

***In vitro* production and biocontrol potential of nematodes associated with molluscs**

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*Dissertation presented for the degree of Doctor of Nematology in the
Faculty of AgriSciences at Stellenbosch University*



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March 2020

Declaration

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This dissertation includes one original paper published in a peer-reviewed journal. The development and writing of the paper was the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

March 2020

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Acknowledgements

First and foremost, I would like to thank my two supervisors, Prof Antoinette Malan and Dr Jenna Ross. This thesis would not have been possible without their help, patience and expertise. I am grateful for the opportunity to have been part of this novel work in South Africa.

I would like to thank Prof. Des Conlong for welcoming me at SASRI in KwaZulu-Natal and organizing slug collections with local growers, as well as Sheila Storey for helping me transport the slugs from KZN. I am thankful to all the people who allowed me to collect slugs from their properties and to colleagues who brought me specimens to help my research (Deidré Odendaal, Caro Kapp and Marinus Geldenhuys). Also, a special thanks to Mr Matthew Addison, Abdul Jacobs, Steffan Hansen, Dona van Eeden and Sonya Beukes for helping me with the difficult task of specimen collection. Thank you to Dr Louwrens Tiedt for always being available to take SEM photos and to Prof Daan Nel for helping me with the statistics of my research.

Thank you to Stellenbosch University, and in particular the Department of Conservation Ecology and Entomology, for the support and wonderful facilities that enabled me to conduct this research.

Lastly, I would like to thank everyone who supported me through my three years as a doctoral student, including Schalk Pienaar, Justine Rudman, Koot Pieterse Jr., Dona van Eeden and my loving parents Morag and Koot Pieterse.

Abstract

Growing concern around the risks associated with the use of some molluscicide chemicals has prompted research into alternative control methods for mollusc pests. One of the most successful biocontrol methods available in Europe is the use of the mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita*, which cannot be used or sold in South Africa under the terms of the Agricultural Pest Amendment Act, No. 18 of 1989. It is, therefore, necessary to isolate local mollusc-parasitic nematodes and to assess their biocontrol potential. Nematodes associated with slugs from the KwaZulu-Natal province of South Africa was surveyed. *Caenorhabditis elegans* was the only nematode isolated, and pathogenicity tests concluded that it was not capable of causing mortality to the slug species *Deroceras invadens*, even when associated with a pathogenic bacterium. During this survey, a number of endemic slug species were collected, including *Chlamydephorus gibbonsi*. A detailed description of *C. gibbonsi*'s feeding process is given. Following the quest to isolate other mollusc-nematodes across Africa, a collaborative project led to the isolation and description of *Phasmarhabditis* sp. (KEN1), a new nematode species from Kenya. *Phasmarhabditis* sp. (KEN1) can be characterised by the females having a conoid tail shape, the presence of males with a bursa bearing nine bilateral pairs of genital bursal papillae, as well as the infective juvenile (IJ) having the longest body length thus far recorded in the genus. The life cycles of five nematodes associated with molluscs were then studied and described. Based on the ease of mass-culturing, the species were ranked in the following order: *C. elegans*, *P. hermaphrodita* and *Phasmarhabditis bohémica*, with *Phasmarhabditis papillosa* and *Phasmarhabditis* sp. (KEN1) being equally difficult to mass-culture. The *in vitro* culturing of the nematodes *P. papillosa*, *C. elegans*, *Phasmarhabditis* sp. (KEN1) and *P. bohémica* was then studied using seven bacterial isolates from slugs and three associated with entomopathogenic nematodes. The results indicated that *Kluyvera* sp. would be a suitable bacterial candidate to support the growth of *C. elegans* and *Phasmarhabditis* sp. (KEN1), while *Pseudomonas* spp. would make suitable bacterial candidates for *P. bohémica* and *P. papillosa*. The nematode *P. bohémica* was then grown in association with the bacterial isolate *Pseudomonas* sp. (1), in liquid media cultures to test the effects of bacterial inoculum density and initial IJ inoculum density on the total nematode yield, the IJ yield and the IJ proportion in the cultures. The results showed that 1% bacteria inoculum and a higher initial inoculum concentration of 3000 IJs/ml led to higher total nematode and IJ yields. Lastly, a novel application method of metaldehyde was tested in an apple orchard on the snail *Cornu aspersum*. Baitchain, a new application method whereby bait pellets can be wrapped around the base of a tree was tested along with Sluggit, a metaldehyde bait pellet applied to the soil, to

compare their effectiveness in protecting apple trees from *C. aspersum*. All treatments caused a significant reduction in snail numbers after 28 days, and all treatments, except Sluggit applied at a concentration of 15 g/kg metaldehyde, caused significant mortality.

