7	9°C 18°C	3	Forest soil/ 50 meter from before	Lat: 50.8711302280426 Lon:-1.5712165832519531 Alt:81.0
56	New 6	Minstead	19/02/2009 12/May/2009	7	10°C 18°C	1	Farm soil	Lat: 50.87125897407532 Lon:-1.5712380409240723 Alt:86.0
57	New 7	Beaulieu	19/02/2009 12/May/2009	6.9	10°C 18°C	3	Forest soil	Lat: 50.85521399974823 Lon:-1.5039092302322388 Alt:91.0
58	New 8	Beaulieu	19/02/2009 12/May/2009	7	10°C 18°C	8	Forest soil	Lat: 50.81950306892395 Lon:-1.4553934335708618 Alt:45.0
59	New 9	Beaulieu	19/02/2009 12/May/2009	7	10°C 18°C	3	Forest soil	Lat:50.82443833351135 Lon:-1.45677774534225464 Alt:56.0
60	New 10	Beaulieu	19/02/2009 12/May/2009	7	10°C 18°C	5	Forest soil	
61	Wey1	Bounemouth	19/02/2009 12/May/2009	6.8	10°C 18°C	1	Sandy	Lat: 50.72188138961792 Lon:-1.8195998668670654 Alt:78.0
62	Wey2	Bounemouth	19/02/2009 12/May/2009	7	10°C 18°C	2	Sandy/playing ground	Lat: 50.72189748287201 Lon:-1.8196159601211548 Alt:75.0
63	Wey3	Bounemouth	19/02/2009 12/May/2009	6.9	9°C 18°C	1	Sandy	Lat: 50.7216078042984 Lon:-1.8195945024490359 Alt:81.0
64	Wey4	Weymouth	20/02/2009 12/May/2009	7	9°C 18°C	7	Sandy/beach	Lat:50.6472247838974 Lon:-2.4058502912521362 Alt:132.0
65	Wey5	Weymouth	20/02/2009 12/May/2009	5.8	9°C 18°C	5	Garden soil	Lat:50.60881555080414 Lon:-2.450900673866272 Alt:53.0
66	Wey6	Weymouth	20/02/2009 12/May/2009	6.1	8°C 18°C	8	Grass land	Lat:50.608364939689636 Lon:-2.451125979423523 Alt:54.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
67	Wey7	Weymouth	20/02/2009 12/May/2009	7	8°C 18°C	8	Vegetation area	Lat:50.62586367130279 Lon:-2.5111430883407593 Alt:86.0
68	Wey8	Weymouth	20/02/2009 12/May/2009	7	12°C 18°C	6	Grass land	Lat:50.63451647758484 Lon:-2.527579665184021 Alt:97.0
69	Wey9	Weymouth	20/02/2009 12/May/2009	7	10°C 18°C	1	Farm soil	Lat:50.63469350337982 Lon:-2.5274670124053955 Alt:104.0
70	Wey10	Weymouth	20/02/2009 12/May/2009	7	11°C 18°C	6	grass land	Lat:50.65370500087738 Lon:-2.540416717529297 Alt:93.0
71	Sid1	Sidmouth	21/02/2009 13/May/2009	7	9°C 18°C	3	Woodland	Lat:50.690537095069885 Lon:-3.2137423753738463 Alt:218.0
72	Sid2	Sidmouth	21/02/2009 13/May/2009	7	9°C 18°C	3	Grass land	Lat:50.68511366844177 Lon:-3.234320282936096 Alt:68.0
73	Sid3	Sidmouth	21/02/2009 13/May/2009	7	10°C 18°C	6	grass land	Lat:50.67898750305176 Lon:-3.240070939064026 Alt:64.0
74	Sid4	Sidmouth	21/02/2009 13/May/2009	7	10°C 18°C	5	Beside tree	Lat:50.681787729263306 Lon:-3.243289589881897 Alt:64.0
75	Sid5	Sidmouth	21/02/2009 13/May/2009	7	8.5°C 18°C	5	Garden soil/100m from sid4	Lat:50.68618655204773 Lon:-3.2450973987579346 Alt:92.0
76	Sid6	Sidmouth	21/02/2009 13/May/2009	7	7.5°C 18°C	2	Beside tree	Lat:50.68524777889552 Lon:-3.2454997301101685 Alt:107.0
77	Sid7	Sidmouth	21/02/2009 13/May/2009	7	8°C 18°C	6.8	Woodland same garden	Lat:50.68523705005646 Lon:-3.245440721511841 Alt:121.0
78	Sid8	Sidmouth	21/02/2009 13/May/2009	7	7°C 18°C	4	Beside tree	Lat:50.685049295425415 Lon:-3.245649933815024 Alt:97.0
79	Sid9	Sidmouth	21/02/2009 13/May/2009	7	-	-	Grass land	Lat:50.6854523582458496 Lon:-3.2448506355285645 Alt:80.0
80	Sid10	Sidmouth	21/02/2009 13/May/2009	7	-	-	Grass land	Lat:50.68460404872894 Lon:-3.24471652507782 Alt:99.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
81	Brid 1	Bridport	20/02/2009 13/May/2009	7	11°C 17° C	5	Woodland	Lat:50.70981681346893 Lon:-2.745707631111145 Alt:76.0
82	Brid 2	Bridport	20/02/2009 13/May/2009	7	11°C 17° C	3	Grass land	Lat:50.70980608463287 Lon:-2.745680809020996 Alt:69.0
83	Brid 3	Bridport	20/02/2009 13/May/2009	7	11°C 17° C	5	Beside tree	Lat:50.726988315582275 Lon:-2.758721709251404 Alt:57.0
84	Brid 4	Bridport	20/02/2009 13/May/2009	7	11°C 17° C	2	River side	Lat:50.73514223098755 Lon:-2.747011184692383 Alt:61.0
85	Brid 5	Bridport	20/02/2009 13/May/2009	7	11°C 16° C	5	River side/50m from Brid4	Lat:50.73479354381561 Lon:-2.747231125831604 Alt:61.0
86	Brid 6	Bridport	20/02/2009 13/May/2009	7	9°C 17° C	4	Woodland	Lat:50.74778079986572 Lon:-2.7439212799072266 Alt:97
87	Brid 7	Bridport	20/02/2009 13/May/2009	7	9°C 17° C	6	Grass land	Lat:50.748188495635986 Lon:-2.746227979660034 Alt:92.0
88	Brid 8	Bridport	20/02/2009 13/May/2009	7	9.5°C 17° C	6	Grass land	Lat:507478666305542 Lon:-2.744109034538269 Alt:104.0
89	Brid 9	Bridport	20/02/2009 13/May/2009	7	9°C 17° C	4	Woodland	Lat:50.74456751346588 Lon:-2.752874493598938 Alt:62.0
90	Brid 10	Bridport	20/02/2009 13/May/2009	7	8°C 17° C	4	Woodland	Lat:50.744588971138 Lon:-2.7528423070907593 Alt:67.0
91	Wey11	Weymouth	20/02/2009 14/May/2009	7	10°C 19° C	5	Vegetative area	Lat:50.66893994808197 Lon:-2.5636768341084453 Alt:120.0
92	Lyme1	Lyme Regis	21/02/2009 14/May/2009	7	6°C 19°C	3	Garden soil	Lat:50.71730554103851 Lon:-3.0025452375411987 Alt:206.0
93	Lyme2	Lyme Regis	21/02/2009 14/May/2009	7	8° C 19°C	3	Beside tree	Lat:50.71730554103851 Lon:-3.0025452375411989 Alt:209.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
94	Lyme3	Lyme Regis	21/02/2009 14/May/2009	7	8°C 19°C	4.5	Farm soil	Lat:50.71730554103851 Lon:-3.0025452375411987 Alt:206.0
95	Lyme4	Lyme Regis	21/02/2009 14/May/2009	7	9°C 17C	6	Woodland	Lat:50.71730554103851 Lon:-3.0025452375411987 Alt:200.0
96	Lyme5	Lyme Regis	21/02/2009 14/May/2009	7	8°C 17° C	4	Grass land	Lat:50.72379648685455 Lon:-2.9416483640670776 Alt:114.0
97	Lyme6	Lyme Regis	21/02/2009 14/May/2009	7	9°C 19° C	4.5	Grass land	Lat:50.7344262466430664 Lon:-2.9554563760757446 Alt:126.0
98	Lyme7	Lyme Regis	21/02/2009	7	8°C 15°C	3	Vegetation area	Lat:50.73640823364258 Lon:-2.959147095680237 Alt:115.0
99	Lyme8	Lyme Regis	21/02/2009 14/May/2009	7	8°C 17° C	4	Old farm vegetable	Lat:50.75165390968323 Lon:-2.9688191413879395 Alt:221.0
100	Lyme9	Lyme Regis	14/May/2009 14/May/2009	7	8°C 17° C	3	Forest soil	Lat:50.744755268096924 Lon:-2.967429757118225 Alt:137.0
101	Lyme10	Lyme Regis	21/02/2009 14/May/2009	7	8°C 17° C	2	Forest soil	Lat:50.74735164642334 Lon:-2.973845601081848 Alt:191.0
102	Chep1	Chepstow	23/02/2009 16/May/2009	7	11°C 19° C	8	Grass land	Lat:51.39174699783325 Lon:-2.352624535560608 Alt:65.0
103	Chep2	Chepstow	23/02/2009 16/May/2009	7	11°C 19° C	4	Woodland	Lat: 51.64244771003723 Lon:-2.661287784576416 Alt:88.0
104	Chep3	Chepstow	23/02/2009 16/May/2009	7	11°C 19° C	4	Grass land	Lat:51.63969576358795 Lon:-2.655220627784729 Alt:74.0
105	Chep4	Chepstow	23/02/2009 16/May/2009	7	10°C 19° C	7	No vegetation	Lat:51.64792478084564 Lon:-2.65174984931958 Alt:80.0
106	Chep5	Chepstow	23/02/2009 16/May/2009	7	9°C 17° C	7	Grass land	Lat:51.65230751037598 Lon:-2.654496431350708 Alt:97.0
107	Chep6	Chepstow	23/02/2009 16/May/2009	7	10°C 17° C	6	Grass beside river	Lat:51.66124999523163 Lon:-2.6364558935165405 Alt:78.0
108	Chep7	Chepstow	23/02/2009 16/May/2009	7	10°C 17° C	5.5	Grass land	Lat:51.66836857795715 Lon:-2.6292461156845093 Alt:69.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
109	Chep8	Chepstow	23/02/2009 16/May/2009	7	7°C 17° C	3	Farm soil	Lat:51.684826612472534 Lon:-2.607659697532654 Alt:81.0
110	Chep9	Chepstow	23/02/2009 16/May/2009	7	7°C 17° C	2	Woodland	Lat:51.695287227630615 Lon:-2.61774480342865 Alt:141.0
111	Chep10	Chepstow	23/02/2009 16/May/2009	7	8°C 17° C	2	Farm soil	Lat:51.69119417667389 Lon:-2.6332801580429077 Alt:231.0
112	Mon1	Monmouth 1	23/02/2009 16/May/2009	7	9°C 17° C	3	Farm soil	Lat:51.71185255050659 Lon:-2.6317083835601807 Alt:239.0
113	Mon2	Monmouth 2	23/02/2009 16/May/2009	7	11°C 17° C	5	Forest soil	Lat:51.73239827156067 Lon:-2.640618681907654 Alt:278.0
114	Mon3	Monmouth 3	23/02/2009 16/May/2009	7	10°C 17° C	5	Forest soil	Lat:51.74553573131561 Lon:-2.651025652885437 Alt:133.0
115	Mon4	Monmouth 4	23/02/2009 16/May/2009	7	10°C 17° C	5	Forest soil	Lat:51.75115764141083 Lon:-2.662886381149292 Alt:90.0
116	Mon5	Monmouth 5	23/02/2009 16/May/2009	7	10°C 17° C	5	Soil beside river	Lat:51.760719818115234 Lon:-2.677820944214 Alt:64.0
117	Lud1	Ludlow 1	24/02/2009 17/May/2009	7	12°C 15℃	4	Vegetation area	Lat:53.08151721954346 Lon:-5.52662193775177 Alt:9714.0
118	Lud2	Ludlow 2	24/02/2009 17/May/2009	7	10°C 15℃	2	Tree root	Lat:52.36801743507385 Lon:-2.722683548927307 Alt:150.0
119	Lud3	Ludlow 3	24/02/2009 17/May/2009	7	11°C 15℃	4	Grass land	Lat:52.367475628852844 Lon:-2.724539637565613 Alt:146.0
120	Lud4	Ludlow 4	24/02/2009 17/May/2009	7	11°C 15℃	3	Grass land	Lat:52.36581802368164 Lon:-2.7251780033111572 Alt:124.0
121	Lud5	Ludlow 5	24/02/2009 17/May/2009	7	10°C 15℃	3.5	Tree beside lake	Lat:52.365732192983164 Lon:-2,7252691984176636 Alt:131.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
122	Lud6	Ludlow 6	24/02/2009 17/May/2009	7	11°C 15℃	3.1	Tree beside lake	Lat:52.36532986164093 Lon:-2.7251029014587402 Alt:128.0
123	Lud7	Ludlow 7	24/02/2009 17/May/2009	7	11°C 15℃	4	Big tree	Lat:52.38530840396881 Lon:-2.724984884262085 Alt:143.0
124	Lud8	Ludlow 8	24/02/2009 17/May/2009	7	12°C 15℃	4	Grass land	Lat:52.366483211517334 Lon:-2.7242928743362427 Alt:164.0
125	Leo1	Leomister1	24/02/2009 17/May/2009	7	14°C	3	Field Vegetable	Lat:52.36660659313202 Lon:-2.7232199907302858 Alt:171.0
126	Leo2	Leomister2	24/02/2009 17/May/2009	7	10°C 15℃	4	Grass land	Lat:52.23905682563782 Lon:-2.7472901344299316 Alt:113.0
127	Leo3	Leomister3	24/02/2009 17/May/2009	7	10°C 15℃	6	Farm soil	Lat:52.23977029323578 Lon:-2.75095939623047 Alt:122.0
128	Leo4	Leomister4	24/02/2009 17/May/2009	7	10°C 15℃	5	Woodland	Lat:52.2442764043808 Lon:-2.763882279396057 Alt:135.0
129	Leo5	Leomister5	24/02/2009 17/May/2009	7	10°C 15℃	4	Woodland	Lat:52.24731266498566 Lon:-2.7706199884414673 Alt:119.0
130	Here1	Hereford1	24/02/2009 17/May/2009	7	10°C 15℃	5	Field Grass	Lat:52.24772036075592 Lon:-2.772529721260071 Alt: 124.0
131	Here2	Hereford2	24/02/2009 17/May/2009	7	9°C 15℃	5	Grass land	Lat:52.03047752380371 Lon:-2.7138590812683105 Alt: 151.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
132	Here3 A	Hereford3	24/02/2009 17/May/2009	6.1	10°C 15℃	6	Garden soil	Lat:52.02930808067322 Lon:-2.7171796560287476 Alt:137.0
133	Here3 B	Hereford3	24/02/2009 17/May/2009	7	10°C 15℃	4	Garden soil two meter from the previous	Lat:52.02930808067322 Lon:-2.7171796560287476 Alt:137.0
134	Here4	Hereford4	24/02/2009 17/May/2009	7	10°C 15℃	5.5	Soil beside field	Lat:52.012120485305786 Lon:-2.7351075410842896 Alt:130.0
135	Ross1	Ross-on-wye 1	24/02/2009 17/May/2009	7	10°C 15℃	5	Hotel Garden	Lat:52.00700283050537 Lon:-2.7333050966262817 Alt:156.0
136	Ross2	Ross-on-wye 2	24/02/2009 17/May/2009	7	11°C 15℃	5.5	Hotel Garden/ 30 meter from the previous site	Lat:51.91306114196777 Lon:-2.5788742303848267 Alt:106.0
137	Ross3	Ross-on-wye 3	24/02/2009 17/May/2009	7	10°C 15℃	5	Garden soil	Lat:51.91340982913971 Lon:-2.5784611701965 Alt:76.0
138	Ross4	Ross-on-wye4	24/02/2009 17/May/2009	7	10°C 15℃	3	Grass beside water stream	Lat:51.913297176361084 Lon:-2.5778603553771973 Alt:86.0
139	Ross5	Ross-on-wye 5	24/02/2009 17/May/2009	7	10°C 15℃	7	Grass beside water stream	Lat:51.9131737947464 Lon:-2.577667236328125 Alt:111.0
140	Bath1	Shaw bath road	23/02/2009 18/May/2009	7	10°C 16℃	5	Grass land	Lat:51.389413475990295 Lon:-2.1609270572662354 Alt:80.0
141	Bath2	Shaw bath road	23/02/2009 18/May/2009	7	10°C 16℃	7	Grass land	Lat:51.3909637928009 Lon:-2.1768593788146973 Alt:103.0
142	Bath3	Shaw bath road	23/02/2009 18/May/2009	7	10°C 16℃	7	Grass land	Lat:51.39098525047302 Lon:-2.1771007776260376 Alt:113.0
143	Bath4	Bath 1	25/02/2009 18/May/2009	6	8°C 16℃	8	Grass land	Lat:51.91247642040253 Lon:-2.5776833295822144 Alt:95.0

