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Is *Serratia marcescens* strain *MCB* an entomopathogenic bacterium?: a focus on genomics

Thesis by

Mahloro Hope Serepa

In Fulfilment for the requirements for the degree of

Doctor of Philosophy



University of the Witwatersrand

Johannesburg, South Africa

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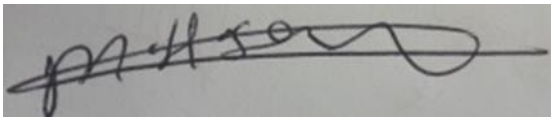
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To my father Mathole, my mother Nape, for instilling the importance of education in me, the love of my life Hetisani for supporting the wildest of my dreams, encouragement and belief in me, and to the brave individuals who have paved the way of knowledge for us who follow.

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Preface

It is through tapping into the untapped world that one learns a new thing. Working on this project has been a challenging, however it is through embarking on a challenging course that one finds growth. This thesis explores whole genomic sequencing of bacteria and nematodes through the next generation sequencing techniques. It serves as an in-depth platform for related studies and research.

Abstract

The phylum nematoda has a variety of functional groups. The parasitic functional group comprise various nematodes some which are parasitic to insects and are known as entomopathogenic nematodes (EPNs). The two most studied genera of EPNs are *Steinernema* and *Heterorhabditis*. These EPNs are associated symbiotically with the two enterobacteria genera; *Xenorhabdus* and *Photorhabdus*, respectively. The explanation of EPNs has been recently expanded to include the genus *Oscheius* which have been found to be associated with *Serratia* species. The bacteria synthesize a range of insecticidal and antimicrobial metabolites which may be useful in various ways as agricultural pest control and medical disease control. An insight into the genome of the nematode-bacterium duo will provide us with information about the symbiosis between the two and parasitism against insect pests. Here in I discuss the isolation and identification of a South African EPN and its symbiotic bacterium. In addition I highlight the production of indole derivatives which are common metabolites produced by entomopathogenic bacteria. The thesis eventually describes and discusses the methods for whole genome sequencing of both the isolated nematode and its symbiotic bacterium, and the genomic content indicate similar genes with other known EPN genera and protein-coding genes involved in symbiosis and parasitism.

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Chapter 1:

Introduction to nematodes and entomopathogenic nematodes*

*This chapter provides an introduction to entomopathogenic nematodes and includes motivation, aims and objectives for the research project.

Introduction

Nematodes. Nematodes belong to the Phylum Nematoda which together with the Phyla Mollusca and Arthropoda represents one of the most species-rich phyla (Blaxter *et al.*, 1998). Nematodes are also one of the most abundant groups of animals on our planet. They are multicellular, triploblastic, pseudocoelomic, non-segmented, bilaterally symmetrical, colourless, slender, worm-like animals that are ubiquitous in nature. The anatomy of nematodes has a "tubular" body structure that has both an open anterior and a sub-terminal anus at the posterior end. The head is radial and has a radial mouth that has either three or six lips with papillae (of which the arrangement can be used as a strong taxonomic attribute) (Adams and Nguyen, 2002).

The outer body is ornamented with ridges which occur in a particular pattern for each kind of genus. The tubular shaped body structure is covered by a tough, elastic, flexible and complex outer cuticular integument. The cuticle consists of many layers of proteinaceous fibres. Beneath the cuticle is the hypodermis and a layer of longitudinal muscles. The longitudinal muscles surround the pseudocoelomic body cavity which functions as a hydrostatic skeleton. The action of the longitudinal muscle against the hydrostatics skeleton results in the generation of the typical whip-lash motion of nematodes. The nematode digestive system consists of three parts which are the stomodaeum (encompass stoma, mouth, buccal cavity and oesophagus); mesenteron (encompass the intestine) and proctoderm (encompass rectum and anus) (Adams and Nguyen, 2002). The stomodaeum subsections are used as taxonomic characters. For example, if the buccal cavity is hollow it can be used to thrust into prey or suck liquids from plants and animals, thus the shape of the buccal cavity indicates if the nematode is a feeder or non-feeder (Adams and Nguyen, 2002; Dorris *et al.*, 1999).

The pharynx connects directly to the intestines, which extends into a rectum and expels waste through the anus (Figure 1 shows anatomical structures of adult hermaphrodite *Caernorhabditis elegans*) (Adams and Nguyen, 2002). The nervous system consists of a circumpharyngeal nerve ring with ventral and dorsal nerve cords running the length of the body. Nerves extending from the circumpharyngeal ganglia also enervate the mouth, lips and anterior sense organs. Nematodes do not have a specialized circulatory system or gas exchange system and excretion occurs via an excretory pore and duct system (Adams and Nguyen, 2002).

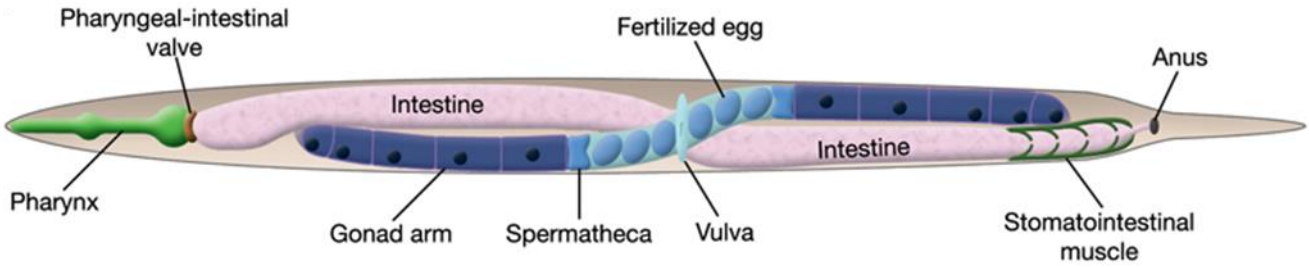


Figure 1.| Schematic drawing. Anatomy of an adult hermaphrodite *Caernorhabditis elegans*. http://www.wormatlas.org

Most nematode species are dioecious, having distinct separate males and females, however some are hermaphrodites which are able to self-fertilize. Both sexes have one or two tubular gonads which in males the end is attached to gubernaculum which guides the protrusion of the spicules (one or two and serve to open the vulva lips and facilitate the transmission of sperms) (Adams and Nguyen, 2002). Some males have bursa and are called bursate and use this structure to attach to the female during copulation (Adams and Nguyen, 2002). The female reproductive system is also tubular which consists of two ovaries each connecting to an oviduct and a uterus which opens to the vagina covered by vulva lips (Adams and Nguyen, 2002). Reproduction in nematodes is usually sexual except for hermaphrodites. The male body length is smaller and has a bent tail as compared to the female one and it has been suggested that this is so, so that the male can be able to attach to the female during copulation (Adams and Nguyen, 2002).

Nematodes inhabit a broad range of environment such as marine, fresh water, mountains, soils, deserts and tropics. The diverse habitats for nematodes lead to various groups of functional nematodes. The various nematode species can be placed into three functional groups which comprise saprophytic, predaceous and parasitic nematodes. Saprophytic nematodes break down compost in the soil; because compost contains a high amount of microorganisms such as bacteria and fungi; the nematodes also feed on these and are thus referred to as bacteriovores and fungivores (Dillman *et al.*, 2012a). Examples include many members of the orders Rhabditida and Aphelenchida.

Predaceous nematodes feed on other nematodes and animals found in the soil, these include orders such as Mononchida and Dorylaimida (Dillman *et al.*, 2012a). Since both the saprophytic and predaceous nematodes are mostly found in the soil, they play a beneficial

role in the soil through their involvement in soil organic matter decomposition and mineral cycling. As soil dwelling organisms, their classification into different function groups and types (Dillman *et al.,* 2012a), allows for analysing their functional relationships with other microorganisms, thereby providing insights into the nature of their functional relationships as saprophytes, predators and parasites (Carrascosa *et al.,* 2014; Neher *et al.,* 2005). They can be used as soil bio-indicators, to assess soil quality and soil fertility in terms of nutrient cycling and nutrition provision to support crop growth (Carrascosa *et al.,* 2014). The presence of certain functional groups of nematodes in the soil during the cropping season may be used as a good indicator for the assessment of soil quality (Carrascosa *et al.,* 2014).

Parasitic nematodes encompass nematodes which are parasitic to animals including humans; an example would be the helminths with the genus *Ascaris* being one of the most studied parasitic nematode. There are plant parasitic nematodes which are also called herbivorous nematodes, an example of these are the Heteroderidae and Solonaceae families. Plant parasitic nematodes are problematic in agriculture because they feed on plants and roots which slow plant growth; this ultimately results in reduced agricultural production. While some parasitic nematodes infect animals and plants, some are parasitic to insects, and these include the so called entomopathogenic nematodes (EPNs). Examples of the EPN genera which have been intensively studied are *Steinernema* and *Heterorhabditis*.

The EPN parasitic associations with insect crop pests are of benefit to the agricultural sector (Dillman and Sternberg, 2012; Kaya and Stock, 1997). EPNs have several deleterious effects on their insect host such as sterility, reduction in fecundity, reduced flight activity, delayed development and death and as such they play a major important role as biological control agents in agriculture (Poinar, 1979; Vashisth *et al.,* 2013). Since EPNs are important in agriculture their presence or absence can indicate the impact of different approaches to agricultural crop production that include mechanical or physical soil disturbances, and if these impacts are detrimental to EPN populations in the soil then crop production practices need to be adapted to reduce the negative impacts (Dong *et al.,* 2008).

The parasitism of insects by the nematodes has been known since the 17th century, but it was only in the 1930s that serious consideration was given to using nematodes as biocontrol agents for insects (Smart, 1995). In 1929 in New Jersey, Glaser and Fox found a nematode infecting a thick-bodied larva of several insects, including the Japanese beetle, *Popillia japonica* (Smart, 1995). Glaser and Fox (1930) performed numerous field trials with the

nematodes isolated from infected grubs of the Japanese beetle, *P. japonica* (Glaser and Fox, 1930). The trials proved to be highly effective and thus the result of the research was implemented into the biological control of insect pests using entomopathogenic nematodes (EPNs).

Entomopathogenic nematodes (EPNs) life cycle. EPNs of the genera, *Steinernem*a and *Heterorhabditis* are associated symbiotically with the insect pathogenic enterobacteria, *Xenorhabdus* and *Photorhabdus* genera, respectively. These are the two most studied bacterial genera associated with EPNs (Forst *et al.,* 1997). The EPNs have four life stages J1, J2, J3 (which is known as the Infective Juvenile) and J4 (which is the adult EPN) (Ehlers, 2001). The infective juvenile (IJ) is the non-feeding and free-living stage in the EPN life cycle. The IJ is analogous to the dauer juveniles of *Caenorhabditis elegans*. The IJs can survive without food in the soil for lengthy periods of time and have evolved different host location strategies when soil conditions become suitable. The emission of carbon dioxide by insects within the soil attracts the IJs which enter the insect through the various natural openings such as the mouth, anus and respiratory spiracles; alternatively they may gain ingress through the insect membranes or outer integument with the aid of enzymatic and mechanical means (Poinar, 1975).

Once inside the insect host, the symbiotic bacteria are released by defecation or regurgitated into the insect haemolymph where they multiply, and produce toxins and hydrolytic enzymes which acting together with the nematode toxins kill the insect host by causing septicaemia (Gaugler, 2002; Batalla-Carrera *et al.,* 2014). *Xenorhabdus* bacteria are released into the haemolymph through the anus of the nematode (Snyder *et al.,* 2007); in contrast, *Photorhabdus* bacteria are released through the mouth (Ciche and Ensign, 2003). Insect mortality occurs within 48 hours following infection (Gaugler, 2002). Following insect infection, EPNs utilise the insect cadaver as a nutrient source, from which they obtain lipids and carbohydrates (Forst and Nealson, 1996). Nematode maturation occurs in the cadaver, where male and female grow, mate and produce offsprings (Surrey and Davies, 1995). *Steinernema* and *Heterorhabditis* production within the insect larvae follow different dynamics. *Heterorhabditis* IJs, upon gaining ingress into insect larvae mature and develop into first generation adults consisting of hermaphrodites which produce the next or second generation adults consisting of amphimictic females and males, with the final generation following nutrient depletion consisting of adult hermaphrodites. In contrast, *Steinernema* first

and second generation consists of amphimictic females and males (Adams and Nguyen, 2002). Figure 2, illustrates the general life cycle of EPNs

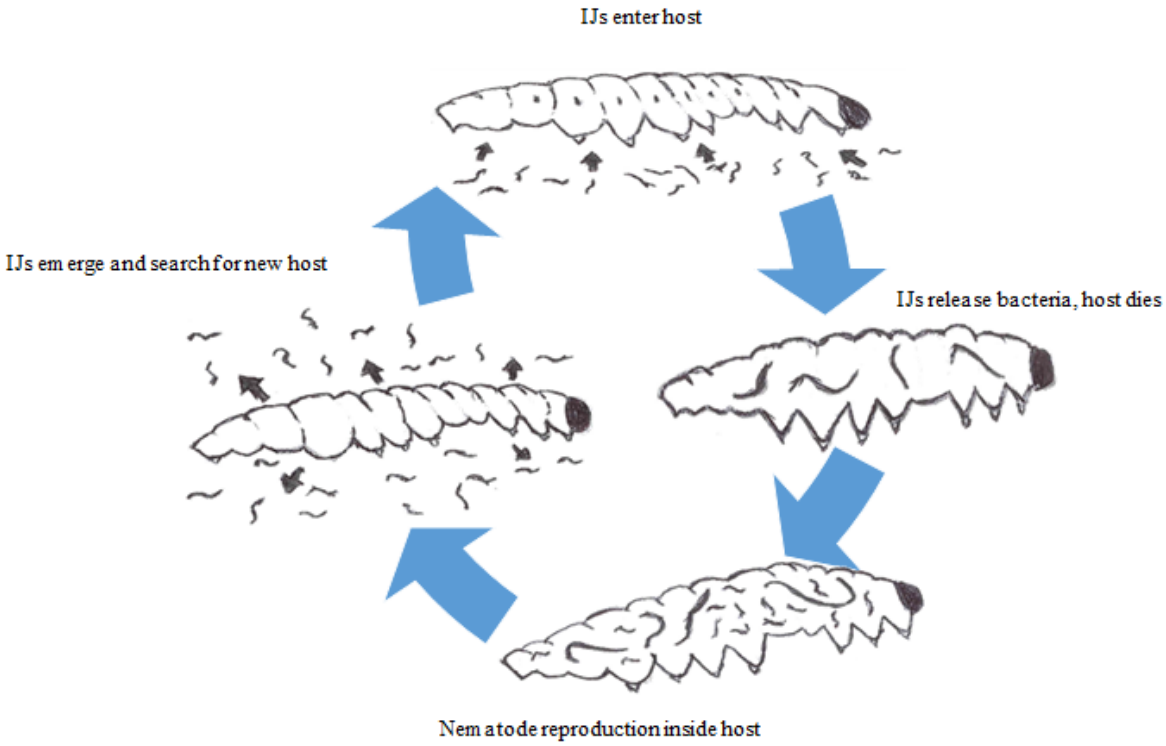


Figure 2. Generalized life cycle of entomopathogenic nematodes.

Advantages of nematode-bacterial relationship. Nematode and bacteria have a mutual relationship, thus both benefits from the relationship. The nematode is the vector for the bacteria and helps to protect the bacteria from the competitive environment of the soil, it transports the bacteria to the nutrient-rich haemolymph and it also helps the bacteria to persist outside the insect host. In turn the bacteria are responsible for causing the death of the insect host by producing a wide range of toxins. The bacteria also produce the hydrolytic enzymes for bioconverting the insect larva into an environment which is ideal for nematode reproduction and growth; the lipids and carbohydrates from the cadaver support the growth of both the nematode and bacteria (Forst and Nealson, 1996; Forst and Leisman, 1997). By entering the haemolymph the bacteria helps overcome the humoral and cellular defences of the insect host and in addition some of the antimicrobial metabolites produced by the bacteria inhibit competing saprophytic microorganisms and scavenging insects, the nematode in turn takes the advantage of the pathogenic potential of the bacteria to help kill the insect host rapidly (Dunphy and Webster, 1991).

Nematodes that lack symbiotic bacteria usually fail to cause insect death or if insect death occurs the infecting nematode fails to grow and reproduce (Boemare, 2002). Each partner of the nematode-bacterial association can be cultured separately, but when combined the nematode-bacterial partnership is more infectious and effective in causing insect mortality (Ciche *et al.,* 2006). This mutual relationship between bacteria and nematodes is very species specific. A given nematode species is associated with a single specific species of symbiotic bacteria; however a specific bacterial species may be associated with more than one nematode species belonging to the same genus, which suggests horizontal transfer of the bacteria between nematode species (Goodrich-Blair, 2007; Clarke, 2008).

Bacterial insect pathogenicity is a prerequisite for the successful establishment and maintenance of the nematode-bacteria symbiosis. In order for a nematode to be an entomopathogen it needs to evolve a symbiotic association with an insect pathogenic bacterium. In addition, the nematode needs to be an effective vector that has the ability to locate and infect various insect hosts associated with the soil environment; the nematode also needs to be adapted to persist in the soil as a non-feeling infective juvenile during which time the bacteria is maintained within the nematode until such time that it is able to re-infect an insect (Adams and Nguyen, 2002; Dillman *et al.,* 2012a).

Bacterial physiological variants. Within the *Steinernema* IJs, the *Xenorhabdus* bacteria are located in intestinal receptacle (Sugar *et al.,* 2012; Snyder *et al.,* 2007); and in contrast, the *Heterorhabditis* associated *Photorhabdus* bacteria are located within the anterior part of the IJs gut (Boemare *et al.,* 1996). When compared with other symbiotic bacteria that are associated with animals, the life cycle of these bacteria are unique as they have a mutualistic relationship with nematodes and a pathogenic relationship with insects, thus the symbiotic bacteria exist in two physiological states, each associated with two different ecological environments. The above two bacteria genera are Gram negative and belong to Enterobacteriacea family.

When cultured *in vitro*, the bacteria occur as two phase variants: primary (phase I) and secondary (phase II), which differ in dye absorption, response to biochemical tests, and metabolite production (Cowles *et al.,* 2007; Akhurst, 1980; Kaya and Stock, 1997). It is the primary phase that supports growth and reproduction of nematodes in *in vitro* culture (Cowles *et al.,* 2007; Akhurst, 1980). Although entomopathogenic nematodes have been reported to grow on secondary phase symbionts or non-symbiotic bacteria, the primary phase

is most conducive for nematode growth and IJs tend to retain only the primary phase symbionts (Cowles *et al.,* 2007; Akhurst, 1980). The two phases, I and II, can be differentiated on MacConkey agar and NBTA (nutrient agar supplemented with 25 mg of bromothymol blue and 40 mg of triphenyltetrazolium chloride per liter) by observing the colour, shape, morphology and size of colonies (Kaya and Stock, 1997). Primary phase variants tend to be smaller in size whereas secondary phase variants are larger in size (Kaya and Stock, 1997). The metabolites and toxins produced by phase I bacteria are responsible for the death of the insect larvae and also the inhibition of competitive saprophytic bacterial and fungal species, which in turn prevents the putrefaction of the insect cadaver (Boemare *et al.,* 1992; Thaler *et al.,* 1998).

Metabolites produced by the symbiotic bacteria. The study of the microbial metabolites, especially the secondary metabolites produced by the EPN symbiotic bacteria both *in vivo* and *in vitro* has increased our understanding of their chemistry, structure, their role in symbiosis and as well as biosynthetic pathways (Akhurst,1982; Webster *et al.,* 2002). There is evidence that some of these metabolites have broad spectrum antibiotic activity against bacterial, fungal, yeast species and they may also be effective against multi-drug resistant human pathogens, thus they have a potential application in pharmaceutical and agroforestry industries (Akhurst, 1982).

More than 30 types of metabolites have been isolated and described from cultures of *Xenorhabdus spp*. and *Photorhabdus spp.*; these include xenorhabdins, xenorxides, xenocoumacins, indoles, indole derivatives, cyclolipopeptide (PAX), xenematide, nematophin and hydroxystilbenes (Ji *et al.,* 2004; Bode, 2009; Lang *et al.,* 2008; Gualtieri *et al.,* 2009). More groups of metabolites have been isolated from *Xenorhabdus spp*. cultures as compared to *Photorhabdus spp.* (Webster *et al.,* 2002). The bacterial phase growth stage does affect secondary metabolite production, in that more metabolites are produced during the phase I growth and none or less are produced during phase II, in addition more metabolites with high concentrations have been reported in insect larvae cadaver than in *in vitro* cultures (Chen *et al.,* 1996).

The metabolites produced by EPN bacterial symbionts are not only diverse in chemical structure but also diverse in bioactivities such as antibiotic, antimycotic, insecticidal and nematicidal, and may be used for new drug development. Each bacterial symbiont produces more than one group of the metabolites; production does however differ with strains and

species, stage of bacterial growth, as well as culture conditions (Chen *et al.,* 1996). No metabolites were detected when *Xenorhabdus spp.* was cultured in 1% peptone water (Chen *et al.,* 1996), in contrast, media such as Luria-Bertani broth (Sundar and Chang, 1993), yeast extract broth (Akhurst,1982) and sea water medium (Paul *et al.,* 1981) resulted in increased metabolite production. The production also differs with time; some metabolite concentrations increase during the first day and decline on subsequent days (Li *et al.,* 1997). In *X. bovienii* cultures, indole derivatives increased significantly on the first two days and declined thereafter, whereas nematophin increased from the first to the second day and remained high thereafter (Chen, 1996).

Aeration and fluctuations in temperature also have an effect on secondary metabolite production; high concentrations of secondary metabolites were also observed with continuous aeration and high temperatures (Chen, 1996). These results indicate that the metabolite production was influenced by many factors with the bacterial growth phase being a significant factor in that it could not be manipulated like other factors such as temperature, aeration and nutrients within media.

Gene-coding enzymes for proteases, lipases, chitinases and other secondary metabolites have been identified and characterized for *P. luminescens spp. laumondii* strain *TT01* (Bode, 2009). Moreover some of the identified coding genes encode for non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) which are involved in biosynthesis of peptide and polyketide secondary metabolites, respectively (Bode, 2009; Staunton and Weissman, 2001). The NRPS and PKS are organized in gene clusters and their products have various biological activities which include antibiotic and antifungal activities for example (Bode, 2009; Sieber and Marahiel, 2005; Staunton and Weissman, 2001). Their function is associated with the chemical structure, which facilitates interactions with specific molecular targets (Sieber and Marahiel, 2005; Staunton and Weissman, 2001). Xenorhabdins and xenorxides produced by *Xenorhabdus* species have been found to be synthesized by NRPS\PKS systems (Bode, 2009).

With the potential of the secondary metabolites produced by the symbiotic bacteria, there is an incentive to isolate new species of EPNs, thus isolating and identifying new species of symbiotic bacteria. Recently a new species belonging to the genus, *Oscheius*, have been isolated and described. Secondary metabolites from some of the entomopathogenic bacteria such as *Serratia* species isolated from *Oscheius* species have not been not fully characterised

as entomopathogenic bacterial nor have all the metabolite or virulence factor protein-coding genes been fully characterized.

***Oscheius*, a new genus of entomopathogenic nematodes.** An *Oscheius* species (*Oscheius tipulae)* was first described by Lam and Webster (1971). The genus was first erected in 1976 by Andrassy (Andrassy, 1976; Sudhaus and Hooper, 1994). *Oscheius* genus presents soil dwelling Rhabditidae family species with evolutionary differences in mode of reproduction and body sizes (Felix, 2006, Felix *et al.,* 2001). The genus comprises two subclades (*Dolichura* and *Insectivora* groups) (Figure 3) (Sudhaus and Hooper, 1994). *Dolichura* clade nematode species are smaller in size and have reduced gonads whereas *Insectivora* clade has species with a large and wide body size (Felix, 2006, Sudhaus and Hooper, 1994). Even though there are striking differences between the two groups of *Oscheius* genus, it still remains difficult to identify *Oscheius* species based solely on morphological data (Sudhaus and Hooper, 1994; Felix *et al.,* 2001). Thus like *Steinernema* and *Heterorhabditis*, *Oscheius* species can also be identified and differentiated with the use of molecular markers such as the internal transcribed spacer region (ITS), D2-D3 expansion segments of the large subunit rDNA gene (LSU) and concise small subunit rDNA gene (SSU) (Felix *et al.,* 2001).

The life cycle of *Oscheius* genus, also has four juvenile life stages (J1, J2, J3 and J4), and IJ (J3) stage which is analogous to dauer juveniles of *C. elegans* (Felix, 2006). The reproduction mode in *Oscheius* genus is also androdioecious like *C. elegans*; where hermaphrodite females can self-fertilise or cross-mate with males (Baille *et al.,* 2008; Sommer and Streit, 2011; Gibson and Fuentes, 2014).

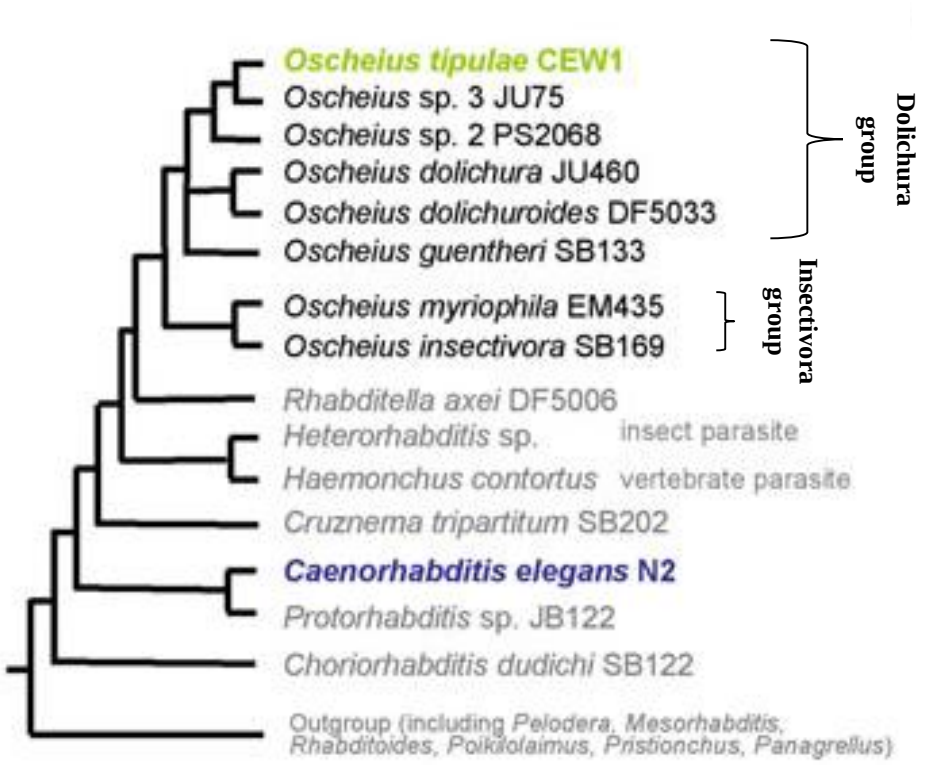


Figure 3. Phylogenetic tree indicating the phylogeny of *Oscheius* species in relation to other nematodes as well as two groups of *Oscheius* genus. Figure use acknowledged by author (Félix, 2006).