Opsomming

Groeiende kommer oor die risiko geassosieer met die gebruik van sommige chemiese slakdoders, het gelei tot soeke na alternatiewe beheermetodes vir slakspesies. Een van die mees suksesvolle biologiese beheer opsies is die gebruik van die slak-parasitiese nematode, *Phasmarhabditis hermaphrodita*, wat nie gebruik of verkoop kan word in Suid-Afrika nie. Dit is daarom belangrik dat plaaslike nematodes gevind word en hul biologiese beheer potensiaal ondersoek word. 'n Opname was gedoen van nematodes geassosieer met slakke van die KwaZulu-Natal provinsie van Suid-Afrika. *Caenorhabditis elegans* was die enigste nematode spesie wat geïsoleer is en toetse het bevestig dat dit nie mortaliteit kan veroorsaak in die slak spesie *Deroceras invadens* nie. Een van die slak spesies wat versamel was gedurende die opname was *Chlamydephorus gibbonsi*. Die slak is endemies aan oostelike Suid-Afrika, maar opnames het getoon dat die spesie besig is om na die Wes-Kaap te versprei. 'n Beskrywing van die proses waarin die slak voed op 'n *Amyntas* spesie erdwurm was volledig beskryf. *Phasmarhabditis* sp. (KEN1), 'n nuwe nematode spesie van Kenia, was ook beskryf en word uitgeken aan die keëlagtige stert van die wyfie, teenwoordige mannetjies met 'n bursa met nege bilaterale, gepaarde genitale bursale papille, asook 'n infektiewe larwe (IL) met die langste liggaamslengte tot dusver gevind in die genus. Die lewensiklusse van vyf nematode spesies geassosieer met slakke was toe bestudeer en beskryf. Gebasseer op die resultate, kan die spesies gelys word van maklik tot moeilik om te massa-produseer soos volg: *C. elegans*, *P. hermaphrodita* en *P. bohémica*, met *P. papillosa* en *Phasmarhabditis* sp. (KEN1) wat ewe moeilik sou wees om te massa-produseer as gevolg van hul stadige lewensiklus en hul voortplantingsmetode. Die *in vitro* produksie van die nematodes *P. papillosa*, *C. elegans*, *Phasmarhabditis* sp. (KEN1) en *P. bohémica* was ondersoek. Die resultate het getoon dat *Kluyvera* sp. 'n gepaste bakteriële kandidaat sou wees vir *C. elegans* en *Phasmarhabditis* sp. (KEN1), terwyl *Pseudomonas* spesies gepaste bakteriële kandidate sou wees vir *P. bohémica* en *P. papillosa*. Die nematode *P. bohémica* was vermeerder in vloeistof medium in assosiasie met die bakterieë *Pseudomonas* sp. (1), om die effek van bakteriële en die invektiewe larwe (IL) inokulum digtheid te toets op die totale nematode opbrengs, IL opbrengs en IL proporsie van die totale nematodes. Die resultate het getoon dat 1 % bakteriële inokulum en 'n hoër inokulum konsentrasie van 3000 ILs/ml gelei het tot hoër totale nematode en IL opbrengste. Laastens was 'n nuwe metode vir die aanwending van metaldehyd in 'n appelboord getoets. Baitchain, 'n produk wat bestaan uit slakpille gerangskik op 'n koord, is ontwerp om om die stam van 'n boom gebind te word. Die produk was getoets tesame met Sluggit, 'n metaldehyd slakpil wat op die grondoppervlak aangewend word, om te toets hoe effektief die behandelinge

is daarin om die appelbome te beskerm teen die dopslak, *Cornu aspersum*. Alle behandelinge het 'n beduidende afname veroorsaak in slak getalle na 28 dae en alle behandelinge, behalwe Sluggit teen 'n konsentrasie van 15 g/kg metaldehyd, het beduidende slakdood veroorsaak.

