Investigating the potential of indigenous nematode isolates to control invasive molluscs in canola

by
Annika Pieterse

Thesis presented in fulfilment of the requirements for the degree of

Master of Agricultural Sciences in the Department of Conservation

Ecology and Entomology at Stellenbosch University

Supervisors: Dr A P Malan and Dr J L Ross

December 2016

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Acknowledgements

I wish to thank the following people and institutions:

This study wouldn't have been possible without the help and guidance from my supervisors, Dr J.L. Ross and Dr A.P. Malan. I would like to thank them for their hard work and patience.

The Department of Conservation Ecology and Entomology, Stellenbosch University.

Prof D. Nel for his help with the statistical analysis of my data.

The Protein Research Fund (PRF) for funding.

Dr W. Sirgel and Prof S.A. Reinecke from the Department of Botany and Zoology, Stellenbosch University, for their help with the identification of species.

My family and friends for their love, support and help throughout the study.

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Abstract

Terrestrial molluscs (Mollusca: Gastropoda) are important economic pests worldwide, causing extensive damage to a variety of crop types, and posing a health risk to both humans and wildlife. In South Africa, the climate is favourable for invasive European molluscs, especially in the Western Cape province, where there are mild, damp winters. One crop that is particularly targeted by the pests concerned is canola (*Brassica napus*), which is a winter arable crop that is commercially produced for its use in cooking, food processing, fertilisers, fuels, pet food, plastics, and animal feed. Molluscs on canola in the Western Cape province are currently controlled using chemical molluscicide pellets. These chemicals have the potential to adversely affect the environment and non-target organisms. The use of mollusc-parasitic nematodes is a possible environmentally-friendly alternative.

Current knowledge indicates that there are eight nematode families that associate with molluscs, including Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae, and Rhabditidae. To date, *Phasmarhabditis hermaphrodita* is the only nematode that has been developed as a biological molluscicide. The nematode, which was commercially released in 1994 by MicroBio Ltd, Littlehampton, UK (formally Becker Underwood, now BASF) under the trade name Nemaslug®, is now sold in fifteen different European countries. Due to current legislation, Nemaslug® cannot be sold or used in South Africa. A survey was therefore conducted in the Western Cape province of South Africa to locate a local nematode isolate capable of causing mortality in invasive mollusc pests.

A total of 1944 slugs were collected from 12 different study sites. On the identification of slugs, they were dissected alive, and examined for internal nematodes. Nematodes were identified using morphological and molecular techniques (18S rRNA). Seven of the 12 sites had nematodes present, with 8% of the slugs being found to be infected with nematodes. Six

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nematode species were identified, including *Angiostoma margaretae*, *Angiostoma* sp., *Caenorhabditis elegans*, a mermitid sp., and *Phasmarhabditis* spp. (SA3 and SA4). Of the six species mentioned, four were previously undescribed. The isolation of new *Phasmarhabditis* spp. indicates the importance of conducting further surveys of mollusc-parasitic nematodes in South Africa.

Nematodes isolated in the survey were tested for their ability to reproduce on decaying organic matter (consisting of dead frozen slugs), with results demonstrating that one of the nematodes, *Phasmarhabditis* sp. SA4, could complete its life cycle under such conditions. In addition, pathogenicity tests illustrated that *Phasmarhabditis* sp. SA4 caused significant mortality of the slug *D. panormitanum.*

Phasmarhabditis sp. SA4 was then fully described and characterised by the shape and length of the female tail, and by the presence of males. Phylogenetic analysis demonstrated that *Phasmarhabditis* sp. SA4 was placed in a monophyletic clade along with *Phasmarhabditis* sp. SA2, *Phasmarhabditis papillosa*, and the mollusc-parasitic nematode, *Angiostoma dentiferum*. The new species brings the total complement of the genus to seven species.

Phasmarhabditis sp. SA4 was then established in monoxenic cultures. Five bacterial isolates were isolated from the intestine of slug hosts, identified using 16S rRNA gene sequences, and their pathogenicity tested by means of injecting directly into the haemocoel of *D. reticulatum*, and monitoring the mortality over time. *Kluyvera* sp., which was found to cause the highest mortality rate among the slugs concerned, was chosen for monoxenic culturing. Cultures containing *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. were optimised using temperatures ranging from 15°C to 25°C, with results showing that 15°C was the optimum growth temperature.

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Opsomming

Landlewende weekdiere (Mollusca: Gastropoda) is wêreldwyd belangrike ekonomiese plae wat aansienlike skade aan 'n verskeidenheid landbou gewasse veroorsaak en kan 'n nadelige effek op die gesondheid van mense en dier hê. Die Wes-Kaap provinsie van Suid-Afrika met sy matige, klam winters is veral 'n gunstige omgewing vir indringer Europese slakke en naakslakke. Een gewas wat veral benadeel word deur die aktiwiteit van hierdie spesies is canola (*Brassica napus*). Canola word kommersieel produseer vir gebruik in die voorbereiding van voedsel, kunsmis, brandstowwe, voer vir troeteldiere, plastiek en veevoer. Slakke word tans beheer in canola in die Wes-Kaap, deur die gebruik van slakpille. Die chemikalieë in hierdie slakpille het die potensiaal om 'n negatiewe effek op die omgewing en nie-teiken organismes te hê. Nematodes wat dien as natuurlike parasiete van slakke is 'n moontlike omgewingsvriendelike biologiese beheer alternatief.

Volgens kennis is daar tans agt nematode families wat assosieer met slakke, naamlik Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae, en Rhabditidae. Tot op datum is *Phasmarhabditis hermaphrodita* die enigste nematode wat al ontwikkel is in 'n biologiese beheermiddel vir slakke. Die nematode is in 1994 kommersieel vrygestel deur MicroBio Ltd, Littlehampton, UK (voorheen Becker Underwood, nou BASF) onder die handelsnaam Nemaslug® en word tans verkoop in vyftien verskillende Europese lande. Huidige wetgewing verbied die verkoop of gebruik van die produk in Suid-Afrika. 'n Opname was daarom gedoen van die nematodes geassosieer met slakke in die Wes-Kaap van Suid-Afrika, om 'n plaaslike nematode te vind met dieselfde biologiese beheer potensiaal.

'n Totaal van 1944 naakslakke was versamel van 12 verskillende studie areas. Nadat hul geidentifiseer was, was hul lewend dissekteer en ondersoek vir interne nematodes.

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Nematodes was geidentifiseer deur gerbuik te maak van morfologiese en molekulêre tegnieke (18S rRNA). Nematodes was teenwoordig by sewe van die twaalf studie areas en 8% van naakslakke was geïnfekteer deur nematodes. Ses nematode spesies was geïdentifiseer, naamlik *Angiostoma margaretae*, *Angiostoma* sp., *Caenorhabditis elegans*, 'n mermitid sp., en *Phasmarhabditis* spp. (SA3 and SA4). Van die ses spesies wat gevind is, was vier nog nie voorheen beskryf nie. Die ontdekking van die nuwe *Phasmarhabditis* spesies is 'n aanduiding van die belangrikheid van verdere opnames vir die voorkoms van nematodes parasiete geassosieer met slakke in Suid-Afrika.

Nematodes wat gevind was in die opname se vermoë om voort te plant op organiese materiaal (bestaande uit gevriesde, dooie naakslakke) was getoets. *Phasmarhabditis* sp. SA4 kon suksesvol sy lewenssiklus voltooi in laboratorium toestande en verdure patogenisiteit toetse het bewys dat die nuwe nematode 'n merkwaardige effek gehad het op sterftes van die indringer naakslak, *D. panormitanum*.

Phasmarhabditis sp. SA4 was toe volledig beskryf en word gekarakteriseer deur die vorm en lengte van die vroulike nematode se stert asook die teenwoordigheid van manlike nematodes. Filogenetiese analise het getoon dat *Phasmarhabditis* sp. SA4 geplaas is in 'n monofiletiese klade saam met *Phasmarhabditis* sp. SA2, *Phasmarhabditis papillosa*, en die slak-parasitiese nematode, *Angiostoma dentiferum*. Die nuwe spesie bring die totale hoeveelheid spesies in die genus na sewe.

Om die patogenisiteit van die nematode te verhoog, kan die nematode gegroei word op 'n bakterieë wat dood veroorsaak van slakke. Vyf bakterieë spesies was geïsoleer vanaf die ingewandes van slak gashere en geïdentifiseer deur die 16S rRNA gene. Die patogenisiteit van die bakterieë was getoets deur dit direk in *D. reticulatum* in te spuit en sterftes te monitor. *Kluyvera* sp. het die meeste sterftes veroorsaak in slakke en was gekies vir verdere formulering met *Phasmarhabditis* sp. SA4. Kulture bestaande uit slegs *Kluyvera* sp. en *Phasmarhabditis* sp.

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SA4 was getoets by 15°C, 20°C en 25°C. Die optimale groeitemperatuur vir *Phasmarhabditis* sp. SA4 was 15°C.

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Summary and Objectives

Nematodes associated with molluscs tend to be understudied, with the majority of work focusing on *P. hermaphrodita*, a mollusc-parasitic nematode that has been successfully developed as a biological molluscicide in Europe. However, the introduction of this biological control agent is prevented in South Africa under the terms of an Agricultural Pests Amendment Act that forbids the introduction of exotic organisms to the country (Ross *et al.*, 2012). Therefore, an alternative method of biological control must be developed in South Africa to control molluscs, especially in such key agricultural crops as canola.

The overall aim of the current study is to develop a nematode as a biological control agent for slugs and snails in South Africa.

The three main objectives of this study are to:

1. Investigate the diversity and distribution of nematodes associated with terrestrial molluscs from canola crops and commercial nurseries in the Western Cape province.
2. Characterise indigenous nematode isolates with biocontrol potential, using a combination of morphological and molecular analysis.
3. Establish *in vitro* monoxenic cultures of indigenous nematode isolates with biocontrol potential, and optimise production efficiency.

The chapters of this thesis have been written as separate publishable papers, and, for this reason, some repetition in the different chapters has been unavoidable. The format of the chapters was written according to the 'instructions for authors' of the *Journal of Helminthology*.

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CHAPTER 1

Literature review

Nematodes associated with Molluscs (slugs and snails) as definitive hosts

Published as: **Pieterse, A., Malan, A.P. & Ross, J.L.** (2016) Nematodes that associate with terrestrial molluscs as definitive hosts, including *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) and its development as a biological molluscicide. *Journal of Helminthology* (doi: 10.1017/S0022149X16000572)

Abstract

Terrestrial molluscs (Mollusca: Gastropoda) are important economic pests worldwide, causing extensive damage to a variety of crop types, and posing a health risk to both humans and wildlife. Current knowledge indicates that there are eight nematode families that associate with molluscs, including Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae, and Rhabditidae. To date, *Phasmarhabditis hermaphrodita* is the only nematode that has been developed as a biological molluscicide. The nematode, which was commercially released in 1994 by MicroBio Ltd, Littlehampton, UK (formally Becker Underwood, now BASF) under the trade name Nemaslug®, is now sold in fifteen different European countries. This paper reviews nematodes isolated from molluscs, with special detailed information on the life cycle, host range, commercialisation, natural distribution, mass production, and the field application of *P. hermaphrodita*.

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1.1 Introduction

Terrestrial gastropod molluscs (slugs and snails) (Mollusca: Gastropoda) constitute approximately 35 000 species, making them one of the most successful animal groups in the terrestrial ecosystem (Barker, 2001). Although no formal method has been developed for differentiating between slugs and snails, an organism is considered to be a slug when it has no external shell, or when the shell is small in comparison to its body size, and it is considered to be a snail when it has a large external shell (Barker, 2001). All slugs have evolved from snails, with such evolution having occurred multiple times throughout evolution (South, 1992).

Terrestrial molluscs lay eggs that hatch into juveniles, which have a similar shape to the adults, albeit varying in colour. As adults, they reproduce by means of either amphimixis or hermaphroditism, with the hermaphrodites either being self-fertilising (e.g. *Arion intermedius* Normand, 1852) or fertilising, by means of outcrossing (e.g. *Deroceras reticulatum* (Müller, 1774)). The timing of the life cycle varies between species, with some being opportunistic breeders (*D. reticulatum*) that can go through multiple generations within one year, whereas some are annual breeders (*Arion* spp.), and some span several years (*Achatina fulica* Bowdich, 1822) (Wilson, 2007). A detailed biology of molluscs has been described by Barker (2001).

Terrestrial molluscs have colonised all inhabited continents, and are important economic pests of a number of different crop types, including pasture, arable, ornamental, and vegetable crops (Glen & Moens, 2002; Moens & Glen, 2002; Port & Ester, 2002; Wilson & Barker, 2011). Molluscs attack plants by destroying their stems and growing points, causing a reduction in their growth and vigour. In addition, they target seedlings and seeds, and decrease the leaf area (South, 1992). In severe cases, the damage that is done to germinating seeds is so extreme that entire fields must be resown, resulting in huge economic losses to both growers

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and farmers (Willis *et al.*, 2006). Harvested crops may also be devalued through the presence of slugs, faeces, eggs, mucus, or feeding damage (Iglesias *et al.*, 2002).

1.2 Molluscs in South Africa

In South Africa, the climate is favourable for invasive European molluscs, especially in the Western Cape province, where there are mild, damp winters. European molluscs were first introduced to South Africa by European settlers in the eighteenth and early nineteenth centuries (Herbert, 2010), and they have now become well-established in their new environment. An estimated 34 invasive terrestrial mollusc species have been introduced to South Africa, with habitat ranging from agricultural land, through woodland and gardens, to glasshouses (Herbert, 2010). Mollusc introductions continue at a rate of approximately two species a decade (Herbert, 2010). The estimated crop loss and costs that are associated with controlling the alien molluscs in South Africa was calculated to be US$1 billion per annum in 2002 (Pimentel, 2002). The economic costs involved include the direct impact of molluscs, such as feeding, the clogging of machinery, and mucus or faecal soiling of crops, as well as indirect impacts, such as the rejection of harvested crops by quarantine officials, the refusal of contaminated pastures by livestock, and the transmission of diseases and parasites (Herbert, 2010).

One crop that is particularly targeted by the pests concerned is canola (*Brassica napus*), which is a winter arable crop that is commercially produced for its use in baking, cooking, food processing, fuels, fertilisers, plastics, pet food and animal feed. Canola is sown between March and May in the Western Cape province, during which time the temperatures begin to fall. The seedlings are mostly susceptible to mollusc damage during the first four weeks, during which time they require protection to prevent whole fields from having to be resown. The canola plants create a cool, damp and shaded environment, which enables the molluscs to reproduce throughout the entire season, and to remain active until the canola is windrowed (Tribe &

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Lubbe, 2010). During the summer months, the molluscs burrow into the soil to depths of approximately 20 cm, re-emerging when the temperatures drop and the moisture levels increase. The three mollusc species that have been recognised as being pestiferous to canola crops are the grey field slug (*D. reticulatum*), the brown field slug (*Deroceras panormitanum* (Lessona & Pollonera, 1882)), and the keeled slug (*Milax gagates* (Draparnaud, 1801)) (Fig. 1.1).

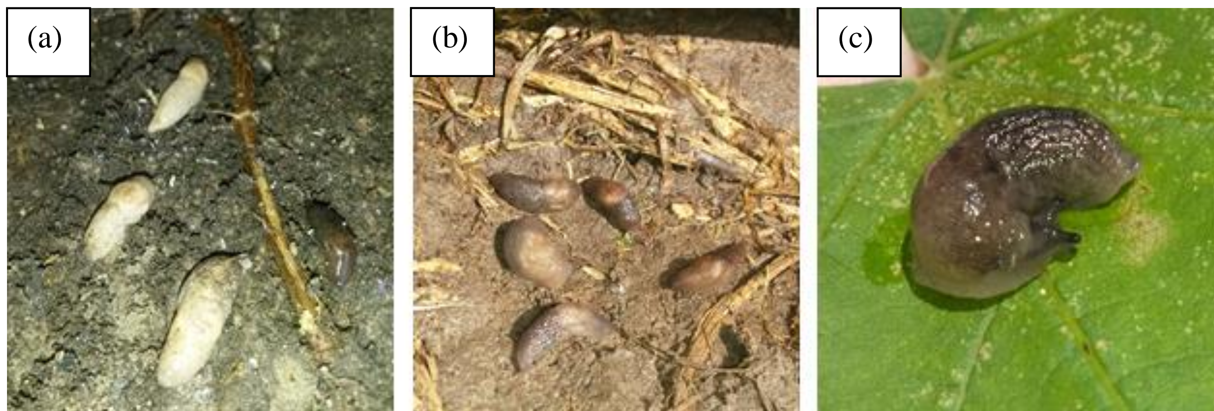

Figure 1.1 The three mollusc species most pestiferous in South African canola: (a) *Deroceras reticulatum*, (b) *Deroceras panormitanum*, and (c) *Milax gagates*.

A possible explanation for the success of invasive terrestrial molluscs in South Africa is the 'enemy release' hypothesis, which refers to the theory that organisms are freed from the effect of their co-evolved natural enemies when invading new areas, thus giving them competitive advantage over the native species (Torchin *et al.*, 2001). Ross *et al.* (2010a) determined that enemy release plays a significant function in the invasion of European molluscs in North America. Comparable findings were also obtained in South Africa (Ross *et al.*, 2012), with the invasive range having low levels of species richness and parasite prevalence.

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1.3 Molluscs as intermediate hosts

In addition to crop pests, many terrestrial mollusc species are intermediate hosts to parasites, making them a potential health risk to humans and wildlife (South, 1992). A detailed review of nematodes using molluscs as intermediate hosts has been described by Grewal *et al.* (2003a).

1.4 Control

1.4.1 Chemical control

Molluscs are currently controlled in South Africa using chemical molluscicide pellets (Fig. 1.2), containing a combination of 30 g kg^{-1} metaldehyde and 20 g kg^{-1} carbaryl, at a proposed application rate of 6-12 kg ha^{-1} (Tribe & Lubbe, 2010). Chemical molluscicide toxins include: iron phosphate, carbamate compounds (methiocarb and thiodicarb), and metaldehyde. See Bailey (2002) for a review of chemical control. However, the use of chemical molluscicides carries a number of environmental risks. Metaldehyde and methiocarb compounds have proven to be toxic to an array of vertebrates (Fletcher *et al.*, 1994), as well as to some species of isopods (Santos *et al.*, 2010), with methiocarb being toxic to a number of beneficial invertebrates, like carabid beetles and earthworms (Purvis & Bannon, 1992). Furthermore, growers often overuse slug pellets, as they are unaware of the intensity of their slug populations, resulting in such exaggerated environmental problems as surface wash-off after heavy rain (O'Brien *et al.*, 2008). As a result, the European Union has voted to ban methiocarb across Europe, due to the effects of the pesticide on key organisms (Jones, 2014). In addition, metaldehyde contamination of drinking water has received much attention since the UK Environmental Agency, in 2008, warned that the metaldehyde levels in some areas exceeded European and UK drinking water standards. Iron phosphate is highly effective in controlling a number of mollusc species and is favoured in organic farming. Recommendations have been made to

combine iron phosphate pellets with nematode applications to effectively control molluscs with a decreased effect on the environment (Rae *et al.*, 2009a).

Figure 1.2 Products sold in South Africa for the chemical control of molluscs in gardens.

1.4.2 Cultural control

Cultural methods of controlling molluscs include the use of physical barriers, irritants, antifeedants, and chemical repellents. The most effective cultural control methods for use against slugs include cinnamamide, garlic, mulch, copper foil, copper ammonium carbonate, aluminium foil, Tex-R®, and Snailban®, although such methods are not cost-effective for extensive use with large-scale crops (Schüder *et al.*, 2003). Slugs can also be controlled by means of trapping, drilling at greater depth, ploughing, crop rotation, increased cropping diversity, and firm seedbed preparation (Glen, 2000).

1.4.3 Biological control

Establishing a method of control for molluscs that is effective, but that is neither harmful nor toxic to its surrounding environment, is important. One such method entails the use of biological control, in terms of which one or more living organisms are used to control another living organism. Although gastropods have a number of predators and parasites that could be

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developed for biological control, to date, the European mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrássy, 1983 (Rhabditida: Rhabditidae), has shown the greatest commercial potential (Rae *et al.*, 2007).

1.5 *Phasmarhabditis hermaphrodita*

1.5.1 Classification

In a revision of the '*Papillosa*' group in the genus *Pellioditis* Dougherty, 1953 *sensu* Sudhaus (Sudhaus 1976, 2011; Sudhaus & Fitch, 2001), Andrássy (1976) proposes a completely different genus, *Phasmarhabditis.* The new genus was conceived of five different species, including *Phasmarhabditis papillosa* (Schneider, 1866) Andrássy, 1983 as the type species, *P. hermaphrodita* and *Phasmarhabditis neopapillosa* (Mengert, 1952) Andrássy, 1983 from the '*Papillosa*' group, and two littoral/marine species (*Phasmarhabditis nidrosiensis* Allgén 1933 and *Phasmarhabditis valida* Sudhaus 1974) (Andrássy, 1983).

In a more recent revision than the above, Sudhaus (2011), moved the *nidrosiensis* and *valida* littoral/marine species to another genus (*Buetschlinema* Sudhaus, 2011). The remaining *Phasmarhabditis* species were transferred to the '*Papillosa'* group, located within the *Pellioditis* genus (Sudhaus, 2011). The fundamental similarity of *Pellioditis sensu* Sudhaus (Sudhaus, 1976, 2011; Sudhaus & Fitch, 2001) and *Phasmarhabditis sensu* Andrássy (Andrássy, 1976, 1983) can be seen in them comprising the stem species of the '*Papillosa*' group (*P. hermaphrodita, P. neopapillosa*, and *P. papillosa*). The nomenclature '*Pellioditis*' has priority over '*Phasmarhabditis*', although both names refer to the same group of rhabditids (*P. hermaphrodita, P. neopapillosa*, and *P. papillosa*), but with the nomenclature concerned being based on different perceptions. In order to avoid taxonomic confusion, new species have been referred to as *Phasmarhabditis*. To date, the *Phasmarhabditis* Andrássy, 1976 genus is known from six different species (*P. hermaphrodita*; *P. neopapillosa*; *P. papillosa*;

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Phasmarhabditis tawfiki Azzam, 2003; *Phasmarhabditis huizhouensis* Huang, Ye, Ren & Zhao, 2015; and *Phasmarhabditis californica* Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine & De Ley, 2016).

1.5.2 Isolation

The first description of *P. hermaphrodita* (referred to as *Pelodytes hermaphroditus*) was undertaken by Schneider in 1859, who found the nematode associating with an *Arion* spp. (Schneider, 1859). Later, Maupas (1900) reisolated the nematode (referred to as *Rhabditis caussaneli*) from the intestine of an *Arion ater* (Linnaeus, 1758) collected in Normandy (France), thereafter maintaining the nematode culture on rotting flesh. Maupas (1900) noted that the nematode, instead of reproducing sexually, produced protandrous autogamous hermaphrodites, with males occurring only very rarely (1 male / 715 females). Mengert (1953), in conducting a study on nematodes associated with terrestrial molluscs, failed to find *P. hermaphrodita*, although the closely related species, *P. neopapillosa*, was isolated from the slug, *Limax cinereoniger* Wolf, 1803. Mengert (1953) suggests that *P. hermaphrodita*, *P neopapillosa*, and an additional species, *P. papillosa*, are all from the same ecological grouping, with them all undergoing the same form of nonparasitic life cycle. Andrássy (1983) found that *P. hermaphrodita* and *P. neopapillosa*, were identical in their morphology, with their only distinguishable feature, in relation to each other, being their sex ratios, with the latter being characterised by its equal numbers of males and females, in contrast to the rare occurrence of males in the former.

In 2003, a new member of the genus was described, being *P. tawfiki*, which was isolated in Egypt, from the terrestrial snail *Eobania vermiculata* (Müller, 1774) and from the slug *Limax flavus* Linneaus, 1758 (Azzam, 2003). In 2015, another new species, *P. huizhouensis*, was described from its location in rotting leaves in the Guangdong Province of China (Huang *et al.*,

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2015). In the following year, *P. californica* was described from where it was found in *D. reticulatum* collected in California, USA (Tandingan De Ley *et al*., 2016). A number of undescribed *Phasmarhabditis* spp. have also been identified across the USA (Tandingan De Ley *et al*., 2014) and South Africa (Ross *et al*., 2012), along with several individual isolates from Slovenia and Tanzania (Ross unpubl.) (Fig. 1). The increase in the number of species discovered, as well as the finding of the, so far, undescribed *Phasmarhabditis* species, indicates that the genus is more diverse than was initially anticipated.