Number	Code	Site name	Sampling Date	PH	Temp	Moisture	Habitation	Co-ordinate epi-collect google
144	Bath5	Bath 2	25/02/2009 18/May/2009	7	9°C 16℃	6	Beside tree	Lat:51.91247642040253 Lon:-2.5776833295822144 Alt:95.0
145	Bath6	Bath 3	25/02/2009 18/May/2009	7	9°C 16℃	5	Grass land beside football	Lat:51.36326193809509 Lon:-2.384456992149353 Alt:201.0
146	Bath7	Bath 4	25/02/2009 18/May/2009	7	9°C 16℃	7	Beside farm	Lat:51.35318219661713 Lon:-2.3810720443725586 Alt:223.0
147	Bath8	Bath 5	25/02/2009 18/May/2009	7	9°C 16℃	6	Park	Lat:51.35688900947571 Lon:-2.383480668067932 Alt:202.0
148	Bath9	Bath 6	25/02/2009 18/May/2009	7	9°C 16℃	6	Prior park	Lat:51.36503219604492 Lon:-2.3603546619415283 Alt:198.0
149	Bath10	Bath 7	25/02/2009 18/May/2009	6	9°C 16℃	6	Soil beside Avon river	Lat:51.373910307884216 Lon:-2.3511385917663574 Alt:72.0
150	Bath11	Bath 8	25/02/2009 18/May/2009	6	9°C 16℃	5	Garden soil	Lat:51.38162970542908 Lon:-2.355837821960449 Alt:75.0
151	Bris1	Bristol 1	26/02/2009 18/May/2009	7	9°C 16℃	5.1	Farm soil	Lat:51.35783851146698 Lon:-2.3748278617858887 Alt:217.0
152	Bris2	Bristol 2	26/02/2009 18/May/2009	7	9°C 16℃	5	Grass/golf field	Lat:51.441110372543335 Lon:-2.4116116762161255 Alt:297.0
153	Bris3	Bristol 3	26/02/2009 18/May/2009	7	8°C 15°C	5	Beside tree	Lat:51.44755303859711 Lon:-2.4161821603775024 Alt:107.0
154	Bris4	Bristol 4	26/02/2009 18/May/2009	7	8°C 16℃	5	Bunker soil	Lat:51.44720435142517 Lon:-2,4164557456970215 Alt:139.0
155	Bris5	Bristol 5	26/02/2009 18/May/2009	7	8°C 16℃	5.5	Beside tree	Lat.51.52466654777527 Lon:-2.5994092226028442 Alt:111.0