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Chapter 1

Literature Review

Nematode control options for molluscs in South Africa and the potential for their future mass production

Molluscs as crop pests

Terrestrial gastropod molluscs (slugs and snails) are one of the most diverse and successful animal groups in the terrestrial environment (Barker, 2004). Crop damage from molluscs has occurred throughout history, however twentieth-century agricultural practices, such as the cultivation of new crops, the intensification of farming systems and the transportation of plant material, has led to molluscs adapting to these modified habitats, thus becoming significant crop pests of tropical and temperate regions (Barker, 2002a). Populations of non-native terrestrial molluscs are continuously being found worldwide (Rowson *et al.*, 2014; Ross, 2019).

Slugs are generalist feeders, making them significant pests of a wide range of crops, including ornamentals, arable crops and horticultural crops (Barker, 2002a). In pastures, slugs not only feed on grasses, but also cause damage to clover, which leads to a reduction in nitrogen fixation (Barker, 2002b). *Deroceras reticulatum* Müller (Agriolimacidae), originally endemic to the Palaearctic, is considered the most economically pestiferous slug species and is widespread in temperate regions, including New Zealand, Europe, Australia, North and South America and Asia (Godan, 1979; South, 1992; Speiser *et al.*, 2001). However, many slug species are under-recorded and are, therefore, often only detected long after they have become established in their new environment (Rowson *et al.*, 2014). The spread of slugs and their eggs is usually caused inadvertently through the movement of plants, soil, vehicles, livestock, or other goods (Rowson *et al.*, 2016; Ross, 2019).

Many snail species have also achieved pest status worldwide. The giant African snail, *Achatina fulica* (Férussac) (Achatinidae), is a serious pest of gardens and crops in subtropical and tropical regions, capable of consuming 10% of its own body weight in a day (Schreurs, 1963). *Cornu aspersum* (Müller) (Helicidae) is a well-known, cosmopolitan pest that has been transported to most parts of the world (Guiller *et al.*, 2012; Peltanová *et al.*, 2012). *Theba*

pisana (Müller) (Helicidae) is a snail species that is mostly found in coastal regions and in agricultural crops, thriving in a wide range of habitats, from grasses through to shrubs and trees (Cowie *et al.*, 2008; Herbert, 2010). Although being native to the Mediterranean coasts of North Africa and Europe, it has since spread to South Africa, Australia, the United States (California), Asia and the Netherlands (Cowie *et al.*, 2008). The snail is known for its high reproductive rate and for its aggregative invasive behaviour, with one report of approximately 3,000 snails surrounding a single citrus tree (Mead, 1971). Snails from the Achatinidae family are not only considered crop pests, but they also pose a human health risk for the transmission of *Angiostrongylus cantonensis* (Chen) and *Angiostrongylus costaricensis* (Morera & Céspedes) (Rhabditida: Angiostrongylidae) (Carvalho *et al.*, 2003; Hollingsworth *et al.*, 2007; Ross 2019).

Molluscs in South Africa

In 2010, it was estimated that approximately 34 non-native terrestrial mollusc species were present in South Africa (Table 1.1). Of these species, 28 are believed to have become established in the country and 13 are regarded as invasive species. Twenty-nine of these species originated in Europe, with nine coming from the Mediterranean. The above is mainly a result of the European colonial history of South Africa, but it is also because of the Mediterranean-like, temperate winter rainfall climate of the Cape area (Herbert, 2010). Some of the most pestiferous introduced terrestrial molluscs in South Africa, although sometimes widely distributed, have fortunately not successfully spread into natural environments, with them being mostly restricted to transformed habitats and to monoculture crops. The snail, *T. pisana*, which was introduced to South Africa prior to 1881, is currently widespread between the West Coast and East London on the east coast of the country (Herbert, 2010). Due to the destruction that it causes, particularly in the grape-producing regions, it is classified as a pest in South Africa and Australia (Swart *et al.*, 1976; Sanderson & Sirgel, 2002). *Theba pisana* (Fig 1.1b) and *C. aspersum* (Fig. 1.1a) are found in South African vineyards, with infestations of up to 400 molluscs having been recorded on a single grapevine (Loubser, 1982). They feed on developing foliar buds and on the young leaves in spring, leading to stunted shoot growth and to decreased yield of the vines. In extreme *C. aspersum* infestations, crop losses of up to 25% have been reported (Sanderson & Sirgel, 2002). *Cornu aspersum* is also the only alien species that has been recorded in all nine provinces of South Africa, with it thriving in almost all areas of human settlement (Herbert, 2010). *Eobania vermiculata* (Müller) (Helicidae) is also an