Some of the described *Oscheius* species include *O. tipulae* (Lam and Webster, 1971), *O. maqbooli* (Tabassum and Shahina, 2002), *O. shamimi* (Tahseen and Nisa, 2006), *O. carlianonsis* (Weimin *et al.,* 2010), *O. amsactae* (Ali *et al.,* 2011), *O. carolinensis* (Ye *et al.,* 2010), *O. siddiqii, O. niazii* ((Tabassum and Shahina 2010) and *O. chongmingensis* (Liu *et al.,* 2012). Among the *Oscheius* species, few of them have been reported to show potential as EPNs; *O. carolinensis* (Ye *et al.,* 2010; Torres-Barragan *et al.,* 2011), *O. siddiqii, O. niazii* (Tabassum and Shahina 2010), *O. chongmingensis* (Liu *et al.*, 2012), *O. rugaoensis* (Zhang *et al.,* 2012) and *O. amsactae* (Ali *et al.,* 2011). The described species were isolated from regions in continents which include North America, South America, Europe and Asia (Baille *et al.,* 2008; Ye *et al.,* 2010, Liu *et al.,* 2012, Zhang *et al.,* 2012). The isolation of these species from tropical to temperate regions indicates that they are widespread and abundant like *Steinernema* and *Heterorhabditis* (Felix, 2006).

The described *Oscheius* species with EPN potential have shown to be symbiotically associated with bacteria of the genus *Serratia*, because more infectivity was observed with the *Oscheius* species carrying *Serratia* bacteria (Torres-Barragan *et al.,* 2011; Zhang *et al.,*

2008; Zhang *et al.,* 2012). In addition to this *Galleria mellonella* larvae were killed following infection with *Oscheius* species carrying *Serratia* bacteria (Torres-Barragan *et al.,* 2011; Zhang *et al.,* 2008; Zhang *et al.,* 2012; Liu *et al.,* 2012, Ye *et al.,* 2010). To date, the three identified *Oscheius* vectoring *Serratia* bacteria are *O. carolinensis, O. chongmingensis* and *O. rugaoensis* (Zhang *et al.,* 2008, Zhang *et al.,* 2012; Torres-Barragan, 2011). *Serratia* species were also isolated from *C. briggsae* and Abebe and colleagues hypothesized *C. briggsae* to be EPN (Abebe *et al.,* 2010). However according to the EPN status explained by Dillman *et al.* (2012a), *C. briggsae* is not an EPN whereas *O. carolinensis* and *O. chongmingensis* are considered EPNs.

In their analysis of the properties or characteristics which define the entomopathogenic status of nematodes Dillman *et al.* (2012a) identified the following criteria: 1) the nematode must be symbiotically associated with an insect pathogenic bacterium to facilitate pathogenesis and 2) insect death must be rapid (insect killing within five days). Moreover the nematode must not only infect and kill the insect host but also be reproductive producing progeny that have been successfully colonized with the symbiotic bacteria which in turn is passed onto the next generation of infective juveniles (Dillman *et al.,* 2012a). In addition the two partners should benefit from the interaction, with bacteria assisting the nematode in killing the insect host and nematode should vector the bacteria to the insect host. The recently isolated *C. briggsae* does not meet the criteria for EPN as the IJs recovered from cadavers were able to re-infect new hosts (*Galleria mellonella*) but with less virulence than with bacterial injection alone, in addition symbiont heritability and speed of insect host death have not been resolved (Dillman *et al.,* 2012a; Abebe *et al.,* 2010). Although some *Oscheius* species such as *O. carolinensis* and *O. chongmingensis* meet the criteria of EPNs some do not such as *O. tipulae*, thus further research is required on other *Oscheius* to assess if these meet the criteria for EPN.

***Serratia* an entomopathogenic bacteria genus**. *Serratia* genus comprises species which are Gram-negative, rod-shaped, enterobacteria of the family Enterobactericeae (Grimont and Grimont, 1978a). The genus *Serratia* was first described by Bizio (1823); where he named the first species of *Serratia* as *Serratia marcescens* (Bizio, 1823; Grimont and Grimont, 2006). At the start of describing and naming *Serratia* species in subsequent years, different numbers of *Serratia* species were listed (Bergey *et al.,* 1923; Breed *et al.,* 1957; Ewing *et al.,* 1959). However with technologies such as genetic comparison and numerical taxonomy of strains obtained from various habitats, *Serratia* genus was reported to have ten known species

(Grimont and Grimont, 1984; 2006). Some of the ten listed and named species include *Serratia marcescens, S. liquefaciens, S. proteamaculans, S. plymuthica* and *S. entomophila* (Grimont and Grimont, 2006). *Serratia* species occupy various habitats such as plants (*S. marinorubra* and *S.* marcescens in vegetables), animals (*S. anolium* found in lizard *Anolis equestris*, water (*S. plymuthica*) and soil (Grimont and Grimont, 1978a, 1984, 2006). Some *Serratia* species such as *S. plymuthica, S. liquefaciens, S. rubidaea, and S. odoriferae* have been found to cause human infections, some of these have been found to be possible cause of contamination within plants used for human consumption (Grimont and Grimont, 1978a).

Grimont and Grimont (1978a), hypothesized that *Serratia* species found on plants might originate from the soil which would explain their contamination in plant food sources which cause human infection. Most non-pigmented *Serratia* marcescens species have been found to be the ones causing human infections and in addition *Serratia* infections in humans occur in immune compromised individuals (Grimont and Grimont, 1978a). According to Grimont and Grimont (1978a), some *Serratia* species (*S. liquefaciens, S. entomophila* and red-pigmented *S. marcescens*) have been recovered from healthy, diseased or dead insect species. The recently described *Serratia* species associated with *O. chongmingensis* (*S. nematodiphila*) and *O. carolinensis* (*S. marcescens*) have also been found to be red-pigmented, (Zhang *et al.,* 2009; Torres-Barragan *et al.,* 2011) respectively. The re-pigmentation is due to prodigiosin (Williams and Hearn, 1967). The above described species are considered EPNs according to status of EPNs described by Dillman *et al.* (2012a), however further studies are required to satisfy the status of *Oscheius-Serratia* as EPN duo. Since some of the *Oscheius* species do not meet Dillman *et al.* (2012a) EPN criteria, study on symbiotic heritability, virulence and time of death for insect host in *Oscheius-Serratia* will satisfy the status of *Oscheius* genus as EPNs.

To use *Oscheius-Serratia* duo as biocontrol agents, it is important to isolate, identify and study more *Oscheius-Serratia* duos. In view of this, since the two known entomopathogenic bacteria genera; *Xenorhabdus* and *Photorhabdus* are known to secrete secondary metabolites, secondary metabolites secreted by *Serratia* species isolated from *Oscheius* EPNs can be studied to ascertain any differences and similarities with the genera *Xenorhabdus* and *Photorhabdus*. In addition to this, growth phases and morphological features can also be compared between the three genera. Knowledge of the whole genome, can shed light into evolutionary similarities and differences between species. Thus comparison of whole genome sequences of *Steinernema, Heterorhabditis* and *Oscheius* as well as *Xenorhabdus,*

Photorhabdus and *Serratia* genera will be informative in determining the evolutionary history of *Oscheius-Serratia* and also shed light on their relation to other known and described EPN duos. In addition this will also reveal evolutionary traits between the known EPN genera.

Motivation of the research. A study of the secondary metabolites produced by the symbiotic bacteria has potential benefits as they have useful applications for the medical, pharmaceutics and agroforestry industries. The availability and analysis of the genome sequence of both the symbiont and host nematode can lead to the identification of genes encoding enzymes for metabolic pathways leading to the production of secondary and other metabolites with antimicrobial and insecticidal properties. Genes encoding enzymes for the biosynthesis of useful biological secondary metabolites could have applications as pest resistant transgenes for the genetic engineering of crops against disease and insect crop pests. Microbial infections that have developed resistance to existing antibiotics could be treated with newly identified antimicrobial metabolites produced by bacteria associated with entomopathogenic nematodes; furthermore the availability of genome sequences of the bacterium-nematode pair can increase our understanding of symbiosis and insect pathogenesis.

Objectives

I. Isolation of a novel entomopathogenic nematode from South Africa, using the *Galleria mellonella* baiting technique.
II. Identification and morphological observations of selected indigenous EPN and symbiotic bacteria using light (LM) and scanning electron microscopes (SEM).
III. Morphometric characterization of the novel entomopathogenic nematode.
IV. Molecular and phylogenetic characterization of the nematode using the sequences of the internal transcribed spacer region (ITS), D2-D3 expansion segments of the large subunit rDNA gene (LSU) and concise small subunit rDNA gene (SSU).
V. Isolation and identification of the insect pathogenic bacteria associated with the nematode.
VI. Identification and morphological observations of selected indigenous EPN and symbiotic bacteria using light (LM)
VII. Molecular phylogenetic characterization of the bacteria using the sequence of the 16S ribosomal RNA gene.
VIII. *In vitro* growth of the symbiotic bacteria in various liquid media.

IX. Sequencing, assembly and annotation of the bacterial genome in order to identify the genes responsible for the synthesis of antimicrobial compounds.

X. Sequencing, assembly and annotation of the EPN genome to increase understanding the mutual relationship between the two genomes.

XI. Comparative genomics of both the sequenced, assembled and annotated whole genomes of the EPN and its symbiotic bacterium with other genera of EPN-symbiotic bacterium duo.

Organization of the thesis.

- Chapter 2: Description of a South African entomopathogenic nematode, *Oscheius safricana*. This chapter focuses on the isolation, morphological and molecular identification of a South African *Oscheius safricana.* The chapter addresses objectives (I-IV) of the research project.
- Chapter 3: Metabolites produced by the symbiotic bacterium, *Serratia marcescens* strain *MCB*. Chapter 3 includes the isolation, morphological as well as molecular identification of the symbiotic bacteria *Serratia marcescens* strain *MCB* associated with *Oscheius safricana*. This chapter further discusses the indole derivatives which are produced by *S. marcescens* strain *MCB.* Objectives (V-VIII) are covered in this chapter.
- Chapter 4: Whole genome sequence of *Serratia marcescens* strain *MCB* reveals it is an entomopathogenic bacterium. This chapter covers objective IX and XI. It includes the whole genome sequence, assembly and annotation of *S. marcescens* strain *MCB* as well as genome comparison with symbiotic bacteria associated with EPNs. Some of the protein-coding genes responsible for metabolite and antibiotic production are identified.
- Chapter 5: Whole genome sequence of *Oscheius safricana*. This chapter includes the whole genome sequence, assembly and annotation of *Oscheius safricana* as well as genome comparison with other EPNs. It addresses objectives X and XI.
- Chapter 6: Conclusion, this chapter includes conclusion of the research project linking all the chapters, in addition it states the contributions and recommendations for future work.
- Appendices A, B, C and D for chapters, 2, 3, 4 and 5 with supplementary methods.

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Chapter 2:

Description of a South African entomopathogenic nematode, *Oscheius safricana**

*This chapter was submitted to Phytopathology and plant protection Journal, titled "A new species of entomopathogenic nematode *Oscheius safricana n. sp*. (Nematoda: Rhabditidae) from South Africa" (2015) and was still under review on submission of this thesis. It was written by M.H Serepa and VM Gray.

Abstract

In a recent entomopathogenic nematode (EPN) survey in the North West province of South Africa, *Oscheius safricana* was isolated from soil samples using the *Galleria mellonella* bait method. Morphological studies using light microscopy and scanning electron microscopy, molecular analysis of the internal transcribed spacer region (ITS), D2-D3 expansion segments of the large subunit rDNA gene (LSU) and concise small subunit rDNA gene (SSU), revealed that it was a new species, described herein as *Oscheius safricana*. *Oscheius safricana* was characterized by unique ribosomal DNA sequences, six separate lips each two bristle-like sensillae, narrow pharynx, valvated basal bulb, lateral field with four lines, leptoderan and closed bursa and fused spicules. This EPN belongs to the group *Insectivora* group, and is morphologically close to *O. necromenus, O. chongmingensis and O. carolinensis. Oscheius safricana n. sp.* is symbiotically associated with *Serratia marcescens* strain *MCB*. 50% of the *Galleria mellonella* population was killed after 24 hours on infection with *Oscheius safricana.*

Introduction

Entomopathogenic nematodes (EPNs) have been found to be suitable biocontrol agents (Kaya and Stock 1997; Poinar 1976). The biocontrol advantages of EPNs have been well established and have encouraged further surveys of EPNs on a global basis. These surveys have shown that EPNs are widely distributed in various soils throughout the world (Adams *et al.*, 2006; Hominick *et al.*, 1997; Hominick 2002). With the biocontrol advantages and useful antimicrobial products produced by the symbiotic bacteria (Wang *et al.*, 2011) associated with EPNs, there exists a strong incentive to discover new EPN species associated with new species of insect pathogenic symbiotic bacteria. Recently described *Oscheius* species such as *Oscheius carolinensis, O. chongmingensis, O. carolinensis* and *O. rugaoensis* have shown that they share many functional similarities and attributes with Steinernematidae and Heterorhabditidae and on these grounds that may be also categorized as entomopathogenic nematodes (Ye *et al.*, 2010; Liu *et al.*, 2012 and Torres-Barragan *et al.*, 2011; Zhang *et al.*, 2008, Zhang *et al.*, 2012; Dillman *et al.*, 2012a).

Thus far, approximately 80 different valid EPN species have been identified, with 63 belonging to the family Steinernematidae, 15 to the Heterorhabditidae. The EPNs in all three genera have evolved mutualistic associations with three different genera of Gram-negative, motile, rod-shaped bacteria belonging to the family Enterobactericeae, all of which are lethal pathogens to a wide range of insects. *Xenorhabdus* bacteria are located in the intestinal

vesicle of the infective juveniles (IJs) of *Steinernema* (Bird and Akhurst, 1983); in contrast *Photorhabdus* bacteria are located throughout the whole intestine of *Heterorhabditis* IJs (Endo and Nickle, 1991) whereas bacteria belonging to the genus *Serratia* have been isolated from *Oscheius carolinensis, O. chongmingensis* and *O. rugaoensis* (Torres-Barragan *et al.,* 2011; Zhang *et al.,* 2009 and Zhang *et al.,* 2012). In this study we report on *Oscheius safricana n. sp.* which was isolated from South Africa.

Material and Methods

Soil sampling and nematode isolation. Soil samples were collected from Brits in the North West province of South Africa GPS co-ordinates S 25° 42′ 16.74′′ E 27° 44′ 19.542′′ (Latitude: -25.70465 | Longitude: 27.738762). Sampling strategy was done according to (Kaya and Stock 1997). Extraction of EPNs from the soil samples was done according to live bait method using laboratory reared late-instar *Galleria mellonella* larvae (Bedding and Akhurst, 1975); see Appendix A for nutrients and growth cultures of the *G. mellonella* larvae. Maintenance of the nematode population was done by re-infecting fresh larvae with the infective juveniles escaping from the cadavers of *G. mellonella* (see Appendix A for supplementary methods). Emerging IJs were recovered from White traps (White, 1927). The nematodes were then harvested from the White traps and washed with 1% sodium hypochlorite (NaClO), rinsed with distilled water twice.

Morphological characterization. The morphology of IJs, males and females, including hermaphrodites and the holotype male, were characterized with respect to the following anatomical attributes: body length (L); largest body diameter (D); stoma length, stoma diameter, distance from anterior end to excretory pore (EP); distance from anterior end to nerve ring (NR); distance from anterior end to end of pharynx (ES); distance from anterior end to vulva/body length x100 (V); tail length with sheath; tail length without sheath; anal body diameter (ABD); spicule length (SP); gubernaculum length (GU) as described by Adams and Nguyen (2002).

Light microscopy. For morphological measurements of the current EPN isolate, one male was selected as a holotype for this new strain, 20 first generation (males, hermaphrodite and amphimictic females) adults and 20 IJs were randomly selected from White traps and heat killed at 60°C for 2 minutes in Ringer's solution. These were then fixed in hot triethanolamine formalin (TAF) in 85°C (Courtney *et al.,* 1955) and kept in this solution for 48h. EPNs were further processed using solutions I and II according to Seinhorst (1959). The

EPNs were then mounted in pure glycerine on slides and observations as well as measurements were made with an Olympus BX63 Fluorescent Microscope, Olympus GmbH equipped with an Olympus DP 80 video camera using The CellSens Dimension 1.9.1 software.

Scanning electron microscope (SEM). IJs and first generation adults (males, hermaphrodite and amphimictic females) were heat killed at 60 °C for 2 minutes in Ringer's solution. IJs were rinsed with Ringers solution three times with 5 minutes interval between each rinse. These were then fixed in 8% glutaraldehyde overnight (glutaraldehyde 25% EM grade, diluted in Ringers' solution). For post fixing, these were rinsed with sterile distilled water three times and dehydrated with 30 %, 50 %, 70 %, 90 %, 95 %, and 100% ethanol at 10 minute intervals. These were then critically point dried with CO_2 for 2 hours and mounted on SEM stubs, coated with gold and palladium, and viewed using the FEI QUANTA 400 ESEM fitted with a digital camera.

Polymerase chain reaction (PCR) amplification and sequencing. Genomic DNA of the EPNs was isolated from IJs inbred thirteen times to eliminate heterozygosity. The IJs were inbred on lipid agar (see Appendix A for composition and preparation) with 13 subcultures. Total genomic DNA was extracted using the protocol from, Pure Gene® DNA Purification Kit (Gentra Systems 2003, the protocol is in Appendix A). Three molecular markers were used to identify the EPN: ITS, 18S rRNA gene and the D2-D3 region on the 28S rRNA gene. The ITS region was amplified using universal primers described by Joyce *et al.* (1994). The 18S rRNA gene was amplified using primers described by (Subbotin *et al.*, 2006) and D2-D3 region on the 28S rRNA gene was amplified using primers described by (Vovlas *et al.*, 2006). All PCR products were purified using the QiaQuick PCR purification kit and sequenced by Inqaba Biotechnical Industries (PTY) Ltd, South Africa. The obtained sequences were deposited in Gen-Bank under the name *Oscheius sp. MCB* and assigned accession numbers KF684370 (for ITS), KM270115 (18S rRNA) and KM270116 (for D2-D3 region on 28S rRNA gene).

Analysis of the phylogenetic relationships. The sequences obtained were used to search for similar rhabditid species using Basic Local Alignment Search Tool (BLAST) on GenBank database and the species with the highest similarity matches were selected and used for phylogenetic analysis. The sequences of the selected species and the EPN isolate were aligned using MUSCLE (Edgar 2004), with default options. Neighbour-joining tree of the

homologous sequences in this study obtained from GenBank database was inferred with 1000 replications and the Jukes-Cantor evolution model used (Saitou and Nei, 1987; Jukes and Cantor, 1969).

Nematode entomophathogenicity. Nematode entomophathogenicity was evaluated using late-instar *Galleria mellonella* larvae. A 10 cm diameter Petri dish lined with two layers of Whatman No.1 filter paper, the filter papers were kept moist with distilled water. Each Petri dish had four larvae which were inoculated with one thousand IJs (i.e., 250 nematodes /larva). Distilled water was used as the control. The Petri dishes were kept at room temperature in the dark and mortality of the larvae was evaluated every 24 hours. Dead larvae were collected and transferred to White traps (Glazer and Lewis, 2000) to determine IJ emergence, this experiments had five replicates and was performed three times.

Results and Discussion

Measurements of *Oscheius safricana n. sp.*

Table 1. Morphometrics in µm of *Oscheius safricana*, presented as the means ± SD and the range.

Character	Holotype	Male	Hermaphrodite female	Female	IJ
n	1	20	20	20	20
Body length (*L*)	1000.63	1048.22 ± 159.89 (864.45-1309)	1988.78 ± 229.30 (1000-2230.89)	1153.61 ± 167.29 (893.89-1413.32)	375.08 ± 64.07 (297.09-460.75)
Greatest body diam (*D*)	52.05	69.28 ± 12.33 (48-93)	274.44 ± 63.31 (109-250)	104.91 ± 33.77 (59-150)	15.29 ± 3.49 (8.73-20.5)
Stoma length			10.32 ± 0.72 (11-20)	7.51 ± 0.74 (6.9-9.9)	
Stoma width			9.80 ± 1.07 (8-17)	5.95 ± 1.34 (5-7)	
EP	166.02	199 ± 17 (111-256)	219 ± 29.69 (178-300)	167.00 ± 16.66 (120-250)	17.36 ± 7.63 (9.96-28.75)
NR	127.4	144.00 ± 15 (86-145)	161.53 ± 24.93 (98-169)	156.45 ± 15.69 (141-179)	43.28 ± 9.84 (30.82-58.67)
ES	146.99	169 ± 14 (108-198)	198.37 ± 24 (137-268)	183.07 ± 39.71 (145-213)	63.36 ± 11.85 (41.47-72.92)
Tail with sheath (*T*)	48.56	46.00 ± 8.48 (38-58)	126.93 ± 28.18 (90-131)	69.70 ± 18.10 (50-89)	84.37 ± 8.75 (26.46-155.53)
Tail without sheath					
Anal body diam (ABD)	32.67	32 ± 2.12 (22-35)	40.15 ± 1.76 (34-51)	30.94 ± 2.88 (25-45)	11.94 ± 2.57 (9.9-15.97)
Spicule length (SP)	52	39 ± 11.31 (28-60)			
Gubernaculum length (GU)	27	24.51 ± 3.53 (21-32)			
V			52.64 ± 0.77	44.69 ± 8.61	

		(20.98-25-12)	(6.89-15)	(13.50-16)	(6.76-16.55)
D%=EP/ES*100	112	117 ± 5.79	110 ± 2.78	90.58 ± 5.84	27.47 ± 10.32
		(109-121)	(76.1-112)	(65.09-91)	(14.79-49.92)
E%=EP/T*100					40.78 ± 13.82
					(28.55-65.74)
SW%=SP/ABD*100	159	121 ± 27.33			
		(110-250)			
GS%= GU/SP*100	51	64 ± 9.56			
		(52-80)			

Number of specimens measured (n); body length (L); largest body diameter (D); stoma length, stoma diameter, distance from anterior end to excretory pore(EP); distance from anterior end to nerve ring (NR); distance from anterior end to end of pharynx (ES); distance from anterior end to vulva/body length x100 (V); tail length with sheath; tail length without sheath; anal body diameter (ABD); spicule length (SP); gubernaculum length (GU)

Description (Figures 1-3)

Adults: Cuticle was smooth with longitudinal striations: lateral field pattern with nine ridges visible from mid-carpus to phasmids in females and near bursa region in males. There are six unfused lips, each bearing two terminal sensillae that are bristle-like. Amphids are present. Buccal cavity present. The stoma was long and narrow. Pharynx was cylindrical containing metacorpus with a valvated basal bulb. Isthmus was present and made up about 20% of the pharynx. Median bulb was absent. Excretory pore was located posterior to nerve ring, before basal bulb. Nerve ring is located 60% of pharynx.

Male: Body was C-shaped after heat killing. Bursa was leptoderan and is closed. Tail short than female and protrude beyond bursa. Spicules are fused and J-shaped with a crochet needle-shaped tip of head. Gubernaculum was thin and boat-shaped.

Female: Body was slightly curved after heat killing. General morphology was similar to male. Reproductive system was amphidelphic with reflexed ovaries extending as far as vulva. Vulva in form of transverse slit with lateral vulval flaps. The tail was pointed and longer than male.

Juveniles: Body was straight after heat killing. Body was slender. Tri-radiate mouth closed with four terminal sensillae. Stoma was long and narrow. Pharynx and isthmus long and narrow. Valvated basal bulb. Tail was pointed and elongate.

Description and Diagnosis. *Oscheius safricana n. sp.* was characterized by having adult stages with six unfused lips each bearing a terminal sensillae, long and narrow stoma, valvated bulb, amphimictic and amphidelphic reproduction. Four lateral field with 10 ridge lines, leptoderan male bursa which was open. The crochet-needle-shaped spicules and leptoderan bursa place *Oscheius safricana* in the *Insectivora* group of *Oscheius* (Sudhaus and Hooper 1994; Sudhaus and Fitch 2001).

Species comparisons in this group are compiled and updated in (Stock *et al.,* 2005; Ye *et al.,* 2010). From the *Insectivora* group of *Oscheius safricana* is morphologically close to *O. necromenus, O. chongmingensis and O. carolinensis*. It closely resembles with *O. necromenus* but differs in larger body length (vs. L=1179 µM); smaller "a" values (vs. a=17.2); larger "b" value (vs. b=5.5); same "c" value (vs. c=11), larger vulva opening (vs. V= 50%), smaller spicule length (vs. SP=40). The two have the same number of lateral lines (vs.=4). *Oscheius safricana* also resembles *O. chongmingensis* but differs in larger body size (vs. 1921); smaller spicule length (vs. SP=51); same stoma length and vulva opening (vs. SL=10) and (vs. V=52%) respectively. *Oscheius safricana* also resembles *O. carolinensis* but differs with larger body length (vs. L=1728 µM); smaller "a" values (vs. a=18.2); larger "b" value (vs. b=5.5); same "c" value (vs. c=11), larger vulva opening (vs. V= 50.3%), smaller spicule length (vs. SP=65).

Type host: It is currently unknown what the host of *Oscheius safricana* is, however several generations were produced using *Galleria mellonella*.

Type locality: Citrus farm, North West Province, South Africa

Entymology: The name is derived from the country of isolation.