1.5.3 Life cycle

Phasmarhabditis hermaphrodita is a facultative parasite that can undergo three type of life cycles: saprobic, necromenic and parasitic. It can live on organic matter, slug faeces, dead earthworms and insects (Nermut' *et al.*, 2014), or exhibit a necromenic strategy in relation to the relatively large slug species (e.g. *A. ater*) (Rae *et al.*, 2009b). The parasitic life cycle of *P. hermaphrodita* has been studied by Wilson *et al*. (1993a) and by Tan & Grewal (2001), using the grey field slug, *D. reticulatum*. Third-stage dauer dauer juvenile larvae, upon entering the slug through the dorsal integumental pouch, travel to the shell cavity lying immediately below the mantle. After recovery, the larvae develop into self-fertilising hermaphrodites, whereupon they reproduce and feed on the bacteria in the host (Wilson *et al*., 1993a; Tan & Grewal, 2001). *Phasmarhabditis hermaphrodita* usually produces approximately 250-300 offspring, with the second generation spreading throughout the slug's body in which it develops. Following the death of the slug concerned, a third generation of nematodes is produced, which feed on the cadaver. On depletion of the food source involved, new dauer juveniles are produced that then enter the soil to find new hosts (Wilson & Rae, 2015). Host death, which usually occurs between 4-21 days after nematode infection, is dependent on factors relating to external temperature, inoculum density of the nematode species and the pathogenicity of its associated bacteria (Wilson *et al*., 1993a; Tan & Grewal, 2001). Under laboratory conditions, *P.*

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hermaphrodita dauer juveniles infect *D. reticulatum* within a period of 8-16 hours (Tan & Grewal, 2001). Although dauer juvenile nematodes act as the dauer stage concerned, Tan & Grewal (2001) have proved that, when such nematodes are injected directly into the slug, both adult and juvenile nematodes are capable of causing the mortality of *D. reticulatum*.

Phasmarhabditis hermaphrodita has a life cycle that is similar to that of the entomopathogenic nematodes (i.e. Steinernematidae and Heterorhabditidae), however, unlike entomopathogenic nematodes, no specific symbiotically associated bacterium has been isolated from this nematode to date. Members of the Steinernematidae and Heterorhabditidae families, which are lethal parasites of a variety of soil-dwelling insects, have been commercially developed as biological control agents (Lacey & Georgis, 2012; Campos-Herrera, 2015). Third-stage dauer juveniles, when applied in a water-dispersible formulation, can infect insect larvae through their natural openings (anus, mouth, and spiracles). The former then release their symbiotic bacteria (Enterobacteriaceae: *Xenorhabdus* Thomas & Poinar, 1979 for Steinernematidae, and *Photorhabdus* (Thomas & Poinar, 1979) for Heterorhabditidae), causing the insects involved to die within 24-48 hours of exposure. The nematodes then recover and continue their life cycle on the insect cadaver, thereby producing two to three generations, depending on the size of the insect involved. On depletion of the food source concerned, new dauer juveniles are produced that move off into the soil, in search of new insect hosts.

1.5.4 Host range

A wide range of slug species is susceptible to *P. hermaphrodita*, including the families Agriolimacidae, Arionidae, Limacidae, Milacidae, and Vagnulidae (Rae *et al.*, 2007). However, a number of species within the above-mentioned families are unaffected, including *Limax maximus* Linnaeus, 1758, *Arion subfuscus* (Draparnaud, 1805) and *Arion hortensis* Férussac 1819 (Grewal *et al.*, 2003b). The immunity of such species could be due to their size,

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with studies showing the juveniles to be susceptible to the nematode concerned, whereas the adults are not (Glen *et al*., 1996). Speiser *et al.* (2001) have demonstrated the failure of *P. hermaphrodita* to kill, or to at least inhibit the feeding of, *Arion vulgaris* Moquin-Tandon, 1855 (also known as *Arion lusitanicus* Mabille, 1968) that are over 1 g in mass, indicating that the nematode can offer crop protection against only the younger stages of the species, thus reducing the effectiveness of its use (Speiser *et al*., 2001; Grimm, 2002; Kozłowski *et al.*, 2014). Field trials with *A. hortensis* and *Arion distinctus* Mabille, 1868 have also revealed that such slugs have low susceptibility to *P. hermaphrodita* (Rae *et al*., 2007).

Snail species that are susceptible to *P. hermaphrodita* include: *Cornu aspersum* (Müller, 1774); *Cepaea hortensis* (Müller, 1774); *Lymnaea stagnalis* (Linnaeus, 1758); and *Monacha cantiana* (Montagu, 1803) (Rae *et al*., 2007). In addition to identifying two strains of *P. hermaphrodita* from the snail species *Theba pisana* (Müller, 1774), and *Trochoidea elegans* (Gmelin, 1791), Coupland (1995) conducted pathogenicity studies on *Cochlicella acuta* (Müller, 1774), *Cernuella virgata* (Da Costa, 1778), and *T. pisana*. The nematode concerned was found to cause 80-100% mortality within 8 days of initial exposure (Coupland, 1995). *Phasmarhabditis hermaphrodita* have also been isolated from the following snail species: *Oxychillus draparnaudi* (Beck, 1837); *Pomatias elegans* (Müller, 1774); *Cepaea nemoralis* (Linnaeus, 1758); *Succinea putris* (Linnaeus, 1758); *Discus rotundatus* (Müller, 1774); *Euomphalia strigella* (Draparnaud, 1801); *Monacha cartusiana* (Müller, 1774); and *Helix pomatia* Linnaeus, 1758 (Morand *et al*., 2004). However, their effect on the snail species in question is, as yet, unknown (Morand *et al.*, 2004). Snail species that are not susceptible to *P. hermaphrodita* include *Cepaea nemoralis* (Linnaeus, 1758), *Physa fontinalis* (Linnaeus, 1758), *P. elegans, Oxychilus helveticus* (Blum, 1881), *Ponentina ponentina* (Morelet, 1845), *Clausilia bidentata* (Ström, 1765), *D. rotundatus*, and *A. fulica* (Rae *et al*., 2007; Williams &

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Rae, 2015). *Achatina fulica* have the ability to encapsulate and destroy invading nematodes with their shell (Williams & Rae, 2015).

Despite the initial studies that have been conducted on freshwater snails having shown *P. hermaphrodita* to be pathogenic to laboratory-reared *L. stagnalis* (Morley & Morritt, 2006), Whitaker & Rae (2015) have demonstrated that wild *L. stagnalis*, *Planorbarius corneus* (Linnaeus, 1758), and *Bithynia tentaculata* (Linnaeus, 1758) can remain unaffected by the nematode, possibly due to an increased immune response.

In addition to molluscs, the commercial strain of *P. hermaphrodita* has also been tested on a range of non-target invertebrates, being found to be non-pathogenic to several insects (Wilson *et al.,* 1994a), acarids, collembolans, and earthworms (Iglesias *et al.*, 2003). Both UK and US strains of earthworms have been tested, including *Lumbricus terrestris* Linnaeus, 1758, *Eisenia andrei* Bouché, 1972, *Eisenia fetida* (Savigny, 1826), *Eisenia hortensis* (Michaelsen, 1890), *Dendrodrilus rubidus* (Savigny, 1826), and the platyhelminth *Arthurdendyus triangulatus* (Dendy, 1894), with all the above-mentioned being unaffected by the nematode concerned (Grewal & Grewal, 2003; DeNardo *et al.*, 2004; Rae *et al.*, 2005). However, the results in question contradict initial studies conducted by Zaborski *et al.* (2001), who found a '*Phasmarhabditis*-like' species infecting *L. terrestris*. Nevertheless, the results obtained in the present instance might have come about due to a misdiagnosis (Rae *et al*., 2007).

1.5.5 Mass production

Methods for mass-producing *P. hermaphrodita* have been adapted from entomopathogenic nematode protocols (Ehlers & Shapiro-Ilan, 2005). The methods concerned include modifying solid-phase foam-chip and deep-liquid cultures (Wilson *et al.*, 1993b). However, the *in vivo* production of *P. hermaphrodita* is more difficult than is that of entomopathogenic nematodes (in the case of the commercially produced *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera:

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Pyralidae) and *Tenebrio molitor* Linnaeus 1758 (Coleoptera: Tenebrionidae)) (Rae *et al.*, 2007), as slug hosts must be either field-collected or laboratory-reared.

In the light of the above, the research that has been undertaken so far has focused on *in vitro* production using xenic (*P. hermaphrodita*, and an unknown mix of bacterial species) or monoxenic cultures (*P. hermaphrodita*, and one known bacterial species) (Wilson *et al.*, 1995a). The latter production has been favoured, as it offers a more predictable result than does the former (Ehlers & Shapiro-Ilan, 2005). The use of monoxenic cultures also helps to ensure the production of high numbers of dauer juveniles, with consistent infectivity (Wilson *et al.*, 1995a).

Wilson *et al.* (1995a) conducted initial monoxenic studies using sixteen different bacterial isolates, comprised of thirteen different species. Results indicated that the bacterial isolates *Providencia rettgeri* (Hadley *et al.*, 1918) (Enterobacteriaceae) and *Moraxella osloensis* (Bøvre & Henriksen, 1967) (Moraxellaceae) produced the highest yields of dauer juveniles, with *P. rettgeri* producing the highest numbers overall (85 000 dauer juveniles ml^{-1}). Investigation of the virulence of the nematodes grown on different bacterial isolates revealed not only that *M. osloensis* produced high yields of dauer juveniles, but that it was also consistently pathogenic to *D. reticulatum*. Therefore, it was chosen as the bacterium for the commercial production of *P. hermaphrodita* (Wilson *et al.*, 1995b). However, it was noted by Rae *et al.* (2010) that *M. osloensis* does not naturally associate with *P. hermaphrodita,* which is in contrast to its association with the commercially available entomopathogenic nematodes (e.g. Heterorhabditidae and Steinernematidae, which have a symbiotic association with *Photorhabdus* spp. and *Xenorhabdus* spp. bacteria, respectively).

To date, *P. hermaphrodita* has been mass produced in large-scale 20 000-litre fermenters, using monoxenic liquid cultures of *M. osloensis*. After each run, dauer juveniles are extracted from the media by means of centrifugation and repeated washing with water.

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Dauer juveniles are then mixed with an inert gel polymer (G. Martin, pers. comm., January 6, 2016) to produce a water-dispersible formulation (Glen *et al.*, 1994). The mixtures are then sealed and packaged in sizes of 30 million and 250 million nematodes per pack. Despite the *P. hermaphrodita* formulation being able to survive for a period of up to six months under refrigerated conditions of -4ºC, the shelf life of the product is much shorter than is the standard two-year shelf life of chemical pesticides (Grewal, 2001).

1.5.6 Commercialisation

In the early 1990s, Wilson *et al*. (1993a) patented the use of *P. hermaphrodita* as a biological molluscicide, after the nematode concerned was found actively reproducing within the mantle cavity of a diseased *D. reticulatum* (Wilson *et al*., 1993a). Further research has shown that *P. hermaphrodita* is capable of infecting a wide range of terrestrial slug species, including the families Agriolimacidae, Arionidae, Milacidae, Limacidae, and Vagnulidae (Rae *et al*., 2007), resulting in an extensive amount of interest in developing the nematode for *in vitro* production. In 1994, the biological molluscicide, Nemaslug® (Fig. 1.3), was commercially released by MicroBio Ltd (which was later acquired by Becker Underwood in 2000, to be taken over by BASF in 2012). Nemaslug® is currently sold in fifteen different European countries, based on its natural distribution: Belgium; the Czech Republic; Denmark; Finland; France; Germany; Ireland; Italy; the Netherlands; Norway; Poland; Spain; Sweden; Switzerland; and the UK (G. Martin, pers. comm., March 24, 2015) (Fig. 1.4).

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Figure 1.3 Nemaslug® is sold in a water-dispersible formulation.

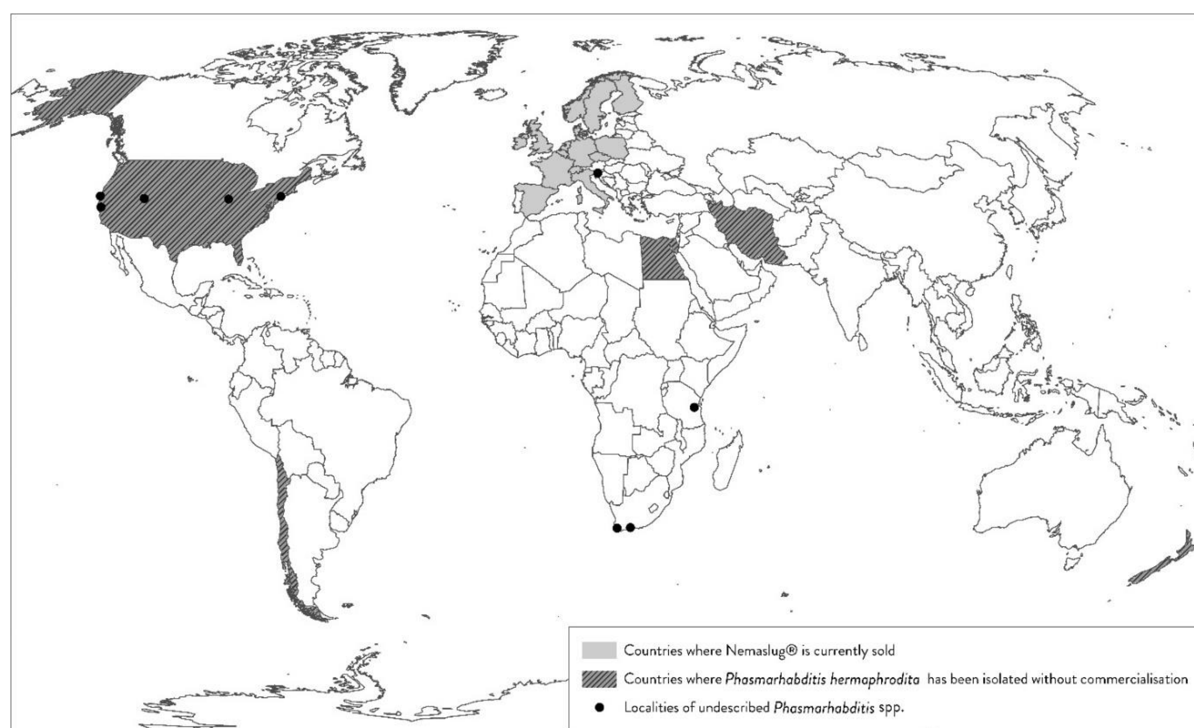

Figure 1.4 Current retail market and distribution of *Phasmarhabditis hermaphrodita*, along with localities of undescribed *Phasmarhabditis* spp.

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The molluscicide concerned is an important part of BASF's nematode business, with current retail sales standing at approximately 1 million euro, although the amount sold tends to fluctuate in response to annual rainfall patterns (G. Martin, pers. comm., March 24, 2015).

Nemaslug® has a number of potential markets outside Europe, with studies documenting the presence of *P. hermaphrodita* in Egypt (Genena *et al.*, 2011), Iran (Karimi *et al.*, 2003), Chile (France & Gerding, 2000), New Zealand (Wilson *et al.*, 2012), and the USA (Tandingan De Ley *et al.*, 2014) (Fig. 1.4). However, to date, *P. hermaphrodita* has not been isolated in South Africa, so its use as a biological control agent is regulated by the Agricultural Pests Amendment Act, No. 18 of 1989 (South Africa, 1989), which prohibits the introduction of exotic animals (Ross *et al.*, 2012).

1.5.7 Field application

Nemaslug® is effective against molluscs in a number of crop types, including winter wheat (Wilson *et al.*, 1994b), oilseed rape (Ester & Wilson, 2005), strawberries (Glen *et al.*, 2000), Brussels sprouts (Ester *et al.*, 2003a), sugar beet (Ester & Wilson, 2005), cabbages (Grubisic *et al.*, 2003), asparagus (Ester *et al.*, 2003b), lettuce (Ester & Wilson, 2005), hostas (Grewal *et al.*, 2001), and orchids (Ester *et al.*, 2003c). The product is sold as a water-dispersible formulation that can be applied with a watering can, with a hydraulic sprayer, or with specialised nematode application equipment, which has been designed to inject *P. hermaphrodita* into water for boom or gun irrigators, while keeping the nematode solution agitated and aerated (Brown *et al.*, 2011).

The standard application rate for Nemaslug® is 3×10^9 dauer juveniles per ha^{-1} (Rae *et al.*, 2007). However, the high production costs involved mean that it is not feasible to use *P. hermaphrodita* on a large scale. However, the use of novel application methods might reduce

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the application costs, thus making such employment of the nematode more appealing than it has been in the past. Although the success of *P. hermaphrodita* has been documented on numerous occasions, the failure of the nematode has been reported, on occasion, to provide adequate protection in this regard (Glen *et al*., 2000). However, such lack of success might have been due to unfavourable environmental factors, poor nematode handling, or the presence of nonsusceptible species (Rae *et al*., 2007).

1.6 Other nematodes associated with molluscs as definitive hosts

Current knowledge of nematodes that use molluscs as definitive hosts is based on surveys that have been conducted in Europe (Ross *et al.*, 2016a), Asia (Ivanova *et al.*, 2016), North America (Ross *et al.*, 2010a), Australia (Charwat & Davies, 1999) and Africa (Ross *et al.*, 2012), along with a number of individual nematode parasites found globally (see Morand *et al.*, 2004 and Grewal *et al.*, 2003a for reviews). The surveys concerned reveal that there are eight families of nematodes that associate with molluscs as definitive hosts, namely: Agfidae; Alaninematidae; Alloionematidae; Angiostomatidae; Cosmocercidae; Diplogastridae; Mermithidae; and Rhabditidae (including the genus *Phasmarhabditis*). Detailed reviews of the characterisation and host range of the above-mentioned nematode families can be found in Morand *et al.* (2004) and Grewal *et al.* (2003a). Ross *et al*. (2010b) has demonstrated that Agfidae, Angiostomatidae and Rhabditidae (*Phasmarhabditis* genus) form a tight monophyletic group, indicating that they evolved from a single mollusc-colonising ancestor.

Mollusc-parasitic nematodes should be identified using a combination of morphological and molecular analysis. Often, field-collected molluscs have been found to contain a mixed nematode infection (Ross *et al.*, 2016a), requiring that molecular analysis should be conducted on individual nematode specimens. There are multiple methods for DNA extraction (see Tandingan De Ley *et al.*, 2007; Ross *et al.*, 2010b), and amplification should

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be based on numerous genes: the small subunits (SSU or 18S) rRNA gene; the D2D3 large subunit (LSU or 28S) rRNA gene; the internal transcribed spacer regions (ITS-1, 5.8S, ITS-2) rRNA gene; and the mitochondrial cytochrome *c* oxidase subunit 1 (mtCOI) gene. Table 1.1 summarises the primers that have been successfully used to identify nematodes associated with molluscs.

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Table 1.1 Primers for amplifying 18S rRNA, 28S rRNA, ITS rRNA and mtCOI that have been successfully used for the molecular identification of mollusc-parasitic nematodes.

Primer name	Sequence (5'-3')	Gene	Reference
G18S4 (nSSU_F_04)	GCTTGTCTCAAAGATTAAGCC	18S rRNA	Blaxter *et al.*, 1998
A (uSSU_F_07)	AAAGATTAAGCCATGCATG	18S rRNA	Blaxter *et al.*, 1998
26R (nSSU_R_26)	CATTCTTGGCAAATGCTTTCG	18S rRNA	Blaxter *et al.*, 1998
22F (NSSU_F_22)	TCCAAGGAAGGCAGCAGGC	18S rRNA	Blaxter *et al.*, 1998
1080JR	TCCTGGTGGTGCCCTTCCGTCAATTTC	18S rRNA	Ross *et al.*, 2010b
24F (nSSU_F_24)	AGRGGTGAAATYCGTGGACC	18S rRNA	Blaxter *et al.*, 1998; Da Silva *et al.*, 2010
18P (nSSU_R_81)	TGATCCWKCYGCAGGTTCAC	18S rRNA	Blaxter *et al.*, 1998
Q39	TAATGATCCWTCYGCAGGTTCACCTAC	18S rRNA	Ivanova *et al.*, 2013
RHAB1350F	TACAATGGAAGGCAGCAGGC	18S rRNA	Haber *et al.*, 2005; Barrière & Félix, 2006
RHAB1868R	CCTCTGACTTTCGTTCTTGATTAA	18S rRNA	Haber *et al.*, 2005; Barrière & Félix, 2006
55F*	GCCGCGAATGGCTCGGTATAAC	18S rRNA	Ross *et al.*, 2010b
920DR*	CTTGGCAAATGCTTTCGCAG	18S rRNA	Ross *et al.*, 2010b
555F*	AGCCGCGGTAATTCCAGCTC	18S rRNA	Ross *et al.*, 2010b
1165SR*	CGTGTTGAGTCAAATTAAGCCGCAGG	18S rRNA	Ross *et al.*, 2010b
18s-5F*	GCGAAAGCATTTGCCAAGAA	18S rRNA	Vandergast & Roderick, 2003
18s-9R*	GATCCTTCCGCAGGTTCACCT	18S rRNA	Vandergast & Roderick, 2003
D2A	ACAAGTACCGTGAGGGAAAGTTG	28S rRNA	Nunn, 1992
D3B	TCGGAAGGAACCAGCTACTA	28S rRNA	Nunn, 1992
No 391	AGCGGAGGAAAAGAAACTAA	28S rRNA	Nadler & Hudspeth, 1998; Nadler *et al.*, 2003
No 501	TCGGAAGGAACCAGCTACTA	28S rRNA	Thomas *et al.*, 1997; Nadler *et al.*, 2003
D2F	CCTTAGTAACGGCGAGTGAAA	28S rRNA	Nguyen, 2007
503R	CCTTGGTCCGTGTTTCAAGACG	28S rRNA	Nguyen, 2007
502F	CAAGTACCGTGAGGGAAAGTTGC	28S rRNA	Nguyen, 2007
536R	CAGCTATCCTGAGGAAAC	28S rRNA	Nguyen, 2007
N93F	TTGAACCGGGTAAAAGTCG	ITS rRNA	Nadler *et al.*, 2005
N94R	TTAGTTTCTTTTCCTCCGCT	ITS rRNA	Nadler *et al.*, 2005
AB28	ATATGCTTAAGTTCAGCGGGT	ITS rRNA	Joyce *et al.*, 1994
TW81	GTTTCCGTAGGTGAACCTGC	ITS rRNA	Joyce *et al.*, 1994
18S	TTGATTACGTCCCTGCCCTTT	ITS rRNA	Vrain *et al.*, 1992
26S	TTTCACTCGCCGTTACTAAGG	ITS rRNA	Vrain *et al.*, 1992
COIF1	CCTACTATGATTGGTGGTTTTGGTAATTG	mtCOI	Kanzaki & Futai, 2002
COI-R2	GTAGCAGCAGTAAAATAAGCACG	mtCOI	Kanzaki & Futai, 2002

*Primers for Mermithidae

1.6.1 Rhabditida: Agfidae

Currently, three known species exist within the Agfidae family: *Agfa flexilis* (Dujardin, 1845); *Agfa morandi* Ribas & Casanova, 2002; and *Agfa tauricus* Korol & Spiridonov, 1991 (Morand *et al.*, 2004). *Agfa flexilis* has been recorded in Europe, the USA and Africa (Ross *et al.*, 2010a, 2016a), whereas *A. morandi* has only been isolated in the French Pyrenees (Ivanova *et al.*,

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2013). *Agfa tauricus* has been found in several locations in the Crimea and Bulgaria (Ivanova *et al*., 2013). Agfids, which are obligate parasites that are isolated in the adult and juvenile stages, are characterised by means of their long, thin neck region. All three agfids parasitize limacid hosts, with the exception of *A. tauricus*, which has been found to associate with agriolimacids and a zonitid snail, and *A. flexilis*, which has been found associating with *A. vulgaris* (Ross *et al*., 2016a). Little is known about the life cycle of the Agfidae family, apart from the fact that they are obligate parasites of molluscs. To date, no study has yet investigated the potential of agfids to serve as a biological control agent for molluscs.

1.6.2 Panagrolaimorpha: Alaninematidae

Little is known about the family Alaninematidae, which consists of only one genus, *Alaninema* Théodoridès, 1957. To date, three species have been described: *Alaninema venmansi* Théodoridès, 1957, which was found in *Amphidromus contrarius* (Müller, 1774) from Indonesia; *Alaninema njoroensis* Puylaert, 1970, which was isolated from an unidentified mollusc host in Kenya; and *Alaninema ngata* Ivanova, Spiridonov, Clark, Tourna, Wilson & Barker, 2013, which is known to parasitise the intestines of endemic leaf-veined slugs in New Zealand (Ivanova *et al*., 2013). Another undescribed species is said to occur in Athoracophoridae in New Zealand (Morand *et al*., 2004). The biology of the nematodes mentioned is unknown, but they associate with the pallial cavity, or digestive tract, of their mollusc host (Morand *et al*., 2004).