Table 2. Number of dead insects and insects producing nematodes in different soil habitats.

Habitat	Number of soil sample (number of Galleria*)	Number of dead insects	Number of dead insects producing nematodes
Bunker	6(18)	12	8
Grass	2(6)	6	6
Wood	6(18)	12	10
Total	14(42)	30	24
Total%		(30/42) 71.42%	(24/42) 57.14%

*Three Galleria per soil sample

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Query:   8       GAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT 67
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 59      GAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT 118


Query:   68      GAWKGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCTTCGGACCAA 127
                 ||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 119     GAT-GGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCTTCGGACCAA 177


Query:   128     AGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT 187
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 178     AGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT 237


Query:   188     GGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACAC 247
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Subject: 238     GGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACAC 297


Query:   248     TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG 307
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Subject: 298     TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG 357


Query:   308     GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC 367
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 358     GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC 417


Query:   368     TTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTGCATTGACGTTACTCGCAGAA 427
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 418     TTTCAGCGAGGAGGAAGGGTTCAGTGTTAATAGCACTGTGCATTGACGTTACTCGCAGAA 477


Query:   428     GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGRRGGKKGCAAGCGTTAAT 487
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                  ||||||||||||||||||||||||||||||||||||||||||||  ||  ||||||||||||
Subject: 478     GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG-AGGGTGCAAGCGTTAAT 536


Query:   488     CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCG 547
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 537     CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCG 596


   Query:   548     CGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTC-TGTAGAGGGG 600
                     |||||||||||||||||||||||||||||||||||||||| ||||||||||