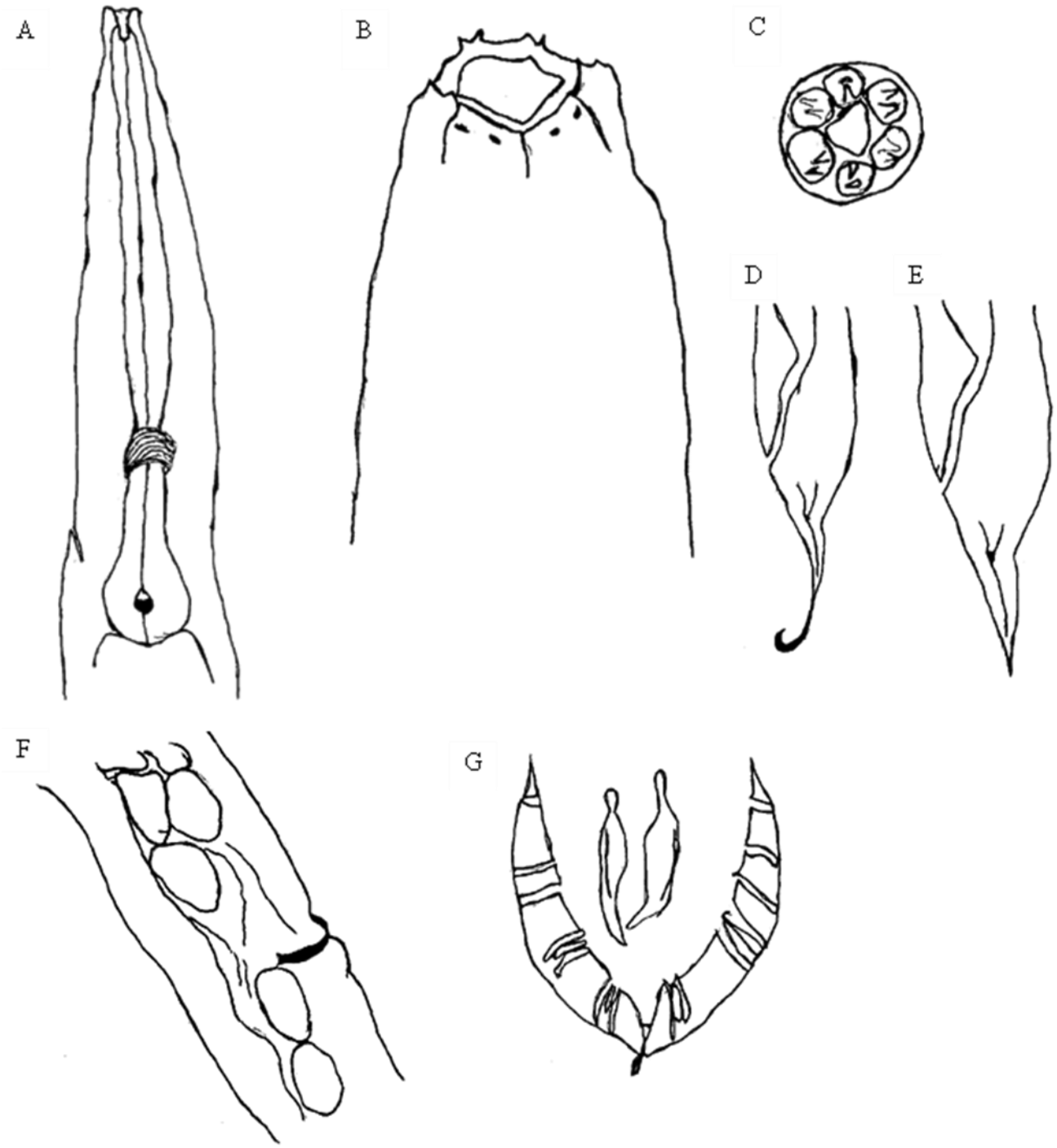


Figure 1. *Oscheius safricana n. sp.* A: Pharyngeal region of female, lateral view; B: Anterior region of male with six lips each with two sensillae; C: Schematic representation view of lip region; D: Lateral view of female tail with rectum; E: Lateral view of male tail with rectum; F: Lateral view of vulva region and eggs and G: Ventral view of bursa and spicules. (Scale bars: A=20µm; B=20µm; C=10µm; D=20µm; E=20µm; F=20µm; G=20µm)

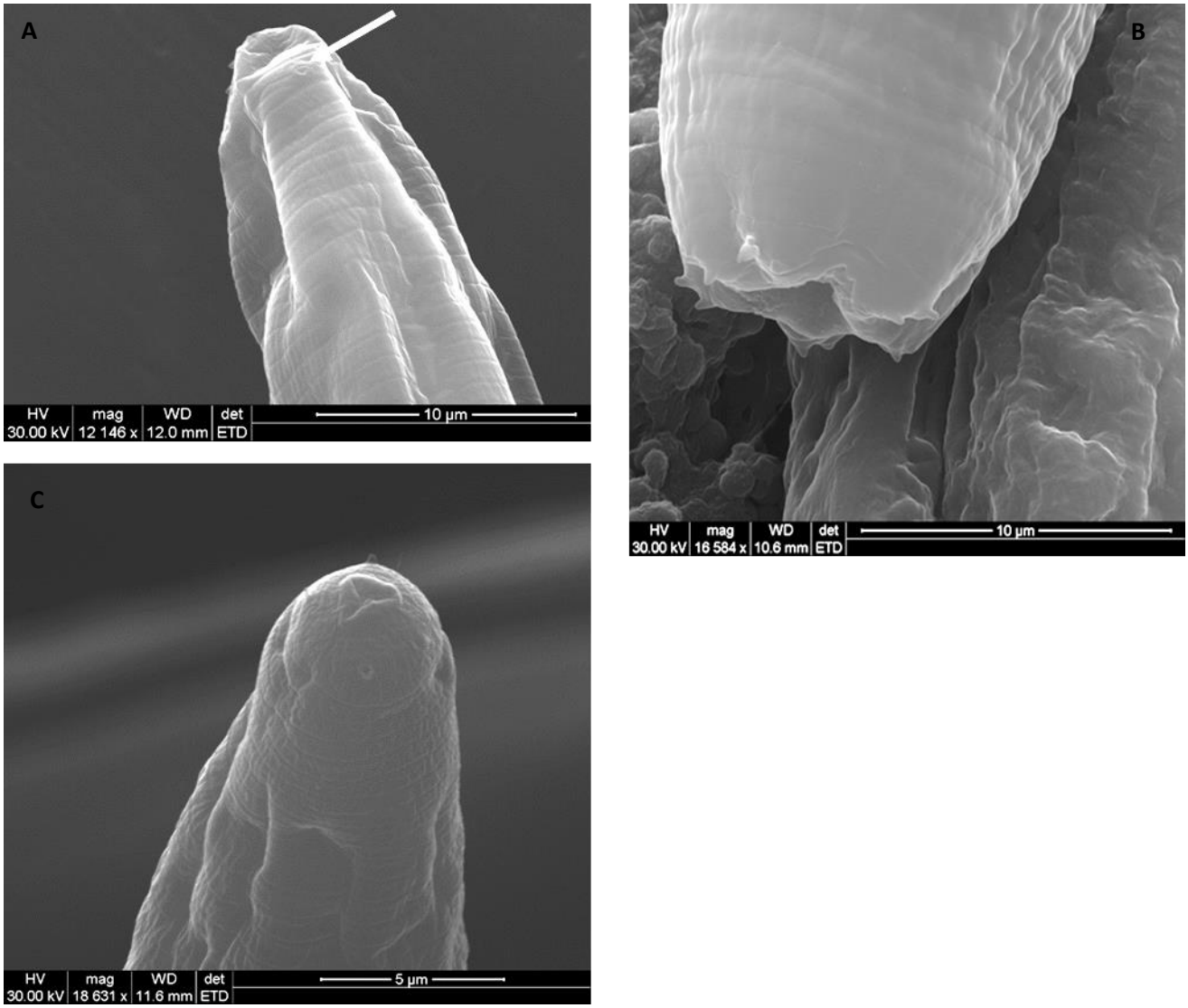


Figure 2. Scanning electron micrographs of *Oscheius safricana n. sp.* A: Lip region showing amphid (arrow); B: anterior region showing six lips each with two sensillae and C: anterior region of IJ showing tri-radiate lips with four terminal sensillae.

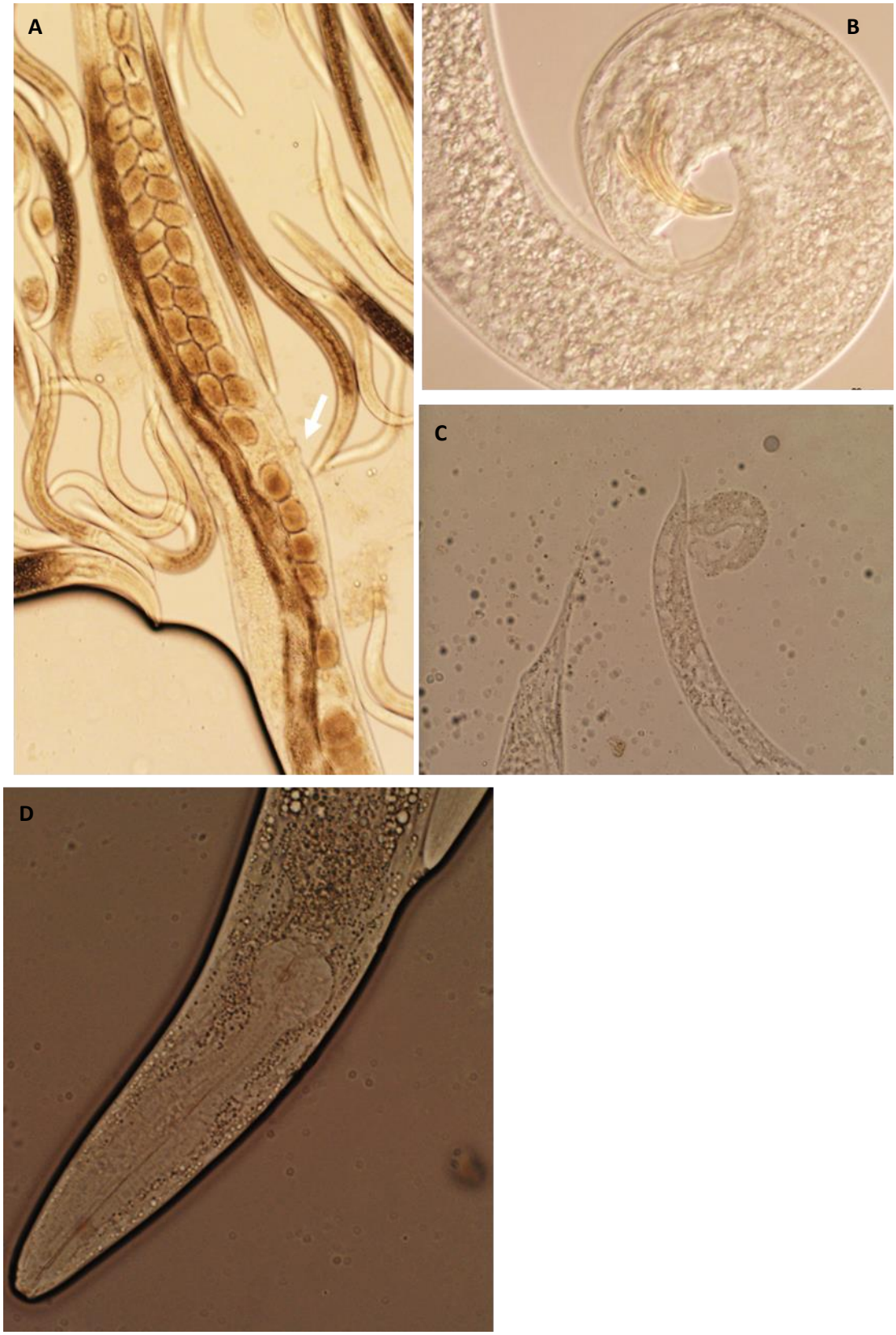


Figure 3. Light micrographs of *Oscheius safricana n. sp.* A: Female adult showing eggs and vulva lips (arrow); also surrounded by IJs; B: Spicules and gubernaculum; C: female and

male tails respectively and D: anterior region of female showing pharyngeal region. (Scale bar= 20µm).

Nematode entomophathogenicity. The results indicate that *Oscheius safricana* was capable of infecting *G. mellonella* within 24-96 hours which is within the five day requirement for EPN criteria (Dillman *et al.*, 2012a), 50% of the larvae population were killed within 24 hours and 100% mortality was reached on day 4 (Figure 4). This is the first study in South Africa to report on *Oscheius* species and the morphometric measurements indicate that it was a new species. In this study the nematodes emerged from the cadaver 2 days after death. Since several generations were propagated under laboratory conditions and 100% infection\mortality of larvae was observed, *Oscheius safricana* shows a potential as a biocontrol agent.

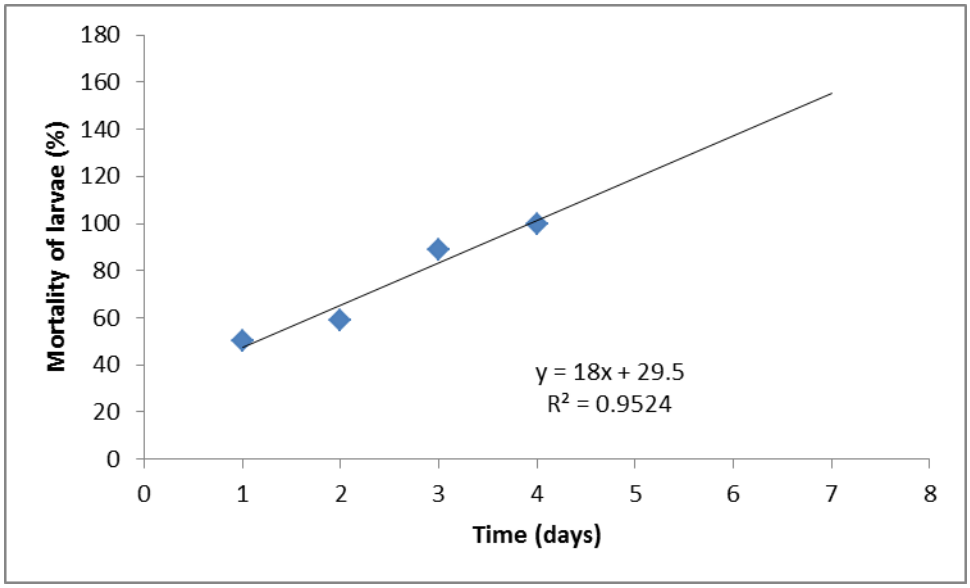


Figure 4. Infectivity of *Oscheius safricana* against *G. mellonella larvae.*

Molecular characterization of the EPN found in the study. Amplification by PCR of the ITS resulted in a DNA sequence of 816 base pairs (bp), the 18S rRNA gene resulted in 1014 bp DNA sequence whilst the D2-D3 region of the 28S rRNA gene had a DNA sequence of 603 bp. The phylogenetic analysis of *Oscheius safricana* using the ITS region, 18S rRNA and D2-D3 region of the 28S rRNA gene with other rhabditids species confirmed that the current nematode species was of the *Oscheius* genus (Figures 5-7). *Oscheius safricana* has unique sequences of ITS, 18S rRNA gene and D2-D3 region of 28S as these are not the same as any of the Rhabditidae species deposited in GenBank database, the BLAST searches of ITS, 18S rRNA gene and D2-D3 region of 28S all resulted in closely related sequences of Rhabditidae

species from GenBank database. The sequences were used in phylogenetic analysis and in addition the Rhabditidae species retrieved from GenBank database after a BLAST search all belong to the *Insectivora* and *Dolichura* groups across all the phylogenetic trees (Figures 5-7).

The ITS phylogenetic tree (Figure 5) revealed that *O. safricana* grouped with LN611142 *Heterorhabditidoides sp.* BRA6 with a 100% bootstrap support confirming that the two are closely related. *Heterorhabditidoides* was initially described as a genus by Zhang *et al.* (2008) describing *Heterorhabditidoides chongmingensis*, the species was however re-described by Liu *et al.* (2012) and confirmed to be *Oscheius chongmingensis*. Furthermore, the analysis shows that *O. safricana* is a sister species to KP792651 *O. myriophilus* strain JU1386 and KM492926 *Oscheius sp.* TEL-2014 of which *Oscheius sp.* TEL-2014 has been reported to infect insect hosts (Lephoto *et al.*, 2015).

The phylogenetic tree based on the 18S rRNA partial gene revealed that *O. safricana* is a sister species to AF082994 *Oscheius sp.* BW282 (undescribed), U81588 *Rhabditis myriophila*, U13936 *Rhabditis myriophila* EM435 and KP756941 *O. myriophilus* with bootstrap support of 99%. *Rhabditis myriophila* and *Rhabditis myriophila* EM435 species grouped with *Oscheius sp.* BW282, and *O. myriophilus* with bootstrap of 92% which could suggest re-examination of the *Rhabditis* species to confirm if they fall within *Oscheius*. Figure 7 shows the phylogenetic tree based on D2-D3 sequences of *Oscheius safricana* and other related *Oscheius* species, which revealed that *O. safricana* grouped with AY602176 *O. myriophila* DF5020 with a 71% bootstrap support. The BLAST search of the 28S rRNA gene resulted in fewer closely related species.

Overall the three phylogenetic trees confirm the current species to be within the genus *Oscheius* and as observed from its infectivity and efficacy against *G. mellonella*, thus meeting the EPN criteria, *O. safricana* clustered within the *Insectivora* group.

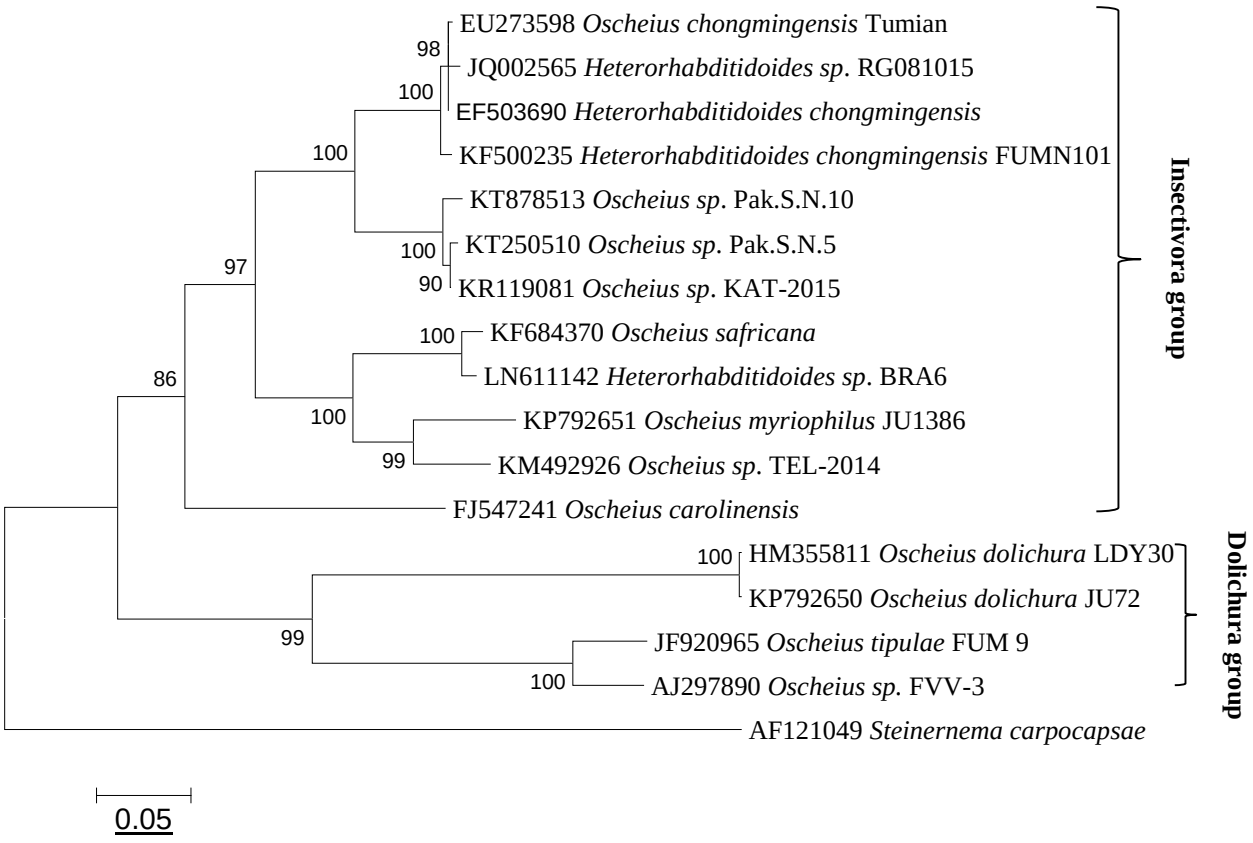


Figure 5. Phylogenetic relationships of *Oscheius safricana* and other closely related Rhabditidae as well as *Oscheius* species within the *Insectivora* and *Dolichura* groups, with *Steinernema carpocapsae* as the outgroup in a neighbour-joining tree based on analysis on ITS sequence data.

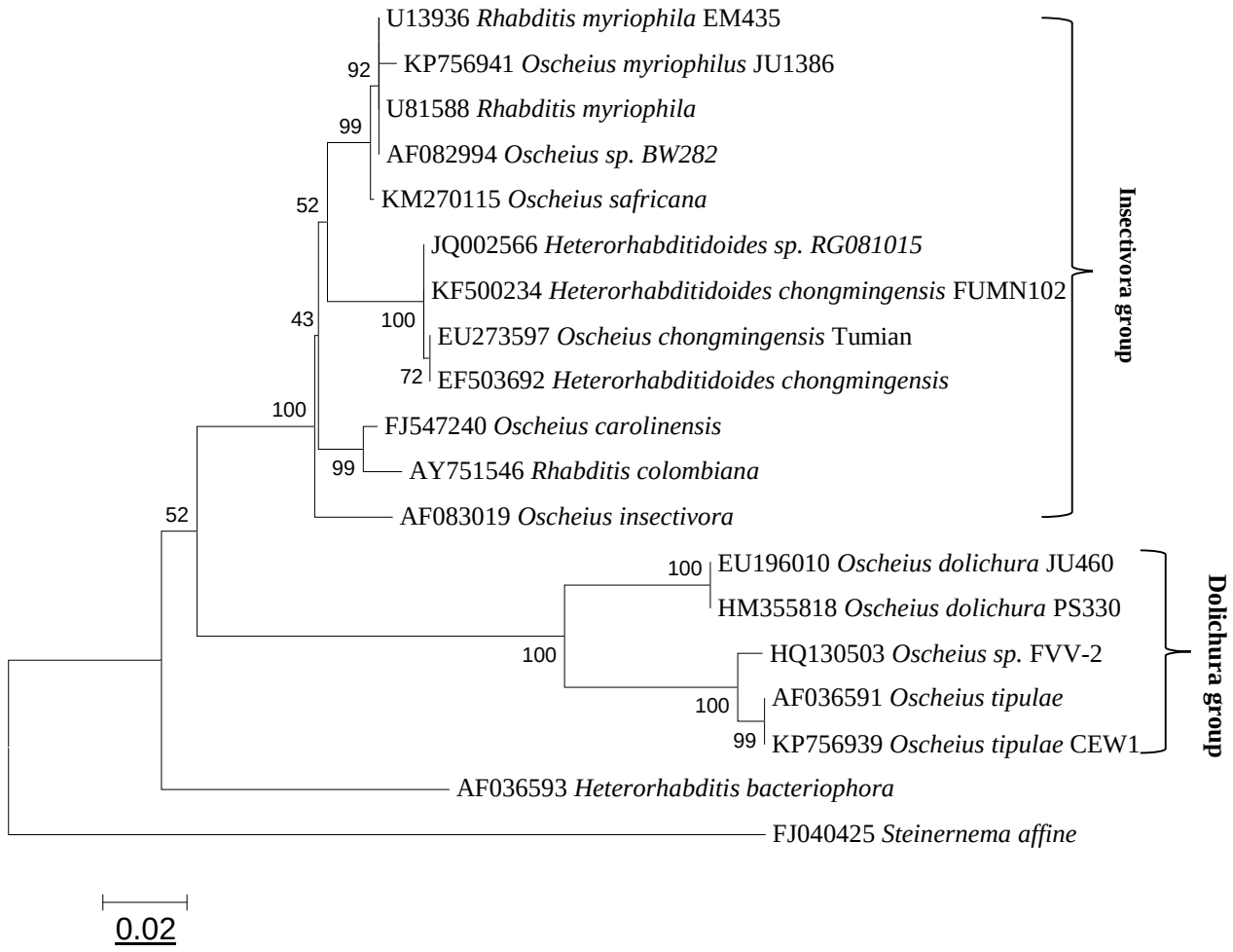


Figure 6. Phylogenetic relationships of *Oscheius safricana* and other closely related Rhabditidae as well as *Oscheius* species within the *Insectivora* and *Dolichura* groups, with *Steinernema affine* as the outgroup in a neighbour-joining tree based on analysis on 18S rRNA sequence data.

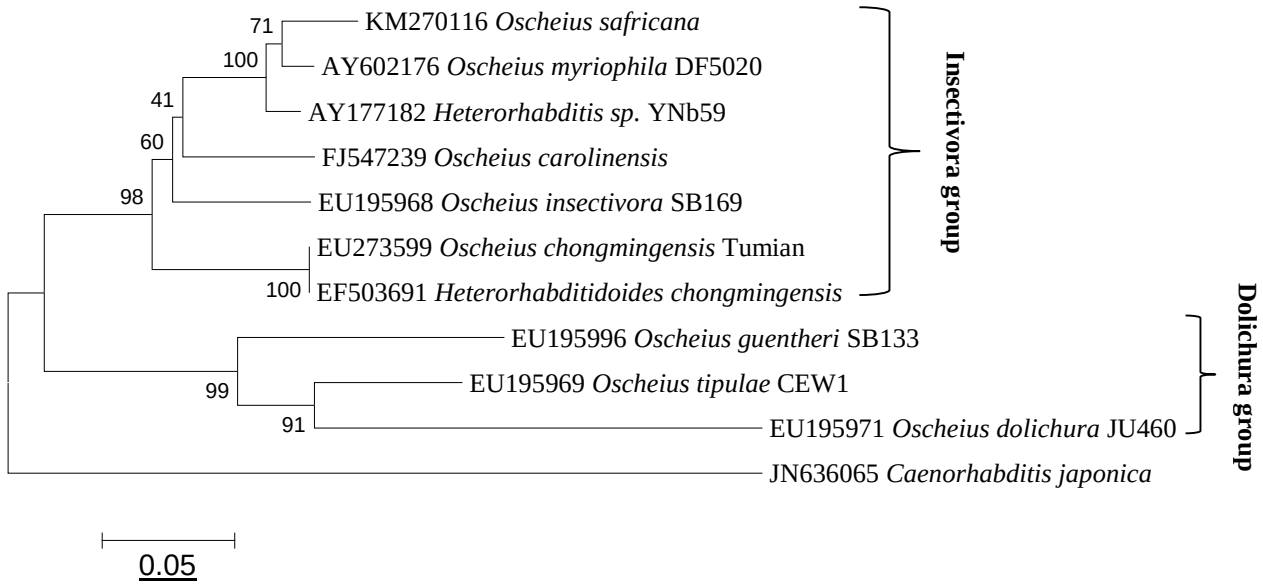


Figure 7. Phylogenetic relationships of *Oscheius safricana* and other closely related Rhabditidae as well as *Oscheius* species within the *Insectivora* and *Dolichura* groups, with *Caernorhabditis japonica* as the outgroup in a neighbour-joining tree based on analysis on D2-D3 region on the 28S rRNA sequence data.