1.6.3 Rhabditida: Alloionematidae

Currently, three known genera exist within the Alloionematidae family: *Rhabditophanes* Fuchs, 1930, which associate with insects, whereas *Alloionema* Schneider, 1859, and *Neoalloionema* Ivanova, Pham Van Luc & Spiridonov, 2016, associate with molluscs.

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Alloionema, which is represented by *Alloionema appendiculatum* Schneider, 1859, was recently redescribed by Nermut' *et al.* (2015). Although the initial reports stated that *A. appendiculatum* is non-pathogenic to hosts (Charwat & Davies, 1999), more recent studies have shown that the nematode is, indeed, capable of attaining significant mortality in Petri dish experiments of *A. vulgaris* (Laznik *et al*., 2009). With the nematode exhibiting both free-living and parasitic life cycles, during its parasitic phase, third- and fourth-stage larvae become encapsulated within the pedal musculature (Morand *et al*., 2004). *Alloionema appendiculatum*, which is widespread, has been isolated in North America, Europe and Australasia (Charwat & Davies, 1999; Ross *et al*., 2010a,b, 2016a).

Neoalloionema is represented by *Neoalloionema tricaudatum* Ivanova *et al.*, 2016, a specialist nematode of *Cyclophorus* sp., which has been isolated in Vietnam. The nematode associates with the pallial cavity of its snail hosts. Despite little being known of its life cycle, the nematode's ability to grow on the tissues of a dead host indicates that it undergoes a free-living stage (Ivanova *et al.*, 2016).

1.6.4 Rhabditida: Angiostomatidae

The Angiostomatidae family consists of two known genera, *Angiostoma* Dujardin, 1845 and *Aulacnema* Pham Van Luc, Spiridonov & Wilson, 2005. The *Angiostoma* genus has eighteen known species, whereas *Aulacnema* is monotypic. Molluscan angiostomatids are obligate parasites of the intestine, the hepatopancreas, the oesophagus, the buccal mass, the crop, the mantle cavity, the salivary gland, and the pallial cavity (Ross *et al*., 2016a,b). In addition to being molluscan angiostomatids, four species have, so far, been recovered from the intestine and bronchi of their amphibian and reptile hosts (Falcón-Ordaz *et al.*, 2008). The potential of angiostomatids as a biological control agent is, as yet, unknown.

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1.6.5 Ascaridida: Cosmocercidae

The Cosmocercidae family has two genera that associate with molluscs, namely *Nemhelix* Morand & Petter, 1986 and *Cosmocercoides* Wilkie, 1930. *Cosmocercoides* associates with terrestrial molluscs in North America, whereas *Nemhelix* associates with European snails (Morand *et al.*, 2004).

Cosmocercoides are represented by *Cosmocercoides dukae* Holl, 1928. Little is known about the potential of this nematode as a biological control agent of molluscs, although a study of the transmission process found that, when third-stage larvae leave the mantle cavity, and are then placed on the foot sole of a new mollusc host, they enter the respiratory pore and mantle cavity to develop further internally. In addition, larvae have also been found to be present in the genital tract and eggs of slugs (Morand *et al.*, 2004).

The *Nemhelix* genera are represented by three known species, of which all three infect the reproductive organs of terrestrial snails in Europe. *Nemhelix bakeri* Morand & Petter, 1966 is a parasite of *Cornu aspersum* (Müller, 1774), with *Nemhelix lamottei* Morand, 1989 being a parasite of *Cepaea nemoralis* (Linnaeus, 1758), and *Nemhelix ludesensis* Morand, 1989 being a parasite of *Cepaea hortensis* (Müller, 1774) (Morand *et al.*, 2004).

Although the Cosmocercidae family is also known to affiliate with reptile and amphibian hosts, it has been suggested that cosmocercids only occur in amphibians due to the ingestion of infected mollusc hosts (Vanderburgh & Anderson, 1987).

1.6.6 Diplogastrida: Diplogastridae

Although Diplogastridae is usually associated with invertebrates, or has a free-living life cycle in soil, nematodes in the adult and juvenile stages have been found in molluscs. *Hugotdiplogaster neozelandia* Morand & Barker, 1995 infects molluscs in its adult stage, whereas Diplogaster Shultze 1857 infect molluscs during their larval stages (Morand *et al.*,

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2004). The potential of the Diplogastridae family as biological control agents of molluscs has not yet been investigated.

1.6.7 Mermithida: Mermithidae

Mermithidae is a family of nematodes of which the members can be extremely large, ranging from 1-10 cm in length (Poinar, 1983). Mermithids produce preparasitic juveniles that infect hosts either through direct penetration, or by means of the ingestion of eggs and paratenic hosts. Although mermithids are usually obligate parasites of insects and arthropods (e.g. spiders and crustaceans), they have also been found to associate with molluscs (Morand *et al.*, 2004). Their potential as biological control agents of molluscs has not yet been investigated.

1.6.8 Rhabditida: Rhabditidae

Rhabditidae are a large family, with species ranging from free-living nematodes to parasites of invertebrates, or commensals of insects (Morand *et al.*, 2004). The several different genera that are associated with molluscs include *Rhabditis* Dujardin, 1845, *Caenorhabditis* (Osche, 1952) Dougherty, 1953 and *Phasmarhabditis* Andrássy, 1976. In contrast to other true parasites (e.g. Agfidae and Angiostomatidae), *Phasmarhabditis* spp. are facultative parasites that live on compost, leaf litter, slug faeces, and dead earthworms and insects (Nermut' *et al.*, 2014). They also exhibit a necromenic strategy in relation to the relatively large slug species (e.g. *A. ater*) (Rae *et al.*, 2009b). In addition, Petersen *et al*. (2015) have demonstrated that *Caenorhabditis elegans* Maupas, 1900 can invade the intestine of molluscs, exiting, while still alive, through the faeces.

1.7 Nematodes associated with molluscs as definitive hosts in South Africa

A survey of nematodes associated with terrestrial slugs that was conducted in the Western Cape province of South Africa by Ross *et al.* (2012) found seven different nematode species,

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including *Agfa flexilis* Dujardin 1845, *Angiostoma* sp. (now described as *Angiostoma margaretae* Ross, Malan & Ivanova, 2011), *Phasmarhabditis* sp. SA1, *Phasmarhabditis* sp. SA2, *C. elegans*, *Panagrolaimus* sp., and *Rhabditis* sp. The nematodes mentioned represented three families, including Agfidae, Angiostomatidae and Rhabditidae. Ross (2010), on investigating the potential of the nematodes as biological control agents, demonstrated that *Phasmarhabditis* sp. SA2 was capable of profuse growth on modified kidney agar, and of causing significant mortality to *D. reticulatum* (Ross, 2010).

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CHAPTER 2

Survey of nematodes associated with terrestrial slugs in the Western Cape province of South Africa and investigation of their pathogenicity

Abstract

A survey of nematodes associated with slugs from canola crops and commercial nurseries was conducted in the Western Cape province with the aim of discovering indigenous nematode isolates for the biological control of molluscs. A total of 1944 slugs were collected from 12 different study sites. On the identification of slugs, they were dissected alive, and examined for internal nematodes. Nematodes were identified using morphological and molecular techniques (18S rRNA). Eight of the 12 sites had nematodes present, with 8% of the slugs being found to be infected with nematodes. Six nematode species were identified, including *Angiostoma margaretae*, *Angiostoma* sp., *Caenorhabditis elegans*, a mermitid sp., and *Phasmarhabditis* spp. (SA3 and SA4). Of the six species mentioned, four were previously undescribed. This is the first record of *A. margaretae* associating with *Deroceras panormitanum* and *Deroceras reticulatum*. In addition, this is the first time that a mermithid has been found associating with molluscs in Africa.

2.1 Introduction

Terrestrial gastropod molluscs (slugs and snails) (Mollusca: Gastropoda) are one of the most successful animal groups in the terrestrial ecosystem (Barker, 2001). They have established themselves as important economic pests in temperate and tropical regions, due to the cultivation of new crops, the intensification of agricultural production systems, and spreading through human activity (Barker, 2002). Major crops that are targeted by molluscs include arable, pasture, ornamental, citrus, vine, and vegetable crops (Glen & Moens, 2002; Hammond

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& Byers, 2002; Moens & Glen, 2002; Port & Ester, 2002; Sakovich, 2002; Sanderson & Sirgel, 2002; Wilson & Barker, 2011). Molluscs reduce the growth of crops through the damage that they inflict on the seedlings, leaves, stems, and growing points (Port & Port, 1986; South, 1992), and they reduce the value of harvested crops, due to their mucus trails, faeces, eggs, and the presence of molluscs (Iglesias *et al*., 2002).

In South Africa, the climate is favourable for European molluscs, especially in the Western Cape province, in which region the winters are mild and damp. European molluscs were first introduced to South Africa by European settlers in the eighteenth and early nineteenth centuries (Smith, 1992), and they have now become well-established in their new environment. One crop that is particularly targeted by these pests is canola (*Brassica napus*), which is a winter-arable crop that is commercially produced for its use in cooking, food processing, fertilisers, fuels, pet food, plastics, and animal feed. Canola is sown between March and May in the Western Cape province, during which period of the year temperatures begin to fall. The seedlings, which are mostly susceptible to mollusc damage during the first four weeks, require protection during this time, so as to prevent whole fields having to be resown. The canola plants create a cool, damp and shaded environment, which enables the molluscs to reproduce throughout the season and to remain active until the canola is windrowed (Tribe & Lubbe, 2010). The three mollusc species that have been identified as being pestiferous in canola are *Milax gagates* (Draparnaud, 1801), *Deroceras panormitanum* (Lessona & Pollonera, 1882), and *Deroceras reticulatum* (Müller, 1774). All three species of slugs (as opposed to snails) are European exotic invaders.

Current methods for protecting the vulnerable seedling stages of canola rely on the use of chemical molluscicide pellets containing 30g/kg metaldehyde and 20g/kg carbaryl, at a dosage of 6-12 kg/ha. To date, such methods have been proved to be most cost-effective for controlling molluscs in South African canola (Tribe & Lubbe, 2010), although studies have

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demonstrated that both metaldehyde and carbaryl have adverse effects on the health of non-target vertebrates (Homeida & Cooke, 1982; Mahajan *et al*., 2014). Iron phosphate pellets are an effective alternative to metaldehyde and could be used in combination with nematodes to control molluscs. The use of mollusc-parasitic nematodes is a possible environmentally-friendly alternative to chemicals. Current knowledge indicates that there are eight nematode families that associate with molluscs as definitive hosts, including Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae, and Rhabditidae. However, to date, the only nematode to be commercially developed as a biological molluscicide is *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrássy, 1983. Although *P. hermaphrodita* was first isolated over 150 years ago, its potential as a biological control agent was only discovered in 1987, when the nematode was found reproducing in the mantle cavity of a diseased *D. reticulatum* (Wilson *et al*., 1993). Further studies found that *P. hermaphrodita* was capable of infecting slug species from the families Agriolimacidae, Arionidae, Milacidae, Limacidae, and Vagnulidae (Wilson *et al*., 1993; Iglesias & Speiser, 2001; Speiser *et al*., 2001; Grewal *et al*., 2003; Rae *et al*., 2008). The nematode was made commercially available by BASF (formally MicroBio Ltd and Becker Underwood) in 1994, under the trade name Nemaslug®, and it is currently sold in fifteen different European countries (G. Martin, personal communication, March 24, 2015).

To date, the above-mentioned product has not been able to be sold in South Africa, due to the legislation that is in place, in terms of which *P. hermaphrodita* is seen as an exotic species (amendment of Act 18 of 1989 under the Agricultural Pest Act 36 of 1947). Therefore, methods of indigenous control require investigation. In 2012, a study was published on nematodes associated with terrestrial slugs in South Africa (Ross *et al*., 2012). The authors identified four true slug-parasitic nematodes, two of which were previously undescribed *Phasmarhabditis* spp., indicating that South Africa is rich in indigenous species (Ross *et al*., 2012). It is important

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to expand this understanding with further surveys, especially with slugs collected from canola crops and nurseries, which often serve as vectors for the movement of slugs (Cowie *et al.*, 2008).

The objective of the study described in the present chapter was to investigate the diversity and distribution of nematodes associated with terrestrial molluscs from canola crops and commercial nurseries in the Western Cape province.

2.2 Materials and Methods

2.2.1 Slugs collected

Terrestrial slugs were collected from 12 sample sites (Fig. 2.1) between 22 September 2014 and 16 October 2015. Slugs were collected as opposed to snails, as the former are the main pests of canola crops, and the lack of an external shell means that they can burrow into the soil, thereby increasing the extent of their exposure to nematode parasites. The sample sites, including those of canola crops and commercial nurseries (Fig. 2.2), were found with the assistance of local farmers and growers. The slugs concerned were stored and transported in plastic containers with perforated lids, and the containers were lined with moist tissue, with approximately 20 slugs per container. The date, location, habitat, and coordinates of the sites from which the collections were gathered were recorded. The washed carrots that were added to each container were replaced every three days. Slugs were identified using morphological characterisation (Ross *et al.*, 2012).

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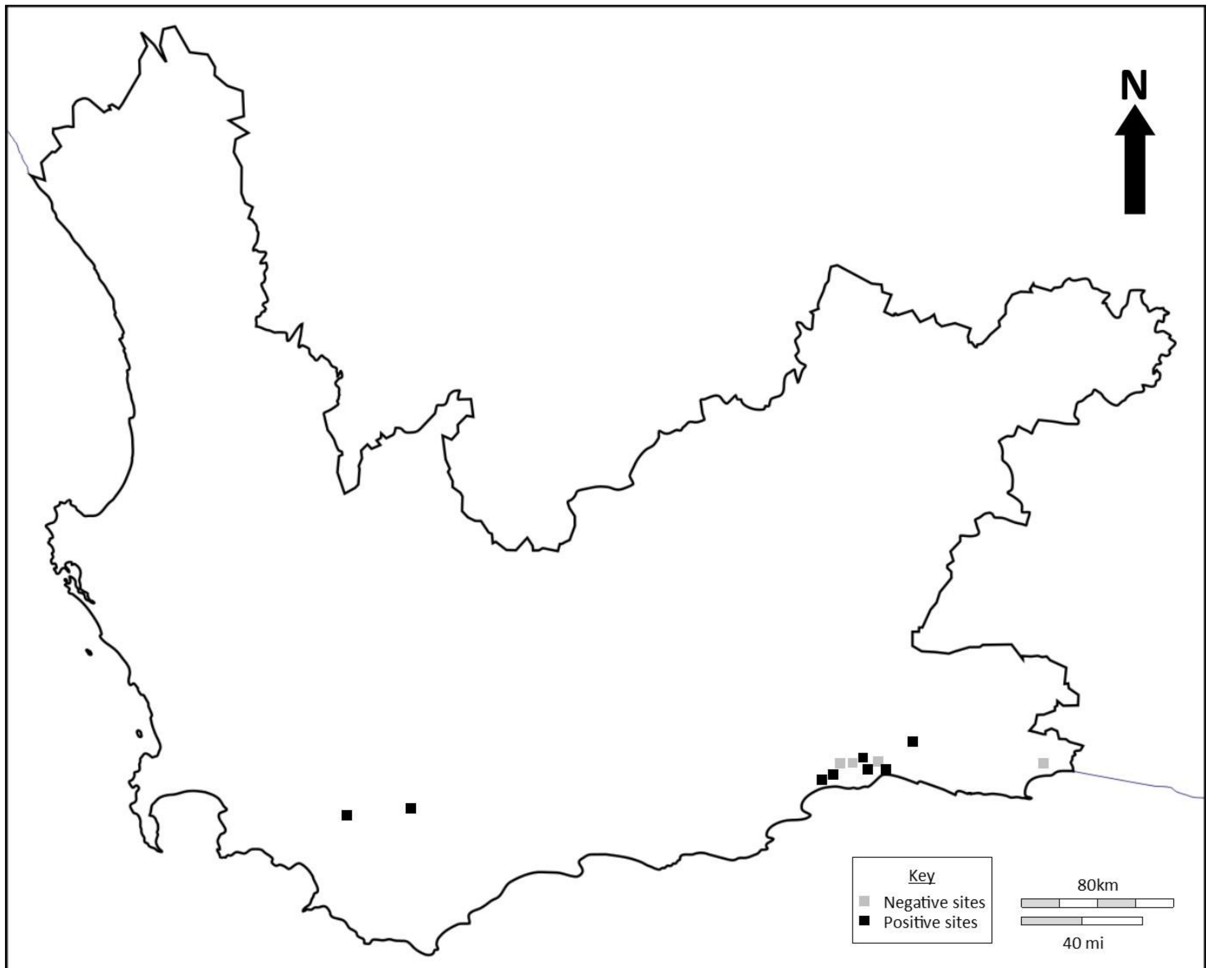

Figure 2.1 Map of negative and positive sample sites in the Western Cape province.

(a) (b)

Figure 2.2 Sample sites consisting of (a) canola fields, and (b) commercial nurseries.

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Figure 2.3 *Deroceras reticulatum* feeding on a canola plant.

2.2.2 Dissection

Prior to dissection, the slugs were washed with a 0.9% saline solution in order to remove any external nematodes. The slugs were dissected alive, using a dissecting microscope (Leica MZ7s), and they were examined for internal nematodes. The nematode intensity of occurrence was recorded.

2.2.3 Morphological identification

After being heat killed, adult nematodes were placed in 5% formaldehyde at 60°C for a minimum of 2 hours, and, after being processed in anhydrous glycerol, they were mounted on microscope slides (Seinhorst, 1959). To assist with the identification of juvenile bacterial-feeding nematodes, and to test their ability to reproduce on decaying organic matter, nematodes were transferred to modified kidney agar plates and frozen slug cadavers. Slides were studied using a compound microscope (Leica DM200, Leica Microsystems, Wetzlar, Germany) that was fitted with a digital camera.

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2.2.4 Molecular identification

Individual nematodes were placed in 70% ethanol, with their DNA then being extracted using a Chelex/Proteinase K mix (Ross *et al*., 2010). Thereupon a polymerase chain reaction was conducted on the 18s rRNA gene (Ross *et al.* 2010), with the sequences involved then being checked for quality, and assembled, using CLC Main Workbench 7.6.4 (https://www.qiagenbioinformatics.com/). Their details were then compared with relevant information on the database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), using the BLASTN search tool (Altschul *et al*., 1990).

2.2.5 Pathogenicity tests

Rhabditid nematodes that showed a similar growth and infection method to *P. hermaphrodita* (Wilson *et al*., 1993) were used in pathogenicity experiments. This included nematodes that formed self-fertilizing hermaphrodites, reproduced on decaying organic matter and that infected the mantle region on the host. In order to investigate this, the isolated nematodes were transferred to frozen and defrosted slugs on modified White traps (Kaya & Stock, 1997), where they were left to reproduce, and harvested after 2 weeks. The pathogenicity of these selected species were then tested. 10 Petri dishes (9-cm) were each lined with a Whatman No.1 filter paper, moistened with 1 ml of distilled water, containing 2000 dauer juveniles, and one *D. panormitanum* host. The total of 10 Petri dishes that served as controls were moistened with 1 ml of distilled water just prior to adding the slugs. One lettuce disc, 3 cm in diameter, was added to each plate as food, and replaced daily. The dishes were incubated at 20°C for 30 days. The feeding and mortality of the slugs was recorded every 2 days. Slugs that died were dissected to determine the cause of death. The experiment concerned was repeated on three different test dates, using different batches of the same nematode.

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2.2.6 Statistical analyses

Normality tests and homogeneity of variances tests were performed using the Shapiro-Wilk test and Levene's test, respectively, and all the data analyses were performed using Statistica 12 (Statsoft, Tulsa, Oklahoma). Slug mortalities were analysed as counts, due to the failure of the data transformation (square-root transformation; Wilson *et al.*, 1994) to produce normally distributed data. A 2 × 2 factorial analysis of variance (ANOVA) test ($P<0.05$) was used to establish the combined effects of time and treatment on mortality. Non-parametric Kruskal-Wallis ANOVA ($P<0.05$) tests were used to compare mortalities between treatments.

2.3 Results

2.3.1 Slugs collected

A total of 1944 slugs were collected from 12 sample sites from around the Western Cape province (Fig. 2.1), with the sample sites concerned including canola crops and commercial nurseries (Fig. 2.2). Slugs were found under plant pots, crops, plastic liners, rocks, and leaf litter. A total of five slug species were identified, representing four different families (Table 2.1). Photographs of the species can be found in Fig. 2.4.

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Figure 2.4: The five slug species collected in the survey: (a) *Deroceras reticulatum*, (b) *Deroceras panormitanum*, (c) *Milax gagates,* (d) *Chlamydephorus gibbonsi* (e) *Lehmannia valentiana.*

2.3.2 Isolated nematodes

Eight of the twelve sample sites examined were found to have slugs infected with nematode parasites, which amounted to 67% of the sample sites (Fig. 2.1; Table 2.2). Eight percent of all slugs were found to be infected with nematode parasites. Nematodes were found parasitising all four exotic species, leaving *Chlamydephorus gibbonsi* Binney, 1879 free from nematode parasites (Table 2.1).

Of the eight sites that had nematodes present, *Caenorhabditis elegans* Maupas, 1900 was found at six sites, *Angiostoma margaretae* Ross, Malan & Ivanova, 2011 at four sites, with

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the other nematodes, namely *Angiostoma* sp., *Phasmarhabditis* sp. SA3, *Phasmarhabditis* sp. SA4, and a mermithid species being found at single sites (Fig. 2.5). The nematodes concerned represented three families: Angiostomatidae: Mermithidae: and Rhabditidae.

Figure 2.5: A mermithid species found after dissecting *Deroceras panormitanum*, collected from a nursery in George in the Western Cape province.

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Table 2.1 Prevalence and intensity of nematodes and their associated hosts collected from sites in the Western Cape province of South Africa.

Slug family/species	Native/ introduced	Number collected	Species	Infection site	Prevalence (%)	Mean intensity
Agriolimacidae:						
Deroceras panormitanum	Introduced	1100	mermitid sp.	Body cavity	0.7 (8)	1
			Angiostoma margaretae	Oesophagus	0.7 (8)	±12
			Caenorhabditis elegans	Intestine	6.5 (72)	±20
			Phasmarhabditis sp. SA3	Buccal mass	0.1 (1)	2
D. reticulatum	Introduced	173	*A. margaretae*	Oesophagus	8.1 (14)	±12
			C. elegans	Intestine	5.8 (10)	±20
			Phasmarhabditis sp. SA4	Buccal mass	0.6 (1)	1
Limacidae:						
Lehmannia valentiana	Introduced	387	*A. margaretae*	Oesophagus	0.3(1)	1
			C. elegans	Intestine	3.9 (15)	±20
Milacidae:						
Milax gagates	Introduced	112	*A. margaretae*	Oesophagus	19.6 (22)	±121
			Angiostoma sp.	Buccal mass	0.9(1)	±20
			C. elegans	Intestine	1.8 (2)	
Chlamydephoridae:						
Chlamydephorus gibbonsi	Native	172	-	-	-	-

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Table 2.2 Nematodes in the Western Cape province of South Africa, and the sites from which they were collected.

Location	Coordinates	Habitat	Nematode infection	Family
Caledon	34.22.93S 19.52.60E	Canola crops	-	-
George	33.99.37S 22.52.09E	Nursery	*Caenorhabditis elegans*	Rhabditidae
George	33.99.09S 22.51.42E	Nursery	*C. elegans*	Rhabditidae
			Angiostoma margaretae	Angiostomatidae
			Angiostoma sp.	
			mermithid sp.	Mermithidae
			Phasmarhabditis sp. SA3	Rhabditidae
George	33.99.62S 22.52.90E	Nursery	-	-
George	33.99.43S 22.53.88E	Nursery	*C. elegans*	Rhabditidae
George	33.99.00S 22.41.34E	Nursery	-	-
George	33.99.27S 22.40.21E	Nursery	-	-
George	33.99.37S 22.39.33E	Nursery	*C. elegans*	Rhabditidae
			A. margaretae	Angiostomatidae
			Phasmarhabditis sp. SA4	Rhabditidae
Great Brak River	34.04.81S 22.29.28E	Nursery	*C. elegans*	Rhabditidae
Plettenberg Bay	33.95.75S 23.47.54E	Nursery	-	-
Riviersonderend	34.14.34S 19.90.69E	Canola crops	*C. elegans*	Rhabditidae
			A. margaretae	Angiostomatidae
Wilderness	33.94.93S 22.66.46E	Canola crops	*A. margaretae*	Angiostomatidae

2.3.3 Pathogenicity tests

Phasmarhabditis sp. SA4 was found to show a similar growth and infection method to *P. hermaphrodita*, so it was therefore chosen for pathogenicity experiments against *D. panormitanum*. Significant interaction occurred between the effects of time and treatment on slug mortality (ANOVA, $F = 34.1$, $P<0.001$), with both time and treatment having a significant effect on mortality (ANOVA, $F = 34.1$, $P<0.001$; and $F = 1288.4$, $P<0.001$, respectively; Figure 2.6). Slugs treated with nematodes (median with quartile ranges: 8.5, 3.0–10.0) had a significantly higher mortality count compared to that of the control (0.0, 0.0–0.0) (Mann–Whitney U: N=42, $U = 126.0$, $z = -6.8$, $P<0.000001$). On the elapse of 14 days, 100% of the slugs were infected, with over half of the inoculated slugs having been infected after the elapse of 5 days. All the dead slugs, on dissection, were found to be infected with nematodes.