     Subject: 597     CGCTTAACGTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGG
```
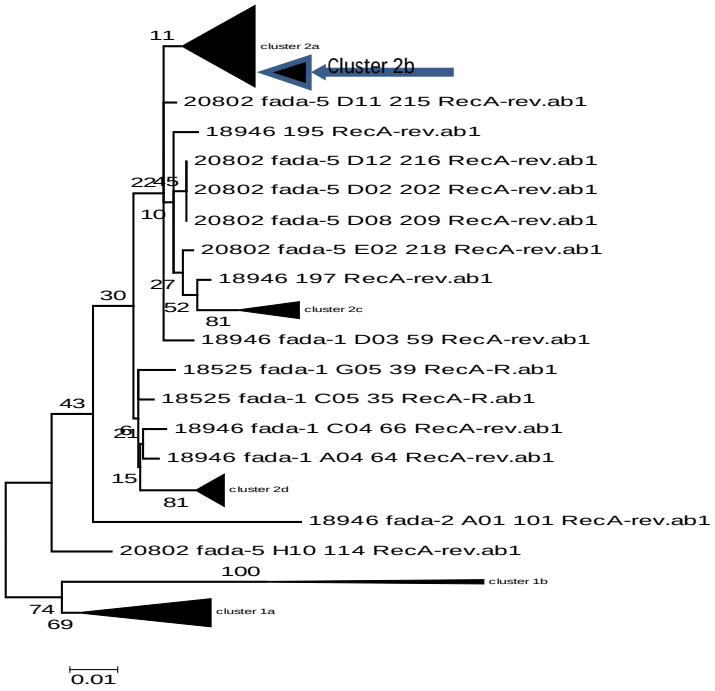

Score = 1121, Identity = (587/594) 98.8% Expect = 0

Figure 1. *Serratia proteamaculans*. 16S rRNA gene (strain DSM 4543). Example of the blast results % identity. ACCESSION AJ233434

Figure 2. Neighbour-joining phylogenetic tree obtained from *recA* gene sequences belonging to *S. proteamaculans*. Numbers within the dendrogram indicate the occurrence (%) of the branching order in 100 bootstrapped tree



Appendix 2:

Table 1. Samples used for MLST.

Strain #	ST	Location	Cultural characteristics	Isolation	Dates of isolation
0	**1**	Bath	yellow colonies	Crushed nematodes	Feb-09
1	2	Brighton1	yellow colonies	Crushed nematodes	Feb-09
3	3	Brighton 1”	Yellow colonies	Crushed nematodes	Feb-09
4	4	Brighton 1”'	Cream colonies	Crushed nematodes	Feb-09
5	5	Brighton 7	creamy colonies	Crushed nematodes	Feb-09
6	6	Brighton 7'	Yellow colonies	Crushed nematodes	Feb-09
7	7	Chepstow8	Yellow colonies	Crushed nematodes	Feb-09
9	8	Chepstow10	Yellow colonies	Crushed nematodes	Feb-09
10	9	Chepstow7’	Yellow colonies	Crushed nematodes	Feb-09
11	10	Chepstow7”	Cream colonies	Crushed nematodes	Feb-09
13	11	Bridport2	yellow colonies	Crushed nematodes	Feb-09
14	11	Bridport2’	cream colonies	Crushed nematodes	Feb-09
15	12	Bridport2''	yellow colonies	Crushed nematodes	Feb-09
16	13	Sidmouth9	cream colonies	Crushed nematodes	Feb-09
17	4	Sidmouth10’	yellow colonies	Crushed nematodes	Feb-09
19	4	Sidmouth10’’	yellow colonies	Crushed nematodes	Feb-09
20	14	Newforest3	cream colonies	Crushed nematodes	Feb-09
21	15	Newforest10	Cream colonies	Crushed nematodes	Feb-09
22	16	Newforest2	Cream colonies	Crushed nematodes	Feb-09
23	17	Weymouth10	cream colonies	Crushed nematodes	Feb-09
25	18	Weymouth4’	Cream colonies	Crushed nematodes	Feb-09
26	19	Chichester1	Cream colonies	Crushed nematodes	Feb-09
27	19	Chichester9	Cream colonies	Crushed nematodes	Feb-09
28	20	Arundal5	Yellow colonies	Crushed nematodes	Feb-09
29	21	Arundel6	Cream colonies	Crushed nematodes	Feb-09
30	11	Arundal5’	yellow colonies	Crushed nematodes	Feb-09
31	21	Arundel6’	cream colonies	Crushed nematodes	Feb-09
32	4	Arundel8	Cream colonies	Crushed nematodes	Feb-09
33	22	Agmering8	yellow colonies	Crushed nematodes	Feb-09
34	4	Agmering8'	cream colonies	Crushed nematodes	Feb-09
35	23	Sidmouth10	Yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
36	24	Sidmouth10’	Cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
38	25	Sidmouth10”'	Cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
39	26	Chichester1	Cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
40	11	Chichester1’	yellow colonies	Haemolymph of Galleria that	Feb-09

				showed nematodes	
41	11	Chichester9	Cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
44	27	Bridport2	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