Oscheius safricana was isolated from South Africa. Both the morphological and molecular identification place it within the *Insectivora* group of *Oscheius*. Several generations of *Oscheius safricana* were produced within laboratory conditions and it was able to infect, hamper and kill *G. mellonella* larvae within 4 days which falls within the five day criteria for EPNs (Dillman *et al.*, 2012a), thus this EPN can be used as a biological control in agriculture. However we recommend a field study and use of other types of larvae to ascertain if infection will occur outside laboratory environments. This was the first study to describe the potential of *Oscheius* species as a potential biocontrol in South Africa.

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Chapter 3:

Metabolites produced by the symbiotic bacteria *Serratia marcescens* strain *MCB**

*This chapter was first published in the African Journal of Bacteriology Research, titled "Purification and characterization of tryptophan and indole-3-acetic acid produced by *Serratia marcescens strain MCB* associated with *Oscheius sp. MCB* (Nematoda: Rhabditidae) obtained from South African soil" (2015). It was written by MH Serepa[1], NT Tavengwa[2] and VM Gray[1]. Please note that on publishing this paper the name of the isolated nematode was not decided, thus it was initially called *Oscheius sp. MCB.*

[1] First co-author

[2] Second co-author

Abstract

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are lethal to insect pests that attack plants. These EPNs are associated symbiotically with the two enterobacteria *Xenorhabdus* and *Photorhabdus sp*., respectively. The bacteria synthesize a range of insecticidal and antimicrobial metabolites which may be useful in agricultural pest control and medical disease control. Recently the function group of EPNs has been expanded to include several newly discovered species of insect pathogenic nematodes that belong to the genus *Oscheius*. We isolated, characterized and identified a symbiotic bacterium and of some the metabolites it produces. The symbiotic bacterium was isolated from a South African nematode, *Oscheius sp. MCB* (GenBank internal transcribed spacer region (ITS) accession number: KF684370). The symbiotic bacterium was identified to be a *Serratia marcescens* strain *MCB* (GenBank 16S rRNA accession number: KF793930). Two metabolites it produced were indole-3-acetic acid and tryptophan, with tryptophan as an aromatic amino acid was assumed to be a precursor substrate for indole-3-acetic acid. While indole-3-acetic acid has been shown to have antimicrobial effects it could also be substrate for the synthesis of an antimicrobial compound yet to be more fully characterized.

Introduction

Entomopathogenic nematodes (EPNs) have been found to have attributes which make them suitable biocontrol agents (Kaya and Stock, 1997; Poinar, 1975). The advantages of using EPNs in biocontrol have been well established and their value as biocontrol agents has encouraged further surveys of EPNs on a global basis. Surveys have established that EPNs are widely distributed and occur ubiquitously in various soils throughout the world (Adams *et al.,* 2006; Hominick *et al.,* 1996; Hominick, 2002). Many species of EPNs are cosmopolitan and are found widely dispersed through the world. With the biocontrol advantages and useful antimicrobial products produced by the symbiotic bacteria associated with EPNs (Wang *et al.,* 2011), there exists a strong incentive to discover new EPN species associated with new species of symbiotic bacteria. Recently it has been shown that nematodes belonging to the genus *Oscheius* possess very similar attributes to those that have been normally associated with the well-known Steinernematidae and Heterorhabditidae, which have become the exemplary model EPNs (Liu *et al.,* 2012; Ye *et al.,* 2010, Zhang *et al.,* 2008; 2009; Torres-Barragan *et al.,* 2011). Several species from the genus *Oscheius,* share similar attributes with two other well-known EPN genera (*Steinernema* and *Heterorhabditis)* and display the characteristic attributes associated with these EPNs (Dillman *et al.,* 2012a; Torres-Barragan

et al., 2011). The genus *Oscheius* was first erected by Andrassy (1976). Liu *et al.* (2012) and Ye *et al.* (2010) used *Galleria mellonella* larvae to test the entomophathogenicity of *Oscheius chongmingensis* and *Oscheius carolinensis* which caused larval mortality in 56 h with mortality rate of 35% and larval mortality within 48 h with 100% larvae mortality, respectively.

The *Oscheius* genus comprises some of the following recently identified novel species of EPNs, *O. chongmingensis, O. rugaoensis* and *O. carolinensis* (Liu *et al.,* 2012; Torres-Barragan *et al.,* 2011; Ye *et al.,* 2010). The EPNs in all three genera have evolved mutualistic associations with three different genera of Gram negative, motile, rod-shaped bacteria belonging to the family Enterobactericeae, all of which are lethal pathogens to a wide range of insects. *Xenorhabdus* bacteria are located in the intestinal vesicle of the infective juveniles (IJs) of *Steinernema* (Bird and Akhurst, 1983); in contrast *Photorhabdus* bacteria are located throughout the whole intestine of *Heterorhabditis* IJs (Endo and Nickle, 1991) whereas bacteria belonging to the genus *Serratia* have been shown to be symbiotically associated with *O. chongmingensis* and are located within a pouch next to the pharyngeal bulb (Liu *et al.,* 2012).

Infective juveniles (IJs) penetrate the insect host through natural openings such as mouth, anus and respiratory spiracles and release the bacteria in the haemocel of the host (Poinar, 1975). In this relationship, EPNs are vectors for the bacteria, which are transported into the haemolymph of insect hosts (Boemare *et al.*, 1993). After releasing the symbiotic bacteria into the insect haemolymph, the bacteria replicates and suppress the insect host's immune response system by producing various metabolites which hamper and kill the insect host and further inhibit the growth of competing microorganism. In doing so, a monoxenic environment is maintained for the EPNs to reproduce, grow and develop (Hussien and Hanan, 2008; Wang *et al.,* 2011). The metabolites produced by the symbiotic bacteria have shown to have antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastic and antiviral properties and may have useful medical and agricultural applications (Wang *et al.,* 2011).

Various metabolites have been reported from entomopathogenic bacteria cell cultures, and these include: indole, dithiolopyrrolones and xenocoumacins, however, very little is known about the ones secreted by *Serratia* species. In addition to the work done thus far, we strongly believe that in further accepting the *Oscheius* as an EPN genus, the metabolites produced by its symbiotic bacteria can be studied and compared with the ones produced by *Xenorhabdus* and *Photorhabdus* sp., thereby examining the similarities and differences in metabolites

produced by the three bacteria. In this paper, we report on metabolites produced by a new strain of *Serratia marcescens; S. marcescens* strain *MCB* isolated from *Oscheius* sp. *MCB* which was obtained from South African soil.

Material and Methods

Isolation and morphological identification of the symbiotic bacterium. The symbiotic bacteria was isolated from *Oscheius* sp. *MCB* (GenBank accession number: KF684370) obtained from soil samples collected in North West province, South Africa. The methods used for isolating the bacteria were those described by Kaya and Stock (1997). The isolated bacteria were then grown and maintained on solid media namely Nutrient Agar supplemented with 0.004% (w/v) triphenyltetrazolium chloride (TTC) and 0.0025% (w/v) bromothymol blue (BTB) (NBTA) and MacConkey agar plates and sub-cultured on the same media on a monthly basis at 25°C (see Appendix B for agar preparation).

Long term maintenance of the bacterial cultures was done by suspending the cultures in 1 mL of 30% glycerol and stored at -70°C. Red colonies were isolated, characterized morphologically and Gram staining performed (Zhang *et al.*, 2008; Torres-Barragan *et al.*, 2011; Bergey *et al.*, 1994). To deduce if the symbiotic bacteria from *Oscheius* sp. *MCB* displays growth phases (primary and secondary) similar to *Xenorhabdus* and *Photorhabdus sp.*, colonies grown on NBTA and MacConkey agar plates were observed for the first 24 h of incubation and compared with colonies at 72-120 h. *Serratia marcescens* species have bioluminescence ability due to prodigiosin (Andreyeva and Ogorodnikova, 2015), thus the fluorescence ability of the symbiotic bacteria was determined with a Fluorescent Microscope equipped with an Olympus DP 80 video camera using the CellSens Dimension 1.9.1 software with (excitation wavelength 490–510 nm).

PCR amplification of the 16S rDNA from symbiotic bacteria. The DNA of the 16S rRNA gene of the symbiotic bacteria was amplified. Genomic DNA was isolated from the NBTA colonies using the ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050) (see Appendix B for protocol). The DNA extracted from the bacterial colonies was quantified using the NanoDrop ND-1000® spectrophotometer (Bio-Rad). The primers used for 16S rDNA amplification and sequencing were the 5`-AGAGTTTGATCCTGGCTCAG-3`f and 5`-AAGGAGGTGATCCAAGCCGCA-3`r (Brosius *et al.*, 1978), corresponding to positions 8-27 and 1521- 1540 in the 16S rDNA sequence of *Escherichia coli*. The PCR

products were cleaned using ExoSAP-it® and sequenced by Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

Sequence and phylogenetic relationship analysis of the symbiotic bacteria. The sequence was screened for chimeras by using DECIPHER tool (Wright *et al.*, 2012). The NCBI-BLAST search tool was used to establish the degree of similarity of the sequence with other sequences of known bacteria taxa in the GenBank database. Bacterial species from GenBank database with the highest matches after a Basic Local Alignment Search Tool (BLAST) search were selected for phylogenetic analysis.

We used *Serratia nematodiphila* strain *DR186* (JQ002568), *Serratia sp. R26 (2012)* (JX082294), *S. marcescens WW4* (NR_102509), *S. marcescens* strain *AU1209* (AY043387), *S. marcescens* clone *HUWU3B* (KC583444), *S. marcescens* strain *DAP33* (EU302858), *Serratia* sp. *C8* (JX258135), *Serratia urelytica* (AJ854062), *Serratia rubidaea* strain *DSM 4480* (AJ233436), *S. entomophila* (NR025338), *Serratia ficaria* strain *DSM 4569* (NR_041979), *P. luminescens* subp. *luminescens* strain *Hb* (NR_037074) and *X. bovienii DSM 4766* (X82252) species to observe the relationship that the current strain (*S. marcescens* strain *MCB* KF793930) has with the other species. *Bacillus cereus* (AB334763) was used to root the tree. The sequences were aligned using MUSCLE (Edgar, 2004), with default options and the distance analysis with closest taxa were conducted in MEGA6 (Tamura *et al.*, 2013). The evolutionary distances were computed using Jukes-Cantor model (Jukes and Cantor, 1969). Neighbour-joining tree of the homologous sequences in this study obtained from GenBank database was inferred (Saitou and Nei, 1987).

Selection of the optimal nutrient medium for metabolite production. Five loopful of log phase of the symbiotic bacteria obtained from NBTA/MacConkey agar plates were inoculated into each 100 mL flasks containing 20 mL of Luria-Bertani broth (LB), Nutrient Broth (NB), Nutrient Broth supplemented with canola oil and glucose and Nutrient Broth supplemented with canola oil only (Torre, 2003) (see Appendix B for broths preparation). The media were adjusted to final pH of 7.0 (Wang *et al.*, 2011). The flasks were incubated at 28°C for 48 h in the dark on an orbital shaker at 160 rpm. At hourly intervals, the cell culture was centrifuged and the supernatant was removed, the bacterial concentration was measured at 600 nm with an S-20 Boeco Spectrophotometer for 24 h. The media with highest bacterial yield was deduced to be the one suitable for the bacterial growth. This experiment was carried out in triplicates.

Metabolite identification and characterization. For purification and characterization of the metabolites, all media were analysed with an HPLC system (Model 510, waters, USA) and the metabolites were separated using a C18 reversed phase column (25 cm x 4 mm x 0.5 µm). A flow rate of 1.0 mL min^{-1} was used and the detection of separated metabolites was achieved using a fluorescence detector at excitation and emission wavelengths of 280 and 435 nm, respectively. The solvent used was a mixture of acetonitrile: water with 0.3% acetic acid (50:50 v/v) (see Appendix B for preparation). After the HPLC development, the method was validated in terms of limit of detection (LOD), limit of quantification (LOQ) and other parameters (Table 1).

Table 1. HPLC determination of tryptophan and indole-3-acetic acid including the concentrations.

Parameter	Analyte name	
	Tryptophan	Indole-3-acetic acid
Retention time (min)	2.00	3.13
Equation	y = 1.9714x + 976.16	y = 25.054x - 338.8
R^2	0.995	1.000
ψLOD (µg L^{-1})	46.7	7.99
ϡLOQ (µg L^{-1})	155.7	26.6

ψLOD is limit of detectation and ϡLOQ limit of quantification.

Role of metabolites on the bacterial growth. Different concentrations (mg L^{-1}) of the identified metabolites were used to determine effects on bacterial growth. Luria-Bertani broths with the supplemented metabolites produced were inoculated with a loopful of *S. marcescens* strain *MCB.* The produced metabolites were then detected with HPLC and the concentrations determined. LB without the metabolites was used as a negative control.

Results

Morphological characteristics. Colony morphology and cell morphology based on Gram staining of these bacteria were determined (Table 2). *Serratia marcescens* strain *MCB* was rod shaped with fluorescing ability and was located next to the pharyngeal bulb as well as throughout the intestines of adult females (Figure 1a-c).

Table 2. Morphological characteristics of symbiotic bacteria isolated from *Oscheius* sp. *MCB.*

Characteristic	**Symbiotic bacteria**
Host EPN	*Oscheius sp. MCB*
Symbiotic bacteria	*Serratia marcescens* strain *MCB*
Colony diameter on NBTA agar plate (mm)	1-4 mm
Colony morphology and pigmentation on NBTA agar (Phase I)	Deep red circular, with regular margins and smaller diameter
Colony morphology and pigmentation on NBTA agar (Phase II)	Brown, flat with irregular margins and larger in diameter
Pigmentation on MacConkey agar	Light pink
Pigmentation in nutrient broth	Light Maroon
Pigmentation in nutrient broth supplemented with canola oil	Light pink
Pigmentation in nutrient broth supplemented with canola oil and glucose	Light pink
Pigmentation in luria broth	Maroon
Optimum temperature	28°C
Gram stain	Gram negative
Fluorescence ability	Present
Morphology	Rod

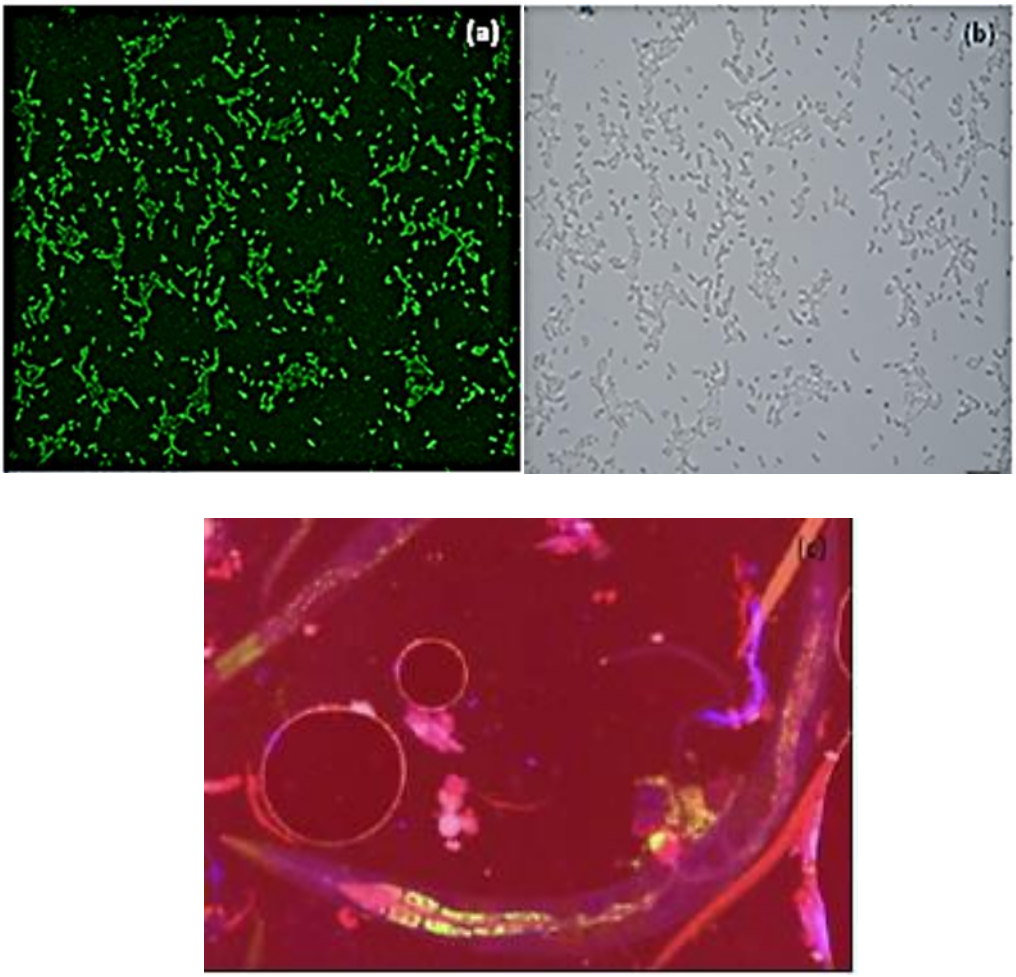


Figure 1. *S. marcescens* strain *MCB* under the light microscope (a) fluorescing rod shaped bacteria, (b) bacteria without fluorescence and (c) fluorescing bacteria (green coloured) within the *Oscheius* sp. *MCB* female adult nematode. (Scale bars: a=10µm; b=10µm; c=100µm)

Molecular identification. The 16S rDNA sequence product was 1152 base pairs (bp) and was deposited in GenBank and assigned the accession number KF793930. The BLAST search of this sequence showed that it belongs to the genus *Serratia*, with high sequence similarity identity with *S. marcescens* strain DAP33, *S. marcescens* strain AU1209, *S. marcescens sp.* clone HUWU3B, *S. marcescens sp.* C816, *S. marcescens* sp. R26 (2012) and *S. marcescens* WW4. Phylogenetic tree revealed a close relationship of *S. marcescens* strain *MCB* with *S. marcescens* strain DAP33 (bootstrap of 70%) as shown in Figure2. Neighbour-joining tree indicated that *S. marcescens* strain *MCB* and *S. marcescens* strain *DAP33* form a polytomy clade with four strains of *S. marcescens* and one *S. nematodiphila* (bootstrap of 52%).

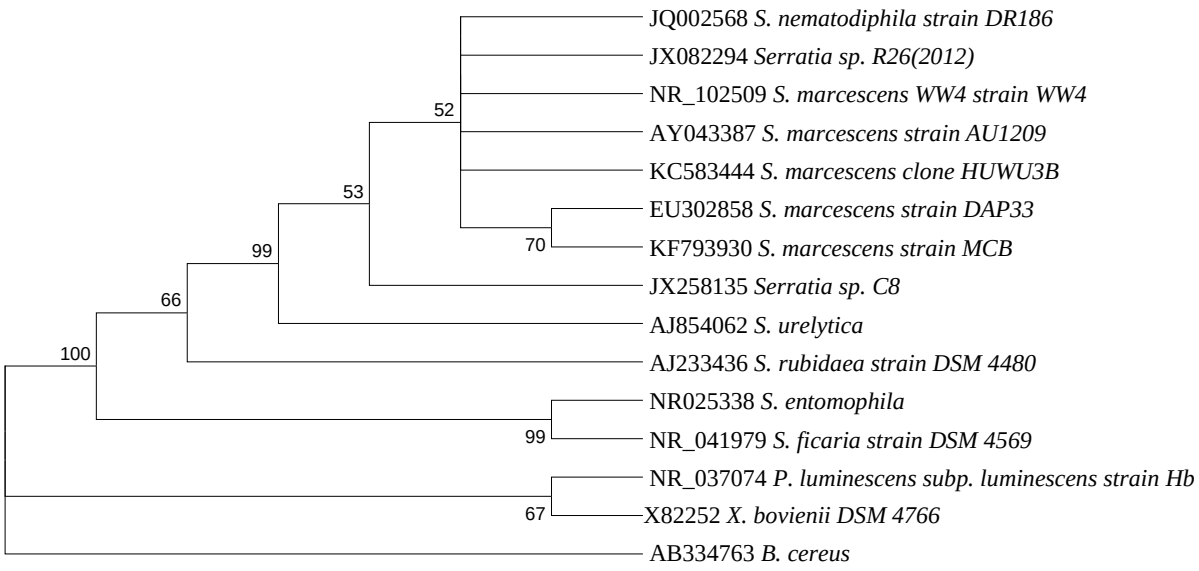


Figure 2. Neighbour-joining tree of *S. marcescens* strain *MCB* with closely related bacteria inferred by homologous sequences based on 16S rRNA gene sequence data. The nodal supports are bootstrap values from 1000 replications.

Selection of the optimal nutrient medium for antibiotic production. Distinctive colour changes were observed in all media types, that are, NB, NB with canola oil, NB with canola oil and glucose and LB inoculated with *S. marcescens* strain *MCB* (results not shown). The colour changes (Table 2) were a result of metabolites secreted by the bacteria as it proliferates and some metabolites have antibiotic activity against opportunistic bacterial and fungi that may be in the media. The colour changes in the media thus serve as a method to visually verify the purity of the bacterial culture.

The different growth media had significant effects on bacterial growth and metabolite production. Luria-Bertani broth (LB) showed a maximum bacterial yield, thus it increases bacterial growth and metabolism, increased bacterial yield results in an even increased antibiotic production due to nutrients available and a highly active metabolism and most antibiotics or metabolites get secreted in the stationery phase of bacterial growth (Thomashow, 1996). LB had a higher bacterial yield with an optical density of 1.5, followed by NB with an optical density of 0.9 at the 24th h and for NB supplemented with canola oil had the highest optical density of 0.6 at 19th h and NB supplemented with canola oil and glucose had optical density of 0.4 at the 16th h (Figure 3a and b).

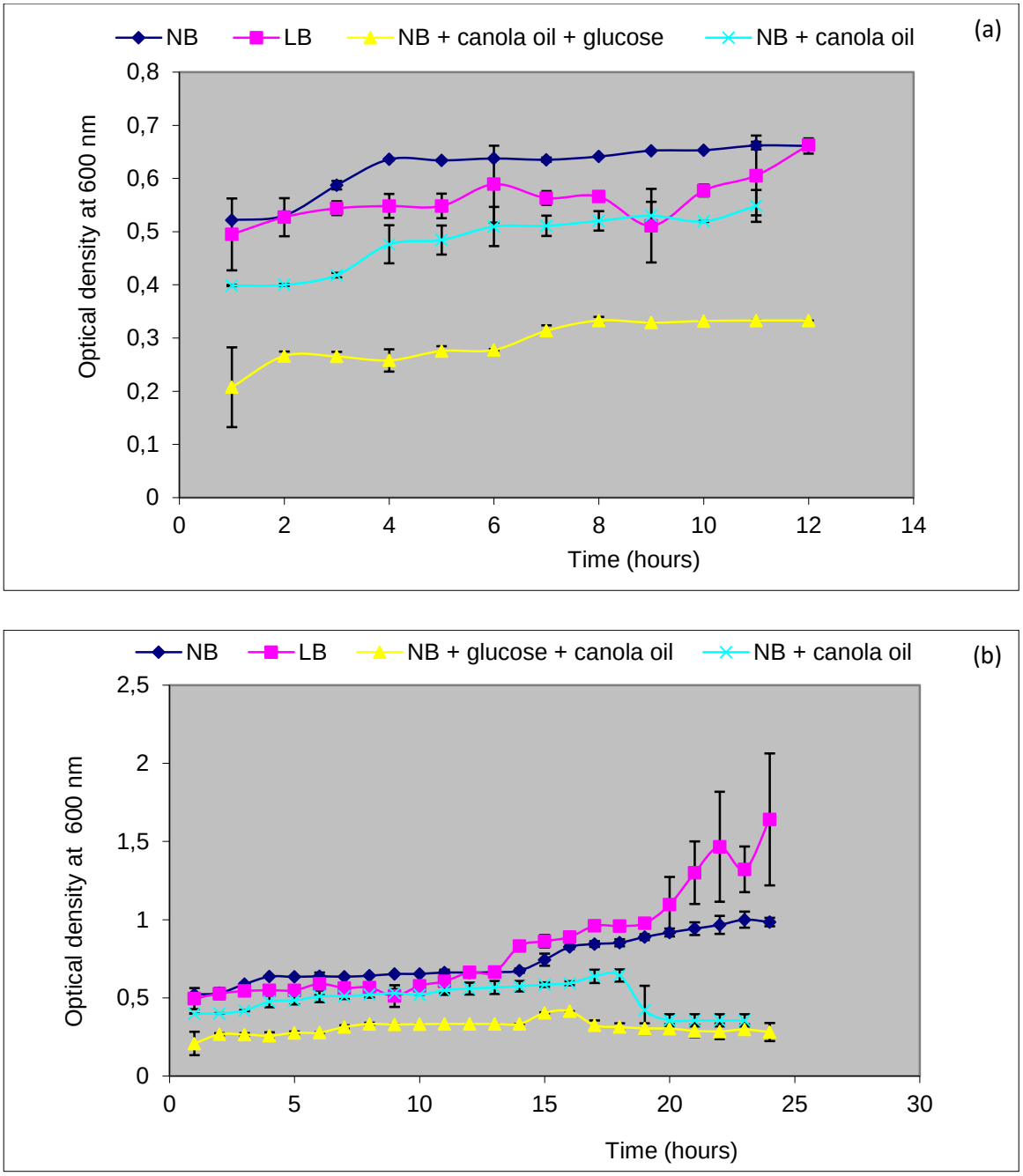
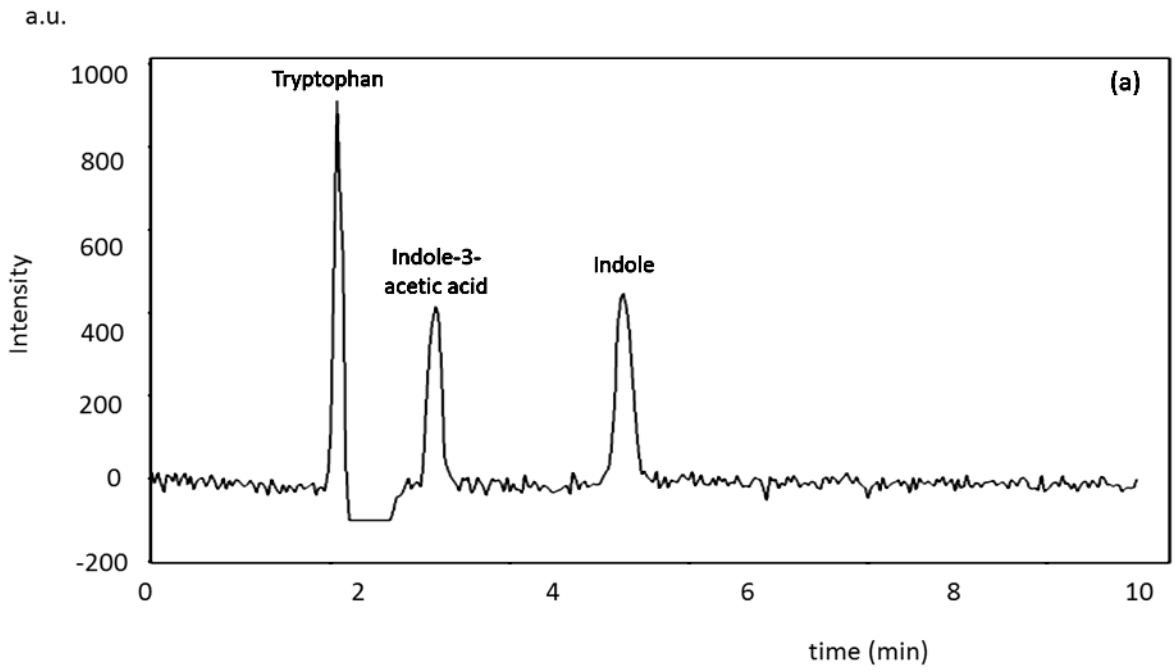
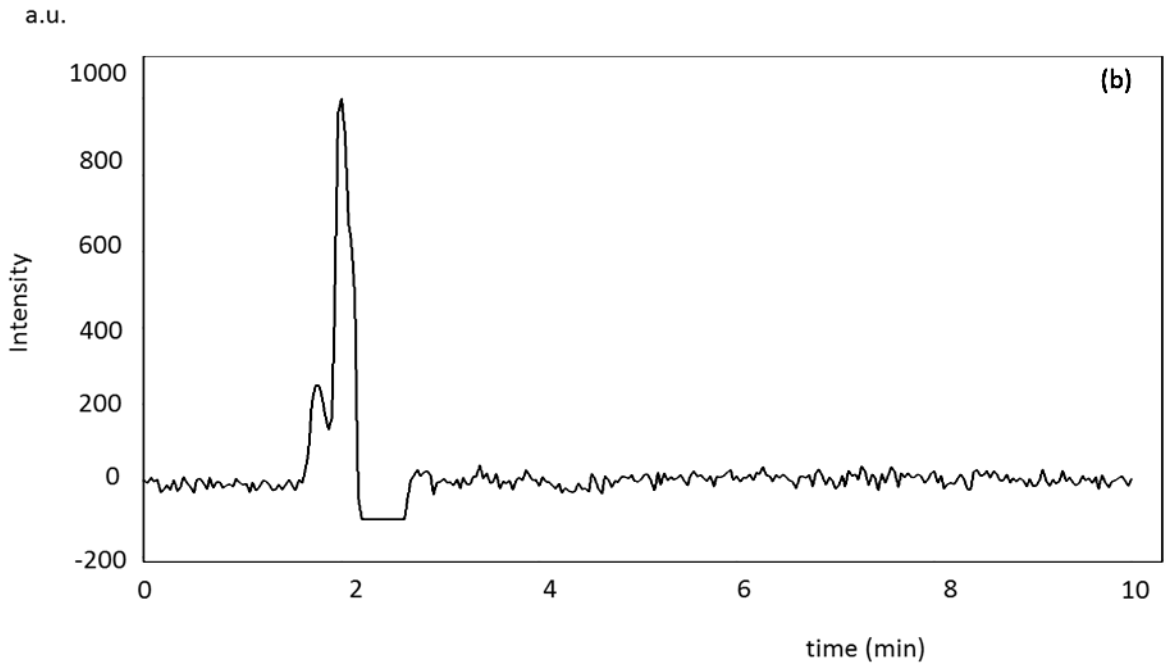