invasive snail species that is found throughout South Africa, with it having spread to relatively undisturbed habitats (Herbert, 2010; Van Elden *et al.*, 2015).

Table 1.1. Alien terrestrial molluscs in South Africa with their date of introduction and status.

(Adapted from Herbert, 2010).

Species	Introduction	Status
<i>Achatina fulica</i>	pre 1900	Not established
<i>Aegopinella nitidula</i> (Draparnaud)	pre 1930	Uncertain
<i>Ambigolimax valentianus</i> (Férussac)	pre 1961	Established, largely synanthropic
<i>Arion hortensis</i> aggr.	pre 1939	Established, locally invasive
<i>A. intermedius</i> (Normand)	pre 1898	Established, locally invasive
<i>Bradybaena similaris</i> (Férussac)	pre 1858	Established, synanthropic
<i>Cochlicella barbara</i> (Linnaeus)	pre 1909	Established, invasive
<i>C. cf. lubrica</i> (Müller)	pre 1978	Established, synanthropic
<i>C. cf. lubricella</i> (Rossmässler) Anderson	pre 1965	Established, synanthropic
<i>Cornu aspersum</i>	pre 1855	Established, largely synanthropic
<i>Deroceras laeve</i> (Müller)	pre 1898	Established, invasive
<i>D. panormitanum</i> (Lessona & Pollonera)	pre 1963	Established, invasive
<i>D. reticulatum</i>	pre 1898	Established, invasive
<i>Discus rotundatus</i> (Müller)	pre 1986	Established, locally invasive
<i>Eobania vermiculata</i> (Müller)	pre 1987	Established, largely synanthropic
<i>Hawaiiia miniscula</i> (Binney)	pre 2006	Established, synanthropic
<i>Lauria cylindracea</i> (da Costa)	pre 1879	Established, locally invasive
<i>Lehmannia nyctelia</i> (Bourguignat)	pre 1939	Established, largely synanthropic
<i>Limacus flavus</i> (Linnaeus)	pre 1898	Established, synanthropic
<i>Limax maximus</i> Linnaeus	pre 1898	Established, locally invasive
<i>Milax gagates</i> (Draparnaud)	pre 1873	Established, synanthropic
<i>Otala punctata</i> (Müller)	pre 1986	Eradicated
<i>Oxychilus alliarius</i> (Miller)	pre 1894	Established, largely synanthropic
<i>O. cellarius</i> (Müller)	pre 1846	Established, largely synanthropic
<i>O. draparnaudi</i> (Beck)	pre 1908	Established, locally invasive
<i>Rumina decollata</i> (Linnaeus)	pre 1897	Eradicated
<i>Subulina octona</i> (Bruguière)	pre 1913	Probably not established in SA
<i>Testacella maugei</i> Férussac	pre 1893	Established, synanthropic
<i>Theba pisana</i>	pre 1881	Established, invasive
<i>Vallonia costata</i> (Müller)	pre 1980	Established, synanthropic
<i>V. pulchella</i> (Müller)	pre 1846	Established, synanthropic
<i>Vitrea contracta</i> (Westerlund)	pre 2004	Established, locally invasive
<i>V. crystallina</i> (Müller)	pre 1898	Uncertain
<i>Zonitoides arboreus</i> (Say)	pre 1898	Established, locally invasive