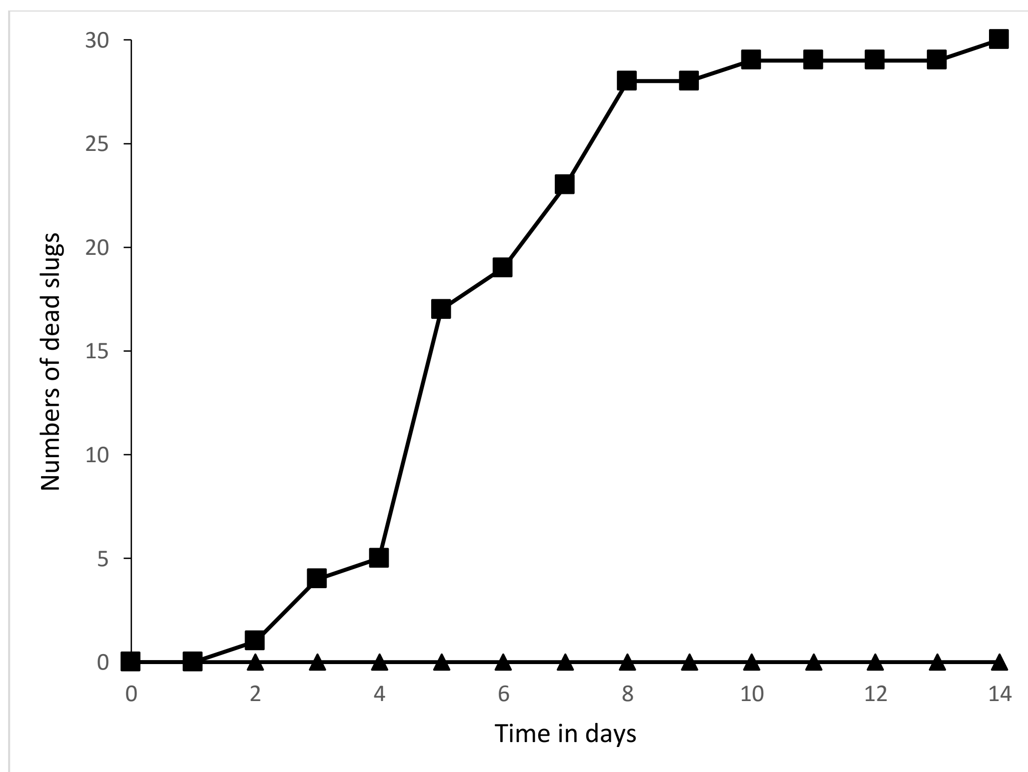

Figure 2.6: Number of dead slugs (n = 30) over 14 days in Petri dish experiments without nematodes (▲), or with 2000 dauer juveniles of *Phasmarhabditis* sp. SA4 (■) at 20°C.

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2.4 Discussion

The current study examined the diversity of nematodes associated with terrestrial slugs as definitive hosts in the Western Cape province, with the aim of finding an indigenous nematode isolate that could be developed as a biological molluscicide in South Africa. A total of five slug species were identified in the Western Cape province, representing four slug families, including Agriolimacidae, Chlamydephoridae, Limacidae, and Milacidae. *Milax gagates* (Milacidae), *D. panormitanum* (Agriolimacidae), and *D. reticulatum* (Agriolimacidae) were collected from both nurseries and canola crops. *Lehmannia valentiana* (Férussac, 1822) (Limacidae) and *C. gibbonsi* (Chlamydephoridae) were, however, only found at the nurseries involved. All four slug families had previously been identified in the Western Cape province, although *C. gibbonsi* had never before been found in such high numbers. Ross *et al*. (2012) found a single specimen of *C. gibbonsi* in the Western Cape, and Herbert (1997) found a single outlying species on Table Mountain. *Chlamydephorus gibbonsi*, which is endemic to Southern Africa, naturally occurs in indigenous forest habitats and savannah/thornveld as far south as the highlands of eastern Zimbabwe, through Mpumalanga and KwaZulu-Natal, to the Eastern Cape (Herbert, 1997). However, in the present survey, 172 specimens were found in the Western Cape, as far west as Stellenbosch. The specimens collected during the study were all associated with nurseries, indicating that the increase in the species numbers in the Western Cape is due to nurseries acting as a vector. The finding was confirmed by Ross *et al.* (2012), whose specimen was found within 1 km of a nursery in Knysna. The natural predators of *C. gibbonsi* include hadedah ibises (Herbert, 1997), and the snail *Natalina wesseliana* Kobelt, 1876, although *N. wesseliana* only occurs in the eastern part of Southern Africa (Herbert & Mousalli, 2010), so the absence of natural predators could have facilitated the success of *C. gibbonsi* in its new habitat in the Western Cape province. The potential of *C. gibbonsi* to become an

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economic pest, as well as its ability to survive outside of nurseries, requires further investigation.

Although a total of 1944 terrestrial slugs were collected during the survey, only 172 individuals were found to be native to South Africa, with the remaining 1772 individuals being European exotics. The total number of non-native terrestrial mollusc species currently present in South Africa stands at 34 (Herbert, 2010), with the first introductions being recorded in 1846, when *Oxychilis cellarius* (Müller, 1774) and *Vallonia pulchella* (Müller, 1774) were found in Cape Town (Benson, 1850). The success of many molluscan colonists is mostly attributed to their ability to thrive in association with humans, and to establish themselves in new habitats without predators, competitors, and diseases (Herbert, 2010). Even though many pestiferous terrestrial molluscs have become widely distributed in South Africa, they are most associated with transformed habitats. However, one exception is the genus *Deroceras*, which, with human aid, has spread into pristine habitats (Herbert, 2010).

One family that is particularly good at invading new habitats is that of Arionidae, with the presence of *Arion hortensis* Férussac, 1819 and *Arion intermedius* Normand, 1852 being recorded in North America, Australia, New Zealand, and South Africa. However, caution should be taken regarding the arionids, as the invasive Iberia slug (*Arion vulgaris* Moquin-Tandon, 1855 - also known as *Arion lusitanicus* Mabille, 1968) has invaded all parts of Europe, and it is now being recorded in some areas of Russia (Sysoev & Schileyko, 2009). *Arion vulgaris* is a major economic pest that is known to damage ornamental and vegetable crops (Frank, 1998; Hofsvang *et al.*, 2008). Therefore, ongoing vigilance is essential to avoid its introduction into South Africa.

A total of six nematode species were identified in the Western Cape, including *C. elegans* and *A. margaretae*, which were found in both canola crops and nurseries, and

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Angiostoma sp., mermithid sp., *Phasmarhabditis* sp. SA3, and *Phasmarhabditis* sp. SA4, which were found to be only present in the latter. Of the six nematode species, four were previously undescribed (mermithid sp., *Phasmarhabditis* sp. SA3, *Phasmarhabditis* sp. SA4, and *Angiostoma* sp.). The isolation of the undescribed species is an indication that South Africa is rich in unidentified species. The finding that was made in this respect is supported by similar findings that have already been made in relation to entomopathogenic nematodes (EPNs). To date, 13 EPNs have been reported from South Africa, of which ten have been described as being new to science (Malan *et al*., 2016). With regards to the biological mining of indigenous species, South Africa is still in its infancy (Malan *et al*., 2006; Hatting *et al*., 2009; Malan *et al*., 2011). Conducting further surveys of nematodes that use molluscs as definitive hosts in South Africa is, therefore, crucial, so as to enable the full investigation of the biological control potential of indigenous species.

The six nematodes that were isolated in the current study are represented by three nematode families, namely Angiostomatidae, Mermithidae, and Rhabditidae. The Angiostomatidae family consists of two genera, *Angiostoma* Dujardin, 1845 and *Aulacnema* Pham Van Luc, Spiridonov & Wilson, 2005. Molluscan angiostomatids are obligate parasites that have been isolated from the buccal mass, crop, hepatopancreas, intestine, mantle cavity, oesophagus, and salivary gland of their hosts (Ross *et al*., 2016). Angiostomatids have, however, also been found in the bronchi and intestines of reptiles and amphibians (Chitwood, 1933; Bursey & Goldberg, 2000; Bursey & Manire, 2006; Falcon-Ordaz *et al*., 2008). In South Africa, *A. margaretae* was described from the oesophagus of the slug host *M. gagates*, collected near Caledon in the Western Cape province (Ross *et al*., 2011). In the present study, *A. margaretae* was found in four new localities, including Riviersonderend (canola crop), Wilderness (canola crop), and two sites in George (commercial nurseries) (Table 2.2). It also has two new host associations, including *D. panormitanum* and *D. reticulatum* (Table 2.1).

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Mermithids are large nematodes that range between 1-10 cm in size, and which are usually found as parasites of invertebrates (usually arthropods) (Poinar, 1976). However, juveniles have been found in the body cavity of mollusc hosts, but it is believed that molluscs are not the usual hosts, and that they act as facultative paratenic hosts (Chitwood & Chitwood, 1937; Théodoridès, 1957; Mienis, 1986; Ross *et al*., 2010). In the current survey, mermithids were found associating with *D. panormitanum*, with the discovery being the first record of a mermithid associating with a mollusc host in Africa.

The Rhabditidae family has a number of genera that associate with slugs, including *Rhabditis*, *Caenorhabditis*, and *Phasmarhabditis*. Until recently, it was thought that *Phasmarhabditis* was the only genus to be considered a true parasite (Morand *et al*., 2004; Pieterse *et al*., 2016), and that *C. elegans* forms a phoretic, or, under laboratory conditions, a facultative necromenic association, with slugs (Kiontke & Sudhaus, 2006). However, Petersen *et al*. (2015) have demonstrated that *C. elegans* can infect the intestine of mollusc hosts, exiting, while still alive, through the faeces. The researchers in question found that slugs are a suitable transport method for a variety of *Caenorhabditis* species in different life stages. Accordingly, they suggested that *Caenorhabditis* species have possible mutualistic, commensal, or even parasitic, associations with slugs (Petersen *et al*., 2015). In the present survey, the most commonly found *C. elegans* was found to be present at six of the 12 sample sites, where they were found to be associating with all the exotic European slug species mentioned.

Only one previous survey has been conducted on nematodes associated with molluscs in South Africa, with the authors collecting 521 slug specimens from an array of diverse habitats (Ross *et al*., 2012). The present survey was more intense than was the earlier survey mentioned, with 1944 slug specimens being collected across the Western Cape province. However, the current survey focused solely on slugs from canola crops and commercial nurseries. The imposition of such a limitation had an impact on the number of slug species

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collected, with Ross *et al.* (2012) identifying 12 species, compared to the five that were identified in the current study. However, one important similarity between the two surveys is the isolation of the new *Phasmarhabditis* species, particularly from the George area. In common with the discovery of two previously undescribed *Phasmarhabditis* species by Ross *et al.* (2012), the present study found an additional two new species (with a full description of *Phasmarhabditis* sp. SA4 being given in Chapter 3).

Of the two new *Phasmarhabditis* species that were found, *Phasmarhabditi*s sp. SA3 was found to be unable to reproduce under laboratory conditions, whereas *Phasmarhabditis* sp. SA4 was successfully reared under such conditions. The latter's pathogenicity could, therefore, be tested, with it being shown to have significant effects on the mortality of the slug *D. panormitanum.*

Future work should be focused on the pathogenicity of the nematodes against pestiferous slugs in canola. In addition, the effects of the nematode on native slug species should also be investigated, to ensure that it has no negative effect on the native slug populations, or on any endangered mollusc species, as well as other nontarget organisms.

The current chapter has presented new data on nematodes that use molluscs as definitive hosts in the Western Cape province, South Africa. Six nematode species were isolated in the survey undertaken in this respect, including *Angiostoma* sp., *A. margaretae*, *C. elegans*, a mermithid sp., and *Phasmarhabditis* spp. (isolates SA3 and SA4). Of the six species, *Angiostoma* sp., the mermithid sp., *Phasmarhabditis* spp. (islates SA3 and SA4) were previously undescribed. *Phasmarhabditis* sp. SA4, which is capable of reproducing under laboratory conditions, has proven to be pathogenic to the slug species, *D. panormitanum*. Further pathogenicity tests require undertaking to investigate the potential of *Phasmarhabditis*

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sp. SA4 as a biological control agent against terrestrial slugs in South Africa. Tests should also be conducted to determine its effect on native mollusc species, as well as non-target organisms.

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CHAPTER 3

Phasmarhabditis sp. SA4 (Nematoda: Rhabditidae), a parasite of the slug *Deroceras reticulatum* from South Africa

Abstract

A new *Phasmarhabditis* species was isolated from the invasive slug, *Deroceras reticulatum*, collected from George in the Western Cape province of South Africa (see Chapter 2). The new species, *Phasmarhabditis* sp. SA4 is characterised based on morphology, morphometrics and molecular analysis. The new species is characterised by the shape and length of the female tail and by the presence of males. The *in vivo*-produced DJ of the new species has a body length of 900 (813-1042) μm, a body width of 32 (29-36) μm, and a tail length of 128 (113-148) μm. The molecular phylogeny of the new species, as inferred from its 18S rRNA gene, placed *Phasmarhabditis* sp. SA4 in the same clade as *Phasmarhabditis* sp. SA2, *P. papillosa* and the mollusc-parasitic nematode, *Angiostoma dentiferum*. The results varied compared to the morphological analysis, which suggested that the nematode was closest to *P. californica.* The new species brings the total complement of the genus to seven species. The isolation of the new *Phasmarhabditis* species indicates the importance of conducting further surveys of mollusc-parasitic nematodes in South Africa.

3.1 Introduction

Terrestrial slugs (Mollusca: Gastropoda) are important economic pests of a broad range of crops worldwide. Although current control relies on chemical molluscicides, they have often been found to be both ineffective and toxic to non-target organisms. An alternative to chemical control are biologicals, with the slug-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrássy, 1983, being the most effective option in Europe. In 1994, the

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nematode was developed as a biological molluscicide by MicroBio Ltd (which was later acquired by Becker Underwood, and then taken over by BASF in 2012), with it being sold in Europe under the trade name, Nemaslug®. The product contains the free-living, nonfeeding, third-stage dauer juvenile (DJ), or dauer larvae, that infects slugs via the dorsal integumental pouch, and which travels to the shell cavity that lies immediately below the mantle. Once inside the slug, the larvae recover and develop into adults and reproduce, killing the slug within 4-21 days, and then feeding on the bacteria on the cadaver (Tan & Grewal, 2001; Wilson *et al.*, 1993). On depletion of the food source, DJs are produced, entering the soil in search of new hosts (Wilson & Rae, 2015). *Phasmarhabditis hermaphrodita* is capable of infecting a wide range of terrestrial slugs, including Agriolimacidae, Arionidae, Milacidae, Limacidae, and Vagnulidae (Rae *et al.*, 2007). The nematode is a facultative parasite that can reproduce on a variety of substrates, including compost, slug faeces, leaf litter, and dead earthworms and insects (MacMillan *et al.*, 2009; Nermut' *et al.*, 2014; Tan & Grewal, 2001).

Phasmarhabditis hermaphrodita (initially referred to as *Pelodytes hermaphroditus* Schneider, 1859) was first isolated in Germany over 150 years ago from an arionid host (Schneider, 1859). Later, the nematode was reisolated by Maupas (1900) (referred to as *Rhabditis caussaneli* Maupas 1900) from the intestine of an *Aron ater* Linnaeus, 1758 collected in France. At the time, it was noted that the nematode produced protandrous autogamous hermaphrodites, with the production of males being rare (one male to 715 females). Mengert (1953) described the closely related species *Phasmarhabditis neopapillosa* (Mengert, 1952) Andrássy, 1983 from the slug *Limax cinereoniger* Wolf, 1803, hypothesising that *P. hermaphrodita*, *P. neopapillosa* and *Phasmarhabditis papillosa* (Schneider, 1866) Andrássy, 1983, were from the same ecological group.

In revising the '*Papillosa*' group in the genus *Pellioditis* Dougherty, 1953 *sensu* Sudhaus (Sudhaus, 1976, 2011; Sudhaus & Fitch, 2001), Andrássy (1976) proposes a new

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genus entitled *Phasmarhabditis.* The genus was regarded as containing five species, including *P. papillosa* as the type species, *P. hermaphrodita* and *P. neopapillosa* from the '*Papillosa*' group, *Phasmarhabditis nidrosiensis* Allgén, 1933 from a marine habitat, and *Phasmarhabditis valida* Sudhaus 1974 from a littoral habitat (Andrássy, 1983). Andrássy (1983) also notes that *P. hermaphrodita* and *P. neopapillosa* are morphologically identical, and that they can only be separated by their sex ratios, with *P. neopapillosa* having an equal number of males and females, with males being rare in *P. hermaphrodita*.

When conducting another revision, Sudhaus (2011) repositioned the *P. nidrosiensis* and *P. valida* species with another genus (*Buetschlinema* Sudhaus, 2011), moving the remaining *Phasmarhabditis* species to the '*Papillosa'* group within the *Pellioditis* genus (Sudhaus, 2011). *Pellioditis sensu* Sudhaus (Sudhaus, 1976, 2011; Sudhaus & Fitch, 2001) and *Phasmarhabditis sensu* Andrássy (Andrássy, 1976, 1983) are essentially the same, both containing the '*Papillosa*' group (consisting of *P. hermaphrodita, P. neopapillosa*, and *P. papillosa*). Despite the nomenclature '*Pellioditis*' having precedence over '*Phasmarhabditis*', in order to avoid taxonomic confusion, new species in the same genus are referred to as *Phasmarhabditis*.

In 2003, the genus expanded with the addition of *Phasmarhabditis tawfiki* Azzam, 2003, which was described from Egypt, following its isolation from the terrestrial snail *Eobania vermiculata* Müller, 1774 and from the *slug Limax flavus* Linneaus (Azzam, 2003). In 2015, *Phasmarhabditis huizhouensis* Huang, Ye, Ren & Zhao, 2015 was described from rotting leaves in the Guangdong Province of China (Huang *et al.*, 2015), while, in 2016, *Phasmarhabditis californica* Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine & De Ley, 2016 was described from *Deroceras reticulatum* (Müller, 1774) collected in California, USA (Tandingan De Ley *et al.*, 2016). In total, there are six described species within the genus, as well as a number of undescribed *Phasmarhabditis* species that have been isolated in the USA (Tandingan De Ley *et al.*, 2014), South Africa (Ross *et al.*, 2012), Slovenia, and Tanzania

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(Ross pers. comm.). The increase in species discovery within the genus indicates that the genus is more diverse than was initially anticipated.

The current paper describes the first new species in the genus *Phasmarhabditis* from South Africa, based on morphological, molecular and phylogenetic analysis. With the new addition, the total complement of the genus has been brought to seven species.

3.2 Materials and methods

3.2.1 Nematode source

Slugs were collected from a nursery near George, Western Cape province, South Africa (33°99'37''S; 22°39'33''E) (see Chapter 2). They were then rinsed with 0.9% saline solution to remove surface-dwelling nematodes, and dissected while alive (Ross *et al.*, 2012). The nematodes were transferred to modified kidney agar plates and frozen slug cadavers, and subcultured until they were ready for identification.

3.2.2 Morphological observations

Nematodes, after being picked alive, were transferred to a drop of water in a paraffin wax ring (to aid with the support of the nematode) on a glass slide, to which a cover slip was added, where they were killed with gentle heat. Measurements were taken using a compound microscope (Leica DM200, Leica Microsystems, Wetzlar, Germany) that was fitted with a digital camera, and using the software Leica Application Suite V3.5.0., with live measurement capability.

For observing the morphology of the bursa, males were fixed in TAF (2% triethanolamine, 8% formalin in distilled water) (Courtney *et al.*, 1955), thereafter being transferred to a small drop of lactophenol containing 0.002% acid fuchsine on a microscope slide. After about 20 min, each male was transferred to a drop of clear lactophenol on a glass

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slide, whereupon its tail was removed with the slanted, sharp edge of a syringe needle. The rest of the nematode was then removed, and a cover slip added to the slide concerned. By means of slightly moving the cover slip, the tail was rotated, so as to obtain a ventral view (Nguyen *et al.*, 2004). The bursa of 20 males was observed using the method involved.

To view the spicules and gubernaculum, the males were transferred from the TAF to a small drop of lactophenol, containing 0.002% acid fuchsine, on a glass slide, whereupon they were left alone for 2 min. The males were then individually transferred to a drop of lactophenol on a glass slide. After which the tail was cut off, using the slanted, sharp edge of a syringe needle. The rest of the nematode was then removed, with a covered slip being added to the slide. The cover slip was gently pressed and moved, while undergoing observation under a stereo microscope, so as to isolate the spicules and gubernaculum from the rest of the body (Malan *et al.*, 2016). A total of 20 males were observed using this method.

3.2.3 Scanning electron microscopy (SEM)

For their use in SEM, males, females and DJs were fixed in TAF fixative for a minimum of 3 days, after which they were washed three times in 0.05M cacodilate buffer for 15 min each, and then washed three times in distilled water for 15 min each. After the above, they were dehydrated in a graded ethanol series (70, 80, 90 and 2 × 100%). The samples were critical point dried with liquid carbon dioxide, mounted on SEM stubs, and sputter coated with 20 nm gold/palladium (66/33%). The samples were viewed with an FEI Quanta 200 ESEM (Duren, Germany), operating at 10kV under high-vacuum mode (Malan *et al.*, 2016).

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3.2.4 Molecular analysis

Individual nematodes were preserved in ethanol prior to molecular analysis. The nematodes were transferred to a Chelex/Proteinase K mix for DNA extraction (Ross *et al.*, 2010), thereafter undergoing a polymerase chain reaction (PCR) of the 18S rRNA gene (Ross *et al.,* 2010), the D2-D3 large subunit (LSU) rRNA gene (Ivanova & Spiridonov, 2010; Nguyen, 2007), the internal transcribed spacer regions (ITS1, 5.8S, ITS2) rRNA gene (Nadler *et al.*, 2005; Vrain *et al.*, 1992), and the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene (Kanzaki & Futai, 2002). Sequence traces were inspected for quality and assembled using the CLC Main Workbench 7.6.4 (https://www.qiagenbioinformatics.com/), whereupon they were uploaded onto the GenBank Database, at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

3.2.5 Phylogenetic analysis

The phylogenetic analysis of *Phasmarhabditis* sp. SA4 was conducted using the 18S rRNA gene. Additional sequences were downloaded from the GenBank, providing a total of 28 18S rRNA gene sequences for analysis. The nematode sequences involved included representatives of the genera *Agfa*, *Angiostoma*, *Phasmarhabditis*, and *Pellioditis*, along with *Oscheius tipulae* Lam & Webster, 1971 and *Oscheius insectivora* (Körner, 1954), which were used as out-groups. Sequences were manually aligned using BioEdit Sequence Alignment Editor (Hall, 1999), prior to the removal of regions of ambiguous alignment. Maximum likelihood (ML), distance and maximum parsimony (MP) analyses were performed on 1058 unambiguously aligned positions, using the software packages PHYML (Guindon & Gascuel, 2003) and PHYLIP (Felsenstein, 2007). Sequence alignments were assessed using Modeltest, with the general time-reversible (GTR) model being utilised, in conjunction with the among-site rate heterogeneity, which was modelled based on an eight-category gamma correction, with a

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fraction of the invariant sites being determined from ML analysis. Bootstrap support was determined using 1000 replicates, with values above 65% being considered.

3.3 Results

Phasmarhabditis sp. SA4

3.3.1 Measurements

Reference to morphometrics given in Table 1.

Table 3.1. Morphometrics of different stages of *Phasmarhabditis* sp. SA4. All measurements are in µm and in the form: mean ± sd (range).