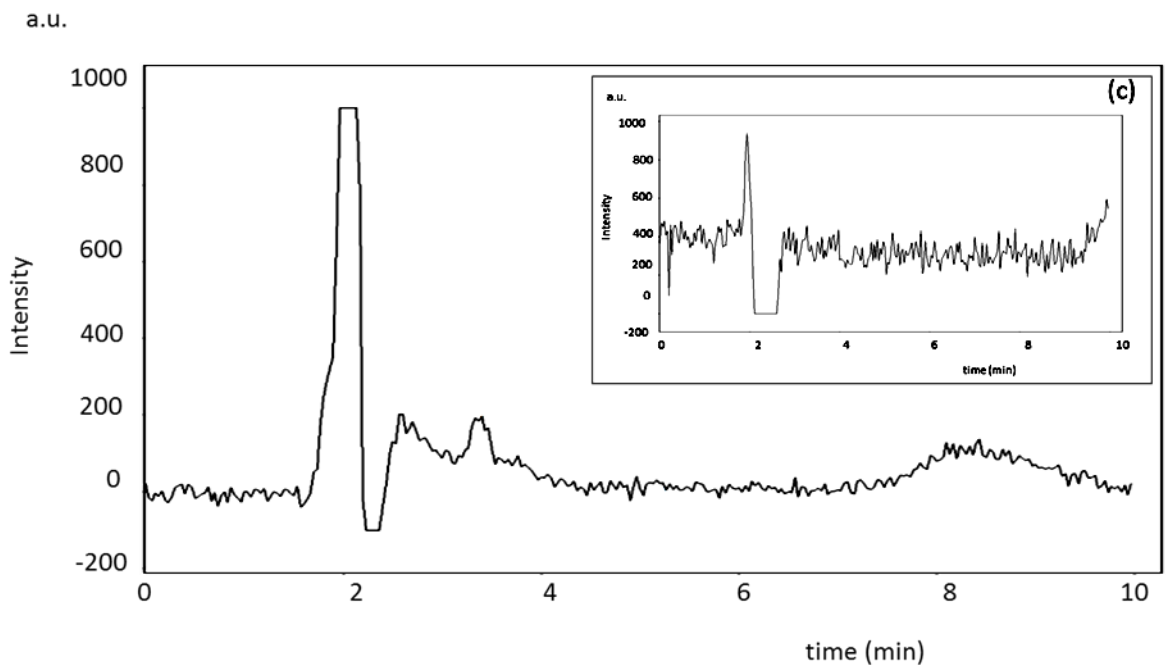
Figure 3. Absorbance over time at 600 nm of *S. marcescens* strain *MCB* in Luria-Bertani broth (LB), nutrient broth (NB), nutrient broth supplemented with canola oil and nutrient broth supplemented with glucose and canola oil at approximately 25°C on a shaker at 160 rpm for (a) 12 h and (b) 24 h. Each point represents the average of three replicates. Bars indicate size exclusion.

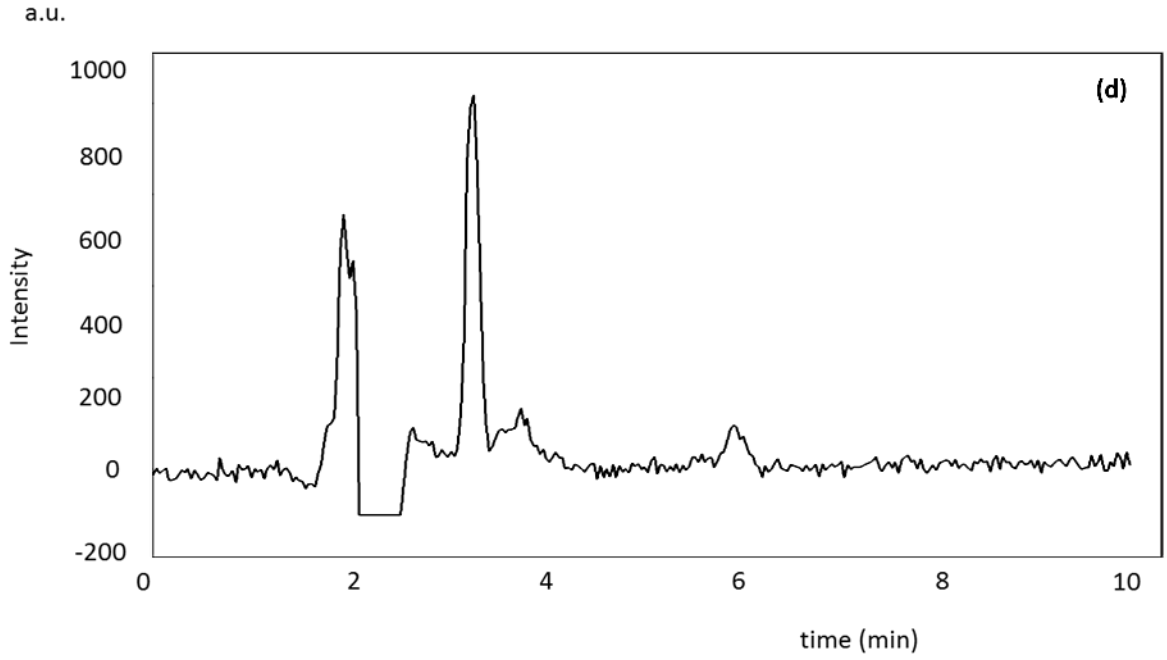
Metabolite identification and characterization. After 48 hours of incubation, the metabolites were analysed from all the four different media used. Three metabolites; tryptophan, indole-3-acetic acid (IAA) and indole were expected to be produced; in this study

only two were identified, tryptophan and IAA produced by *S. marcescens* strain *MCB* (Figure 4a to e). Tryptophan was detected first with high quantity concentration as compared to IAA (Table 3) and it was only produced in all the media whilst IAA was produced in LB and NB + canola oil.






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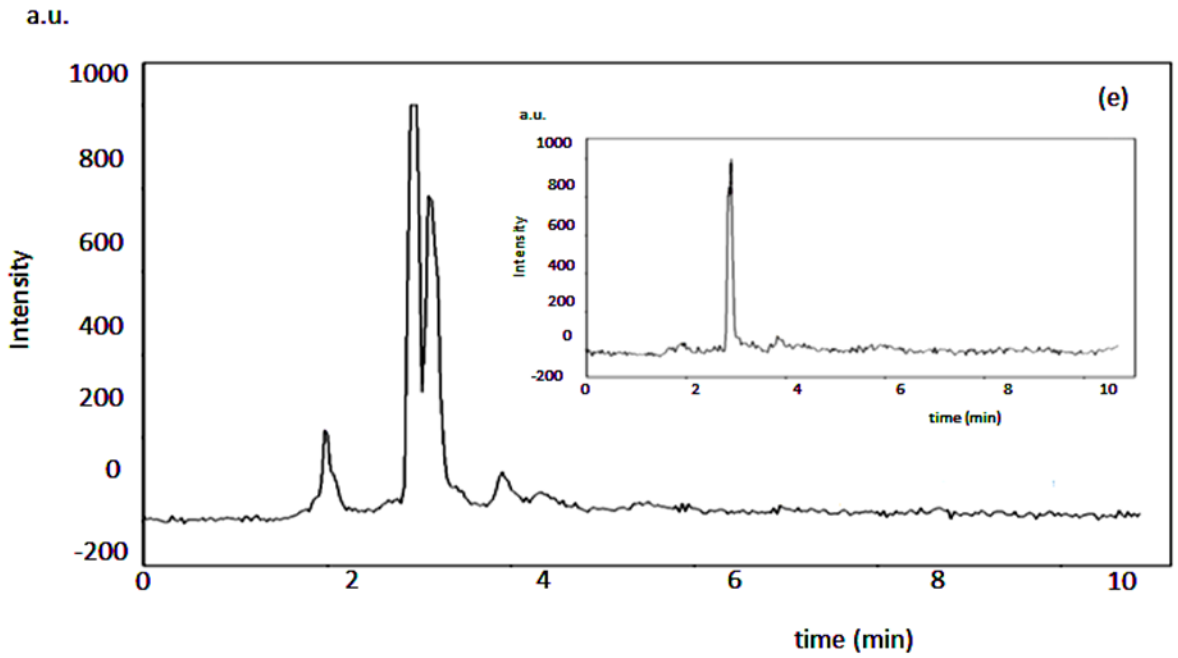


Figure 4. Chromatograms showing: (a) retention times of tryptophan, indole-3-acetic acid and indole standards, (b) 250X diluted NB sample, (c) 100X diluted NB + canola oil + glucose sample with 200X diluted, (d) 100X diluted LB sample and (e) 150X diluted NB + canola oil sample with 250X diluted.

Table 3. Concentrations of the metabolite from each media.

Media name	Concentration (mg L^{-1})		
	Tryptophan	Indole-3-acetic acid	Indole
LB	380	32	-
NB+ canola oil + glucose	42	11	-
NB	1194	-	-
NB+ canola oil	148	54	-

Role of metabolite on the bacterial growth. The bacterial growth increased with each increase in the increment in tryptophan concentration (results not shown). It was assumed that the aromatic amino acid tryptophan was the precursor for IAA production (Dewick, 2002), because from the metabolite analysis, the IAA production increased with an increase in

tryptophan, however there was no relationship between IAA in the broth and tryptophan production (Tables 4 and 5).

Table 4. Effects of tryptophan concentrations on tryptophan and indole-3-acetic acid production.

Concentration of tryptophan in LB (mg L^{-1})	Bacterial yield of (mg L^{-1})	
	Tryptophan	Indole-3-acetic acid
0	1123	45
10	1342	78
20	1402	83
30	1409	94

Table 5. Effects of indole-3-acetic acid concentrations on tryptophan and indole-3-acetic acid production.

Concentration of indole-3-acetic acid in LB (mg L^{-1})	Bacterial yield of (mg L^{-1})	
	Tryptophan	Indole-3-acetic acid
0	1124	45
10	1124	51
20	1134	55
30	1123	69

Discussion

A new species of *Oscheius* genus of entomopathogenic nematodes (EPNs) (Ye *et al.,* 2010) had been accepted as a genus displaying characteristic EPN adaptation which have been acquired through convergent evolution (Dillman *et al.,* 2012a); however more research needs to be done to support its acceptance as an EPN genus. One of the criteria for EPNs, is for their symbiotic bacteria to produce metabolites which not only kill the insect host but also other competitive microorganisms, thus, we strongly believe that analysing metabolites produced by symbiotic bacteria from *Oscheius* species, will shed light as to whether *Oscheius* symbiotic bacteria is similar to *Xenorhabdus* and *Photorhabdus* sp. In this paper, we managed to identify two compounds produced by *S. marcescens* strain *MCB,* namely tryptophan and IAA.

Indole derivative compounds produced by *Xenorhabdus* and *Photorhabdus* sp. have been identified and shown to inhibit growth of insect larvae and other microorganisms (Sundar and Chang, 1993; Hu and Webster, 2000; Paul *et al.,* 1981). Tryptophan was recently added to growth medium for bacteria to induce production of other antimicrobial metabolites (Sundar and Chang, 1993), we identified tryptophan as one of the metabolites produced and we strongly believe that tryptophan is a precursor for IAA production and other compounds (Sundar and Chang, 1993; Manulis *et al.,* 1991; Paul *et al.,* 1981; Martino *et al.,* 2003). Previous studies indicate that both *Xenorhabdus* and *Photorhabdus* sp. are biochemically and physiologically suitable as symbionts of entomopathogenic nematodes (Thaler *et al.,* 1998; Thomas and Poinar, 1979; Akhurst, 1980; 1982), due to production of these metabolites. The metabolites produced by these bacteria also contribute towards the symbiotic relationship with the entomopathogenic nematodes in providing a monoxenic growth environment and nutrients for the nematodes which in turn act as vectors.

In this study, various liquid media for *S. marcescens* strain *MCB* were used and it was found that LB resulted in higher bacterial yield which in turn lead to an increased metabolite synthesis and production (Figure 3a-b). Increased bacterial yield results in an increased level of metabolite production due to nutrient availability and a highly active metabolism (Thomashow, 1996). Luria-Bertani broth contains sodium chloride, yeast extract and tryptone, whereas nutrient broth contains meat extract, yeast extract, peptone and sodium chloride. Sodium chloride increases metabolite production due to its osmolarity and peptone, tryptone as well as yeast extract are good nitrogen sources (Wang *et al.,* 2011), which explains the high bacterial growth which resulted in high metabolite quantity and

concentrations in both media (Table 3). Peptone has been found to be a better nitrogen source as compared to tryptone (Wang *et al.,* 2011), this is validated by the high concentration of IAA produced in NB as compared to LB. Results reported here are similar to those reported in previous studies which indicate that both NB and LB are suitable for metabolite production of *S. marcescens* strain *MCB* due to presence of NaCl, peptone, tryptone and yeast extract which are good sources of nitrogen and also increase metabolite production (Wang *et al.,* 2011).

We have showed that media composition affects bacterial yield and quality and quantity of metabolite production. Tryptophan is a precursor of IAA which is produced by *S. marcescens* strain *MCB. S. marcescens* strain *MCB* isolated from *Oscheius sp. MCB* has similar attributes with *Xenorhabdus* and *Photorhabdus* species, thus it can be accepted as an entomopathogenic bacterium. Future studies may be focused on determining the genetic characteristics, mechanism, and the pathway that are responsible for the production of metabolites in *Serratia marcescens* strain *MCB* as well as genome comparison with *Xenorhabdus* and *Photorhabdus* species to ascertain evolutionary differences and similarities.

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Chapter 4:

Whole genome sequence of *Serratia marcescens* strain *MCB* reveals it is an entomopathogenic bacteria*

*This chapter was first published in American Society of Microbiology-Genome Announcements, titled "Draft Whole-Genome Sequence of *Serratia marcescens* strain *MCB*, Associated with *Oscheius sp. MCB* (Nematoda: Rhabditidae) Isolated from South Africa" (2014). It was written by MH Serepa and VM Gray. Please note that on publishing this paper the name of the isolated host nematode was not decided, thus it was initially called *Oscheius sp. MCB.*

**The supplementary methods for this chapter can be found in Appendix C.

Abstract

Here we report on the draft genome sequence of *Serratia marcescens* strain *MCB* associated with *Oscheius sp. MCB* (Nematoda: Rhabditidae) isolated from South African soil. *Serratia marcescens* strain *MCB* has 5,304,212 bp genome size with 4,877 genes and a G+C content of 59.1%. The genome comparison of *Serratia marcescens* strain *MCB* with other entomopathogenic bacteria indicates a high similarities of protein coding genes involved in parasitism against insect pests.

Introduction

Serratia represents a genus of bacteria that are Gram negative, motile, rod-shaped and belongs to the Enterobacteriacea family. *Serratia* species have been isolated from plants, vertebrates and invertebrates with over seventy species found to be associated with insects (Grimont *et al.,* 1979; Chung *et al.,* 2013). Recently *Serratia* bacteria were found to be mutually associated with the newly discovered entomopathogenic nematodes (EPNs) genera, *Oscheius carolinensis, O. chongmingensis* and *O. rugaoensis* (Liu *et al.,* 2012; Ye *et al.,* 2010; Torres-Barragan *et al.,* 2011; Zhang *et al.,* 2012). The mutual relationship of this third group of EPNs, *Oscheius* with *Serratia* species has many similarities with the association that *Steinernema* and *Heterorhabditis* have with the symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, respectively. *Serratia* species associated with *Oscheius* EPN species also secretes various metabolites some of which have the ability to kill insect hosts and inhibit growth of competing bacterial and fungal species, as is the case with *Xenorhabdus* and *Photorhabdus* (Wang *et al.,* 2012). One metabolite with antibiotic activity secreted by *Serratia marcescens* is prodigiosin (Wang *et al.,* 2012; Harris *et al.,* 2004). Here we present the description of the draft genome sequence and the annotation of *Serratia marcescens* strain *MCB* associated with *Oscheius sp. MCB* (GenBank accession number: KF684370) (Nematoda: Rhabditidae) which was isolated from South Africa.

Materials and Methods

DNA isolation and Sequencing. *Serratia marcescens* strain *MCB* was isolated from *Oscheius sp. MCB* nematodes according to methods described by Kaya and Stock (1997). Genomic DNA was isolated from solid bacterial colony cultures using the ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050). The DNA extracted from the bacterial colonies was quantified using the NanoDrop ND-1000® spectrophotometer (Bio-Rad) and then cleaned with ZR fungal\bacterial DNA clean and concentrator™-5 (catalog #D4003S). Genomic DNA (50µg) paired-end libraries were

generated with NextEra DNA sample kit (Illumina) and indexed using NextEra DNA index kit (Illumina) as per the manufacturer's instructions. The main libraries (Paired-end) ranged between 200-600bp. Paired-end (2 × 300bp) sequencing was performed on MiSeq (Illumina) using the MiSeq reagent kit v3 at the Agricultural Research Council (ARC) Biotechnology Platform.

Genome assembly and annotation. Quality adapter trimming was performed on CLC Genomics Workbench v7 (CLC bio). A total of 2,169,542 paired-end reads at 197 × coverage were obtained from this workflow. The genome was assembled using the *de novo* assembly tool in the CLC bio, which produced 104 contigs with an average length of 51,002 bp and an N_{50} of 157,248 bp. Genome annotation was performed using NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP). For annotation comparison with other bacteria, Rapid annotation using subsystem technology (RAST) was also used to annotate the genome (Aziz *et al.,* 2008). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JPQY00000000.

Results and Discussion

The genome of *S. marcescens* strain *MCB* has 5,298,782 bp, with G+C (59.1%) content that is similar to other *Serratia* species (Neupane *et al.,* 2012; Chung *et al.,* 2013; Kwak *et al.,* 2015). *S. marcescens* strain *MCB* genome has 4,877 genes, among the identified genes 4,756 are protein coding sequence genes (CDS) and 21 are pseudogenes (Tables 1 and 2). The genome also has 14 rRNA genes with five operons (5S, 16S and 23S) and 76 tRNAs genes.

Comparison of the annotation pipelines, PGAAP and RAST resulted in minor differences (Table 1). We registered the annotation results from PGAAP on GenBank because the PGAAP pipeline screens the submitted assembly for possible contamination and there is also an option for the submitter to remove the contaminated assembled sequences before annotation proceeds, whilst RAST does not offer this platform. There are however some features that RAST has and PGAAP does not have such as Subsystem Information as gown in Figure1, which shows the subsystem feature counts, with the highest counts observed in carbohydrates, amino acids and derivatives, RNA metabolism and cofactors, vitamins and pigments. In addition RAST offers genome comparison of the annotated genome based on function, sequence and comparison via Kyoto Encyclopedia of Genes and Genomes (KEGG) and as well as Basic Local Alignment Search Tool (BLAST) against other closely related microorganisms (Aziz *et al.,* 2008).

We compared the assembled genomes of *Serratia marcescens* strain *MCB* and *Photorhabdus luminescens subsp. laumondii TTO1*, using the latter as a reference genome and as shown in Figure 2 (a) and (b), *Serratia marcescens* strain *MCB* has a high number of both reverse and forward hits (bidirectional best hits) with approximately 80% similarity in protein sequences to *Photorhabdus luminescens subsp. laumondii TTO1*. Few hits with protein sequence similarity of above 95% were observed. About 15% of *Serratia marcescens* strain *MCB* genome shows 10% bidirectional hits with 10-20% protein sequence similarity.

A hundred and eleven subsystem features were counted to be involved in virulence, disease and defence (Figure 1), we suggest the high number of may be due to interrelation between genes. We found ten genes responsible for antibiotic synthesis and five virulence factors. Genomic comparison of *Serratia marcescens* strain *MCB* with *Serratia nematodiphila* (Table 2) indicate the two are highly similar; these are the only two *Serratia* species fully sequenced which are associated with nematodes. Eight of the predicted antibiotic genes are similar to *Photorhabdus luminescens subsp. laumondii TTO1* which was fully sequenced and annotated as well as some *Xenorhabdus* species (Table 3) (Duchaud *et al.,* 2003). These may be involved in the biosynthesis, metabolism, transcription, translation and transport of the antimicrobial metabolites (Duchaud *et al.,* 2003; Bender *et al.,* 1999). *S. marcescens* strain *MCB* genome encodes for a cytotoxin which might play a role in insect killing or acting synergistically with the nematode host in doing so, in addition antitoxin and toxin-antitoxin system were also predicted and these are efficient in killing other bacterial and fungal species (Duchaud *et al.,* 2003). A number of bacteriocins encoding proteins have been identified such as colicin V.

Table 1.Outcome comparison of *Serratia marcescens* strain *MCB* genome annotation using PGAAP and RAST. *

Feature	PGAAP	RAST
Genome size	5,298,782 bp	5,304,212 bp
Total number of genes	4,877	4,969
Protein coding genes	4,756	4,879
Number of RNAs	90	90
G+C %	59.1	59.7
Number of contigs (with PEGs)	103	104

*Only the PGAAP results were registered with GenBank.

Table 2. Comparison of *Serratia marcescens* strain *MCB* genome with *Serratia nematodiphila* (Kwak *et al.*, 2015).