45	28	Bridport2'	Cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
46	29	Bridport2''	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
47	30	Chepstow7	cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
50	31	Chepstow10'	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
52	32	Chepstow8'	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
53	33	Weymouth2	cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
54	34	Weymouth3	cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
57	35	Brighton 1	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
59	36	Brighton 1'	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
60	37	Brighton 1'	creamy colonies	Haemolymph of Galleria that showed nematodes	Feb-09
61	38	Brighton 1"	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
63	39	Arundal5	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
64	40	Arundal5'	cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
65	41	Arundel8	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
67	42	Agmering9	cream colonies	Haemolymph of Galleria that showed nematodes	Feb-09
68	43	Newforest10	yellow colonies	Haemolymph of Galleria that showed nematodes	Feb-09
80	11	Bridport10	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
97	44	Chepstow 2	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
105	45	Ludlow4	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
117	46	Ditchling1	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
119	47	Bridport4	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
125	48	Bristol4	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
127	49	Bristol1	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09

129	50	Hereford3	cream colonies	Haemolymph of Galleria that not showed nematodes	Feb-09
134	51	Bath2	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
143	51	Bath11	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
144	51	Bath12	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
147	52	Bath14'	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
173	53	Bath23	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
174	54	Bath25	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
201	55	Bath135	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
202	56	Bath148	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
211	57	Bath159	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
212	58	Bath157	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
216	59	Bath153	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
217	60	Bath151	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
224	61	Bath58	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
225	62	Bath59	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
226	63	Bath55	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
228	64	Bath49	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