Character	Phasmarhabditis sp. SA4							
	In vitro				*In vivo*			
	DJ	Female		Male	DJ	Female		Male
		Paratypes	Holotype	Paratype		Paratypes	Holotype	Paratype
N	25	25		25	25	25		25
Body length (L)	864 ± 49	1476 ± 105	1452	1209 ± 106	900 ± 63	1923 ± 171	1935	1529 ± 79
	(789-965)	(1238-1727)		(973-1408)	(813-1042)	(1622-2380)		(1390-1679)
A = L/BW	25 ± 2.40	20 ± 1.53	19	22 ± 1.61	28 ± 1.62	18 ± 2.04	16	25 ± 2.00
	(22-32)	(17-23)		(18-26)	(26 -33)	(14 - 22)		(21-28)
B = L/ES	5.37 ± 0.36	6.17 ± 0.48	6.14	5.67 ± 0.50	5.41 ± 0.38	7.43 ± 0.72	7.28	6.85 ± 0.60
	(4.76-5.94)	(5.00-6.99)		(4.73-6.91)	(4.85-6.30)	(6.45-9.47)		(5.94-8.06)
C = L/T	8.40 ± 1.30	15 ± 1.52	14	24 ± 2.41	7.04 ± 0.42	18 ± 1.91	18	30 ± 2.13
	(6.87-12)	(11-18)		(19-28)	(6.45-8.44)	(14-21)		(26-35)
c' = T/ABW	5.31 ± 0.42	2.70 ± 0.27	2.67	1.40 ± 0.12	6.42 ± 0.50	2.00 ± 0.21	2.21	1.25 ± 0.09
	(4.39-6.10)	(2.23-3.38)		(1.17-1.67)	(4.86-7.11)	(1.55-2.36)		(1.12-1.49)
V%	–	52 ± 3.56	53	–	–	49 ± 4.20	49	–
	–	(42-60)		–	–	(40-56)		–
Body width (BW)	35 ± 2.77	75 ± 5.85	77	54 ± 3.77	32 ± 1.98	107 ± 17	124	61 ± 4.74
	(27-39)	(64-88)		(48-66)	(29-36)	(79-145)		(53-74)
Stoma length	22 ± 1.37	21 ± 1.31	22	19 ± 1.36	23 ± 1.56	23 ± 4.14	22	20 ± 1.10
	(19-24)	(19-24)		(16-22)	(21-26)	(20-34)		(18-22)
Stoma diameter	–	5.04 ± 0.46	5.33	4.31 ± 0.50	–	5.53 ± 1.09	6.93	5.40 ± 0.52
	–	(4.09-5.98)		(3.07-5.06)	–	(3.02-7.24)		(4.57-6.81)
Excretory pore	–	193 ± 17	185	194 ± 9.82	–	230 ± 16	234	205 ± 13
	–	(165-230)		(163-208)	–	(200-287)		(178-226)
Pharynx length	161 ± 8.83	240 ± 11	236	214 ± 16	167 ± 14	260 ± 19	266	224 ± 14.17
	(147-177)	(217-262)		(198-278)	(147-207)	(232-302)		(201-257)
Anterior pharynx	–	140 ± 6.19	137	126 ± 11	–	148 ± 16	149	130 ± 8.01

	–	(125-150)		(116-173)	–	(108-183)		(118-148)
Posterior pharynx	–	99 ± 8.56	99	87 ± 7.01	–	111 ± 15	117	94 ± 6.59
	–	(84-117)		(74-105)	–	(86-153)		(85-109)
Diameter of median bulbus	12 ± 1.22	26 ± 2.94	25	24 ± 1.90	10 ± 1.06	35 ± 4.14	38	30 ± 2.68
	(10-14)	(21-34)		(20-28)	(9.18-14)	(29-43)		(24-34)
Diameter of basal bulbus	16 ± 1.24	36 ± 3.35	38	32 ± 2.60	15 ± 1.15	45 ± 4.36	47	38 ± 2.58
	(14.-19)	(30-45)		(27-40)	(13-18)	(36-57)		(34-44)
Nerve ring (NR)	117 ± 6.25	165 ± 8.10	167	154 ± 14	115 ± 9.47	185 ± 16	187	165 ±10
	(108-133)	(153-185)		(132-204)	(102-145)	(163-217)		(149-184)
Hemizonion	139.82±7.01	179 ± 14	171	183 ± 8.31	142 ± 14	213 ± 6.96	218	191 ± 14
	(129-154)	(153-215)		(166-198)	(120-183)	(187-221)		(154-212)
Anal body width (ABW)	20 ± 2.04	38 ± 2.92	39	37 ± 2.94	20 ± 1.94	54 ± 4.92	50	41 ± 2.26
	(15-23)	(34-45)		(31-42)	(18-25)	(46-69)		(37-45)
Tail (T)	105 ± 13	102 ± 10	103	51 ± 4.07	128 ± 6.78	108 ± 12	110	51 ± 3.21
	(73-127)	(86-123)		(43-57)	(113-148)	(87-143)		(46-59)
Length of female pointed tip	–	63 ± 8.51	72	–	–	63 ± 7.77	59	–
	–	(51-82)		–	–	(48-80)		–
Vulva-to-anus distance	–	647 ± 67	650	–	–	890 ± 99	939	–
	–	(524-794)	–	–	–	(690-1094)		–
Vulva to stoma	–	766 ± 65	763	–	–	939 ± 90	946	–
	–	(615-897)	–	–	–	(801-1231)		–
Hyaline portion	35 ± 5.20	–	–	–	44 ± 4.54	–	–	–
	(25-44)	–	–	–	(32-51)	–	–	–
Spicule length	–	–	–	49 ± 6.67	–	–	–	62 ± 3.42
	–	–		(36-61)	–	–	–	(55-69)
Gubernaculum length	–	–	–	25 ± 3.67	–	–	–	31 ± 2.18
	–	–		(18-31)	–	–	–	(27-34)
Gubernaculum length as % of spicule length	–	–	–	51 ± 4.84	–	–	–	49 ± 3.25
	–	–		(40-62)	–	–	–	(45-56)
D% = EP/ES	–	81 ± 7.84	78	91 ± 4.63	–	89 ± 5.97	88	91 ± 5.77
	–	(68-99)		(75-98)		(77-100)		(83-106)
E% = EP/T	–	191 ± 30	180	382 ± 35	–	214 ± 27	213	400 ± 34
	–	(147-258)		(319-440)		(155-270)		(336-464)
H% = T/Hyaline portion	3.01 ± 0.46	–	–	–	2.95 ± 0.33	–	–	–
	(2.11-4.22)	–	–	–	(2.62-4.16)	–	–	–

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3.3.2 Description

Females

Body elongate and almost straight when heat-killed, tapering toward a blunt anterior end (Fig. 3.1G; 3.2A). Cuticle smooth when observed through light microscope, but with striations under SEM. Lateral field absent. Anterior end bluntly rounded, with six lips grouped in pairs, each lip with a prominent inner labial papilla and with four less prominent outer labial papilla. Amphidial apertures pore-like, perioral disc not observed (Fig. 3.2C) Mouth triangular, cylindrical, with convex sides (Fig. 3.3A). Stoma moderately cuticularised, 23 (20-34) μm in length, and 5.53 (3.02-7.24) μm in diameter. Pharynx with cylindrical procorpus and swollen metacorpus, narrow isthmus surrounded by nerve ring located at anterior end of basal bulb (Fig. 3.1A; 3.2B). Excretory pore opposite anterior part of basal bulb with well-cuticularised excretory duct. Cardia inconspicuous. Vulva located near the middle of the body, V% = 49 (40-56), a transverse slit with slightly protruding lips (Fig. 3.1D; 2.2C). Reproductive system didelphic, amphidelphic, and reflexed. In mature females, uteri often filled with round oocytes, which commonly hatch inside the body. Anus an arcuate slit with slight postanal swelling (Fig. 3.2G). Anal body diameter 50% of tail length. Tail cupola-shaped, with a slender pointed tip forming 58% of the tail length, with inconspicuous phasmids at the junction of the tail and the pointed tip (Fig. 3.1G; 3.2H).

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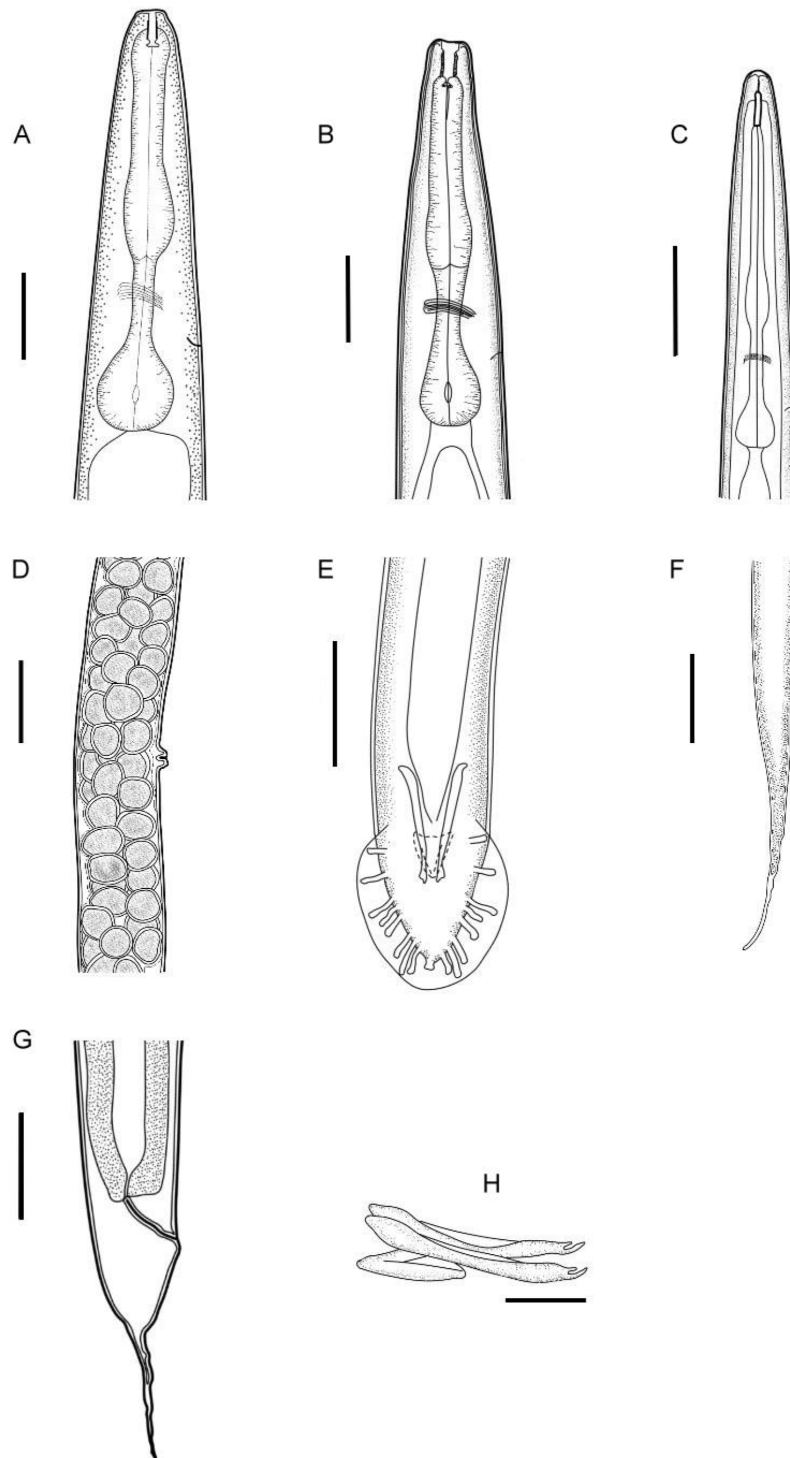

Figure 3.1 *Phasmarhabditis* sp. SA4. A-C: Anterior region. A: Female; B: Male; C: Juvenile; D: Vulva of female; E-G: Tail region; E: Male; F: Juvenile; G: Female; H: Spicules and gubernaculum. (Scale bars: A-C, E-G = 50 μm; D = 100 μm; H = 20 μm.)

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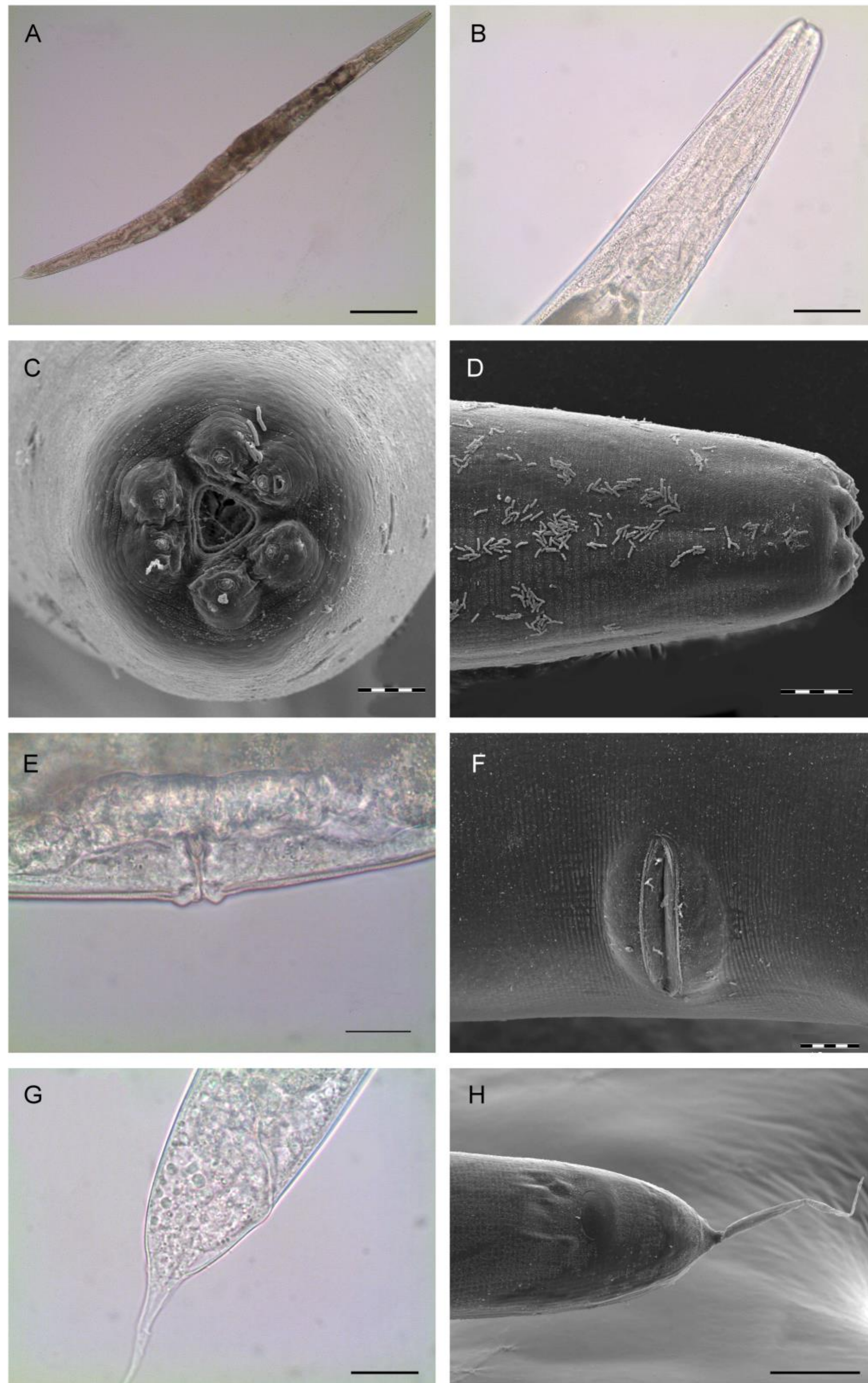

Figure 3.2 Micrographs of *Phasmarhabditis* sp. SA4 female. (A) Overview of female adult. (B) Pharyngeal region of an adult female. (C) Vulva. (D) Tail. (Scale bars: A = 200µm; B = 50µm; C = 5µm; D, F = 10µm; E, G, H = 20µm).

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Males

Males of *Phasmarhabditis* sp. SA4 are common during *in vitro* culture. Body generally shorter and smaller than female. Body curved posteriorly and J-shaped. Cuticle smooth, with very slight striation under SEM. Lateral field has five ridges and four lines (Fig. 3.36C). Cephalic extremity slightly swollen, with six lips grouped in pairs, each lip with a prominent inner labial papilla, four less prominent cephalic papillae present as well as two slitlike amphidial apertures (Fig. 3.3C). Perioral disc not observed. Stoma cylindrical, length 20 (18-22) μm and 5.53 (3.02-7.24) μm in width, moderately cuticularised. Pharynx has cylindrical procorpus, swollen metacorpus, narrow isthmus surrounded by nerve ring, and swollen basal bulb. Nerve ring surrounds middle of isthmus (Fig. 3.1B). Excretory pore opposite anterior end of basal bulb, with well-cuticularised excretory duct. Cardia inconspicuous. Genital system monorchic, reflexed. Posterior region has single precloacal midventral papilla. Copulatory apparatus consists of spicules, gubernaculum and peloderan, proximally open bursa, a lateral cuticular extension from tail, holding nine bilateral pairs of bursal papillae. The first three papillae precloacal, fourth and fifth adcloacal, and last four postcloacal. First and eighth papillae extend to the edge of the bursa. Prominent ventral papilla on the precloacal lip, and a pair of cone-shaped sublateral papillae present posterior to cloacal opening (Fig. 3.1E; 3.3E). Spicules cephalated, paired and separate, 62 (55-69) μm in length, head longer than it is wide (Fig. 3.). Spicule blade slightly curved, gradually tapering to small forked terminus, with dorsal tip longer than ventral tip. Velum present, not reaching the tip of the blade (Fig. 3.1H). Gubernaculum short, 49% of spicule length. Tail point short and conoid (Fig 3.3G).

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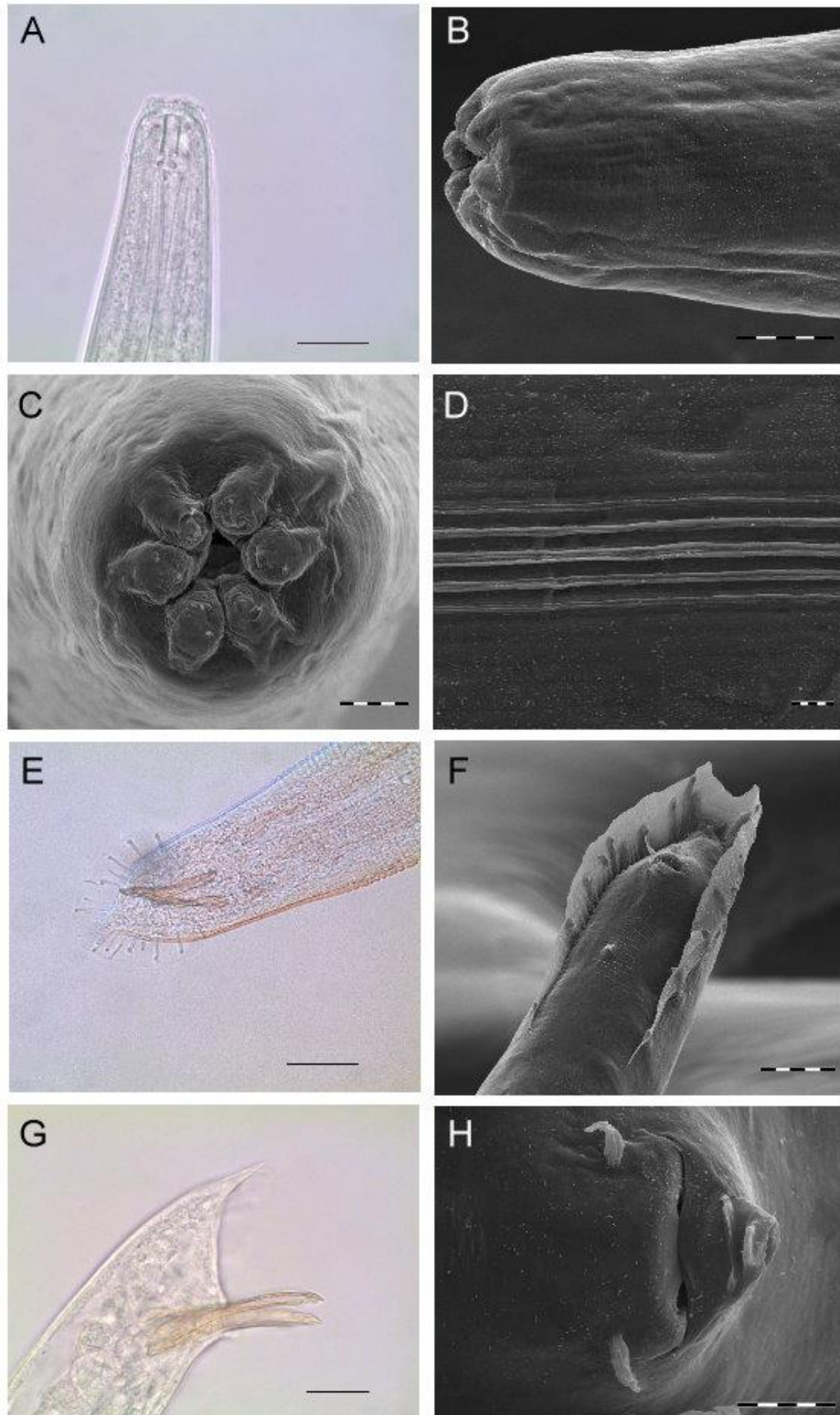

Figure 3.3 Micrographs of *Phasmarhabditis* sp. SA4 males. (A) *En face* view. (B) and (C) Lateral fields of male. (D) Male caudal region with an open bursa and 9 pairs of genital papillae. (Scale bars: A, F, G = 20µm; B = 10µm; C, H = 5µm; D = 2µm; E = 50µm).

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Dauer juveniles

Body shorter and more slender than adults, almost straight when heat-killed (Fig. 3.4). Cuticle striated under SEM. Body tapers to bluntly rounded anterior end. Lip region continuous with body shape, and divided into six lips. Stoma closed. Head annulated, with four cephalic papillae and two slitlike amphidial apertures. Buccal cavity cylindrical, longer and narrower (Fig. 3.1A) than in adults. Pharyngeal region weaker than in adults, excretory pore in the vicinity of the isthmus. Genital primordium is visible as a light, elongate patch in the middle of the body. Seven to eight lateral lines in mid-body (Fig. 3.4D). Towards posterior, body gradually tapers to an elongate conoid tail, with filiform terminus.

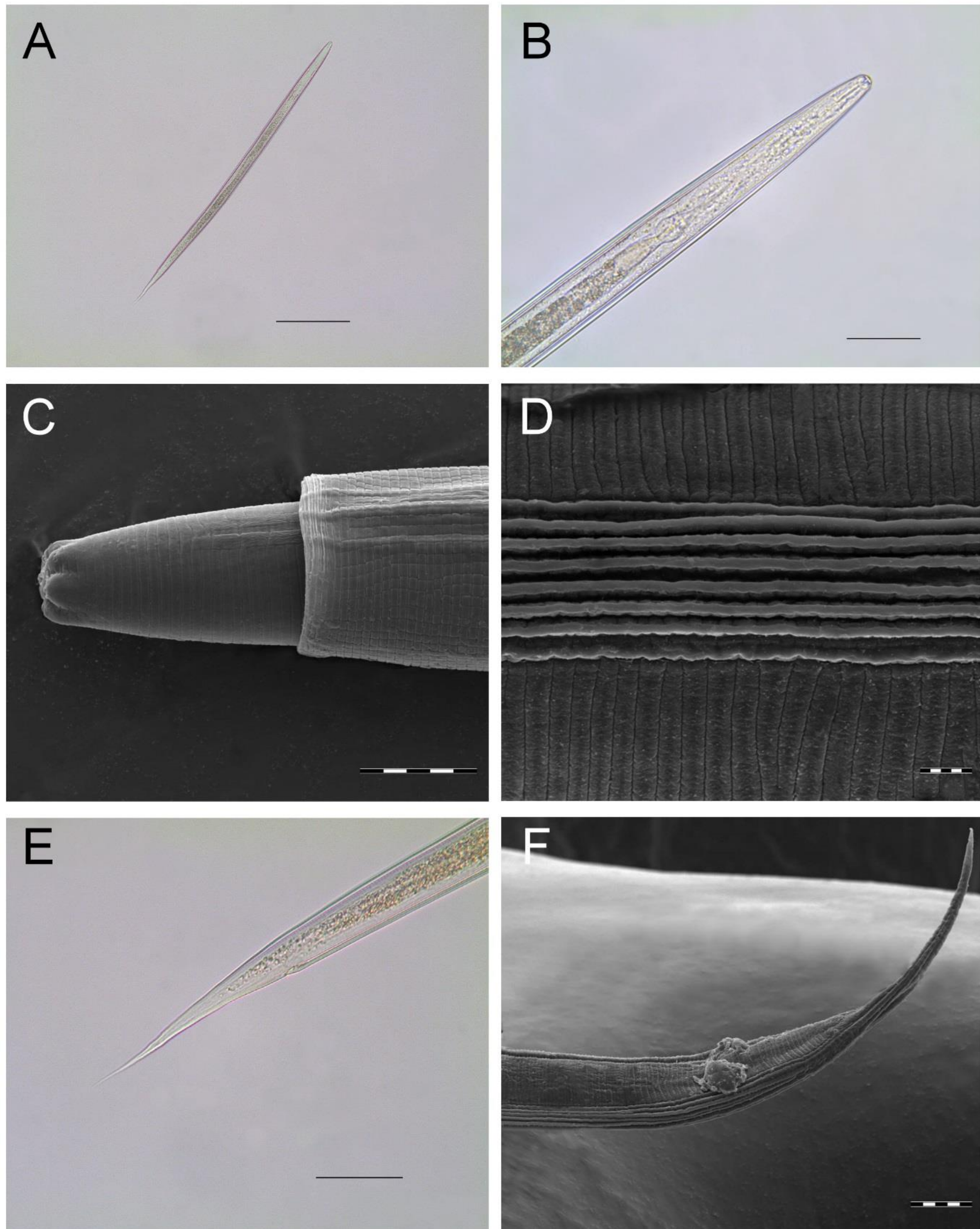

Figure 3.4 Micrographs of the dauer juvenile of *Phasmarhabditis* sp. SA4.
(A) Overview of juvenile; (B) Anterior end; (C) Exsheathed anterior end; (D) Lateral lines in mid-body region; (E-F) Tail region. (Scale bars: A = 200 µm; B, E = 50 µm; C, F = 10 µm; D = 2 µm).