Feature	***Serratia marcescens* strain *MCB***	***Serratia nematodiphila***
Genome size	5.3Mb	5.2Mb
Total number of genes	4 877	4 789
Protein coding genes	4 756	4 629
Antimicrobial producing gene	10	n/a
Virulence factors	5	n/a
G+C %	59.1 %	59.5%

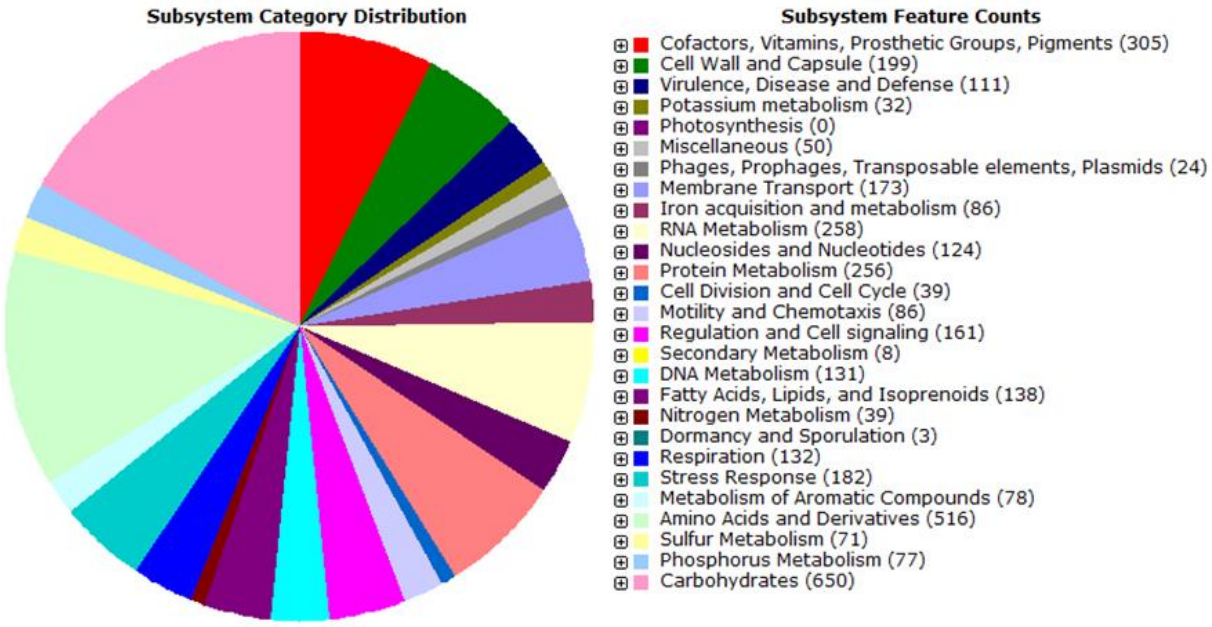
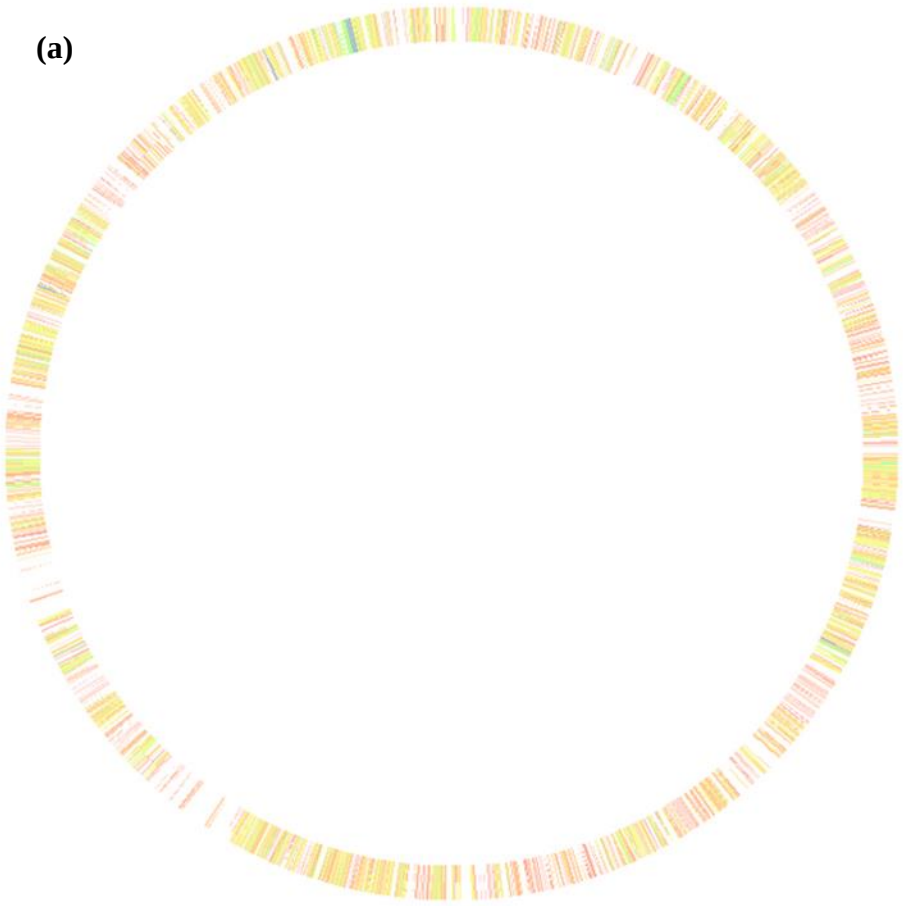


Figure 1. Subsystem feature counts of *Serratia marcescens* strain *MCB* generated by RAST annotation database.

(a)



(b)

	Percent protein sequence identity															
Bidirectional best hit	100	99.9	99.8	99.5	99	98	95	90	80	70	60	50	40	30	20	10
Unidirectional best hit	100	99.9	99.8	99.5	99	98	95	90	80	70	60	50	40	30	20	10

Figure 2. (a) Alignment of *Serratia marcescens* strain *MCB* against *Photorhabdus luminescens subsp. laumondii TTO1* (used as reference genome), colour co-ordination similarity can be viewed in figure2b, empty space indicate no similarity of *S. marcescens* strain *MCB* to *Photorhabdus luminescens subsp. laumondii TTO1* genome . (b) The protein sequence similarity percentage, bidirectional best hit refers to both forward and reverse hits.

*Further information on genome sequence comparison analysis can be found at http://theseed.org/wiki/SEED_Viewer_Manual/MultiGenomeCompare (Overbeek *et al.*, 2013).

Table 3. *Serratia marcescens* strain *MCB* potential genes involved in antibiotic biosynthesis.

Contig number	Gene name/locus	Size (aa)	Product name	*Similar Organisms
5, 26, 37 and 43	IY40_04900 ,{IY40_17535 IY40_17095},IY40_21040 and IY40_22800 respectively	181, 100 x 2, 105 and 115 respectively	Antibiotic biosynthesis monooxygenase	*Xenorhabdus bovienii* *Serratia marcescens subsp. marcescens Db11* *S. proteamaculans 568* *Photorhabdus luminescens subsp. laumondii TTO1*
6 and 29	IY40_05405 and IY40_19210 respectively	74 and 68 respectively	MbtH-like protein	*X. bovienii SS-2004* *P. luminescens subsp. laumondii TTO1* *S. plymuthica S13* *S. marcescens subsp. marcescens Db11* *P. asymbiotica*
18	IY40_12625	210	Antibiotic Acetyltransferase	*X. bovienii* *P. luminescens subsp. laumondii TTO1* *S. plymuthica S13* *P. asymbiotica*
13	IY40_10210	384	Antibiotic ABC Transporter permease	*S. marcescens subsp. marcescens Db11* *P. luminescens subsp. laumondii TTO1* *S. plymuthica S13*
4	IY40_03395	602	Antibiotic ABC transporter substrate-binding protein	*S. marcescens subsp. marcescens Db11* *S. plymuthica S13*
12	IY40_09610	197	Antibiotic transporter	*S. marcescens subsp. marcescens Db11*

*Similar microorganisms were obtained through BLAST of the protein sequences on the NCBI protein database.

We identified two genes which encode for invasion proteins involved in bacterial adhesion to nematode gut; these have also been identified in *P. luminescens subsp. laumondii TTO1* and *Yersinia* species (Duchaud *et al.,* 2003; Gustavsson *et al.,* 2002). An example is leucine-responsive regulatory (Lrp) which is involved in nematode colonization and development as well as in regulating factors involved in pathogenesis and mutualism; this was also identified in *X. nematophila* (Cowles *et al.,* 2007). *Serratia marcescens* strain *MCB* has 10 genes encoding fimbrial proteins as well as type IV pili protein, these proteins assist the bacteria to colonize the nematode gut as well as invading different insect cells and have been identified in *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, and *P. luminescens* TT01 (Duchaud *et al.,* 2003; Srimanote *et al.,* 2002; Zhao *et al.,* 1997). *S. marcescens* strain *MCB* encode for many enzymes and proteins which assist in killing the insect host and also bioconvert the insect cadaver for nematode nutrition, growth and development (Duchaud *et al.,* 2003). Ten lipases were identified and a large number of phospholipase A-like proteins, in addition twenty-six proteases were identified such as CAAX protease, serine proteases and metalloproteases.

Chitin is an important biopolymer in nature, it is found in fungi, arthropods and nematodes; in insects it forms a supporting protective layer of the epidermis (Merzendorfer and Zimoch, 2003). For *S. marcescens* stain *MCB* to be an insect pathogen, it must produce enzymes which can degrade chitin, and as expected, chitinase and chitin-binding proteins were identified, in addition to these, holin protein coding genes were identified which are involved in degrading host's cell wall (Wang *et al.,* 2000). Some *Serratia* species are human pathogens, however it was reported that pigmented *Serratia* are insect pathogens and also produce chitinase, and these only cause human infection occasionally or accidentally (Grimont *et al.,* 1979). It is only nonpigmented *Serratia* species which cause human infections and these do not produce chitinase (Grimont *et al.,* 1979).

Since the symbiotic bacteria has two ecological environments; the nematode gut and the insect haemolymph, a lot of regulation is required to ensure bacterial adaptation and survival within these environments, thus changes in the environments have to be detected, we identified more than 250 transcriptional regulators that are tandemly organized, in addition two sigma factors were identified such as RpoD and RpoS. A luciferase coding gene has been identified and it is responsible for production of bioluminescence (Kaya and Stock, 1997). *Serratia marcescens* strain *MCB* has similar protein coding genes as *P. luminescens TT01*,in

the current and other previous studies *Serratia* genus has shown potential as a biocontrol bacteria (Liu *et al.*, 2012; Ye *et al.*, 2010; Torres-Barragan *et al.*, 2011; Zhang *et al.*, 2012).

This is one of the two studies that have sequenced *Serratia* species associated with nematodes. The genome of *Serratia marcescens* strain *MCB* reveals the mechanisms of endosymbionts and useful factors which can be applied in agriculture such as biological pest control, and its genome comparison with that of *P. luminescens TT01* indicate that *Serratia marcescens* strain *MCB* is an entomopathogenic bacterium as most of the genetic elements are similar. We believe the information arising here will shed light as to the global function of *Serratia* species, as previously there was uncertainty regarding this (Grimont and Grimont, 1978a).

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Chapter 5:

Whole genome sequence of *Oscheius safricana**

*****This chapter is not yet published but is currently in preparation and the current form is solely written by Mahloro H. Serepa. It will ultimately include additional information as further sequencing and analysis is underway.

**The supplementary methods for this chapter can be found in Appendix D.

Abstract

The explanation of entomopathogenic nematodes (EPNs) was recently expanded to include the genus *Oscheius.* Further studies could prove the stance of *Oscheius* genus as entomopathogenic nematodes. Here we report on the genome of *Oscheius safricana* (Nematoda: Rhabditidae) isolated from South African soil. *Oscheius safricana* has 128,821,054 bp genome size and a G+C content of 45.9 %. The genome has 19,825 protein coding genes, of which some code for chitinases, lipases and proteases. These indicate that *Oscheius safricana* is an EPN as most of these genetic elements are similar to *Heterorhabditis bacteriophora.* The genome sequence of *Oscheius safricana* provides genetics, genomics and evolutionary studies into parasitism and symbiosis of EPNs.

Introduction

The phylum Nematoda comprises more than 100 000 species, which inhabit various ecological environments and have also been classified into different functional groups (Blaxter *et al.,* 1998). The complete genome assembly and annotation for *Caernorhabditis elegans* and *C. briggsae* provides us with reference genomes for comparative genomics. Comparative genomics is important for investigating not only evolutionary relationships but also for investigating the molecular genetics underlying the symbiotic and pathogenic relations between nematodes and other organism. So far the genomes of approximately thirty different nematode species have been sequenced; however more access to nematode genomic information will be required to increase our understanding of nematode evolution and nematode interactions with other species and various functional groups.

To date most of the word done on entomopathogenic nematodes (EPNs) has focus on species belonging to the two genera, *Steinernema* and *Heterorhabditis*. Species belonging to these two genera are associated symbiotically with Enterobactericea falling under *Xenorhabdus* and *Photorhabdus* genera; respectively (Adams and Nguyen, 2002). The nematode-bacterium duo is very specific and is under the differential genetics since the symbiotic bacteria have functional relations with two different kinds of ecological environments, one within the non-feeding infective juvenile (IJ) and the other within insects. We believe that the molecular genetic study of both the nematode and its symbiotic bacterium will reveal many insights into the nature of the symbiosis and parasitism. Comparative genomes will assist in increasing our understanding of the genes responsible for the regulation of the bacterial symbiotic and pathogenic relations with the nematode and insect host; respectively. Protein-coding genes involved in secondary metabolic biosynthetic

pathways for antimicrobial and insect virulence factors may have relevance to drug development and application in crop engineering.

In 2012 the functional group comprising the EPNs was expanded to include the genus *Oscheius* (Dillman *et al.,* 2012a). In this study we include the genomic information of *Oscheius safricana* which was isolated from South Africa; we believe the information arising here will determine if this genus is a true EPN with comparison of other sequenced EPN species such as *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* (Bai *et al.,* 2013; Dillman *et al.,* 2015).

Materials and Methods

Nematode culture. *Oscheius safricana* infective juveniles (IJs) were isolated from soil samples from collected North West province of South Africa. The nematodes were extracted from the soil samples by the baiting technique using late-instar *Galleria mellonella* larvae (Kaya and Stock, 1997). *Oscheius safricana* was inbred up to thirteen generations to eliminate heterozygosity when sequencing the genomic DNA (see Appendix D, for supplementary method).

DNA Isolation and Sequencing. Genomic DNA was extracted from IJs using the protocol from Pure Gene® DNA Purification Kit (Gentra Systems 2003). The extracted DNA was quantified using the NanoDrop ND-1000® spectrophotometer (Bio-Rad). DNA Clean & Concentrator™-100-Zymo Research kit was used to clean and concentrate the DNA. Genomic DNA (50µg) paired-end libraries were generated with NextEra DNA sample kit (Illumina) and indexed using NextEra DNA index kit (Illumina) as per the manufacture's instruction. The main libraries (Paired-end) had inserts of ~200bp. Paired-end (2 × 125bp) sequencing was performed (Illumina) using the HiSeq at the Agricultural Research Council (ARC) Biotechnology Platform.

Genome assembly. Quality scores of the raw sequence data were obtained with FastQC software (Andrews, 2010) and further sequence processing (quality trimming) was conducted with Trimmomatic (Bolger *et al.,* 2014), with trimming parameters ILLUMINACLIP, MAXINFO and MINLEN used. The ILLUMINACLIP parameter removes adapters and Illumina-specific adapters (Bolger *et al.,* 2014). MAXINFO is an adaptive quality trimmer that balances read length and error rate to maximise the value of each read and MINLEN was used to specify the minimum length of reads to keep after trimming (Bolger *et al.,* 2014). A *de novo* genome assembly was performed using Velvet v1.2.10 (Zerbino, 2008) and assessed

for genome completeness using BUSCO software v1.1 (Felipe *et al.*, 2015). BUSCO software determines the completeness of a genome assembly or genome annotation using assemblies or annotations of available sequenced genomes (Felipe *et al.*, 2015). In this case we used *C. elegans* as the reference genome for assessing the genome completeness of *O. safricana*. The results of this whole genome shotgun project have been deposited at DDBJ/EMBL/GenBank under the accession JYHL00000000*.

* JYHL00000000, has not yet been released as we are still working on increasing the genome completeness.

Filtering bacterial symbiont DNA and other bacterial DNA contaminants from nematode genome. The assembled genome of *Oscheius safricana* was blasted on GenBank database and all the contigs with non-nematode identity were removed and genome assembly repeated.

Genome annotation. The scaffolds were masked for repeats using RepeatMasker v3.3.0 (Smit and Hubley, 2010). Transfer RNA (tRNA) coding genes were predicted using tRNAscan-SE (Lowe and Eddy, 1997). Protein coding genes were predicted with parameters optimized for *Caenorhabditis elegans* (options: --strand=both --genemodel=complete --species=*caernorhabditis* --outfile=gff inputfile) in AUGUSTUS v2.6 software (Stanke *et al.*, 2004). The protein sequences were searched against *C. elegans* protein database using BLASTP program with an *E*-value cutoff value of 1e-10. The *C. elegans* protein database was downloaded from ftp://ftp.ensembl.org/pub/release-82/fasta/caenorhabditis_elegans/pep/ (Ensembl Release 82). Protein domains of the predicted protein-coding genes were predicted by using HMMscan program from HMMER by searching against the Pfam database (downloaded from ftp://ftp.ebi.ac.uk/pub/databases/Pfam/) (Finn *et al.*, 2008). An *E*-value cutoff of 1e-6 was used in the prediction.

Results and Discussion

Genome assembly. A total of 70,514,418 paired end reads totalling 181,050,796 base pairs were obtained from *Oscheius safricana*. After quality trimming and assembly, the draft genome had 3500 scaffolds with N_{50} of 12281 bp. The genome of *Oscheius safricana* has 128,821,054 bp with G+C (45.9%) content. The G+C content of *Oscheius safricana* is very high compared to *C. elegans* (35.4) and *H. bacteriophora* (32.2%) (Table 1), it is however similar to *O. tipulae* nematode with G+C content of 43.3% (Ahn and Winter, 2006) and *Steinernema* species (45.53% *S. carpocapsae*, 47.98% *S. scapterisci*, 46.99% *S. feltiae*,

47.63% *S. glaseri* and 42.01% *S. monticolum*) (Dillman *et al.,* 2015). The genome assembly of *Oscheius safricana* is 62% partially complete.

Table 1. Comparison of *Oscheius safricana* genome with genomes of *Heterorhabditis bacteriophora* (Bai *et al.*, 2013), *Caenorhabditis elegans* (Stein *et al.*, 2003)

Feature	*Oscheius safricana*	*C. elegans*	*H. bacteriophora*
Genome size Mb	128	100	80
Contigs	73296	n/a	n/a
Scaffolds	3500	n/a	1263
Genome completeness %	62%PC	100%C	80%PC
Protein coding genes	19 825	21 193	21 250
Exons	114980	n/a	n/a
Introns	94556	n/a	n/a
Non-protein coding genes	881	927	258
G+C %	45.9	35.4	32.2

PC is partial completeness, C is complete

Protein-coding genes. *Oscheius safricana* genome has 19,825 protein coding genes. 14,795 of the predicted protein-coding genes had significant homology to *C. elegans* (Ensembl Release 82) (Figure 1) with an *E*-value cutoff of 1e-10 while 5,030 had no homolog and these did not have any sequence similarity to known proteins in the GenBank database which could mean they are unique to *O. safricana*. The number of predicted proteins in *O. safricana* is similar to other sequenced nematode species such as *C. elegans* and *C. briggsae* (Stein *et al.,* 2003). Although the genome *Oscheius safricana* was partially complete most of the protein-coding genes were accounted for.

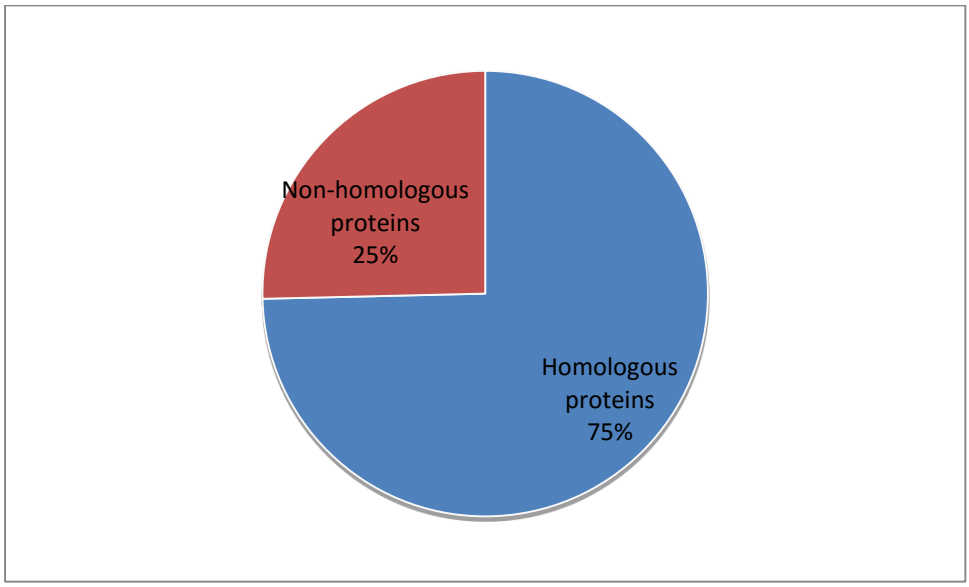


Figure 1. Pie chart showing the fraction of protein coding genes of *Oscheius safricana* with homology (~75%) and non-homology (~25%) to *C. elegans* protein database (Ensembl Release 82).

Non-coding RNA (ncRNA) and Regulatory elements. A total of 738 small RNA (smRNA) genes were identified in *O. safricana* genome. In addition U1, U6 and U5 small nuclear RNA (snRNA) have been identified. The ncRNAs are involved in regulation of transcription, translation and other biological processes such as gene regulation, DNA replication and RNA splicing. A total of 140 transfer RNA genes were predicted in *Oscheius safricana* genome for all 18 of the 20 standard amino acids. The tRNA-Selenocysteine and tRNA-Asparagine genes were not identified. No pseudo tRNA genes were predicted. The number of detected tRNA genes is very low as compared to *C. elegans* (659 tRNA genes and 29 tRNA pseudo genes) and *Heterorhabditis bacteriophora* (254 tRNA genes and 1 pseudo tRNA gene) (The *C. elegans* Sequencing Consortium, 1998; Bai *et al.,* 2013). However, the

number of tRNA genes are intermediate between those identified in human parasitic nematode *Brugia malayi* (233 tRNA and 26 pseudo tRNA) and plant parasitic nematode *Meloidogyne incognita* (28 tRNA and 120 pseudo tRNA) (Bai *et al.,* 2013). In total the number of identified ncRNA is closer to 962 and 838 ncRNA genes in *C. briggsae* and *C. elegans*, respectively (Stein *et al.,* 2003).

Protein domains. The predicted proteins have a total of 12,978 Pfam domains with 4,741 different Pfam accession numbers. We compared top 20 most prevalent Pfam domains in *Oscheius safricana* with *C. elegans* and *H. bacteriophora* (Fig2) (Bai *et al.,* 2013; Stein *et al.,* 2003). In comparison with the two nematode species *Oscheius safricana* appears to have a higher number of Pfam protein domains enriched with various families of G protein-coupled receptors (GPCRs), collagen triple helix repeat and immunoglobulin I-set domains among others (Figure 2).

GPCR gene families are involved in chemosensory actions within nematodes, so we compared the GPCR gene families of *Oscheius safricana* with those of *C. elegans* and *H. bacteriophora* (Figure 3) and discovered that there are various counts and families within the three genomes, the GPCR-Srbc gene family is only present in *C. elegans* and absent in the two EPN species; *O. safricana* and *H. bacteriophora.* This can account for the different life styles and functional groups such as cruisers and ambushers, with cruisers having a high number and diversity of the GPCR gene families. The same observation has been made in a previous study where cruisers such as *Brugia malayi* had low number and diversity of the GCPR gene families (Ghedin *et al.,* 2007).

The main survival and reproduction strategy of EPNs depends on their search behaviour, where an insect host must be located and necessary resources for growth, development and maintenance of reproduction be acquired from the host (Wilson *et al.,* 2012).This however depends on the type of foraging strategy the EPN has. Foraging strategies of EPNs have been classified into cruise forager and ambush forager, with undefined foragers lying in between as intermediate foragers (Wilson *et al.,* 2012). Cruise foragers move through the soil in search of insect hosts and in contrast, ambush foragers have adopted a sit-and-wait strategy for insect hosts (Lewis *et al.,* 1992; Wilson *et al*., 2012). In previous studies it was stated that differences in foraging behaviour may influence the efficacy of EPNs as biocontrol agents (Gaugler, 1988; Lewis *et al.,* 1992). Thus the different number and diversity of the GPCR gene families observed in this study and previous studies mentioned above will have to be

studied further to determine if this play a role in determining the type of insect that can be attracted to the EPN.

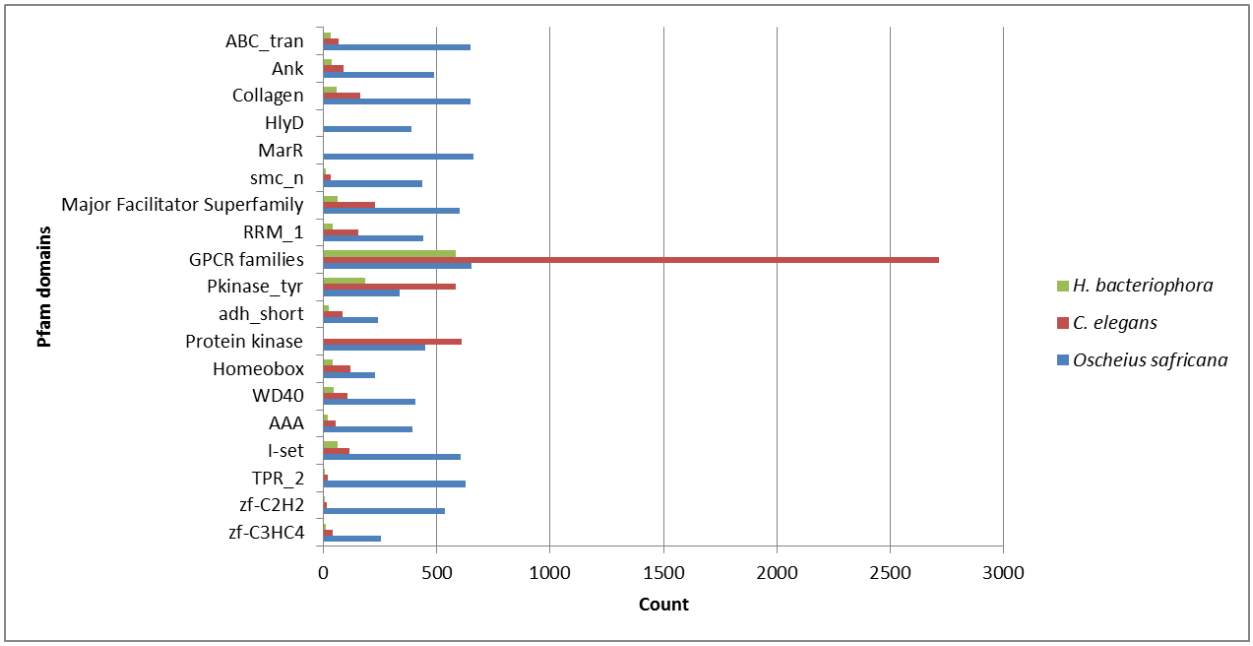


Figure 2. Comparison of most prevalent Pfam domains in *Oscheius safricana* genome with those in *Heterorhabditis bacteriophora* and *Caenorhabditis elegans* (Bai *et al.*, 2013).