233	65	Bath61	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
237	66	Bath38	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
240	67	Bath93	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
242	68	Bath104	cream colonies	Haemolymph of Galleria that not showed nematodes	2008
246	68	Bath47	cream colonies	Haemolymph of Galleria that not showed nematodes	2008

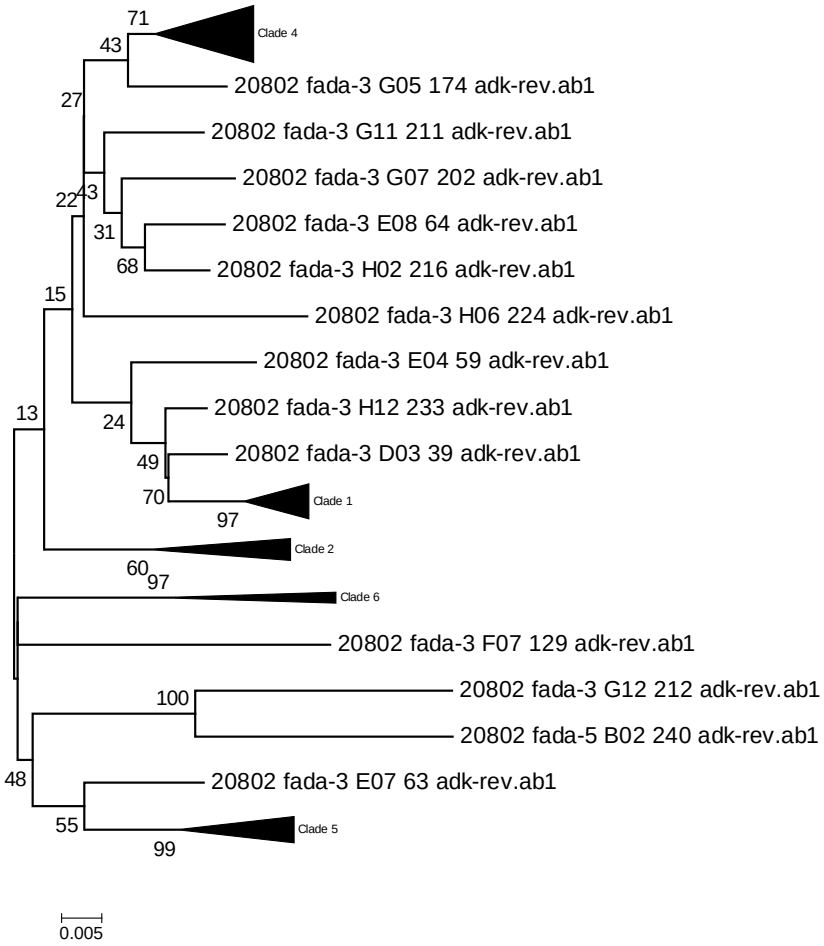


Figure 1. Neighbour-joining phylogenetic tree of 84 *S*. *Proteamaculans* obtained from the concatenated genes. Numbers within the dendrogram indicate the occurrence (%) of the branching order in 1000 bootstrapped trees.

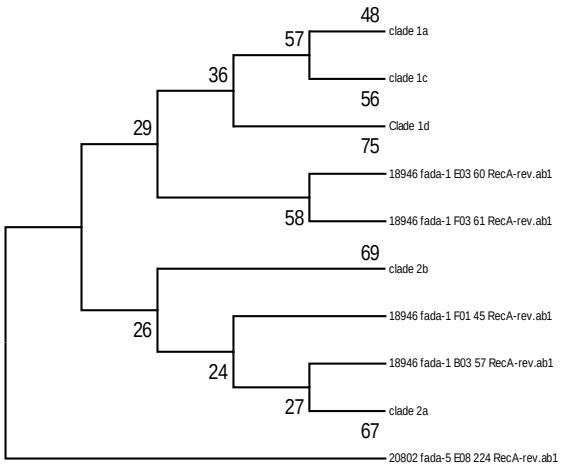


Figure 2. Neighbour-joining phylogenetic tree based on *recA* gene sequence of 84 strains indicate the numbers occurrence (%) of the branching order in 1000 bootstrapped trees.

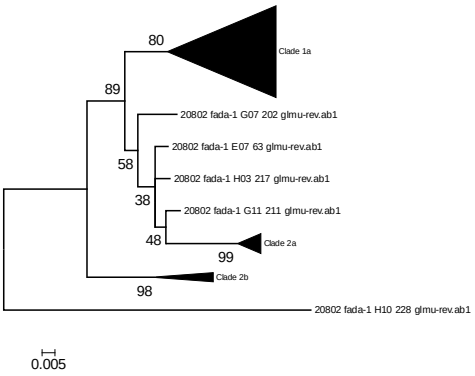


Figure 3. Neighbour-joining phylogenetic tree based on *glmU* gene sequence of 84 strains indicate the numbers occurrence (%) of the branching order in 1000 bootstrapped trees.

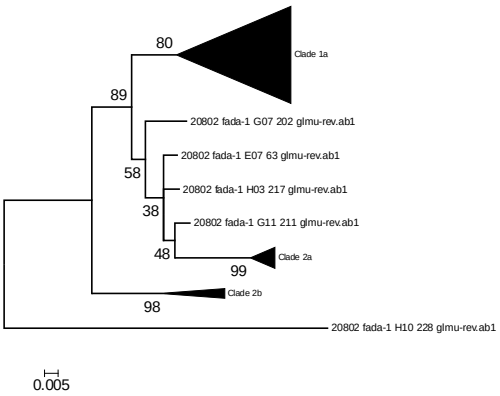


Figure 4. Neighbour-joining phylogenetic tree tree based on *adK* gene sequence of 84 strains indicate the numbers occurrence (%) of the branching order in 1000 bootstrapped trees.

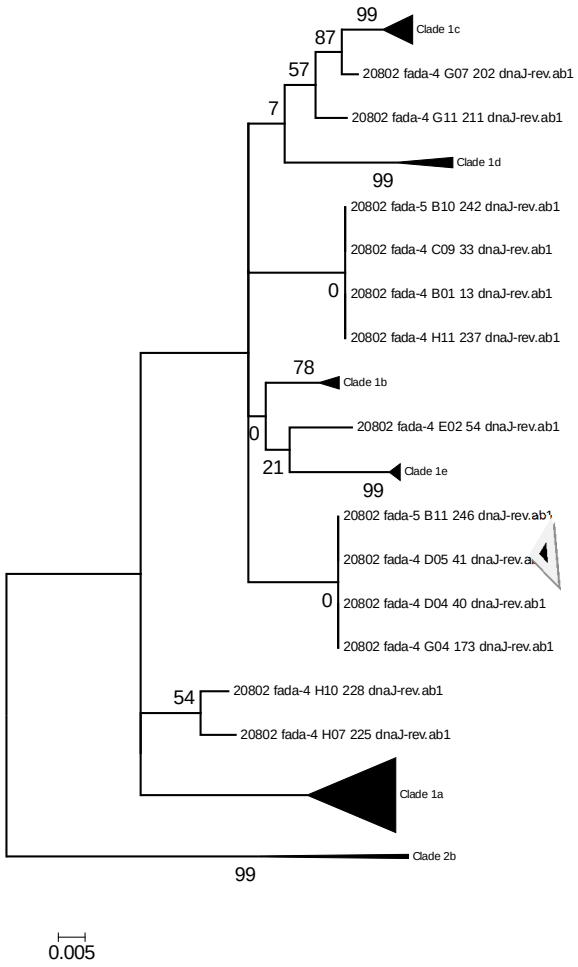


Figure5. Neighbour-joining phylogenetic tree based on *dnaJ* gene sequence of 84 strains indicate the numbers occurrence (%) of the branching order in 1000bootstrapped trees.

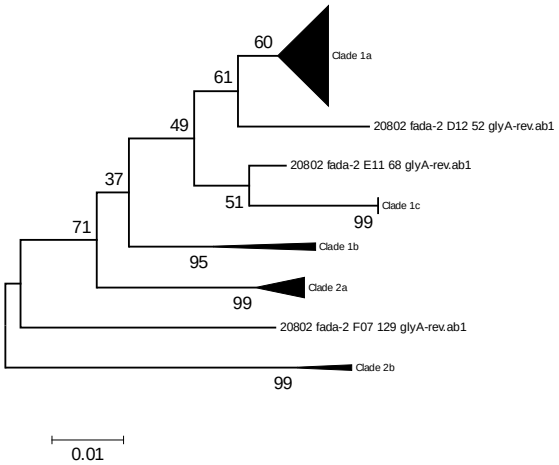
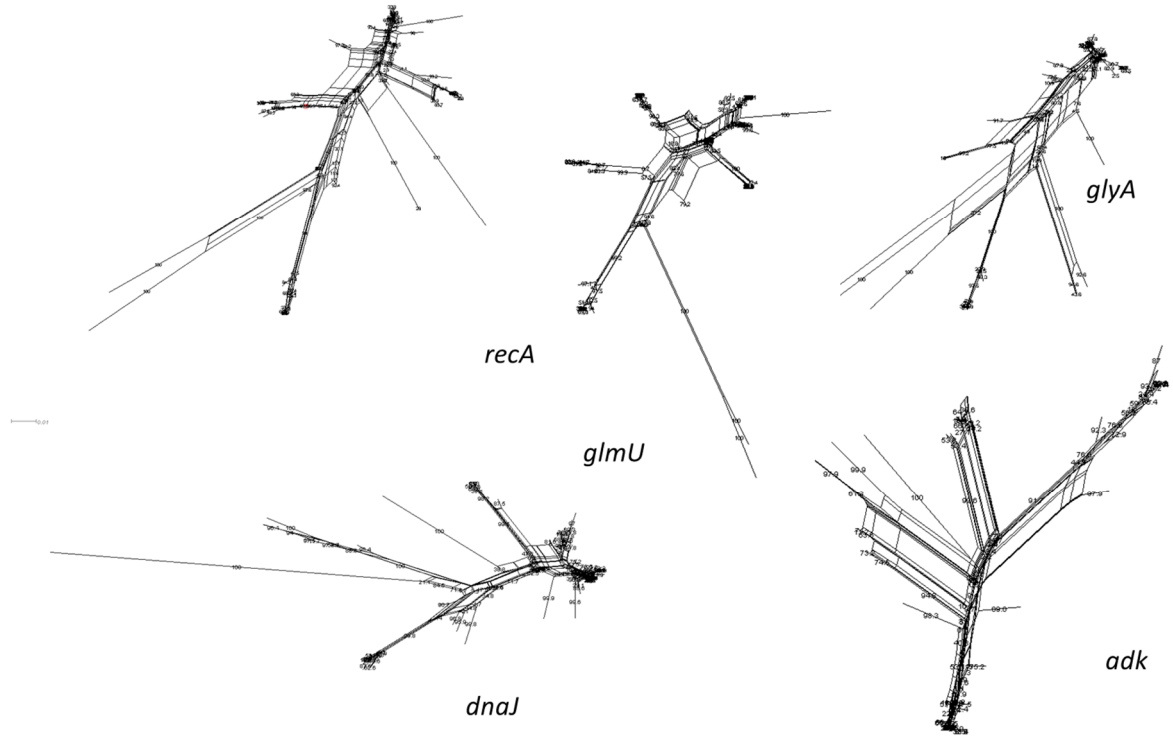
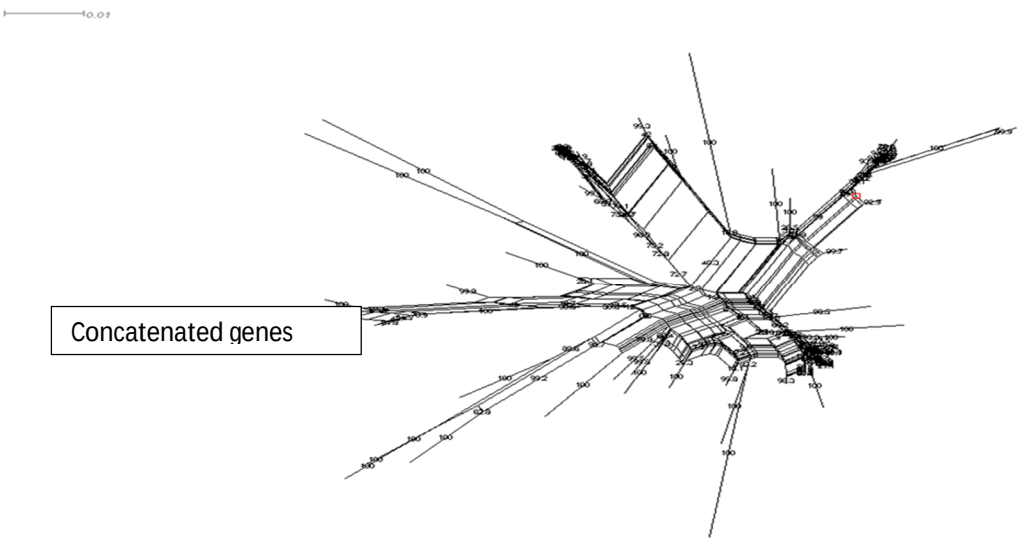


Figure 6. Neighbour-joining phylogenetic tree based on *glyA* gene sequence of 84 strains indicate the numbers occurrence (%) of the branching order in 1000 bootstrapped trees



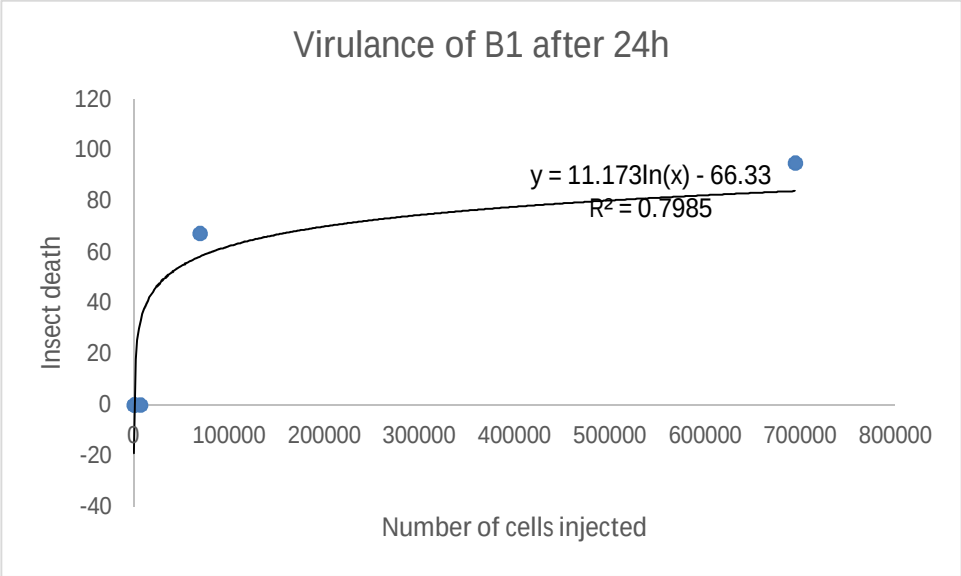
A.



B.

Figure 7. Unrooted phylogenetic networks of 84 *S. proteamaculans* isolates constructed from: A. the five housekeeping genes used in MLST. B. The concatenated sequences of the five genes. Using the SpitsTree 4 software.

Appendix 3



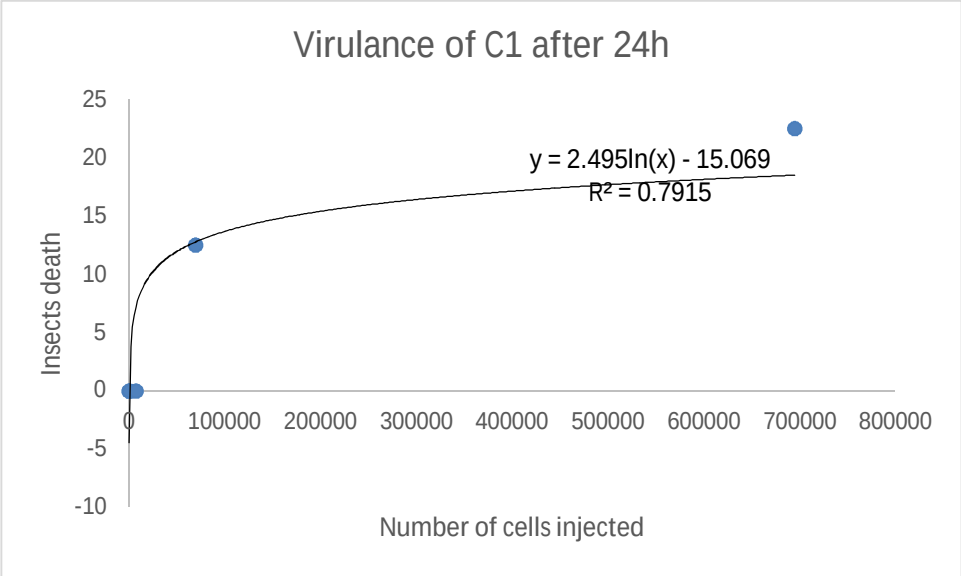


Figure 1. The logarithmic graph estimating the LD_{50} of A. Bath isolates B1 and B. Chepstow isolates C1 virulence test after 24h of injection.

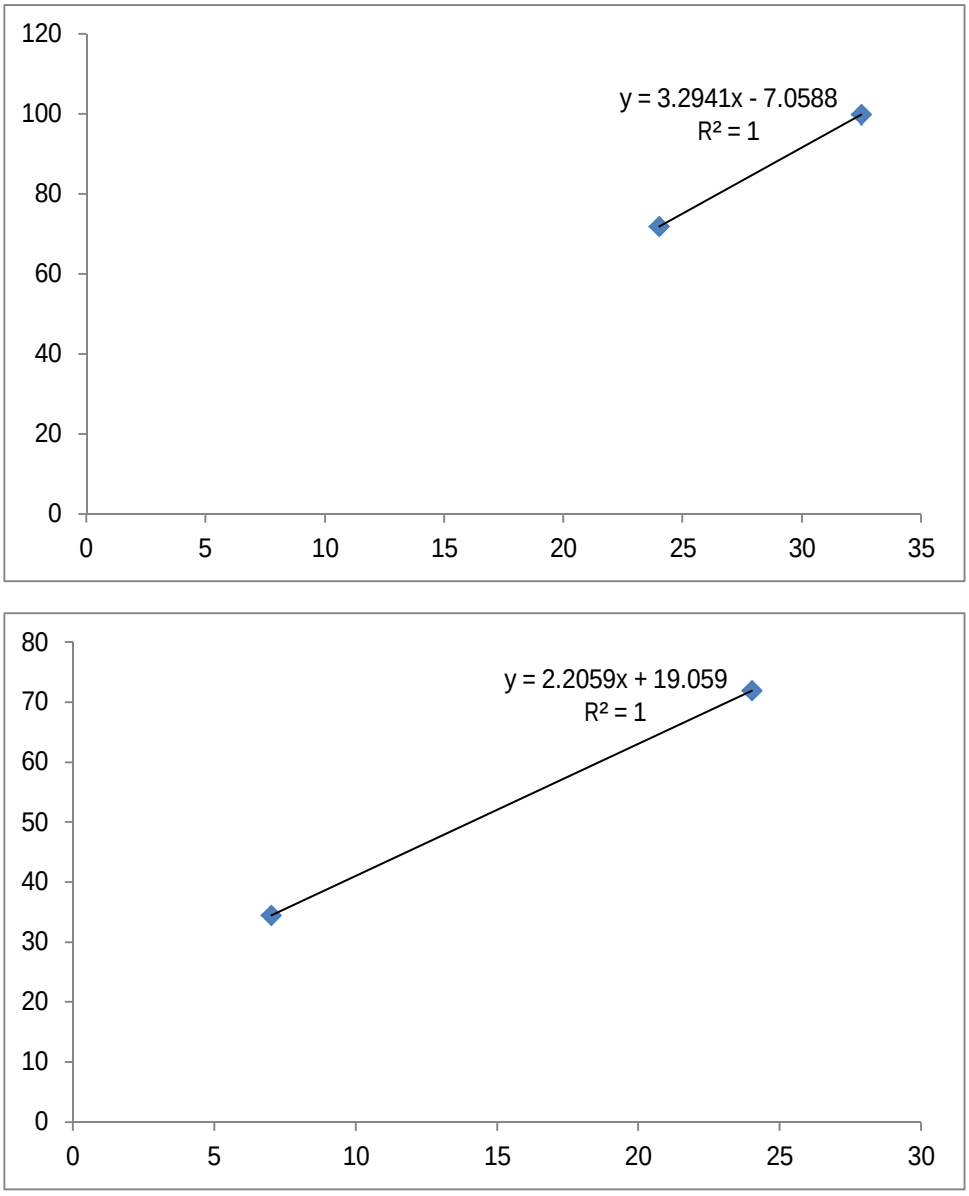


Figure 2. The linear graph estimating the LT50 of A. Bath isolates B1 and B. Chepstow isolates C1 virulence test in 24h and 72h post injection.

Appendix 4:

CD 1. Confocal microscopic images verified that the bacteria were indeed within the nematode tissues using Z-stack 3D reconstruction.

Appendix 5.

BLASTN analysis of the *recA* amplicon sequences of the eggs colonies were identified as *Serratia spp.*

Score	Expect	Identities	Gaps	Strand	Frame
865 bits(468)	0.0()	477/481(99%)	2/481(0%)	Plus/Plus	

Features:
recA protein