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3.3.3 Type host and locality

Phasmarhabditis sp. SA4 was isolated from *D. reticulatum*, collected near George, Western Cape province, South Africa (33°99'37''S; 22°39'33''E). The slug was collected from an ornamental nursery.

3.3.4 Type material

Holotype (first-generation female), isolated from a slug, deposited in the United States Department of Agriculture Nematode Collection (USD ARS), Beltsville, Maryland, USA, as a permanent slide. Five permanent slides of each stage, with paratypes of males of the first (15) and second generation (16); females of the first (20) and second generation (18); and DJs (27) were deposited in the same collection (T-6728p to T-6752p). Specimens of each of the generation (20 each) and numerous DJs in cryotubes with TAF were deposited in the National Collection of Nematodes, Biosystematics Division, Plant Protection Research Institute, Agricultural Research Council, Pretoria, South Africa.

3.3.5 Diagnosis and relationships

The new species is characterised by the morphology and morphometrics of the female, male and DJs (Table 1). The female of *Phasmarhabditis* sp. SA4 is characterised by the presence of a slightly protruding vulva, a body length of 1923 (1622-2380) μm, a body width of 107 (79-145) μm, and a cupola-shaped tail, with a pointed tip of 63 (48-80) μm, consisting of 58% of the tail length. The male is characterised by a bursa with nine pairs of papillae, a spicule length of 62 (55-69) μm, and a gubernaculum length of 31 (27-34) μm, which is 49% of the size of the spicules. The DJs of the new species can be characterised by a body length of 900 (813-1042) μm, a body width of 32 (29-36) μm, and a tail length of 128 (113-148) μm.

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Nutrition, and bacterial species used as a food source, affect the size of the *Phasmarhabditis* species. For this reason, *Phasmarhabditis* sp. SA4 was grown on both decaying organic matter (in the form of frozen dead slugs) and bacterial cultures, and measured to illustrate this effect, and to compare *Phasmarhabditis* sp. SA4 with other *Phasmarhabditis* species, some of which were grown on bacterial cultures (*in vitro*), whereas the others were grown on slugs (*in vivo*).

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Table 3.2 Comparison of females of *Phasmarhabditis* sp. SA4, *P. huizhouensis, P. californica, P. hermaphrodita* (UK), *P. neopapillosa*, *P. hermaphrodita* (US), *P. papillosa*, and *P. tawfiki*. All measurements are in µm and in the form: mean ± sd (range).

Character	*Phasmarhabditis* sp. SA4		*P. huizhouensis*	*P. californica*	*P. hermaphrodita*		*P. neopapillosa*	*P. hermaphrodita*	*P. papillosa*	*P. tawfiki*
	In vitro SA	*In vivo* SA	Rotting leaves China	*In vitro* US	*In vivo* UK	*In vitro* UK	*In vitro* UK	*In vitro* US	*In vitro* US	*In vivo* Egypt
n	25	25	16	20	20	20	20	10	20	20
Body length (L)	1476 ± 105	1923 ± 171	1875 ± 341	1501 ± 117	1799 ± 279	1354 ± 115	2227 ± 190	1542 ± 161	1590 ± 195	1716 ± 347
	(1238-1727)	(1622-2380)	(1333-2341)	(1298-1757)	(1509-2372)	(1186-1525)	(1817-2449)	(1284-1721)	(1202-1932)	(1150-2370)
A = L/BW	20 ± 1.53	18 ± 2.04	14 ± 0.94	19 ± 2.9	20 ± 3.1	15 ± 1.6	16 ± 1.8	17 ± 1.6	20 ± 1.7	17 ± 2.5
	(17-23)	(14 - 22)	(13-16)	(15-23)	(14-29)	(12-18)	(15-16)	(15.1-19.5)	(16-23)	(12-20)
B = L/ES	6.17 ± 0.48	7.43 ± 0.72	8.33 ± 1.05	7.0 ± 0.4	7.2 ± 1.1	5.9 ± 0.4	7.7 ± 0.5	8.0 ± 0.6	7.1 ± 0.9	6.5 ± 1.0
	(5.00-6.99)	(6.45-9.47)	(6.39-10)	(6.3-8.0)	(5.9-9.3)	(5.1-6.4)	(7.2-8.4)	(6.9-8.9)	(5.4-9.0)	(4.9-8.7)
C = L/T	15 ± 1.52	18 ± 1.91	21 ± 3.3	18 ± 2.2	16 ± 2.8	13 ± 0.7	14 ± 1.2	16 ± 1.2	16 ± 4.3	11 ± 3.3
	(11-18)	(14-21)	(16-27)	(14-23)	(13-24)	(12-14)	(12-17)	(13-17)	(11-26)	(5.9-16)
c' = T/ABW	2.70 ± 0.27	2.00 ± 0.21	1.61 ± 0.17	2.8 ± 0.5	3.0 ± 0.3	2.9 ± 0.2	3.9 ± 0.5	2.5 ± 0.2	2.8 ± 0.5	_
	(2.23-3.38)	(1.55-2.36)	(1.28-2.02)	(2.4-4.0)	(2.4-3.6)	(2.4-3.2)	(3.3-5.0)	(2.2-3.0)	(1.9-3.6)	_
V%	52 ± 3.56	49 ± 4.20	52 ± 0.85	52 ± 0.9	51 ± 2.6	50 ± 1.6	51 ± 1.1	50 ± 1.5	50 ± 2.5	52 ± 0.9
	(42-60)	(40-56)	(51-54)	(51-54)	(48-60)	(48-55)	(48-52)	(48-53)	(41-53)	(51-54)
BW	75 ± 5.85	107 ± 17	134 ± 24	81 ± 12	94 ± 16	90 ± 11	141 ± 19	90 ± 8.3	81 ± 15	95 ± 13
	(64-88)	(79-145)	(85.5-171)	(60-99)	(71-118)	(75-106)	(101-174)	(82-107)	(57-116)	(70-110)
Stoma length	21 ± 1.31	23 ± 4.14	20 ± 1.98	19 ± 2.1	19 ± 0.8	18 ± 1.3	21 ± 1.2	18 ± 1.0	20 ± 2.4	20 ± 1.7
	(19-24)	(20-34)	(17-23)	(16-22)	(17-20)	(16-21)	(19-24)	(17-20)	(16-25)	(17-24)
Stoma diameter	5.04 ± 0.46	5.53 ± 1.09	6.53 ± 0.47	_	_	18 ± 0.7	19 ± 0.5	18 ± 1.9	16 ± 1.7	19 ± 2.4
	(4.09-5.98)	(3.02-7.24)	(5.91-7.89)	_	_	(17-19)	(18-19)	(14-20)	(13-20)	(17-20)
Excretory pore	193 ± 17	230 ± 16	_	197 ± 6.6	212 ± 11	172 ± 13	216 ± 11	_	187 ± 14	222 ± 24.2
	(165-230)	(200-287)	_	(187-204)	(192-231)	(157-189)	(199-231)	_	(157-208)	(165-270)
Pharynx length	240 ± 11	260 ± 19	215 ± 20	213 ± 13	251 ± 10	231 ± 8.9	290 ± 8.0	193 ± 9.3	168 ± 6.5	_

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	(217-262)	(232-302)	(184-251)	(180-230)	(235-270)	(212-242)	(277-303)	(175-209)	(152-178)	_
Anterior pharynx	140 ± 6.19	148 ± 16	117 ± 10	108 ± 9.0	115 ± 5.6	107 ± 5.2	144 ± 11	97 ± 6.1	116 ± 7.5	126 ± 18
	(125-150)	(108-183)	(101-139)	(83-119)	(109-126)	(96-114)	(126-168)	(86-107)	(102-130)	(100-156)
Posterior pharynx	99 ± 8.56	111 ± 15	99 ± 10	_	_	_	_	_	_	_
	(84-117)	(86-153)	(85-117)	_	_	_	_	_	_	_
Diameter of median bulbus	26 ± 2.94	35 ± 4.14	38 ± 7.11	30 ± 3.3	_	_	_	23 ± 2.2	26 ± 2.8	_
	(21-34)	(29-43)	(24-52)	(26-38)	_	_	_	(18-25)	(22-35)	_
Diameter of basal bulbus	36 ± 3.35	45 ± 4.36	53 ± 9.64	40 ± 2.5	_	_	_	30 ± 1.9	35 ± 3.3	_
	(30-45)	(36-57)	(39-71)	(35-44)				(27-32)	(30-41)	
Nerve ring (NR)	165 ± 8.10	185 ± 16	_	149 ± 13	166 ± 7.4	141 ± 6.9	188 ± 11	139 ± 7.2	168 ± 6.5	168 ± 38
	(153-185)	(163-217)	_	(115-165)	(154-177)	(131-154)	(168-205)	(126-149)	(152-178)	(135-200)
Anal body width (ABW)	38 ± 2.92	54 ± 4.92	57 ± 5.89	30 ± 4.1	_	_	_	40 ± 4.3	38 ± 3.9	_
	(34-45)	(46-69)	(43-67)	(22-39)	_	_	_	(34-48)	(31-45)	_
Tail (T)	102 ± 10	108 ± 12	91 ± 6.46	83 ± 8.9	114 ± 7.8	104 ± 8.6	157 ± 15	99 ± 10	106 ± 17	128 ± 23
	(86-123)	(87-143)	(81-106)	(66-94)	(99-129)	(82-113)	(141-174)	(85-117)	(73-130)	(85-140)
Length of tail tip	63 ± 8.51	63 ± 7.77	56 ± 4.99	_	_	_	_	_	_	_
	(51-82)	(48-80)	(48-64)	_	_	_	_	_	_	_
Distance from vulva to anus	647 ± 67	890 ± 99	800 ± 169	_	_	_	_	_	_	_
	(524-794)	(690-1094)	(518-1053)	_	_	_	_	_	_	_
Distance from vulva to stoma	766 ± 65	939 ± 90	_	_	_	_	_	776 ± 71	_	_
	(615-897)	(801-1231)	_	_	_	_	_	(673-866)	_	_

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Table 3.3 Comparison of males of *Phasmarhabditis* sp. SA4, *P. neopapillosa*, *P. huizhouensis*, *P. papillosa*, and *P. tawfiki*. All measurements are in µm and in the form: mean ± sd (range).

Character	*Phasmarhabditis* sp. SA4		*P. neopapillosa*	*P. huizhouensis*	*P. papillosa*	*P. tawfiki*
	in vitro	*in vivo*				
n	25	25	20	14	12	10
Body length (L)	1209 ± 106	1529 ± 79	1585 ± 90	1282 ± 248	1233 ± 184	1337 ± 159
	(973-1408)	(1390-1679)	(1432-1771)	(908-1669)	(1011-1565)	(980-1535)
a	22 ± 1.61	25 ± 2.00	19 ± 1.3	15 ± 1.44	21 ± 2.6	18 ± 1.7
	(18-26)	(21-28)	(17-21)	(12-17)	(16-24)	(15-20)
b	5.67 ± 0.50	6.85 ± 0.60	6.5 ± 0.6	6.6 ± 0.79	6.5 ± 0.9	5.8 ± 0.9
	(4.73-6.91)	(5.94-8.06)	(5.9-7.6)	(5.3-7.76)	(5.5-7.8)	(4.8-7.6)
c	24 ± 2.41	30 ± 2.13	32 ± 2.7	27 ± 3.54	29 ± 7	28 ± 4.6
	(19-28)	(26-35)	(28-34)	(21-34)	(23-50)	(18-35)
c'	1.40 ± 0.12	1.25 ± 0.09	1.1 ± 0.1	1.14 ± 0.18	1.2 ± 0.3	-
	(1.17-1.67)	(1.12-1.49)	(0.9-1.2)	(0.90-1.47)	(0.6-1.5)	-
Body width (BW)	54 ± 3.77	61 ± 4.74	84 ± 7.8	88 ± 15	61 ± 16	81 ± 9.1
	(48-66)	(53-74)	(78-94)	(65-114)	(45-88)	(65-100)
Stoma length	19 ± 1.36	20 ± 1.10	19 ± 0.8	18 ± 1.78	18 ± 2.4	21 ± 2.1
	(16-22)	(18-22)	(17-20)	(15-20)	(16-22)	(17-25)
Stoma diameter	4.31 ± 0.50	5.40 ± 0.52	-	5.66 ± 0.48	-	-
	(3.07-5.06)	(4.57-6.81)	-	(5.03-6.49)	-	-
Excretory pore	194 ± 9.82	205 ± 13	196 ± 11	-	170 ± 3.0	191 ± 36.3
	(163-208)	(178-226)	(185-220)	-	(168-172)	(113-250)
Pharynx length	214 ± 16	224 ± 14.17	246 ± 11	189 ± 12	139 ± 15	-
	(198-278)	(201-257)	(223-261)	(169-211)	(95-151)	-
Anterior pharynx	126 ± 11	130 ± 8.01	119 ± 4.5	105 ± 5.91	93 ± 9.5	114 ± 24
	(116-173)	(118-148)	(114-128)	(92 -112)	(80-109)	(80-160)
Posterior pharynx	87 ± 7.01	94 ± 6.59	-	86 ± 7.3	-	-
	(74-105)	(85-109)	-	(78-103)	-	-
Diameter of median bulbus	24 ± 1.90	30 ± 2.68	-	29 ± 2.98	24 ± 5.0	-
	(20-28)	(24-34)	-	(25-34)	(20-37)	-
Diameter of basal bulbus	32 ± 2.60	38 ± 2.58	-	41 ± 6.41	31 ± 4.6	-
	(27-40)	(34-44)	-	(28-53)	(25-40)	-
Nerve ring (NR)	154 ± 14	165 ±10	164 ± 8.8	-	139 ± 15	152 ± 27
	(132-204)	(149-184)	(151-177)	-	(95-151)	(113-200)
Anal body width (ABW)	37 ± 2.94	41 ± 2.26	-	41 ± 6.99	38 ± 7.5	-
	(31-42)	(37-45)	-	(31-55)	(30-50)	-
Tail (T)	51 ± 4.07	51 ± 3.21	49 ± 2.8	47 ± 8.06	43 ± 7.5	49 ± 3.9
	(43-57)	(46-59)	(45-54)	(36-61)	(28-52)	(44-54)
Spicule length (SL)	49 ± 6.67	62 ± 3.42	68 ± 2.8	70 ± 5.62	56 ± 7.1	63 ± 8.2
	(36-61)	(55-69)	(60-71)	(61-82)	(47-67)	(54-75)
Gubernaculum length (GL)	25 ± 3.67	31 ± 2.18	33 ± 1.7	35 ± 3.07	28 ± 5.2	36 ± 3.2
	(18-31)	(27-34)	(31-37)	(30-41)	(22-37)	(33-40)
GS% = GL/SL × 100	51 ± 4.84	49 ± 3.25	-	50 ± 2.03	-	-
	(40-62)	(45-56)	-	(48-55)	-	-

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Table 3.4 Comparison of dauer juveniles of *Phasmarhabditis* sp. SA4, *P. hermaphrodita* (UK), and *P. neopapillosa*. All measurements are in µm and in the form: mean ± sd (range).

Character	*Phasmarhabditis* sp. SA4		*P. hermaphrodita* (UK)	*P. neopapillosa*
	Dauer juvenile			
	In vitro	*In vivo*	*In vitro*	*In vivo*
n	25	25	20	20
Body length (L)	864 ± 49 (789-965)	900 ± 63 (813-1042)	1097 ± 36 (1016-1140)	1010 ± 26 (955-1063)
a	25 ± 2.40 (22-32)	28 ± 1.62 (26 -33)	27 ± 1.2 (24-30)	24 ± 0.7 (22-25)
b	5.37 ± 0.36 (4.76-5.94)	5.41 ± 0.38 (4.85-6.30)	6.3 ± 0.2 (5.9-6.6)	5.6 ± 0.2 (5.4-6.0)
c	8.40 ± 1.30 (6.87-12)	7.04 ± 0.42 (6.45-8.44)	7.7 ± 0.7 (6.6-8.8)	7.2 ± 0.4 (6.2-7.8)
c'	5.31 ± 0.42 (4.39-6.10)	6.42 ± 0.50 (4.86-7.11)	5.6 ± 0.6 (4.5-6.7)	5.8 ± 0.5 (4.8-6.8)
Body width (BW)	35 ± 2.77 (27-39)	32 ± 1.98 (29-36)	41 ± 1.4 (38-43)	42 ± 1.2 (40-44)
Stoma length	22 ± 1.37 (19-24)	23 ± 1.56 (21-26)	22 ± 1.0 (20-23)	20 ± 0.8 (19-22)
Pharynx length	161 ± 8.83 (147-177)	167 ± 14 (147-207)	175 ± 7.6 (158-185)	180 ± 7.3 (168-187)
Diameter of median bulbus	12 ± 1.22 (10-14)	10 ± 1.06 (9.18-14)	- -	- -
Diameter of basal bulbus	16 ± 1.24 (14.-19)	15 ± 1.15 (13-18)	- -	- -
Nerve ring (NR)	117 ± 6.25 (108-133)	115 ± 9.47 (102-145)	127 ± 3.9 (123-135)	119 ± 4.1 (111-123)
Hemizonion	139.82±7.01 (129-154)	142 ± 14 (120-183)	- -	- -
Anal body width (ABW)	20 ± 2.04 (15-23)	20 ± 1.94 (18-25)	- -	- -
Tail (T)	105 ± 13 (73-127)	128 ± 6.78 (113-148)	143 ± 14 (120-168)	141 ± 7.7 (131-163)
Hyaline portion	35 ± 5.20 (25-44)	44 ± 4.54 (32-51)	- -	- -
H% = T/Hyaline portion	3.01 ± 0.46 (2.11-4.22)	2.95 ± 0.33 (2.62-4.16)	- -	- -

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Phasmarhabditis huizhouensis was grown on bacterial cultures, and it is therefore compared to the *in vitro*-cultured *Phasmarhabditis* sp. SA4 specimens. The female of *Phasmarhabditis* sp. SA4 can be distinguished from the female of *P. huizhouensis* by its shorter length of 1476 (1238-1727) μm vs the 1875 (1333-2341) μm of the latter, with the former also having a smaller body width of 75 (64-88) μm vs the 134 (86-171) μm of the latter (Table 3.2). The female of the new species has a cupola-shaped tail with slightly visible phasmids, whereas *P. huizhouensis* females, which also have a cupola-shaped tail, possess one with a relatively slender tip and with 'rod-like' papilliform phasmids flanking the junction of the tail, as well as its tip. The male of *Phasmarhabditis* sp. SA4 can be distinguished from the male of *P. huizhouensis* by its shorter spicule 49 (36-61) μm vs the 70 (61-82) μm of the latter, as well as by its gubernaculum of 25 (18-31) μm vs the latter's gubernaculum of 35 (30-41) μm.

Although the females of *Phasmarhabditis* sp. SA4 are very similar to those of *P. californica*, the former have a relatively long pharynx with a length of 240 (217-262) μm vs the 213 (180-230) μm of the latter's pharynx, and the former have a longer tail of 102 (86-123) μm vs the 83 (66-94) μm of the latter. The vulva of the new species is a transverse slit with slightly protruding lips, whereas the vulva of *P. californica* is only visible as a transverse slit, without a protrusion.

Phasmarhabditis sp. SA4 females are very similar to the females of *P. hermaphrodita* (UK), with only their tail shapes being different. The female of the new species has a cupola-shaped tail that constricts into a thin pointed tip at 38% of the tail length, whereas the tail of the female *P. hermaphrodita* (UK) is conical, gradually tapering to a filiform terminus. The DJs of *Phasmarhabditis* sp. SA4 have a shorter body length of 864 (789-965) μm vs the 1097 (1016-1140) μm of the *P. hermaphrodita*, a thinner body width of 35 (27-39) μm vs the 41 (38-

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43) μm of the latter, and a shorter tail length of 105 (73-127) μm vs the 143 (120-168) μm of the latter.

Females of *Phasmarhabditis* sp. SA4 are thinner than are those of *P. neopapillosa*, with the former having a body width of 107 (79-145) μm vs 141 (101-174) μm, and a shorter tail length of 108 (87-143) μm vs 157 (141-174) μm. Males of the new species have similar morphometrics to the males of *P. neopapillosa*, with a thinner body width of 61 (53-74) μm vs 84 (78-94) μm. DJs of the new species have a body width of 32 (29-36) μm, whereas the juveniles of *P. neopapillosa* have a body width of 42 (40-44) μm.

Females of the new species have a vulva that is visible as a transverse slit with slightly protruding lips, whereas the vulva of *P. hermaphrodita* (US) is a flat transverse slit. Female *Phasmarhabditis* sp. SA4 have a thinner body and a longer pharynx than do the females of the *P. hermaphrodita* from the US, with a body width of 75 (64-88) μm vs 90 (82-107) μm, and a pharynx length of 240 (217-262) μm vs 193 (175-209) μm. In addition, the former have a more posteriorly positioned nerve ring of 165 (153-185) μm from the anterior end vs the 139 (126-149) μm of the latter.

The males and females of the new species have similar morphometrics to those of the *P. papillosa* that were isolated in the USA, but only with a female pharynx length of 240 (217-262) μm vs the 168 (152-178) μm of the latter, and a male pharynx length of 214 (198-278) μm vs the 139 (95-151) μm of the latter.

Males of the new species have a thinner body and a shorter gubernaculum than do the males of *P. tawfiki*, with a body width of 54 (48-66) μm vs the 81 (65-100) μm of the latter, and a gubernaculum length of 25 (18-31) μm vs the 36 (33-40) μm of the latter. The females concerned are also thinner than are the females of *P. tawfiki*, with the former possessing a body width of 75 (64-88) μm vs the 95 (70-110) μm of the latter.

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3.3.6 Molecular differentiation and phylogenetic relationships

The sequences that were obtained for *Phasmarhabditis* sp. SA4 were deposited in the NCBI GenBank under KX267673 for the 18S rRNA, under KX267674 for the D2D3 LSU rRNA gene, under KX267675 for the ITS gene, and under KX263471 for the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene. As the sequences of *Phasmarhabditis* sp. SA4 were identical across each gene, only one representative sequence was submitted.

Phylogenetic analysis was conducted using the 18S rRNA gene of 28 nematode taxa employing representatives of the genera *Agfa*, *Angiostoma*, *Phasmarhabditis*, and *Pellioditis*, along with *O. tipulae* and *O. insectivore* as an out-group. The tree topologies that were obtained by way of ML, MP, and distance analyses were identical, so only the ML tree is presented below (Fig. 3.5). Bootstrap support for each method of analysis is also indicated.

Phylogenetic analyses placed *Phasmarhabditis* sp. SA4 in a strongly supported clade (98/97/95), comprising *Phasmarhabditis* sp. SA2, *P. papillosa* and the mollusc-parasitic nematode, *Angiostoma dentiferum* (Mengert, 1953). The nematodes mentioned, along with *P. huizhouensis*, *P. californica*, and other *Phasmarhabditis* spp., formed a sister group *to P. hermaphrodita*, *P. neopapillosa*, *Phasmarhabditis* sp. SA1, *Angiostoma norvegicum* Ross, Haukeland, Hatteland & Ivanova, 2016, and *Angiostoma margaretae* Ross, Malan & Ivanova, 2011. Together, the nematodes form a sister group to *Agfa flexilis* (Dujardin, 1845) (99/98/98), with *Angiostoma limacis* Dujardin, 1845 as the basal taxon (100/100/100) (Fig. 3.5). The results obtained demonstrated that the three morphologically diverse families of Agfidae, Angiostomatidae and Rhabditidae formed a tight monophyletic clade under strong bootstrap support (100/100/100) (Ross *et al.*, 2010).