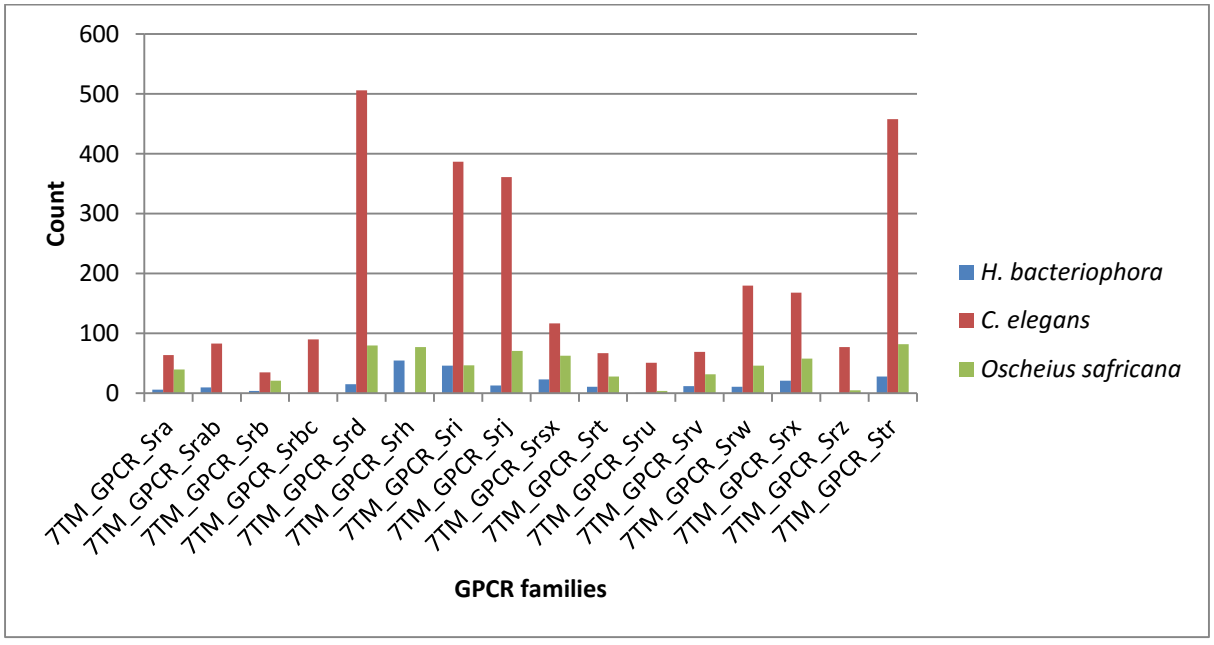


Figure 3. Comparison of most prevalent GPCR protein domain families in *Oscheius safricana* genome with those in *Heterorhabditis bacteriophora* and *Caenorhabditis elegans* (Bai *et al.*, 2013).

As an insect pathogen, we identified various genes and enzymes which play a role to ingress the insect host, toxins that counteract the insect immune system and bacterial adhesion genes that enhance the symbiosis between the nematode and bacteria. Nine chitinases were identified and these are believed to play a role degrading the chitin within the epidermis of the insect (Figure 4). We identified 470 lipase, 315 proteases with a high number of cysteine and serine proteases and 10 protease inhibitors. Proteases, lipases and chitinases have also been identified in the *Serratia marcescens* strain *MCB* genome and this may indicate synergistic activity of the nematode-bacterial duo in killing the insect. We believe that the abundance of these factors within a nematode genome will determine the type of insect host that the EPN is capable of infecting. Bacterial adhesion and high number regulatory protein-coding genes were also identified, the former may play a role in the interaction between the nematode and symbiotic bacteria and the high number of regulatory genes may play a role in exchanging between the two ecological environments; within and outside the insect larvae.

Most of the identified genes within *H. bacteriophora* and *S. carpocapsae* are also present *in O. safricana,* which indicates a high similarity between the three EPN species, thus *Oscheius* can be accepted as one of the EPN genera. Full mode of action of the most identified genes remains unknown, thus we believe comparative genomics with functional genomics can deepen our understanding of the identified genes including the pathways and interaction with each other.

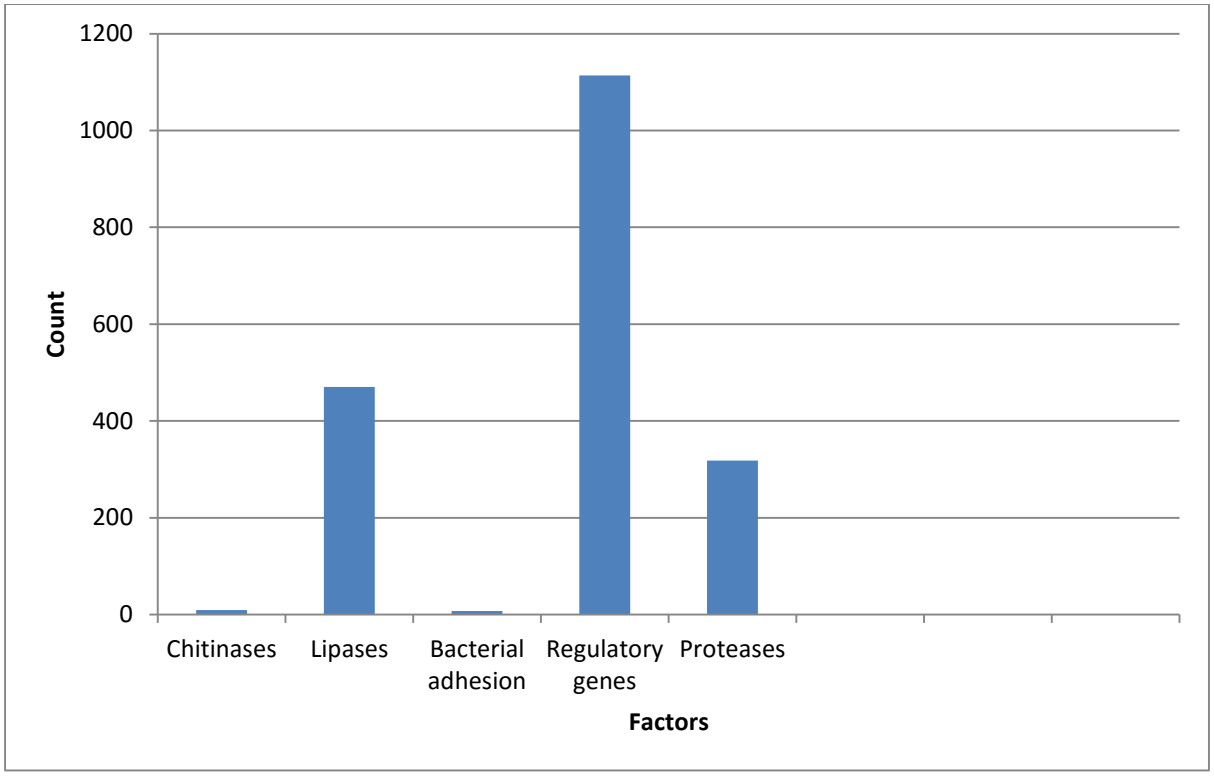


Figure 4. Top 5 factors involved in pathogenesis and symbiosis for *Oscheius safricana*.

Repetitive elements. A total of 4,397,752 bp accounting 3.29% of the genome was masked; this indicates that of the 62% partial completeness of the whole genome only 3% was duplicated. The majority repeat families are simple and low complexity repeats. Simple repeats serve as informative genetic markers to resolve relationships among closely related species because of their high mutation rate and low complexity regions are amino acid sequences that contain repeats of single amino acids or short amino acid motifs. The number of repeats in *Oscheius safricana* is very low as compared to other sequenced nematodes and this might be due to the incomplete genome assembly.

Further sequencing and analysis is underway, and we are working on increasing the genome completeness of *Oscheius safricana*, so we can have most genomic information to compare it other sequenced entomopathogenic nematodes. Currently we had 62% partial completeness; however we were able to account for approximately 70% of the protein coding genes. The genome of *Oscheius safricana* indicates it is an EPN as most elements present in *H. bacteriophora* and *Steinernema carpocapsae* are also present in the former. The G+C% of *Oscheius safricana* is similar to that of *Steinernema* species. Identification of chitinase, proteases and lipases protein coding genes further indicate the importance of these in parasitism against insects.

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Chapter 6:

Conclusion*

*This chapter includes conclusions, recommendations and contributions of the research project.

Conclusions

In a mutualistic relationship, for the interaction to thrive and be of benefit, for both, each partner should increase its compatibility with the other partner so that the two genotypes become more compatible even if the two partners originate from different populations (Sicard *et al.,* 2004). Within the nematode host the symbiotic bacteria have a high number of close relatives to compete with, in addition symbiotic contributions such as helping to establish insect infection and thereby promoting nematode reproduction and development must be achieved. The symbiotic bacteria do so through the transmission of the symbiotic bacteria to the next generation of EPNs and also by producing various antimicrobial compounds which limit the growth of competitive microbes (Murfin *et al.,* 2012; Sicard *et al.,* 2004).

The transmission of the bacterial symbiont in EPNs occurs by bacterial colonization of the alimentary tract of the infective juveniles (IJs), which in turn transmit the bacterial symbionts to the next insect host following its infection by the IJs. The bacterial colonization of the IJs is highly selective and species specific in that a given nematode species is associated with a single specific species of symbiotic bacteria; however a specific bacterial species may be associated with more than one nematode species belonging to the same genus, which suggests horizontal transfer of the bacteria between nematode species (Goodrich-Blair, 2007; Clarke, 2008). In this study *Serratia marcescens* strain *MCB* was transmitted successfully to subsequent generations of IJs which were subsequently able to re-infect *Galleria mellonella*; thus confirming the maintenance of virulence and infectivity through the nematodes persistent association with *Serratia marcescens* strain *MCB*. The time taken for killing *G. mellonella* larvae was 4 days and this falls within the EPN criteria as described by Dillman *et al.* (2012a).

In the current study *Serratia marcescens* strain *MCB* was isolated from *Oscheius safricana*, this was also discovered in other studies where *Serratia nematodiphila, Serratia sp.* and *Serratia marcescens* were isolated and identified from *Heterorhabditidoides chongmingensis, Heterorhabditidoides rugaoensis* and *Oscheius carolinensis*, respectively (Zhang *et al.,* 2008; Zhang *et al.,* 2012; Torres-Barragan *et al.,* 2011). The antimicrobial compounds such as indole derivatives which are produced by *Xenorhabdus* species are also produced by *Serratia marcescens* strain *MCB* (Bode, 2009). One of the advantages of whole genome sequencing and analysis, is the provision of insights into evolutionary patterns of convergent or similar adaptions in organisms which have evolved symbiotic associations which facilitate the

pathogenesis of insect hosts. Whole genome comparison of *Serratia marcescens* strain *MCB* and *Oscheius safricana* with other known EPN-bacterium duo reveals similar protein-coding genes involved in symbiosis and entomophathogenicity. Initially when *Oscheius* genus was isolated and described it was not referred to as an EPN genus but over time, with its behaviour towards insect hosts it evolved\developed traits which are similar to EPNs. In addition putative genes involved in entomophathogenicity identified in *Serratia marcescens* strain *MCB* and *Oscheius safricana* are also present in other EPN-bacterium duo.

The 16S rRNA gene analysis of *Serratia marcescens* strain *MCB* placed it within the *Serratia* polytomy group. Within the group there is *S. nematodiphila* which is also a nematode related symbiotic bacterium. The genome comparison of the two indicate similarities in genome size, number of protein-coding genes, G+C% and protein coding genes involved in symbiosis and pathogenesis. The genome of *Serratia marcescens* strain *MCB* also showed to have a high number of putative protein-coding genes involved in symbiosis and pathogenicity in the tri-partite interaction between symbiotic bacteria, nematode and insect host.

The putative protein-coding-genes encode for products such as toxins, antitoxins, secondary metabolites, fimbrial biogenesis and toxin secretion systems just to name a few. Moreover the genome of *Oscheius safricana* indicates it is an EPN as most elements present in *H. bacteriophora* and *Steinernema carpocapsae* are also present in the former. In addition identification of chitinase, proteases and lipases protein coding genes in both the genomes of *Oscheius safricana* and *Serratia marcescens* strain *MCB* further indicate the importance of these in parasitism against insect hosts. The differences of the 16S rRNA genes between *Serratia marcescens* strain *MCB* with *Xenorhabdus* and *Photorhabdus* species indicate convergent evolution as there are homologous proteins observed across the three (Chaston *et al.*, 2011).

In the current study novel entomopathogenic nematode, *Oscheius safricana* was isolated from South Africa and identified in this research. Its symbiotic bacteria; *Serratia marcescens* strain *MCB* was also isolated and identified; furthermore some of the metabolites it produces were identified. The whole genomes of the *O. safricana* and *S. marcescens* strain *MCB* were sequenced, assembled and annotated. Putative protein-coding genes responsible for synthesis of metabolite or antimicrobial compounds and symbiosis were identified from both genomes. Genome comparison with other EPN-bacterium duo indicates that *Oscheius safricana* and

Serratia marcescens strain *MCB* share similarities with other EPN duo with regards to symbiosis and parasitic protein-coding genes. Major contributions of this research project are summarized as follows:

- *Oscheius safricana* was successfully isolated and identified. The morphological data indicates it is an *Oscheius* species closely related to *O. necromenus, O. chongmingensis and O. carolinensis* in the *Insectivora* group. The identification was further supported by molecular data the 18S, ITS and 28S rRNA. Since several generations of *Oscheius safricana* were produced within laboratory conditions and it was able to infect, hamper the development of and kill *Galleria mellonella* larvae, thus *Oscheius safricana* shows a positive potential as an EPN.
- *Serratia marcescens* strain *MCB* which is the symbiotic bacteria of *Oscheius safricana* was successfully isolated and identified. I have indicated that indole derivatives are also produced by *S. marcescens* strain *MCB* and may be one of common precursor metabolites produced by symbiotic bacteria of entomopathogenic nematode. I have also indicated that the contents of media play a role in metabolite production, which means that the infection of insects by the EPNs is also dependant on the nutrients available within the insect.
- I have successfully sequenced, assembled and annotated the whole genomes of both *Serratia marcescens* strain *MCB* and *Oscheius safricana*. The work here has highlighted steps for next generation sequence analysis for both prokaryotes and eukaryotes.
- The work here provides a platform for studies conducting and taking advantage of whole genome sequencing.
- Genomic comparison with other EPNs and symbiotic bacteria indicates there is a high similarity with other EPN-symbiotic bacterium duo.
- I have identified putative protein coding genes that play an important role in metabolite biosynthesis and transport as well as symbiosis and parasitism in both genomes. Similar genomic contents such as proteases, lipases, chitinases and regulatory protein coding genes in both genomes indicate a synergistic effect of the duo in overcoming the insect immune system. The presence of similar genes in other nematode-bacterium duo might indicate that *Oscheius safricana* is an entomopathogenic nematode. The genomic information has provided a lot of data about the duo.

- The morphological data of the nematode-bacterium duo is not enough to conclude that *Oscheius safricana* and *Serratia marcescens* strain *MCB* can be used against insects, thus in answering the question; "Is *Serratia marcescens* strain *MCB* an entomopathogenic bacterium?: a focus on genomics", based on the genome similarities with *Xenorhabdus* and *Photorhabdus* species I can say it is an entomopathogenic bacterium and its host nematode *Oscheius safricana* can be accepted as an EPN as transmission of the symbiotic bacteria was successful, upon re-infection virulence was maintained and insect host death occurred within 4 days. With further studies on various insect groups, the behaviour of *Oscheius safricana* and other *Oscheius* species will be revealed and its stance as an EPN further explained. It has been a great pleasure working on this project; it has provided me with skills that I never thought I would possess. Next generation sequencing techniques are very rare techniques for one to possess and I believe these will also play an important role in other sectors and not only bacteriology and nematology.

Recommendations for future work

Currently in nematology there is still an uncertainty of Oscheius genus as an entomopathogenic genus. Various Oscheius species have been isolated and identified, including in this study. It is thus recommended that other types of insects from different families be used to test the viability\efficacy of Oscheius as an EPN. Next generation sequencing genome analysis with genome comparison has provided a mild stone in the knowledge of genome contents and evolution of EPN-bacterium duo as well as other species. A combination of comparison genomics and functional genomics can deepen our understanding of the interaction of the genes and their pathways.

Contributions of the research project

Published papers

- ✓ Serepa MH*, Gray VM. 2014. Draft whole-genome sequence of *Serratia marcescens* strain *MCB*, associated with *Oscheius sp. MCB* (Nematoda: Rhabditidae) isolated from South Africa. Genome Announcements. 2(5): e0091114.doi: 10.1128/genomeA.00911-14.
- ✓ Serepa MH*, Tavengwa N & Gray VM. 2015. Purification and characterization of tryptophan and indole-3-acetic acid produced by *Serratia marcescens* strain *MCB*

associated with *Oscheius sp. MCB* (Nematoda: Rhabditidae) obtained from South African soil. African Journal of Bacteriology Research. 7(4): pp. 42-51.

Conference proceedings

- ✓ Gauteng Department of Agriculture and Rural Development (GDARD) 8th Research Symposium in Johannesburg, South Africa, 4th June 2015, Oral presentation.
- ✓ SciFest Africa Science slam in Grahamstown, Eastern Cape, South Africa, 12th-18th March 2014, oral presentation.
- ✓ University of the Witwatersrand Science slam, Johannesburg, South Africa, 28th-30th July 2014, oral presentation.

Awards

- ✓ Best student oral presentation in crop production category at the (GDARD) 8th Research Symposium in Johannesburg, South Africa 4th June 2015.
- ✓ 3rd science slammer at the SciFest Africa Science slam in Grahamstown, Eastern Cape, South Africa, 12th-18th March 2014

Papers under review and in preparation

- ❖ A new species of entomopathogenic nematode *Oscheius safricana n. sp.* (Nematoda: Rhabditidae) from South Africa. Submitted to Archives Of Phytopathology And Plant Protection Journal in March 2015.
- ❖ Genome of *Oscheius safricana*, still under preparation and will be submitted to Genome Biology Journal.

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Appendices

Chapter 2: Appendix A*

*Supplementary methods for chapter 2

1. Nematode isolation and maintenance

Soil samples were collected in North West province of South Africa GPS co-ordinates S 25° 42´ 16.74´´ E 27° 44´ 19.542´´ (Latitude: -25.70465 | Longitude: 27.738762. Two litre containers were used for storage of the soil samples during transport to the lab (Figure A). Late instar larvae of *Galleria mellonella*, the greater wax moth were used as bait for isolation of the nematodes. The insects were reared in the laboratory. The insect larvae were embedded in the soil and the container turned over. This method is also called baiting technique which was first described by Bedding and Akhurst (1975.

The baiting period lasted for 2-3 days as the nematodes take 24-48 hours to kill their hosts. 10 larvae was placed on soil in 2L plastic containers separately and water was added to soil to bring moisture level to 8% which corresponds to a water potential in the range of 7-10 kPa (KiloPascal). This degree of soil moisture is sufficient to facilitate or aid nematodes movement between soil particles when searching for the hosts. These were inverted and stored at 25 °C as it is the optimum temperature for both the hosts and nematodes. Dead larvae were collected every 3-4 days and live ones were placed to extract more nematodes from soil, the cadavers were rinsed with sterile water and each placed separately on moist Whatman No 1 filter paper (50 mm) supported on a 60 mm inverted Petri dish which in turn was placed in a 90 mm Petri dish (Figure B) (White, 1927). Water to a depth of 2mm was added to 90 mm Petri dish to trap the IJs. After 2-3 days in Petri dish, dead larvae with signs of infection by nematodes were placed on a modified White trap for collection of nematodes.



Figure A: The soil samples collected in the North West Province, South Africa, GPS co-ordinates S 25° 42´ 16.74´´ E 27° 44´ 19.542´´ (Latitude: -25.70465 | Longitude: 27.738762).

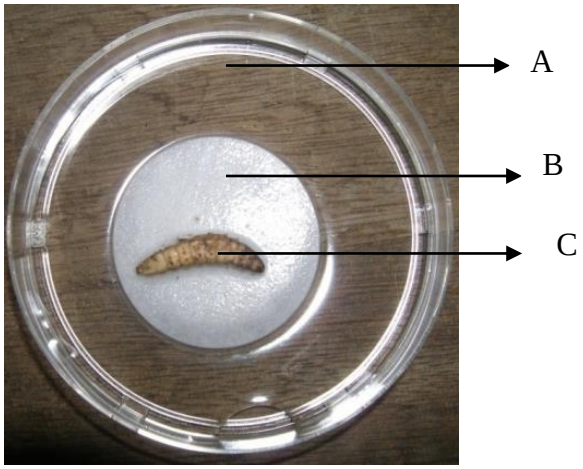


Figure B: Illustration of White trap used to collect Infective Juveniles from insect cadavers. A- Petri dish (90mm diameter) with distilled water, B- Inverted Petri dish cover (60mm diameter) with moist Whatman No 1 Filter paper, C- Infected *Galleria mellonella* larva.

Emerging IJs were then used to re-infect fresh and healthy *Galleria mellonella*. Sterile Petri dishes were filled with autoclaved loam soil. The soil was moistened to 8.0 % water content (w/w) (7-10kPa) with distilled water and inoculated with isolated IJs. The insect larvae were placed onto the soil surface and larval mortality was observed for 2-3 days. After 2 days the dead larvae were transferred to a sterile Petri dish, after 5-7 days from initial soil exposure the larvae were placed on White traps for collection of IJs.

2. Lipid agar

[Method adapted from Woodring and Kaya (1988)]

- Corn syrup was replaced with honey
- Liver oil was replaced with vegetable oil

Composition:

10g honey

5g yeast extract

28g nutrient agar

2.5ml cold vegetable oil

2g MgCl 2 .6h 2 0

1000ml sterile distilled water

Method of preparation

1. Dissolve dry ingredients in water.
2. Add and mix honey and vegetable oil.
3. Autoclaved at 121 °C and 15psi for 20 minutes.
4. Cooled to 50 °C and aseptically poured into Petri dishes.

The lipid agar plates were then spread with isolated *Serratia marcescens strain MCB* (Kaya and Stock, 1997) and incubated at room temperature for 24 hours. Several IJs of the isolated *Oscheius safricana* obtained from White traps were then spread on the bacterial lawn and allowed to grow for two days. After two days, adult IJs were selected and subcultured on fresh lawn of *Serratia marcescens strain MCB*. Subculturing was performed thirteen times.

3. *Galleria mellonella* nutrient Media.

(Method adapted from (Woodring and Kaya, 1988).

- ProNutro original whole wheat was used instead of multivitamin bran.
- Calcium propionate substituted with benzoate.

Composition:

500g ProNutro (original whole wheat)

200ml Glycerol

200ml Honey

200ml Boiled distilled water

5 teaspoons yeast extract powder

1 teaspoon of benzoate

Method of preparation

1. Dry ingredients were mixed together.
2. Mix honey and glycerol in boiled distilled water.
3. Mix with dry ingredients.
4. Wrap mixture in heavy duty aluminium foil and autoclave for 20 minutes at 121°C and 15psi.
5. The media was allowed to cool for 24 hours.

The adult moths and larvae were obtained from infested beehives and these moths were developed from eggs to adults in the same environment. Adult wax moths (male and female) were kept in 3 L glass bottles (Consol® glass jars) (11cm diameter and 23 cm height) at 25°C on an artificial medium. The metal Consol ® jar lids were modified by cutting an 8 cm diameter circular opening into the lid (Fig C). A 9 cm diameter circular stainless steel mesh screen (350µm) was placed on the inside or underside of the modified lid. The metal screen was clamped in place sealing off the opening by screwing the lid tightly onto the jar. The screen facilitated heat and air exchange while preventing larval or moth escape from the jar. Pleated or crumpled wax paper was inserted into the jar to be used as an oviposition site. The moths were allowed to mate and lay eggs. Eggs were also laid on the edge of the lid or on the

paper and were collected by placing a razor blade gently under one side and lifting the eggs. Eggs were placed in another bottle filled with sterilized, cooled *Galleria* media. Eggs hatched within 3-4 days. Larvae were fed weekly with fresh media. Adult wax moths (male and female) and larva kept in 3L glass bottles.



Figure C: A 3L (Consol® glass bottle) with the artificial media and *G. mellonella* larvae inside.

4. Nematode genomic DNA extraction

Protocol from Fermentas Life Sciences Puregene DNA purification Kit, Gentra systems 2003

[(# D-7000A)]

1) Wash nematodes 3 times in sterile double distilled water

2) Pellet nematodes in a microfuge tube by spinning at 14000 rpm for 10 minutes. Remove excess water. Drop microfuge tube in liquid nitrogen and pulverize frozen nematode pellet with a mini pestle.

3) Resuspend pulverized nematodes in 200µl of sterile distilled water buffer

4) Mix 200µl sample with 600µl lysis solution (Fermentas kit) and invert several times to mix.

5) Add 3 µl Protinase K solution and mix by inverting 2.5 times. Incubate at 55 ºC for 3 hours to overnight.

6) Add 3µl RNase a solution to the cell lysate.

7) Mix the sample by inverting the tube 25 times and incubate at 37ºC for 15-60 minutes.

8) Cool sample to room temperature.

9) Add 200 µl Protein Precipitation Solution to the RNase A- treated cell lysate.

10) Vortex vigorously at 6.000 rpm for 3 minutes high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate.

11) Centrifuge at 13.000-16.000 rpm for 3 minutes. The precipated proteins should form a tight pellet.

12) Pour the supernatant containing the DNA (leaving behind the precipited protein pellet) into a 1.5 ml centrifuge tube containing 600 µl 100% Isopranol (2- propanol).

13) Mix the sample by inverting gently 50 times.

14) Centrifuge at 13.000-16.000 rpm for 1 minute; the DNA will be visible as white pellet.

15) Pour off supernatant and drain tube on clean absorbent paper.

16) Add 600 µl of 70% Ethanol and invert tube several times to wash the DNA pellet.

17) Centrifuge at 13.000-16.000 rpm for 1 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.

18) Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.

19) Add 100 µl Hydration solutions.

20) Dehydrated DNA by incubating samples 1 hour at 65ºC or overnight at room temperature.

21) Store DNA at 4 ºC. For long-term storage, store at -20 ºC.

References

1. Bedding, R. A., and Akhurst, R. J. (1975). A simple technique for the detection of insect parasitic rhabditid nematodes in soil. Nematologica. 2, 109-110.
2. Kaya, H.K., and Stock, S.P. (1997) .Techniques in insect Nematology. In *Manual of Techniques in Insect Pathology*, B.H. Knowles, M.R. Blatt, M. Tester, J.M. Horsnell, J. Carroll, G. Menestrina, and D.J Ellar, eds. Academic Press, New York, pp. 281-324.
3. White, G.F. (1927). A method for obtaining infective nematode larvae from cultures. Science. 66, 302–303.
4. Woodring, J.L., and Kaya, H.K. (1988). Steinernematid and heterorhabditid nematodes. In A handbook of biology and techniques, R.H. Fayetteville, ed. Southern Cooperative Series Bulletin 331, pp. 30.

Chapter 3: Appendix B*

*Supplementary methods for chapter 3

1. Protocol for the isolation of symbiotic bacteria associated with EPNs

(Adapted from Akhurst, 1980; Kaya and Stock, 1997)

1) Surface sterilize infective juveniles (IJs) by immersing then in 0.1% sodium hypochlorite (JIK® contains 3.5% sodium hypochlorite) for 1 hour.