```
Query  2       GCGTCTGGGTG-AGACCGTTCTATGGACGTAGAAACGATCTCTACCGGCTCACTGTCACT  60
               |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  937153  GCGTCTGGGTGAAGACCGTTCTATGGACGTAGAAACGATCTCTACCGGCTCACTGTCACT  937212

Query  61      GGACATCGCATTGGGTGCGGGCGGCCTGCCAATGGGTCGTATCGTTGAAATTTATGGCCC  120
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Sbjct  937213  GGACATCGCATTGGGTGCGGGCGGCCTGCCAATGGGTCGTATCGTTGAAATTTATGGCCC  937272

Query  121     GGAGTCTTCCGGTAAAACTACCCTGACGCTGCAGGTTATCGCTGCGGGCACAGCGCGAAG  180
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Sbjct  937273  GGAGTCTTCCGGTAAAACTACCCTGACGCTGCAGGTTATCGCTGC-GGCACAGCGCGAAG  937331

Query  181     GTAAAACCTGTGCGTTTATCGATGCCGAGCATGCGCTGGATCCGATTTATGCGAAAAAGC  240
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Sbjct  937332  GTAAAACCTGTGCGTTTATCGATGCCGAGCATGCGCTGGATCCGATTTATGCGAAAAAGC  937391

Query  241     TGGGTGTTGATATCGACAACCTGCTGTGTTCGCAGCCGGACACCGGTGAGCAAGCGCTGG  300
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Sbjct  937392  TGGGTGTTGATATCGACAACCTGCTGTGTTCGCAGCCGGACACCGGTGAGCAAGCGCTGG  937451

Query  301     AAATCTGTGATGCCTTGACCCGCTCTGGCGCGGTTGACGTGATCATCGTTGACTCCGTAG  360
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Sbjct  937452  AAATCTGTGATGCCTTGACCCGCTCTGGCGCGGTTGACGTGATCATCGTTGACTCCGTAG  937511

Query  361     CGGCGCTGACGCCAAAAGCGGAAATCGAAGGTGAAATTGGTGACTCACACATGGGCCTGG  420
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Sbjct  937512  CGGCGCTGACGCCAAAAGCGGAAATCGAAGGTGAAATTGGTGACTCACACATGGGCCTGG  937571

Query  421     CGGCGCGTATGATGAGCCAGGCGATGCGTAAGCTGGCAAGTAACCTGAAAAACGCCAACA  480
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Sbjct  937572  CGGCGCGTATGATGAGCCAGGCGATGCGTAAGCTGGCCGGTAACCTGAAAAACGCCAACA  937631

Query  481     C  481
               |
Sbjct  937632  C  937632
```

Serratia proteamaculans 568, complete genome
Sequence ID: gb|CP000826.1|Length: 5448853Number of Matches: 1
Related Information

Score	Expect	Identities	Gaps	Strand	Frame

608 bits(329) 1e-170() 423/469(90%) 3/469(0%) Plus/Plus

Features:

```
Query  14      AGACACGATC-ATGGACGT-GCAACGATCTCCACCGGCTCGCTGTCACTTGATCTCGCAC  71
              |||| || || |||||||| | ||||||||| |||||||||||||||||||||| ||||||
Sbjct  773772  AGAC-CGTTCTATGGACGTAGAAACGATCTCTACCGGCTCGCTGTCACTTGATATCGCAC  773830

Query  72      TGGGCGCAGGCGGCTTGCCAATGGGCCGTATCGTCGAGATTTATGGCCCGGAATCCTCCG  131
              |||| ||||||||| |||||||||||||||||| || |||||||| |||||||| ||||
Sbjct  773831  TGGGTGCAGGCGGCCTGCCAATGGGCCGTATCGTTGAAATTTATGGTCCGGAATCTTCCG  773890

Query  132     GTAAAACCACCCTGACTCTGCAAGTTATTGCCGCTGCGCAGCGCGAAGGTAAAACCTGTG  191
              ||||||||||||||| ||||| ||||| || || ||||||||||||||||||||||||||
Sbjct  773891  GTAAAACCACCCTGACGCTGCAGGTTATCGCTGCGGCGCAGCGCGAAGGTAAAACCTGTG  773950

Query  192     CGTTTATCGATGCCGAACATGCGCTGGATCCTATCTACGCCAAGAAACTGGGCGTGGATA  251
              | ||||||||||||| || |||||||||| || || || ||||| |||||||| ||||
Sbjct  773951  CCTTTATCGATGCCGAGCACGCGCTGGATCCGATTTATGCGAAGAAGCTGGGCGTTGATA  774010

Query  252     TCGATAACCTGCTGTGTTCTCAGCCGGATACCGGTGAGCAGGCGCTGGAAATCTGTGATG  311
              |||| |||||||||||||||||||||| |||||||| |||||| ||||||||||||||||
Sbjct  774011  TCGACAACCTGCTGTGTTCTCAGCCGGACACCGGTGAACAGGCGTTGGAAATCTGTGATG  774070

Query  312     CGCTGACCCGTTCCGGCGCGGTTGACGTCATCATCGTCGACTCCGTAGCGGCGCTGACGC  371
              |  | |||||||||||||||||||||| |||||||| |||||||| |||||  ||||||
Sbjct  774071  CCTTAACCCGTTCCGGCGCGGTTGACGTGATCATCGTTGACTCCGTGGCGGCCTTGACGC  774130

Query  372     CGAAGGCGGAAATCGAAGGCGAAATCGGTGACTCACATATGGGGCTGGCGGCACGTATGA  431
              |||| ||||||||||||| |||||||||||||||| ||||| ||||||||||||||||
Sbjct  774131  CGAAAGCGGAAATCGAAGGTGAAATCGGTGACTCACACATGGGTCTGGCGGCACGTATGA  774190

Query  432     TGAGCCAGGCCATGCGTAAATTGGCCGGTAACCTGAAAAACGCCAACAC  480
              |||||||||| ||||||||  ||||||||||||||||||||||||||||
Sbjct  774191  TGAGCCAGGCGATGCGTAAGCTGGCCGGTAACCTGAAAAACGCCAACAC  774239
```

Serratia liquefaciens ATCC 27592, complete genome

Sequence ID: gb|CP006252.1|Length: 5238612Number of Matches: 1

Score **Expect** **Identities** **Gaps** **Strand** **Frame**

568 bits(307) 2e-158() 417/471(89%) 3/471(0%) Plus/Plus

Features:

DNA strand exchange and recombination protein with protea...