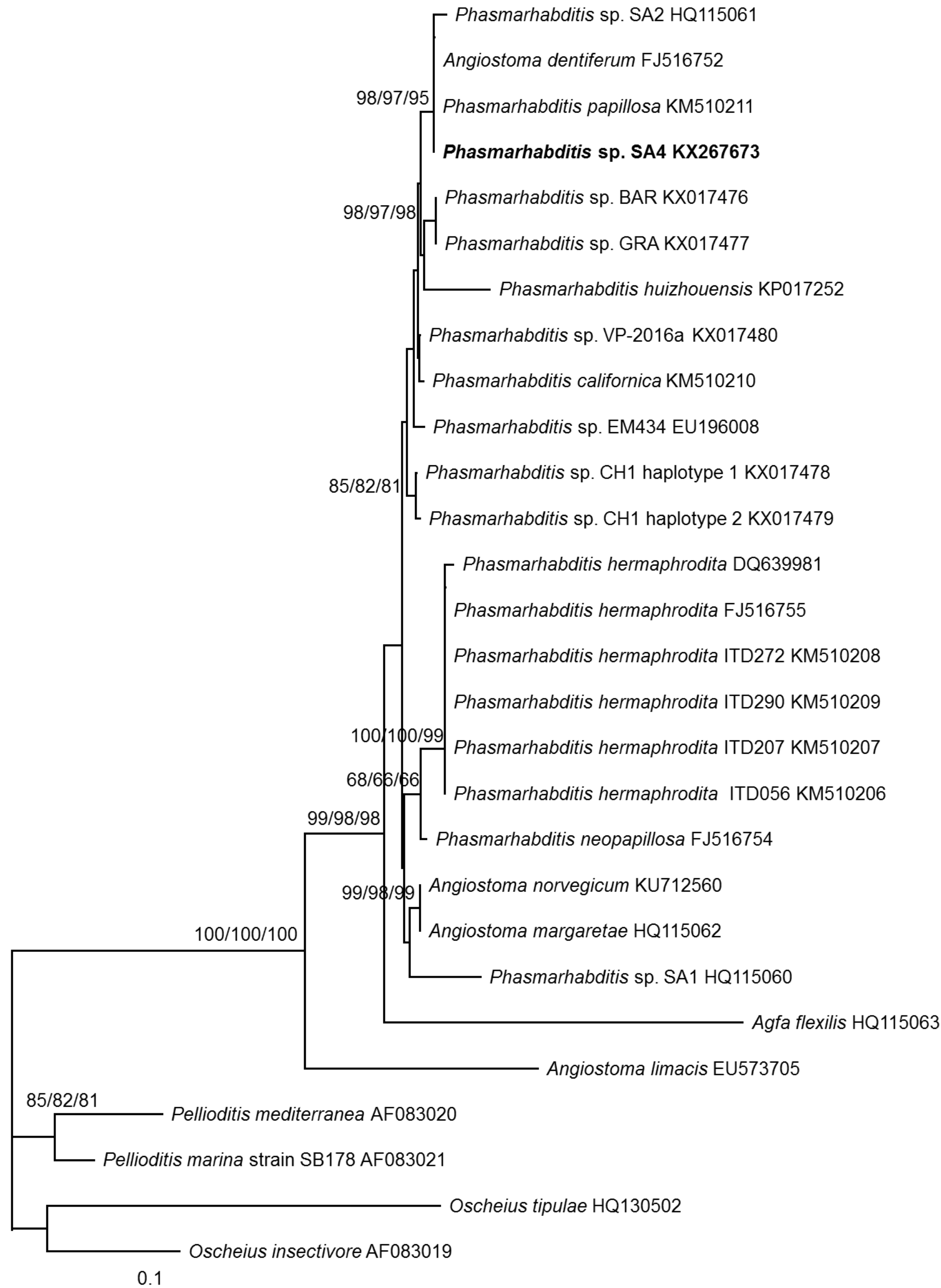

Figure 3.5 The maximum likelihood (ML) phylogenetic tree of 18S rRNA gene sequences, using representatives from the genera *Agfa*, *Angiostoma*, *Phasmarhabditis*, and *Pellioditis*, along with *Oscheius tipulae* and *O. insectivore* as out-groups. The phylogenetic analysis of 1058 unambiguously aligned nucleotide positions used the GTR correction model with eight gamma rates and invariable sites. Bootstrap support was calculated on the basis of 1000 replicates, using ML, distance, and MP methods, respectively. Only bootstrap values above 65% are included.

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3.4 Discussion

The new species, *Phasmarhabditis* sp. SA4, is described from the slug host *D. reticulatum* that was collected from a nursery in George, South Africa. *Deroceras reticulatum* is a European invasive pest that has been dispersed throughout the world, with it currently being found in areas including North and South America, Europe, Australia, the islands of the Atlantic and Indian oceans, New Zealand, and South Africa (Barker, 1999; Godan, 1983). *Deroceras reticulatum*, which is thought to have been introduced to Southern Africa prior to 1898, has invaded a vast number of habitats, including gardens, agricultural land, montane *Podocarpus* forest, and even such dry habitats as *Spirostachys* woodland (Herbert & Kilburn, 2004).

Phasmarhabditis species are usually distinguished within the genus by means of their morphometrics, the female tail characteristics, the presence of males, the bursal papillae, and the spicule size. Identification is difficult, as males are only present in a few of the species, and the *Phasmarhabditis* species are morphologically conservative (Tandingan De Ley *et al.*, 2016). Therefore, species identification heavily relies on molecular identification.

Phasmarhabditis sp. SA4 is characterised by the cupola shape and length of the female tail, and the presence of males. When it is grown *in vitro* on bacterial plates, the nematode is shorter than if it were grown *in vivo* (on decaying frozen slugs). A similar observation was made by Hooper *et al.* (1999) regarding *P. hermaphrodita*. The characteristic has also been observed in the juveniles of the entomopathogenic nematode *Heterorhabditis bacteriophora*, as well as in several of the *Steinernema* species (Nguyen & Smart, 1995).

Phylogenetic analyses placed *Phasmarhabditis* sp. SA4 in the same clade as *Phasmarhabditis* sp. SA2, *P. papillosa*, and the mollusc-parasitic nematode, *A. dentiferum*. The results of such analyses varied in comparison to the morphological analysis, which suggests that the nematode is closest to *P. californica* of all other nematodes.

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The identification of *Phasmarhabditis* sp. SA4 brings the total complement of the genus to seven species. Six of the species were recorded from mollusc hosts, with *P. huizhouensis* having been isolated from rotting plant tissue. However, the close phylogenetic relationship of *P. huizhouensis* with other *Phasmarhabditis* spp. indicates that it might be a facultative parasite (Huang *et al*., 2015).

The isolation of the new *Phasmarhabditis* species in the survey described in the current study indicates the vast species richness in South Africa. Prior to the survey, only one other survey had been conducted on slug-parasitic nematodes in South Africa (Ross *et al.*, 2012). Therefore, future work should focus on sampling for additional *Phasmarhabditis* species in South Africa, and on investigating the biological control potential of new isolates. Sampling protocols could also be improved by means of adopting the method described by Wilson *et al.* (2016), in which slugs are field-collected and decapitated, after which they are incubated for a week prior to being examined for nematodes. Adoption of the method concerned is likely to be less labour-intensive than are live dissections, but care must be taken not to lose the related information regarding infection sites and other parasitic nematodes.

In addition to the above, future work should focus on the pathogenicity of *Phasmarhabditis* sp. SA4, so as to determine its effectiveness at controlling invasive European slug species, as well as indigenous and non-target species. Further research should also be done on establishing monoxenic cultures, and on the optimisation and mass production of the nematode.

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CHAPTER 4

Monoxenic culturing and temperature optimisation of *Phasmarhabditis* sp. SA4 (Nematoda Rhabditidae)

Abstract

Phasmarhabditis sp. SA4, a South African nematode parasitising slugs, has shown promise at controlling invasive European slug species. This study investigated the establishment of *in vitro* monoxenic cultures of *Phasmarhabditis* sp. SA4, as well as temperature optimisation. Five bacterial isolates were obtained by means of swabbing the intestines and internal cavities of *Deroceras reticulatum*, and identifying the different bacteria that were isolated by means of using 16S rRNA gene sequences. The pathogenic effects of the bacteria were tested by means of injecting directly into the haemocoel of *D. reticulatum*, and monitoring the mortality over time. *Kluyvera* sp., which was found to cause the highest mortality rate among the slugs concerned, was chosen for monoxenic culturing. Cultures containing *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. were optimised using temperatures ranging from 15° to 25°C. Results indicate the optimum growth temperature for *Phasmarhabditis* sp. SA4 to be 15°C.

4.1 Introduction

Terrestrial slugs (Mollusca: Gastropoda), which are pests of a broad range of agricultural and horticultural crops in South Africa, are particularly damaging in canola crops that are grown in the Western Cape province. Despite them currently being controlled with the help of chemical pesticides, in Europe a more environmentally friendly approach has been adopted involving the mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrássy, 1983. The nematode is capable of parasitising a wide range of mollusc species, but particularly slugs from the families Agriolimacidae, Arionidae, Limacidae, Milacidae, and Vagnulidae (Wilson *et al.*, 1993; Iglesias & Speiser, 2001; Speiser *et al.*, 2001; Grewal *et al.*, 2003; Rae *et*

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al., 2008). Due to its high virulence and broad host range, *P. hermaphrodita* has been developed into a biological molluscicide by BASF (formally known as MicroBio and Becker Underwood), being marketed under the name Nemaslug® (Rae *et al*., 2007). The product is sold as a water-dispersible formulation containing third-stage dauer juveniles (DJ) that, once applied, search out and infect slugs, with death occurring within 4-21 days (Wilson *et al*., 1993; Tan & Grewal, 2001). *Phasmarhabditis hermaphrodita* is a facultative parasite that can reproduce on a variety of substrates, including compost, slug faeces, leaf litter, dead earthworms, and dead insects (Tan & Grewal, 2001; MacMillan *et al*., 2009; Nermut *et al*., 2014).

Although methods of mass-producing *P. hermaphrodita* have included both *in vivo* and *in vitro* techniques, the former mode of production is considered to be problematic, as mollusc hosts must either be field-collected or laboratory-reared. Consequently, the majority of research thus far has focused on *in vitro* production using xenic (*P. hermaphrodita*, and an unknown mix of bacterial species) or monoxenic cultures (*P. hermaphrodita*, and one known bacterial species) (Wilson *et al*., 1995a). Monoxenic cultures are favoured, as they offer a more predictable result (Ehlers & Shapiro-Ilan, 2005), and they help to ensure that high numbers of DJs, with consistent infectivity, are produced (Wilson *et al*., 1995a).

Initial monoxenic studies were conducted by Wilson *et al*. (1995a), who experimented with 16 bacterial isolates, comprised of 13 different species. Bacterial isolates were collected from infected *Deroceras reticulatum* (Müller, 1774) cadavers, xenic foam chip cultures, and the DJs of *P. hermaphrodita* (Wilson *et al*., 1995a), and tested for their ability to grow in monoxenic liquid cultures. The bacterial isolates, *Providencia rettgeri* (Hadley, Elkins & Caldwell, 1918) Brenner, Farmer, Fanning, Steigerwalt, Klykken, Wathen, Hickman & Ewing, 1978 (Enterobacteriaceae) and *Moraxella osloensis* Bøvre & Henriksen, 1967 (Moraxellaceae), produced the highest yields of DJs, with *P. rettgeri* producing the highest numbers, overall. However, subsequent studies demonstrated that *M. osloensis* produced constant high yields of

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DJs, with consistent pathogenicity. Therefore, *M. osloensis* was chosen as the bacterium that was suited for the commercial production of *P. hermaphrodita* (Wilson *et al.*, 1995b).

The nematode–bacterial combination is mass-produced in large-scale fermenters, with the overall aim being to maximise the production of nematodes in the metabolically suppressed DJ stage. This life stage forms when the food source becomes depleted, or when stressful environmental factors (e.g. temperature, overcrowding) are optimised. On completion of the life cycle, DJs are extracted from the fermented media, being mixed with an inert gel polymer (G. Martin, personal communication, January 5, 2016). The mixtures are then sealed and packaged in sizes of 30 million and 250 million nematodes per pack.

To date, the European biological molluscicide, Nemaslug®, cannot be sold in South Africa, due to the current legislation (amendment of Act 18 of 1989 under the Agricultural Pest Act 36 of 1947). Therefore, methods of indigenous biocontrol should be investigated. In Chapter 2, a *Phasmarhabditis* sp. SA4 that was isolated from a *D. reticulatum*, collected from George, South Africa, showed promise at controlling *Deroceras panormitanum* (Lessona & Pollonera, 1882), which is a species that has been identified as being pestiferous in canola crops. In Chapter 3, the nematode concerned was described as *Phasmarhabditis* sp. SA4.

The objective of the current study was to establish *in vitro* monoxenic cultures of *Phasmarhabditis* sp. SA4, and to optimise production efficiency, using a temperature curve from 15°C to 25°C.

4.2 Materials and methods

4.2.1 Nematode strains

This experiment was conducted using *Phasmarhabditis* sp. SA4. The nematode was isolated in George (33°99’37’’S; 22°39’33’’E) (see Chapter 2), and the description of the morphological and molecular analysis of the new species can be found in Chapter 3.

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4.2.2 Bacterial strains

Bacterial strains were collected by means of swabbing the intestine and internal cavity of 10 *D. reticulatum* specimens collected from the original sample site in George (33.99.37S 22.39.33E). Bacterial isolates were cultured on nutrient agar plates (3 g beef extract, 5 g Tryptone, 8 g NaCl, 15 g agar per litre, autoclaved for 20 min at 121°C) and subcultured until each plate contained a single species of bacteria. Bacterial strains were identified by means of extracting bacterial genomic DNA, using a ZR fungal/ bacterial DNA kit (Zymo Research), and by means of amplifying the partial 16S rRNA gene, using the primer pair 8F and 1512R (Felske *et al*., 1997).

4.2.3 Culturing bacterial strains

Each bacterial species identified was established in liquid cultures by preparing 5 ml of LB (Luria Broth) (10 g NaCl, 10 g Tryptone, 5 g yeast extract per litre, autoclaved for 20 min at 121°C), and incubated for 12 h. A total of 1 ml was then removed from each culture and added to 50 ml of fresh LB in 250 ml Erlenmeyer flasks, with the extracts then being incubated in an orbital shaker (150 rpm) at 30°C for from 24 to 36 h.

4.2.4 Pathogenicity of bacterial strains

One hundred µl of the bacterial culture in LB was then removed and added to 10 ml of fresh LB, which was then incubated at 30°C. Twenty µl of each bacterial culture was removed, and injected directly into the haemocoel of a *D. reticulatum* host. Ten *D. reticulatum* were injected for each bacterial strain concerned, and mortality was recorded daily for 10 days. The experiment was repeated with fresh bacterial cultures on a different test date.

4.2.5 Monoxenic nematode cultures

To establish monoxenic nematode cultures, 20-30 adult nematodes were ruptured to remove their eggs. The rupturing was done by washing the adult nematodes with sterile water, and by means of adding a mixture of 0.5 ml 5 N NaOH mixed to 1 ml bleach in Eppendorf tubes. The

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tubes were then shaken every 2 minutes for 10 minutes, after which they were centrifuged at 2000 rpm for 30 seconds, so as to pellet the released eggs. Each tube was then aspirated to 100 µl, with 900 µl sterile water being added. The latter action was repeated three times. The eggs were then added to kidney agar plates (Wilson *et al.*, 1993) that had been preinoculated with the desired bacteria, whereupon they were incubated at 15°C for 20 days. After 20 days, monoxenic nematode cultures were transferred to 50 ml liquid culture medium (LCM) (9 g pig kidney, 17.4 g yeast extract, 8.6 g egg yolk powder, 52.6 g sunflower oil per litre; autoclaved for 20 min at 121°C) in 250 ml Erlenmeyer flasks, preinoculated with 1 ml of desired bacteria at a density of 1×10^{10} cells ml^{-1}. Flasks were incubated in an orbital shaker (150 rpm) at 15°C for 20 days. After 20 days, the nematodes were collected by means of centrifugation (800 g, 20 min), washed with Ringer's solution (Merck), and centrifuged again, so as to remove the culture debris. The nematodes involved were used as inoculum for the temperature experiment.

4.2.6 Monitoring contamination

Monoxenic cultures were monitored for contamination weekly. A small sample that was extracted from each flask was streaked out on nutrient agar plates. After undergoing incubation for 48 h, the bacterium was visually observed for contamination.

4.2.7 Temperature experiment

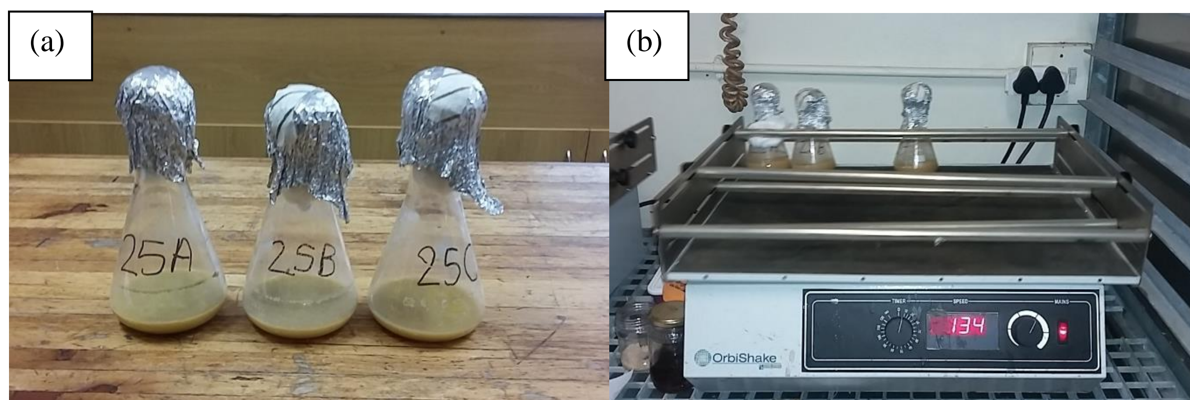

Figure 4.1 (a) Three flasks with 50ml LCM, bacteria and 1500 nematodes ml^{-1} were incubated at each temperature (15, 20 and 25°C) (b) on an orbital shaker.

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The temperature experiment was prepared by inoculating 50 ml LCM with 1 ml of the most pathogenic bacteria, as identified in Subsection 4.3.4, at a density of 1×10^{10} cells ml^{-1}. A total of 1500 nematodes ml^{-1} were then added to each flask (see Subsection 4.3.5). Flasks were incubated at three different temperatures (15, 20 and 25°C), with three replicates being used for each temperature (Fig. 4.1). The population density was then monitored every five days for 20 days post-inoculation (dpi), by means of assessing the total nematode density and the DJ density and proportion. The experiment was repeated and monitored for contamination, using the method described in Subsection 4.3.6.

4.2.8 Pathogenicity test

After the temperature experiment, nematodes from the experiment were used as inoculum for the undertaking of a pathogenicity test. This was done to determine the pathogenicity of *Phasmarhabditis* sp. SA4, with the most pathogenic bacteria identified in Subsection 4.3.4. Ten Petri dishes (9-cm) were each lined with a Whatman No.1 filter paper, moistened with 1 ml of distilled water containing 2000 DJs, and with one *D. panormitanum* added. The total of 10 Petri dishes that served as control were moistened with 1 ml distilled water just before adding the slugs under investigation. One carrot disc, 3 cm in diameter, was added to each plate as food, and replaced daily. The dishes were incubated at 20°C for 14 days. The feeding and mortality of the slugs were recorded every 2 days (Fig. 4.2). Slugs that died were dissected to determine cause of death. This experiment was repeated on five different test dates, with fresh batches of nematodes on each date.

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Figure 4.2 A slug infected by DJs (left) and a slug used as control (right).

4.2.9 Statistical analyses

A chi-squared test was performed to determine which bacteria caused the highest slug mortality. The data for the temperature experiment was tested using the analysis of variance (ANOVA). For the pathogenicity tests, the homogeneity and normality among means was assessed using normality tests, with homogeneity of variances tests being performed using the Shapiro–Wilk test and Levene's test, respectively. All data analyses were performed using Statistica 12 (Statsoft, Tulsa, OK). Slug mortalities were analysed as counts, due to the failure of the data transformation (square root transformation; Wilson *et al*., 1994) to produce normally distributed data. A 2 × 2 factorial ANOVA test ($P < 0.05$) was used to establish the combined effects of time and treatment on mortality. Non-parametric Kruskal–Wallis ANOVA ($P < 0.05$) tests were used to compare the mortalities between treatments.

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4.3 Results

4.3.1 Bacterial strains

Five bacterial isolates were identified, including *Flavobacterium* sp., *Shewanella* sp., *Pseudomonas* sp., *Kluyvera* sp., and *Acinetobacter* sp. These bacterial isolates have not previously been isolated from slugs. The sequences generated were edited and submitted to the GenBank (for the relevant accession numbers and closest matches see Table 4.1).

Table 4.1 The partial 16S rRNA gene accession numbers of five bacterial isolates from *Deroceras reticulatum* collected in George with NCBI matches, with ranges in identity and coverage between 99-100%.

Bacterial isolate	Genbank Accession Number	Habitat	NCBI match	
			Species/strain	GenBank Accession Number
Flavobacterium sp.	KX531094	Soil and fresh water	*Flavobacterium frigidimaris* KUC-1	NR041057
Shewanella sp.	KX531095	Marine bacteria	*Shewanella putrefaciens* S T TSA 99	JX860525
Pseudomonas sp.	KX531096	Wide habitat	*Pseudomonas fragi* M0421	KF924232
Kluyvera sp.	KX531097	Water bacteria and unpolluted soil	*Kluyvera intermedia* TCM1238	NR112007
Acinetobacter sp.	KX531098	Soil bacteria	*Acinetobacter* sp. D14(2011)	JN228315

4.3.2 Pathogenicity of bacterial strains

A significant difference in pathogenicity was found between the bacterial treatments ($\chi^2 = 209$, $N = 20$, $df = 4$, $P < 0.001$). *Post-hoc* pairwise comparisons indicate that there was a significant difference between the slugs that were treated with *Kluyvera* sp. and all other treatments ($P \leq 0.001$). In addition, there was a significant difference between the slugs that were treated with *Flavobacterium* sp. and the control ($P = 0.01$) (Fig. 4.3).

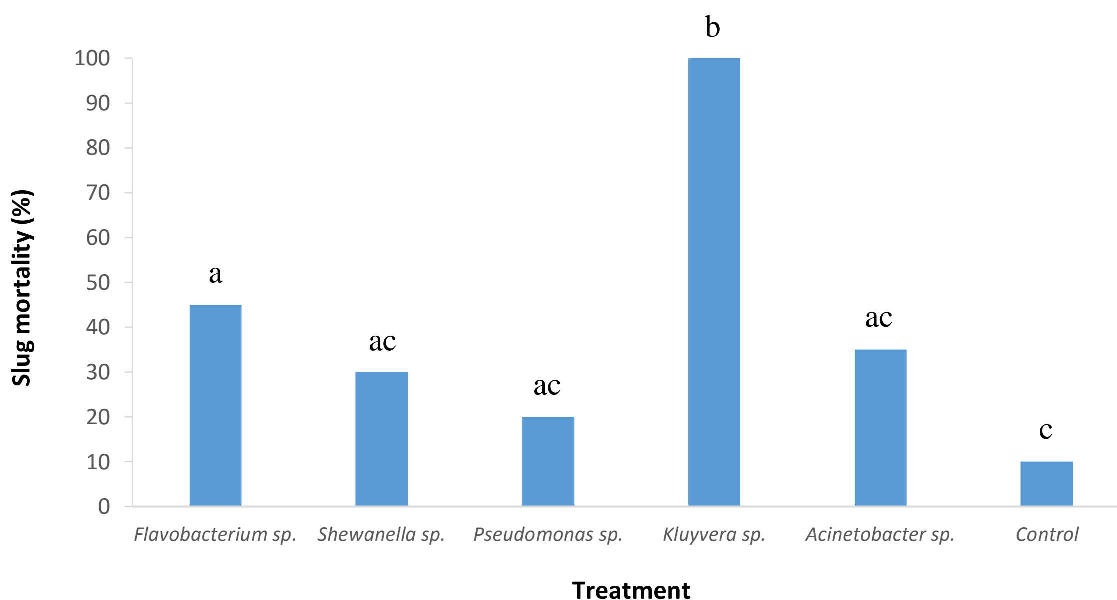


Figure 4.3 Slug mortality (%) 5 days after injection with bacteria, or with saline water as control. Different letters above bars indicates significant differences ($\chi^2 = 209$, $N = 20$, $df = 4$, $P < 0.001$).