2) Transfer the infective juvenile in fresh 0.1% sodium hypochlorite for a further 3 hours.

3) Rinse the IJs twice with Ringer's solution under a laminar flow

4) Suspend surface sterilized IJ in a small volume of nutrient broth

5) Homogenate suspension in a sterile mortar and pestle

6) Aseptically transfer the homogenate to a sterile McCartney bottle

7) Allow bacteria to multiply in the dark on a shaker for 24-48 hours at 25ºC

8) Streak onto MacConkey agar and NBTA and incubate in the dark for 24 hours at 28ºC to allow colony growth.

9) Subculture on to fresh agar plates until there is isolated colonies of the desired bacteria.

2. NBTA (adapted from Akhurst, 1980)

Composition:

1 litre nutrient agar

0.04g triphenyltetrazolium chloride (TTC)

0.025g bromothymol blue (BTB)

Method of preparation:

1. Mix 1 litre nutrient agar and BTB.

2. Autoclave at 121 °C and 15 psi for 15 min, and cool to 50 °C

3. Add TTC, just before pouring into petri dishes, however ensure the autoclaved medium is less than 50 °C. TTC will break down if added when medium is too hot.

4. Swirl to mix.

5. Dispense into sterile Petri dishes and leave to solidify.

MacConkey agar

Composition (g/l)

20.0g Peptone

10.0g Lactose

1.5g Bile Salts

5.0g Sodium Chloride

0.03g Neutral Red

0.0001g Crystal Violet

13.5g Agar

Method of preparation:

1. Weigh out MacConkey agar powder and suspend in 1000ml distilled water.

2. Boil whilst stirring until completely dissolved.
3. Autoclave at 121ºC and 15 psi for 15 min. Cool to 45 – 50ºC.
4. Mix well, dispense into sterile Petri dishes and leave to solidify.

3. Isolation of genomic DNA from bacterial cells (Protocol from ZR Fungal/Bacterial DNA Kit, catalog # D6005)

1) Pick an isolated bacterial colony from a previously streaked NBTA\MacConkey agar plate and suspend in a ZR BashingBeadTM Lysis Tube.
2) Vortex at maximum speed for 5 minutes.
3) Centrifuge the ZR BashingBeadTM Lysis Tube in a microcentrifuge at 10 000 (rpm) for 1 minute.
4) Transfer up to 400µl supernatant to a Zymo-Spin TM IV Spin Filter in a Collection Tube and centrifuge at 7000 rpm for 1 minute.
5) Add 1200µl of Fungal/ Bacterial DNA binding buffer to the filtrate in the Collection Tube from Step 4.
6) Transfer 800µl of the mixture from Step 5 to a Zymo-SpinTM II Column in a Collection tube and centrifuge at 10000rpm for 1 minute.
7) Discard the flow through from the Collection Tube and Repeat Step 6.
8) Add 200µl DNA Pre-Wash Buffer to the Zymo-SpinTM II Column in a new Collection Tube and centrifuge at 10000rpm for 1 minute.
9) Add 500µl Fungal/Bacterial DNA Wash Buffer to the Zymo-SpinTM II Column and centrifuge at 10000rpm for 1 minute.
10) Transfer the Zymo-SpinTM II Column to a clean 1.5 ml microcentrifuge tube and add 100µl DNA Elution Buffer directly to the column matrix. Centrifuge at 100000rpm for 30 seconds to elute the DNA.

4. Nutrient broth

Composition (g/l):

1g Meat extract

2g Yeast extract

5g Peptone

8g Sodium chloride

Method of preparation:

1. Weigh out nutrient broth powder and suspend in 1000ml distilled water.
2. Mix well and dispense adequate amounts into volumetric flasks.
3. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation one

Nutrient Broth

4.0% (W/V) Canola oil

1. Weigh out nutrient broth powder and suspend in 1000mL of distilled water.
2. Mix well and dispense adequate amounts into volumetric flasks.
3. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth.
4. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation two

Nutrient Broth

4.0% (W/V) Canola oil

25mg/ml glucose

1. Weigh out nutrient broth powder and suspend in 1000mL volume of distilled water.
2. Add glucose.

3. Mix well and dispense adequate amounts into volumetric flasks.

4. Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth.

5. Autoclave at 121˚C and 15 psi for 15 min.

Luria broth

Composition (g\l):

10g NaCl

5g Yeats extract

10g Tryptone

1. Weigh out the dry ingredients and suspend in 1000mL volume of distilled water.

2. Mix well.

3. Autoclave at 121˚C and 15 psi for 15 min.

5. HPCL solvent preparation

The solvent used was 50%:30% v\v acetonitrile: water with 0.3 % acetic acid. The mobile solution was then placed in an Ultrasonic bath (with water) to obtain a uniform solution.

6. 1 Preparation of bacterial cells for viewing under the fluorescence light microscope

1) Bacterial colonies were collected from NBTA agar plates.
2) Serial dilution of 10-1, 10-2 and 10-3 performed with sterile nutrient broth.
3) Bacterial cells were mounted on slides, covered with cover slips and viewed under the microscope.

6.2 Preparation of bacteria within nematodes for viewing under the fluorescence light microscope

1) Live adult hermaphrodite female nematodes were selected from White traps and washed three times with sterile distilled water.
2) The nematodes were mounted on slides, covered with cover slips and viewed under the microscope.

References

1. Kaya, H.K., and Stock, S.P. (1997) .Techniques in insect Nematology. In *Manual of Techniques in Insect Pathology*, B.H. Knowles, M.R. Blatt, M. Tester, J.M. Horsnell, J. Carroll, G. Menestrina, and D.J Ellar, eds. Academic Press, New York, pp. 281-324.
2. Akhurst, R.J. (1980). Morphological and functional dimorphism in Xenorhabdus spp., bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis. J. Gen. Microbiol. 121, 303-309.

Chapter 4: Appendix C*

*Supplementary methods for chapter 4

1. CLC bio report of *Serratia marcescens* strain *MCB* genome assembly.

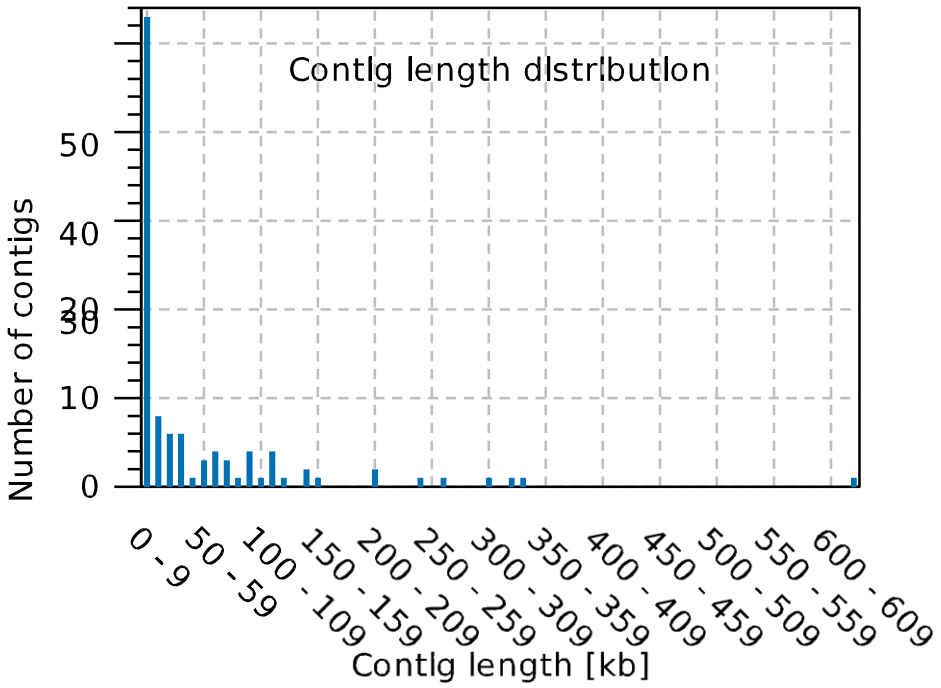
1. Bacteria4H_S4_L001_R1_001 (paired) trimmed (paired) merged trimmed (paired) assembly summary report

1.1 Nucleotide distribution

N	Count	Frequency
Adenine (A)	1,082,804	20.4%
Cytosine (C)	1,552,626	29.3%
Guanine (G)	1,579,560	29.8%
Thymine (T)	1,085,650	20.5%

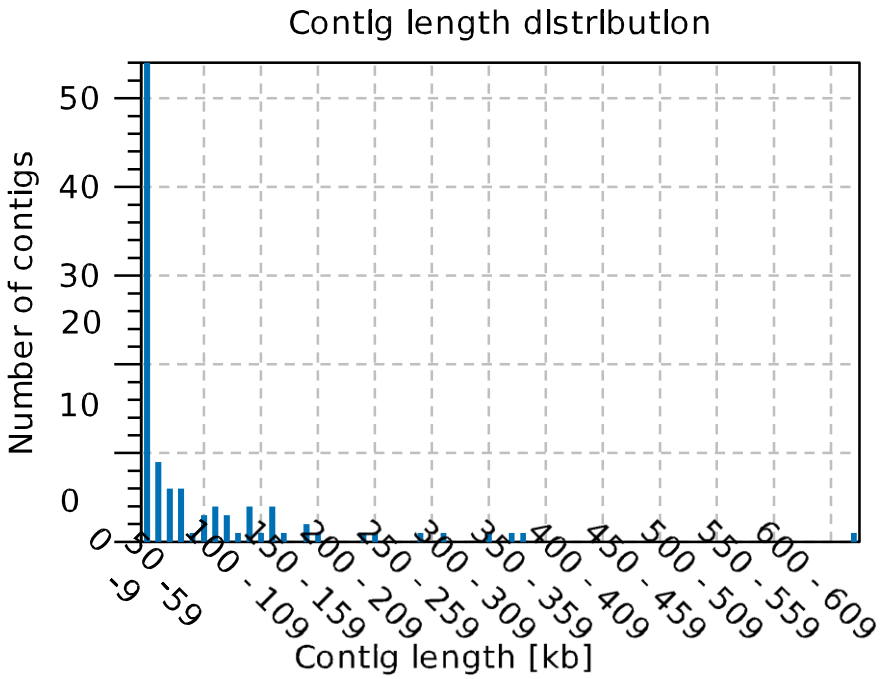
1.2 Contig measurements (including scaffolded regions)

N75	90,107
N50	154,868
N25	306,152
Minimum	265
Maximum	621,746
Average	50,006
Count	106
Total	5,300,640

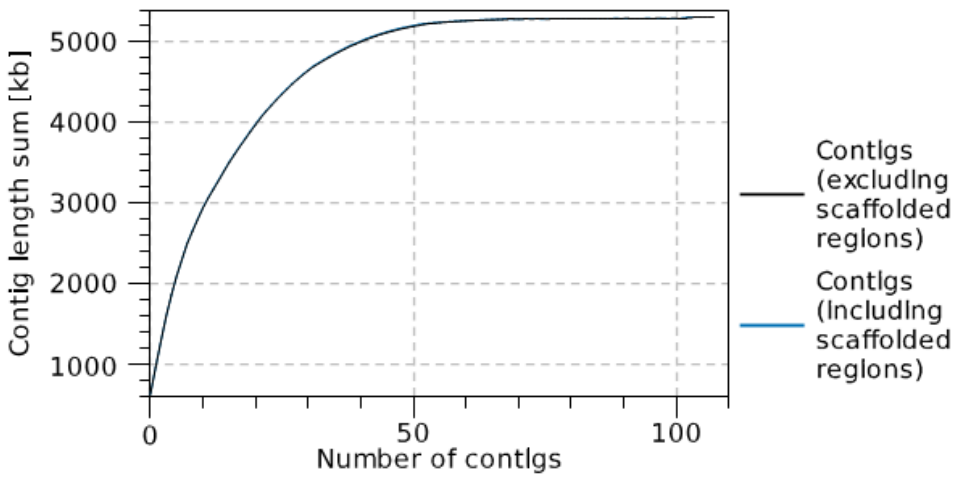


1.3 Contig measurements (excluding scaffolded regions)

N75	88,279
N50	149,128
N25	306,152
Minimum	209
Maximum	621,746
Average	49,080
Count	108
Total	5,300,636



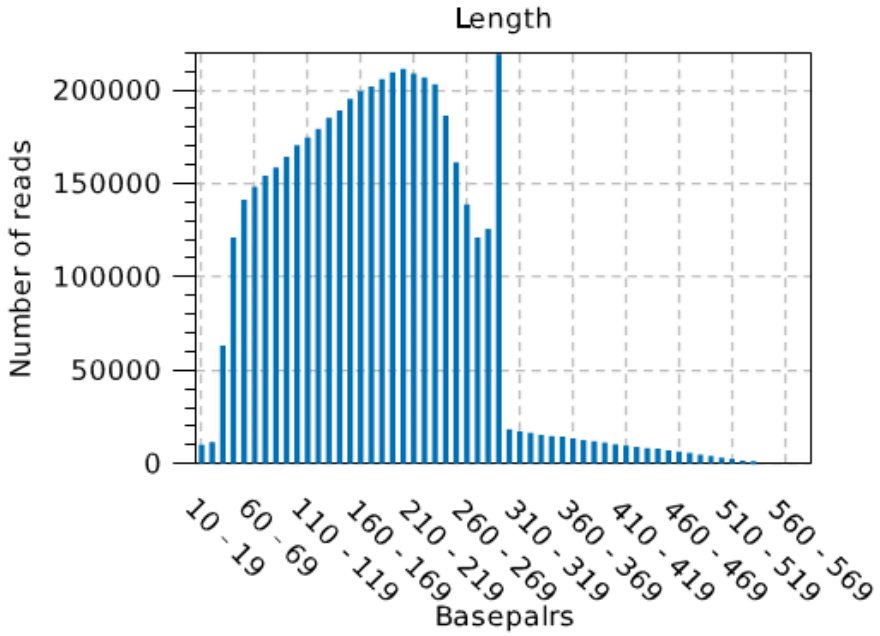
1.4 Accumulated contig lengths



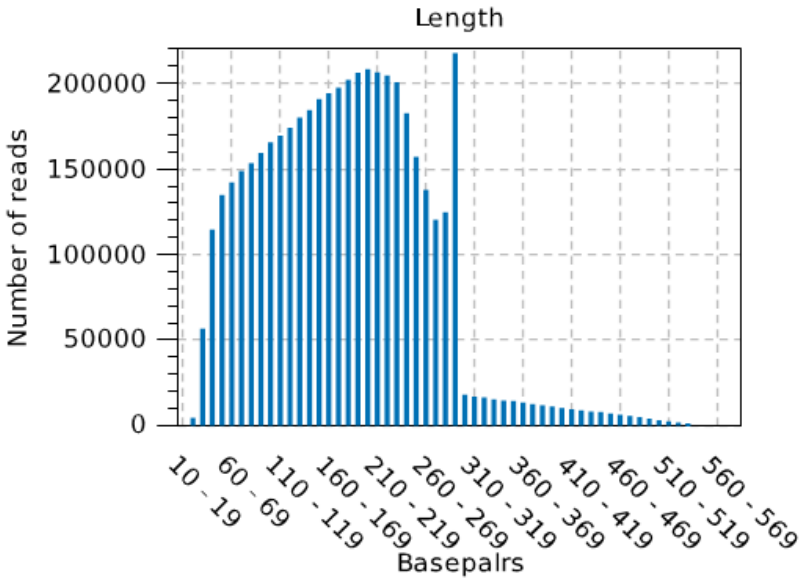
1.5 Statistic report

	Count	Average	Total bases
Reads	4,894,79	180.15	881,812,61
Matched	4,768,13	181.73	866,512,89
Not matched	126,656	120.8	15,299,71
Contigs	106	50,006	5,300,64
Reads in pairs	3,474,97	154.39	
Broken paired reads	926,347	236.5	

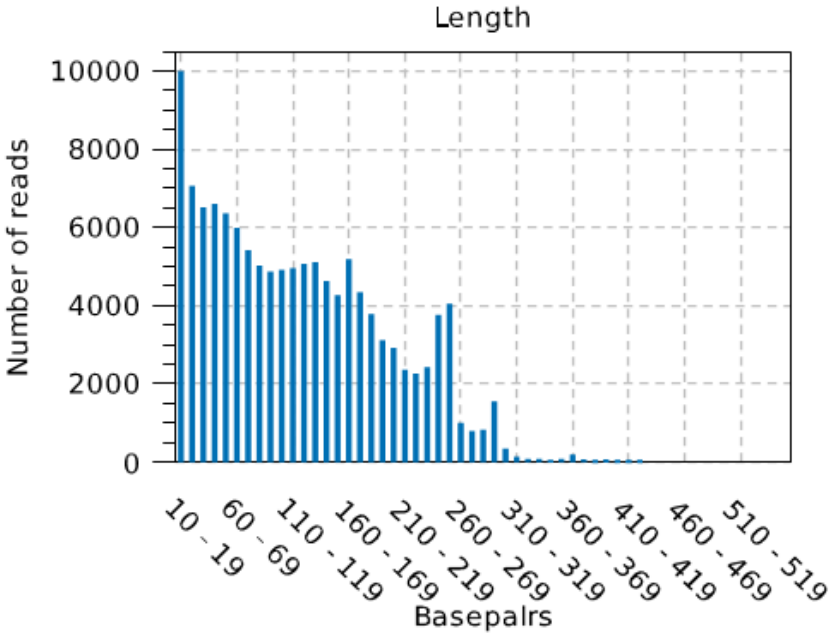
1.6 Distribution of read length



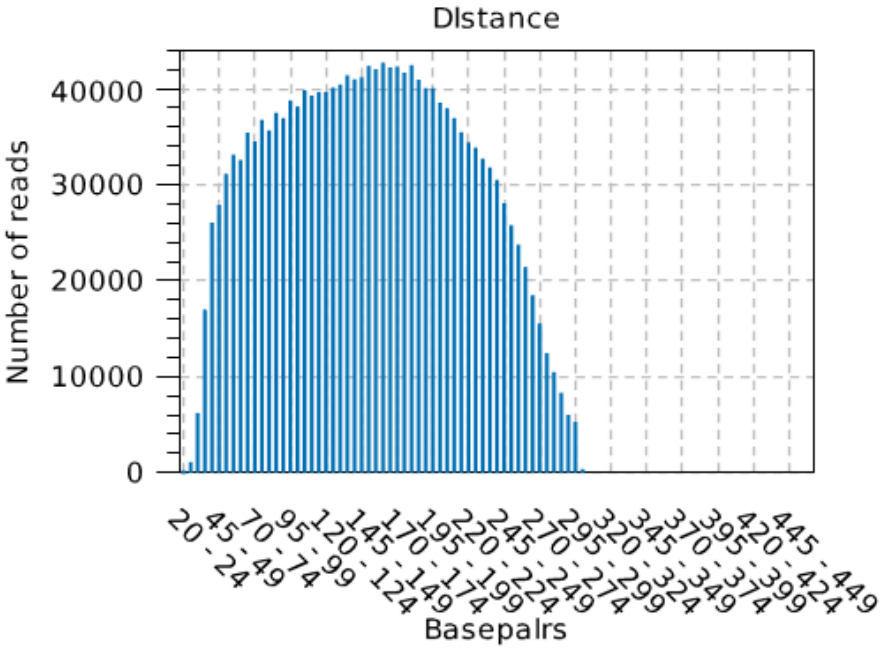
1.7. Distribution of matched read length



1.8 Distribution of non-matched read length



1.9 Paired reads distance distribution



Chapter 5: Appendix D*

*Supplementary methods for chapter 5

A: **Lipid agar**

[Method adapted from Woodring and Kaya (1988)]

- Corn syrup was replaced with honey
- Liver oil was replaced with vegetable oil

Composition:

10g honey

5g yeast extract

25g nutrient agar

2.5ml cold vegetable oil

2g MgCl 2 .6h 2 0

1000ml sterile distilled water

Method of preparation

1. Dissolve dry ingredients in water.
2. Add and mix honey and vegetable oil.
3. Autoclaved at 121 °C and 15psi for 20 minutes.
4. Cooled to 50 °C and aseptically poured into Petri dishes.

The lipid agar plates were then spread with isolated *Serratia marcescens* strain *MCB* (Kaya and Stock, 1997) and incubated at room temperature for 24 hours. Several IJs of the isolated *Oscheius safricana* obtained from White traps were then spread on the bacterial lawn and allowed to grow for two days. After two days, adult IJs were selected and subcultured on fresh lawn of *Serratia marcescens* strain *MCB.* This was performed thirteen times.

B: All the genome analysis of *Oscheius safricana* was performed using Linux command line through the cluster network that had all the software's and programmes installed. Below are all the scripts (with parameters for each software) that were compiled using Linux.

1. **FastQC script**

```
#!/bin/bash

 #PBS -N FastQC_Job_Hope
 #PBS -q WitsLong
 #PBS -l walltime=02:30:00,mem=2gb
 #PBS -l nodes=1:ppn=2
 #PBS -o /home/mserepa/HOPESEREPA/logs/output.log
 #PBS -e /home/mserepa/HOPESEREPA/logs/error.log

 WORK_DIR==/home/mserepa/HOPESEREPA

 cd $WORK_DIR
fastqc $(ls /path/to/the/files/*fastq.gz) -o $WORK_DIR –noextract
```

2. **Trimmomatic**

```
#!/bin/bash

#PBS -N Trimm_Job
#PBS -q WitsLong
#PBS -l walltime=02:00:00,mem=24gb
#PBS -l nodes=1:ppn=1
#PBS -o /home/mserepa/HOPESEREPA/TRIM/logs/output.logs
#PBS -e /home/mserepa/HOPESEREPA/TRIM/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM

cd $WORK_DIR

for read in $(ls *R1*)
do
   (( ++rep ))
   base_in=$read
   base_out="trimmed_rep_00"$rep

   java -jar /opt/exp_soft/bioinf/trinity/trinity-plugins/Trimmomatic/trimmomatic.jar PE -phred33 \
      -trimlog report.log \
```

```
    -basein $base_in \
    -baseout output/$base_out \
    MAXINFO:40:0.7

done
```

3. Velvet

```
#!/bin/bash

#PBS -N assembly_job
#PBS -q WitsLong
#PBS -l nodes=1:ppn=6,walltime=100:00:00,mem=150gb
#PBS -o /home/mserepa/HOPESEREPA/TRIMM/logs/output.logs
#PBS -e /home/mserepa/HOPESEREPA/TRIMM/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM

cd $WORK_DIR

velveth /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY  31 -fastq FILES
velvetg  /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY  -amos_file yes -cov_cutoff 8 -exp_cov 8 -min_contig_lgth 400
```

4. Busco

```
#!/bin/bash

#PBS -N Genome_Assessment
#PBS -q WitsLong
#PBS -l walltime=400:00:00,mem=20gb
#PBS -l nodes=1:ppn=7
#PBS -o /home/phelelani/gray/hope/3_assembly_quality/logs/output_busco.log
#PBS -e /home/phelelani/gray/hope/3_assembly_quality/logs/error_busco.log

WORK_DIR=/home/phelelani/gray/hope/3_assembly_quality
DATA_DIR=/home/phelelani/gray/hope/2_assembly/output_49_3_9

cd $WORK_DIR

start=$(date +"%s")

/home/phelelani/applications/Python-3.4.3/python /opt/exp_soft/bioinf/BUSCO/BUSCO_v1.1.py \
   -in $DATA_DIR/contigs.fa \
```

```
    -l metazoa \
    -o nematode -f \
    -m genome \
    -sp caenorhabditis \
    -c 6

end=$(date +"%s")
diff=$(($end-$start))

echo -e "\n##########\nAnalysis took $(($diff / 3600)) hours, $(($diff % 3600 / 60)) minutes and $(($diff % 60)) seconds to finish!\n##########\n"
```

5. RepeatMasker

```
#!/bin/bash

#PBS -N RepeatMasker
#PBS -q WitsLong
#PBS -l walltime=20:00:00,mem=20gb
#PBS -l nodes=1:ppn=7
#PBS -o /home/phelelani/gray/hope/5_repeatmasker/logs/output_repeatmasker.log
#PBS -e /home/phelelani/gray/hope/5_repeatmasker/logs/error_repeatmasker.log

WORK_DIR=/home/phelelani/gray/hope/5_repeatmasker
DATA_DIR=/home/phelelani/gray/hope/2_assembly/output_49_3_9

cd $WORK_DIR

start=$(date +"%s")

RepeatMasker \
    -species elegans \
    -pa 6 \
    -dir $WORK_DIR/repeat_masker_out \
    $DATA_DIR/contigs.fa

end=$(date +"%s")
diff=$(($end-$start))

echo -e "\n##########\nAnalysis took $(($diff / 3600)) hours, $(($diff % 3600 / 60)) minutes and $(($diff % 60)) seconds to finish!\n##########\n"
```

6. tRNASCAN

```
#!/bin/bash

#PBS -N tRNAscan
#PBS -q WitsLong
#PBS -l nodes=1:ppn=1,walltime=20:00:00,mem=12G
#PBS -o /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/output.logs
#PBS -e /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA

cd $WORK_DIR

tRNAscan-SE -O -T -o tRNA_out -m summary contigs.fa.masker
```

7. BLASTP

```
#!/bin/bash

#PBS -N blastp
#PBS -q WitsLong
#PBS -l nodes=1:ppn=1,walltime=200:00:00,mem=12G
#PBS-o /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/output.logs
#PBS-e /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA

cd $WORK_DIR

blastp -query proteinsequences.fasta -db /data/gray/databases/c_elegans_pep.fasta -out blastpoutput -evalue 1e-6 -outfmt 6
```

8. Augustus

```
#!/bin/bash

#PBS -N annotation_job
#PBS -q WitsLong
#PBS -l nodes=1:ppn=6,walltime=200:00:00,mem=30G
#PBS -o /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/output.logs
#PBS -e /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA

cd $WORK_DIR
```

augustus --species=caenorhabditis --strand=both --genemodel=complete --species=caernorhabditis --outfile=gff inputfile

9. HMMSCAN

```
#!/bin/bash

#PBS -N HMMSCAN
#PBS -q WitsLong
#PBS -l nodes=1:ppn=6,walltime=200:00:00,mem=12G
#PBS -o /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/output.logs
#PBS -e /home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA/logs/error.logs

WORK_DIR=/home/mserepa/HOPESEREPA/TRIMM/output/ASSEMBLY/CEGMA

cd $WORK_DIR

hmmscan --domT 1e-6 --cpu 6 --domtblout  hmmscan.out /data/gray/databases/pfam/Pfam-A.hmm proteinsequences.fasta > pfam.log
```

References

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2. Woodring, J.L., and Kaya, H.K. (1988). Steinernematid and heterorhabditid nematodes. In *A handbook of biology and techniques*, R.H. Fayetteville, ed. Southern Cooperative Series Bulletin 331, pp. 30.