```
Query  12      GGTG-AGA-CGCATCATGGACGTAGTAACGATCTCCACCGAGCTCGATGTCACTTGATCT  69
               ||||  |||  |||  ||||||||| | |||||||||||||| ||||  ||||||| ||| |
Sbjct  925781  GGTGAAGACCGCTCCATGGACGTGGAAACGATCTCCACCG-GCTCACTGTCACTCGATAT  925839

Query  70      CGCACTGGGCGCAGGCGGCTTGCCAATGGGCCGTATCGTCGAGATTTATGGCCCGGAATC  129
               |||  ||||||||  ||||||  ||||  ||||||||  |||||  ||  ||  ||  ||||||||||
Sbjct  925840  CGCCCTGGGCGCCGGCGGCCTGCCGATGGGCCGCATCGTAGAAATCTACGGCCCGGAATC  925899

Query  130     CTCCGGTAAAACCACCCTGACTCTGCAAGTTATTGCCGCTGCGCAGCGCGAAGGTAAAAC  189
                ||||||||||||||  ||||  |||||  ||  ||  ||||||||||||||||||||  |||||
Sbjct  925900  GTCCGGTAAAACCACTTTGACGCTGCAGGTGATCGCCGCTGCGCAGCGCGAAGGCAAAAC  925959

Query  190     CTGTGCGTTTATCGATGCCGAACATGCGCTGGATCCTATCTACGCCAAGAAACTGGGCGT  249
               |||||||||  |||||  ||||||||  ||||||||||||||||||  ||  ||  ||  ||||||||
Sbjct  925960  CTGTGCGTTCATCGACGCCGAACACGCGCTGGATCCTATCTATGCGAAAAAGCTGGGCGT  926019

Query  250     GGATATCGATAACCTGCTGTGTTCTCAGCCGGATACCGGTGAGCAGGCGCTGGAAATCTG  309
                ||||||||  ||||||||||  ||  ||||||||||||||  ||||||||||||||||||||
Sbjct  926020  CGATATCGACAACCTGCTGTGCTCCCAGCCGGATACCGGCGAGCAGGCGCTGGAAATCTG  926079

Query  310     TGATGCGCTGACCCGTTCCGGCGCGGTTGACGTCATCATCGTCGACTCCGTAGCGGCGCT  369
               ||||||||||||||  ||||||||||||||  ||||||||||||||||||||  ||||||||
Sbjct  926080  TGATGCGCTGACCCGCTCCGGCGCGGTTGATGTCATCATCGTCGACTCCGTGGCGGCGCT  926139

Query  370     GACGCCGAAGGCGGAAATCGAAGGCGAAATCGGTGACTCACATATGGGGCTGGCGGCACG  429
               ||||||||||||||||||||||||  ||||||||  ||  ||  ||  |||||  ||||||||  ||
Sbjct  926140  GACGCCGAAGGCGGAAATCGAAGGTGAAATCGGCGATTCGCACATGGGCCTGGCGGCGCG  926199

Query  430     TATGATGAGCCAGGCCATGCGTAAATTGGCCGGTAACCTGAAAAACGCCAA  480
                ||||||||||||||  |||||||||  |||||||  ||||||||||||||||||
Sbjct  926200  CATGATGAGCCAGGCGATGCGTAAACTGGCCGGCAACCTGAAAAACGCCAA  926250
```

Serratia marcescens WW4, complete genome

Sequence ID: gb|CP003959.1|Length: 5241455Number of Matches: 1

Appendix 6.

Table 1. Types of soil, nematodes, habitats, soil pH and temperature in sites sampled in the two transects E-W and S-N.

Associated nematodes	Region	Habitat	Time of isolation	Soil type	Soil PH	Soil Temperature	Associated bacteria
Pristionchus entomophagus20	Brighton1	Woodland	February	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	6	8°C	*Serratia proteamaculans*
Pristionchus entomophagus28	Chepatow7	Grassland	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Pristionchus Iheritieri 46	Brighton7	Woodland	February	Shallow lime-rich soils over chalk or limestone	7	7°C	*Serratia proteamaculans*
Pristionchus entomophagus42	Agmering8	Woodland	February	Freely draining slightly acid loamy soils	7	9°C	*Serratia proteamaculans*
*Pristionchus entomophagus*23	Newforest2	Forest soil	February	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	6	9°C	*Serratia proteamaculans*
Pristionchus entomophagus27	Chichester1	Grassland	February	Loamy soils with naturally high groundwater	7	10°C	*Serratia proteamaculans*
*Pristionchus entomophagus*18	Brighton7	Woodland	February	Shallow lime-rich soils over chalk or limestone	7	7°C	*Serratia proteamaculans*
Pristionchus entomophagus36	Bath SB	Golf coarse	October	Shallow lime-rich soils over chalk or limestone	7	14°C	*Serratia proteamaculans*
Pristionchus entomophagus26	Newforest3	Forest soil	February	Slowly permeable seasonally wet slightly	6	10°C	*Serratia proteamaculans*

				acid but base-rich loamy and clayey soils			
Pristionchus uniformis64	Bridport4	Riverside	May	Loamy and clayey floodplain soils with naturally high groundwater	7	17°C	*Serratia proteamaculans*
Pristionchus entomophagus56	Newforest1	Forest soil	February	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	7	9°C	*Serratia proteamaculans*
Pristionchus entomophagus60	Bridport4'	River side	May	Loamy and clayey floodplain soils with naturally high groundwater	7	17°C	*Serratia proteamaculans*
Pristionchus entomophagus31	Bristol4	Golf coarse	May	Lime-rich loamy and clayey soils with impeded drainage	7	16°C	*Serratia proteamaculans*
Diplogasteroides magnus	Weymouth10	grass land	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Diplogasteroides magnus	Chepstow7	Grass land	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Diplogasteroides magnus	Chepstow7'	Grass land	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Diplogasteroides magnus	Chepstow7''	Grass land	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Diplogasteroides magnus	Chepstow8	Farm soil	February	Freely draining slightly acid but base-rich soils	7	7°C	*Serratia proteamaculans*
Diplogasteroides magnus	Weymouth4	Sandy/ beach	February	Freely draining slightly acid but base-rich soils	7	11°C	*Serratia proteamaculans*

Steinernema glaseri	Sidmouth 10''	Grassland	February	-	7	-	*Serratia proteamaculans*
Steinernema glaseri	Sidmouth 7	Woodland	February	-	*7*	-	*Serratia proteamaculans*
Steinernema glaseri	Sidmouth 10'	Grassland	February	-	*7*	-	*Serratia proteamaculans*
Steinernema glaseri	Newforest 10 Beaulieu	Forest soil	February	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	*7*	*10C*	*Serratia proteamaculans*
Steinernema glaseri	Weymouth 2 Bounemouth	Sandy soil	February		*7*	*10C*	*Serratia proteamaculans*
Steinernema glaseri	Bridport 2	Grassland	February	Loamy and clayey floodplain soils with naturally high groundwater	*7*	*11C*	*Serratia proteamaculans*
Steinernema glaseri	Bridport 2'	Grassland	February	Loamy and clayey floodplain soils with naturally high groundwater	*7*	*11C*	*Serratia proteamaculans*
Steinernema glaseri	Bridport 2''	Gassland	February	Loamy and clayey floodplain soils with naturally high groundwater	*7*	*11C*	*Serratia proteamaculans*
Steinernema glaseri	Agmering 8	Grassland	February	Freely draining slightly acid loams soils	*7*	*10C*	*Serratia proteamaculans*
Steinernema glaseri	Arundel 5	Grassland	February	Naturally wet, loamy and clay soil, slightly acidic soil.	7	8°C	*Serratia proteamaculans*
Steinernema glaseri	Arundel 5'	Grassland	February	Naturally wet, loamy and clay soil, slightly acidic soil.	7	8°C	*Serratia proteamaculans*
Steinernema glaseri	Arundel 5''	Grassland	February	Naturally wet, loamy and clay soil, slightly acidic soil.	7	8°C	*Serratia proteamaculans*
Steinernema glaseri	Chepstow8'	Farm soil	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*

Steinernema glaseri	Chepstow 7''	Grassland	February	Freely draining slightly acid but base-rich soils	7	10°C	*Serratia proteamaculans*
Steinernema glaseri	Bath 8	Grassland	May	Shallow lime-rich soils over chalk or limestone	7	9°C	*Serratia proteamaculans*
Steinernema glaseri	Newforest 2 Minstead	Forest soil	February	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils	6.6	9°C	*Serratia proteamaculans*