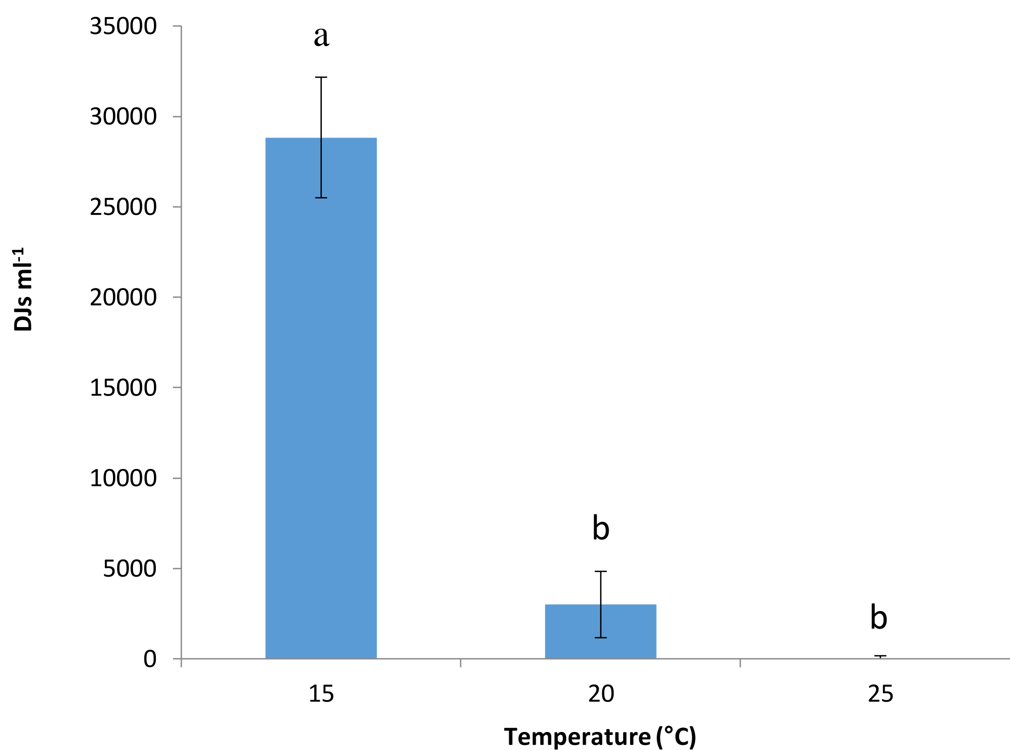
4.3.3 Temperature experiment

The DJ density recorded at 20 dpi (Fig. 4.4a) varied significantly for the different temperatures (Kruskal–Wallis ANOVA: $H_{2,9} = 7.3$, $P = 0.03$). The highest mean density of DJs was recorded at 15°C (31 166 DJs ml^{-1}). The density of DJs was significantly higher at 15°C than it was at 20°C ($P < 0.05$) and 25°C ($P = 0.02$), however there was no significant difference in the DJ density between 20°C and 25°C ($P = 0.54$). Zero DJs were recorded at 25°C (0 DJs ml^{-1}).

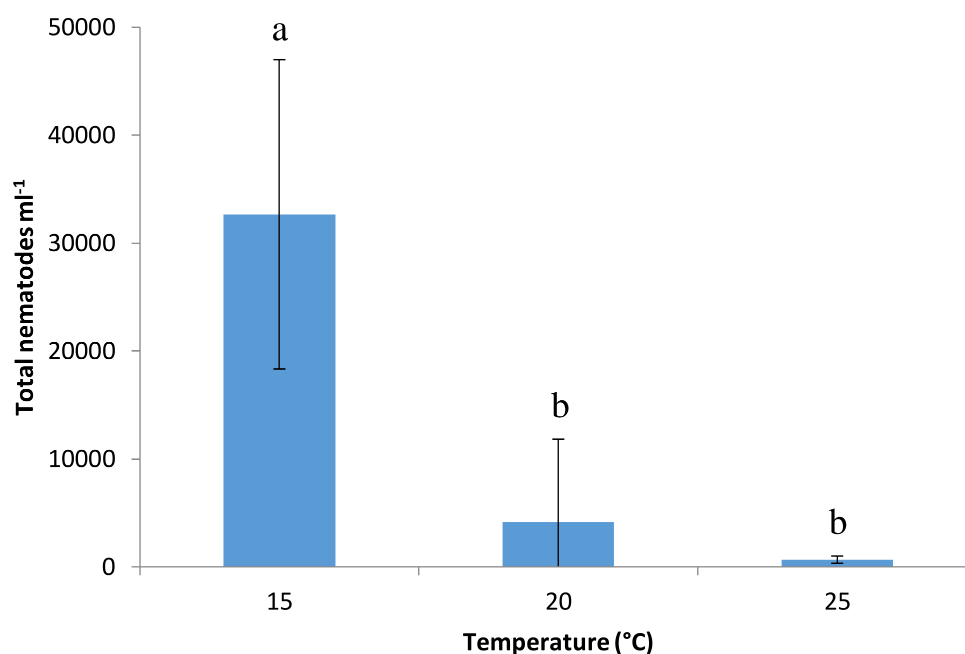
The total nematode density recorded at 20 dpi (Fig. 4.4b) varied significantly for the different temperatures ($H_{2,9} = 7.3$, $P = 0.03$). The highest total nematode density was recorded at 15°C (38 000 nematodes ml^{-1}), and the lowest at 25°C (333 nematodes ml^{-1}). The nematode density was significantly higher at 15°C than at 20°C (<0.05) and 25°C ($P = 0.02$), however there was no significant difference between 20°C and 25°C ($P = 0.54$).

The DJ proportion recorded at 20 dpi (Fig. 4.4c) varied significantly for the different temperatures ($H_{2,9}$ = 6.5, P = 0.04). The highest mean DJ proportion was recorded at 15°C (95.4%). At 25°C, the DJ proportion was significantly reduced, compared to what the proportion was at 15°C (P = 0.03). The lowest DJ proportion of zero was found at 25°C. No significant difference was found in the DJ proportion between 15°C and 20°C (P = 0.89), and between 20°C and 25°C (P = 0.4).

(a) Dauer juvenile (DJ) density at 20 dpi



(b) Total nematode density at 20 dpi



(c) DJ proportion as percentage of nematode population at 20 dpi

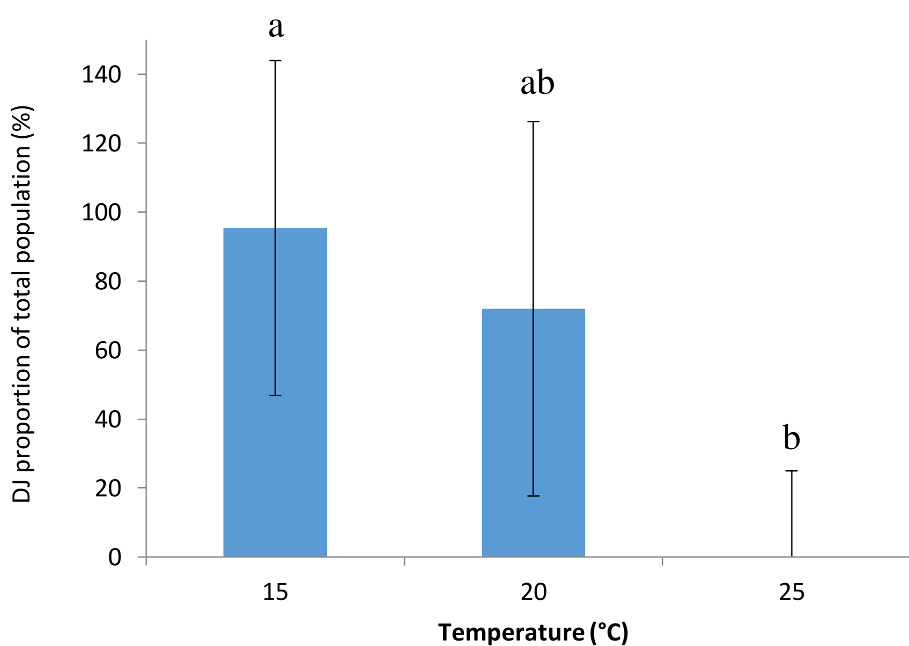


Figure 4.4 The influence of different incubation temperatures on (a) mean dauer juvenile (DJ) density, (b) total nematode density, and (c) the proportion of DJs as a percentage of the total nematode population of *Phasmarhabditis* sp. SA4.

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4.3.4 Pathogenicity tests

A significant interaction was noted between the effects of time and treatment on slug mortality ($F = 109.8$, $P < 0.001$), with both time and treatment having a significant effect on mortality ($F = 120.7$, $P < 0.001$; and $F = 4335.5$, $P < 0.001$, respectively) (Figure 4.5). Slugs treated with nematodes (median with quartile ranges: 8.0, 3.0–10.0) had a significantly higher mortality count compared to the controls (Mann–Whitney U: $N = 70$, $U = 324$, $z = -9.5$, $P < 0.001$).

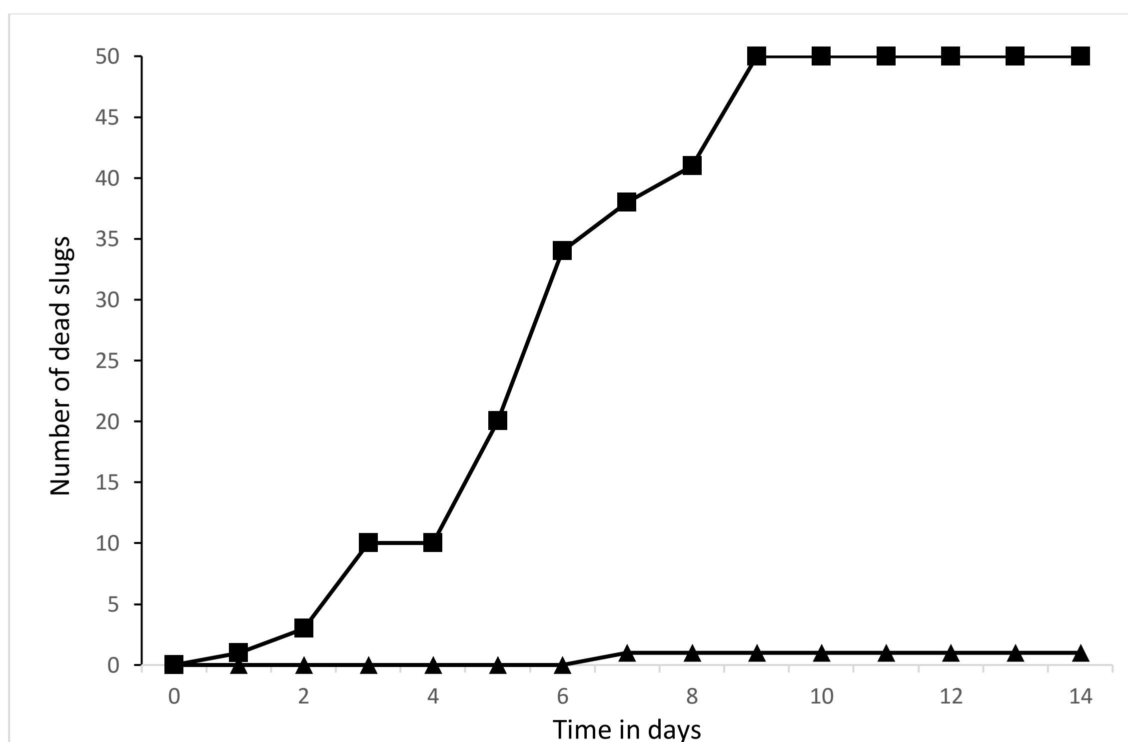

Figure 4.5 Numbers of dead slugs (out of 30) during 14 days in Petri dishes without nematodes (▲), and with 2000 dauer juveniles of *Phasmarhabditis* sp. SA4 (■), when kept at 20°C.

4.4 Discussion

The current study investigated the establishment of monoxenic cultures of *Phasmarhabditis* sp. SA4, using protocols described for the *in vitro* production of *P. hermaphrodita* (Wilson *et al.*, 1995a). Monoxenic cultures are an effective way of mass-producing nematodes with a high proportion of DJs that are consistently dauer (Wilson *et al.*, 1995a; Ehlers & Shapiro-Ilan,

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2005). In the present study, five bacterial isolates (*Flavobacterium* sp., *Shewanella* sp., *Pseudomonas* sp., *Kluyvera* sp., and *Acinetobacter* sp.) were isolated from the intestines and internal cavities of *D. reticulatum* specimens collected from George. Pathogenic studies of the aforementioned bacterial isolates demonstrated that a *Kluyvera* sp. caused the highest mortality rate when injected into the host, resulting in it being used for monoxenic establishment.

The closest match to the *Kluyvera* sp. was *Kluyvera intermedia* (Izard, 1980) Pavan, 2005. *Kluyvera intermedia* has been isolated from a number of sources, including molluscs, soil, surface water, drinking water, and human samples (e.g. blood, stool, bile, wounds, and gall bladder) (Pavan *et al*., 2005). It is Gram negative, with the straight rod occurring in both singles and pairs. No evidence supports the contention that the bacterium concerned can cause human disease (Pavan *et al.*, 2005).

The pathogenicity of monoxenic cultures containing *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. were tested against *D. panormitanum*. The results demonstrated that the monoxenic combination causes significant mortality of the host, thus making the monoxenic union an ideal candidate for future mass production development. However, future research should focus on investigating the pathogenic effect of the nematode/bacterial combination on other host species, including European invasive molluscs, indigenous molluscs, and nontarget organisms (e.g. earthworms, carabid beetles).

Monoxenic cultures containing *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. were optimised using three temperatures, 15, 20 and 25°C. The results showed that the optimum growth temperature was 15°C, which correlates with the findings of the closely related species, *P. hermaphrodita* (Ross, 2010). Although BASF commercially mass-produces *P. hermaphrodita* at 15°C, the high proportion of non-DJ nematode stages that is produced at the temperature identified has resulted in the firm introducing extra clean-up protocols in order to improve the quality of the Nemaslug® product, thus adding extra costs to the production thereof.

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The optimum growth temperature of *Phasmarhabditis* sp. SA4 also correlates with the optimum temperatures of the original host, *D. reticulatum*. Studies have shown that the optimum growth and movement temperature of *D. reticulatum* is between 17°C and 18°C (Dmitrieva, 1969; Stern, 1975; South, 1982), therefore suggesting that the nematode in question has evolved an ability to infect its host during the period of such optimum conditions.

The overall aim for mass production is to produce as many DJ nematodes as possible, as this is the stage that has the ability to locate and infect mollusc hosts. Many soil nematodes, especially from the Rhabditidae family, have a similar stage. The stage was first studied by Schneider (1866), who recorded rhabditid nematodes with a different cuticle from other nematode stages. The DJ stage is similar to the diapause stage that occurs in many organisms under unfavourable conditions (Clutter, 1978), and it is differentiated from other stages by means of varying its physiological, morphological, behavioural, and biochemical characteristics (De Wilde, 1962; Tauber & Tauber, 1976).

An understanding of how the DJ of *Phasmarhabditis* sp. SA4 is formed can be studied using the model organism, *Caenorhabditis elegans* (Maupas, 1900). Under ideal conditions, *C. elegans* develop from an egg, going through four juvenile stages (J1 to J4) to the stage of adulthood. However, if the nematode is exposed to poor conditions during the J1 stage, it can develop into the special nonfeeding third-stage juvenile called the DJ, or dauer, stage (Golden & Riddle, 1984). Poor environmental conditions, including high temperatures, food scarcity, and high population density (Golden & Riddle, 1984), trigger the development of the DJ stage.

In addition to studying *C. elegans*, it is also possible to achieve an understanding of the DJ stage from entomopathogenic nematodes (EPNs) (Shapiro-Ilan & Gaugler, 2002; Ehlers & Shapiro-Ilan, 2005), which are used to control a wide range of insect pests (Ehlers & Shapiro-Ilan, 2005; Georgis *et al.*, 2006). In nature, as EPNs are obligate parasites of insects (Adams & Nguyen, 2002), they require the insect host to complete their life cycle. The mass production

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of EPNs is focused on the optimum production of DJ, which has been achieved using various environmental stresses, including food availability, overcrowding, ammonia production, desiccation, ammonia, pH, low nutrient content, and growth temperatures (Nguyen & Smart, 1992; Patel *et al.*, 1997; Ryder & Griffin, 2002; San-Blas *et al.*, 2008; Hirao & Ehlers, 2009a; Hirao & Ehlers, 2009b).

Although environmental parameters can influence the formation of DJs in both *C. elegans* and EPNs, it is not known what effect they are likely to have on *Phasmarhabditis* sp. SA4. Results from the study showed that DJ numbers were highest at 15°C, possibly due to the stress induced from the overcrowding of other nematode stages. Future work should focus on optimising the *in vitro* production of *Phasmarhabditis* sp. SA4 and *Kluyvera* sp., by means of manipulating the growth conditions.

Additional bacterial isolates should also be collected and tested for their ability to grow alongside *Phasmarhabditis* sp. SA4. The pathogenicity of new bacterial isolates should also be tested, both on invasive and native mollusc species, as well as on nontarget organisms. Doing so should determine whether a negative environmental impact can be expected with the inundative application of DJs to control slugs in canola fields. Such application would also be likely to have an effect on the future possibility of selling a locally produced product in other countries.

Once a suitable monoxenic culture protocol is established, and growth conditions have been optimised, production should be scaled up to mass production level, using large- scale fermenters. Formulations should then be tested to determine the rate of nematode survival, and the application rates should be established. In addition, the nematode should be assessed on various host types across various industries (oilseed, viticulture, citrus and deciduous fruit culture).

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CHAPTER 5

Conclusion

The Western Cape province of South Africa has a unique climate, with warm, damp winters that provide the ideal conditions in which European molluscs can thrive. One of the many crops in the Western Cape province that is affected by molluscs is canola, which is targeted by the slug species *Milax gagates* (Draparnaud, 1801), *Deroceras panormitanum* (Lessona & Pollonera, 1882), and *Deroceras reticulatum* (Müller, 1774). The most common mollusc control method that is currently used in canola is the surface broadcasting of molluscicide pellets containing metaldehyde and carbaryl. However, the chemicals concerned have been shown to cause adverse effects on nontarget organisms. Therefore, a control method is required that can be effective against molluscs, but which is non-harmful to both the environment and to nontarget organisms. Iron phosphate pellets are also effective at controlling slugs and could be used in conjunction with a biological control species. The mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrássy, 1983, has been developed into a highly successful, commercially available biological molluscicide that is known as Nemaslug®. Although the product is currently sold in fifteen different European countries, based on its natural distribution, the nematode has recently been isolated outside of Europe, opening up potential new markets for the product. To date, the nematode has not yet been isolated in South Africa, and, for the reason mentioned, its importation is prohibited under the amended Act 18 of 1989, which falls under the Agricultural Pests Act (No. 36 of 1947). Therefore, the objectives of the current study were to conduct a survey of slugs so as to isolate the local nematode isolates, to determine their mollusc control capabilities, to describe their morphology, biology and phylogeny, and to establish monoxenic cultures, with optimum growth temperatures. The results that have been obtained in response to the objectives have been analysed in each chapter, with the current conclusion serving to highlight the most important findings of the study, and to point out areas requiring future research.

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The first objective involved a survey of parasitic nematodes associated with slugs in the Western Cape province. Slugs were investigated as opposed to snails, as slugs have been identified as being the main pests of canola crops, and with the absence of an external shell meaning that they can burrow deep into the soil, thus increasing their exposure to nematode parasites. Sample sites focused on canola crops and commercial nurseries. A total of six nematode species were identified, including *Angiostoma margaretae* Ross, Malan & Ivanova, 2011; *Angiostoma* sp.; *Caenorhabditis elegans* Maupas, 1900; mermithid sp., *Phasmarhabditis* sp. SA3; and *Phasmarhabditis* sp. SA4. Of these species, four were previously undescribed (*Angiostoma* sp., mermithid sp., *Phasmarhabditis* sp. SA3, and *Phasmarhabditis* sp. SA4). The discovery of these undescribed nematode species indicates that South Africa is rich in biological diversity, and that additional surveys are required to increase the understanding of nematodes that are associated with molluscs in Africa.

Nematodes isolated in the survey were tested for their ability to reproduce on decaying organic matter (consisting of dead frozen slugs), with the results showing that one of the nematodes, *Phasmarhabditis* sp. SA4, could complete its life cycle under such conditions. In addition, preliminary pathogenicity tests demonstrated that *Phasmarhabditis* sp. SA4 caused significant mortality in *D. panormitanum* specimens. Therefore, future work in the area involved should focus on investigating the pathogenic effect of *Phasmarhabditis* sp. SA4 on both invasive and native mollusc species, as well as on nontarget organisms.

The third chapter was focused on the description of *Phasmarhabditis* sp. SA4, which was isolated from the slug host *D. reticulatum*, collected from a nursery in George, South Africa. *Phasmarhabditis* sp. SA4 is characterised by the shape and length of the female tail, and by the presence of males. However, identification within the genus is difficult, as males are rare, and the *Phasmarhabditis* species are morphologically conservative. Therefore, molecular identification and phylogenetic analysis play a key role in the identification of these species. Phylogenetic analysis demonstrated that *Phasmarhabditis* sp. SA4 was placed in a

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monophyletic clade along with *Phasmarhabditis* sp. SA2, *Phasmarhabditis papillosa* (Schneider, 1866) Andrássy, 1983, and the mollusc-parasitic nematode, *Angiostoma dentiferum* (Mengert, 1953). The results varied compared to the morphological analysis, which suggested that the nematode was closest to *Phasmarhabditis californica* Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine & De Ley, 2016. The new species brings the total complement of the genus to seven species.

Future work in the area concerned should focus on sampling for additional *Phasmarhabditis* species in South Africa. Sampling protocols could be improved by means of adopting a new published method, in terms of which slugs are field-collected and decapitated, and then incubated for one week before being examined for nematodes. The use of such a method would be less labour-intensive than dissections, however information regarding the infection sites of the nematode in the slug, as well as regarding the presence of other parasitic nematodes, would not be detected.

The final chapter focused on establishing monoxenic cultures of *Phasmarhabditis* sp. SA4, using methods described for the *in vitro* production of *P. hermaphrodita.* Monoxenic cultures are known to offer a relatively predictable result, and to help to create high numbers of dauer juveniles with consistent infectivity. In the chapter concerned, bacterial isolates were obtained by means of swabbing the intestines and internal cavities of ten *D. reticulatum* specimens collected from George. The pathogenic effects of the bacteria were tested by means of injecting them directly into the haemocoel of *D. reticulatum* hosts, and then monitoring the mortality. Five bacterial isolates were identified using molecular characterisation using the 16S rRNA (*Flavobacterium* sp., *Shewanella* sp., *Pseudomonas* sp., *Kluyvera* sp., and *Acinetobacter* sp.). These bacterial isolates have not previously been found associating with slugs. *Kluyvera* sp., which had the highest mortality rate was chosen for the monoxenic cultures. The monoxenic cultures were then optimised, using growth temperatures. Three different temperatures were tested, being 15°C, 20°C, and 25°C. The total number of nematodes and the number and

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proportion of dauer juveniles were recorded every 5 days for 20 days. The results showed that dauer juvenile numbers were highest at 15°C. The pathogenicity of the *in vitro* nematodes was then tested, with the results showing that *Phasmarhabditis* sp. SA4 grown in monoxenic cultures with *Kluyvera* sp. was found to cause significant mortality in *D. panormitanum.*

Future work should focus on optimising the *in vitro* production of *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. by means of manipulating the growth conditions concerned, such as extreme temperatures, overcrowding, inoculum density, alteration of the growth medium, and variation of the pH. Additional bacterial isolates should also be collected and tested for their ability to grow along with *Phasmarhabditis* sp. SA4. The pathogenicity of new bacterial isolates should also be tested, both on invasive and native mollusc species, as well as on nontarget organisms.

Once the *in vitro* production of *Phasmarhabditis* sp. SA4 and *Kluyvera* sp. and the growth conditions have been optimised, the production should be scaled up to mass production level, using fermenters. Formulations should then be tested to determine nematode survival, with application rates also being established. The nematode should then be assessed on various crop types, using various application methods.