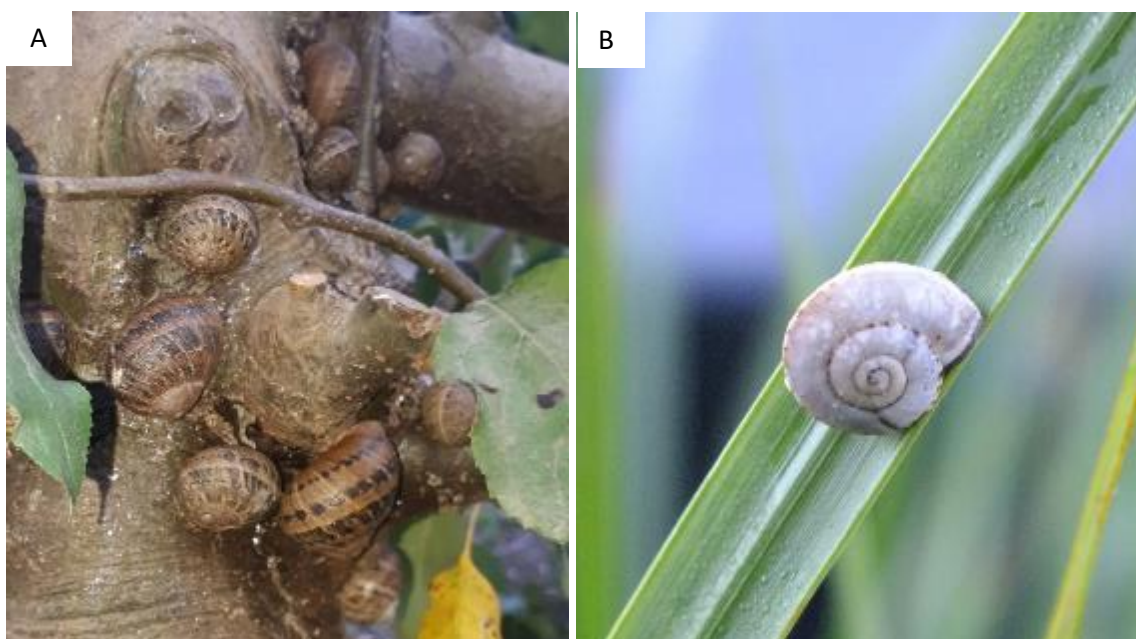


Figure 1.1. A: *Cornu aspersum* clustering on an apple tree; and B: a young *Theba pisana*.

Some slug species, like those of *Deroceras*, have spread further than transformed habitats, and even into relatively pristine environments. *Deroceras reticulatum*, *Deroceras laeve* (Müller) and *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Agriolimacidae) have all been recorded from many different parts of South Africa (Herbert, 2010). The grey garden slug, *D. reticulatum*, which is a serious pest of agriculture, horticulture and gardens, is a non-specific herbivore that is capable of laying up to 500 eggs in one year (South, 1992; Herbert, 2010). *Deroceras laeve* has been recorded as a pest in ornamental crops, vegetables, legumes, pasture and maize, with it being a possible vector of human angiostrongyliasis, while *Deroceras invadens* is considered as a pest in greenhouses, pasture, commercial crops, suburban gardens and nurseries (Altena & Smith, 1975; Barker, 1999, 2002b; Hollingsworth *et al.*, 2007). The black keeled slug, *Milax gagates* (Draparnaud) (Milacidae), is believed to be indigenous to the Mediterranean, but it has been recorded in the Western and Eastern Cape and in KwaZulu-Natal (Wiktor, 1987; Herbert, 2010). It is a known pest of native plants, pasture and arable land in various parts of the world (Cowie, 1997; Barker, 2002b), with it having been reported as an episodic pest in South African barley, wheat, canola (Tribe & Lubbe, 2010) and lucerne (W. Sirgel pers. comm.). The slug *Urocyclus flavescens* Kerferstein (Urocyclidae) was first classified as a pest in Mpumalanga in 1967 (De Villiers, 1973), but it has, since, become known in all the banana-producing regions of South Africa (De Jager & Daneel, 2002).

Current control

CHEMICAL CONTROL

Terrestrial mollusc pests are primarily controlled through the use of chemical molluscicide pellets (Fig. 1.2). The pellets consist mainly of wheat, bran or barley flour, which serves as an attractant, as well as 2-8% of an active ingredient or toxicant, usually metaldehyde, methiocarb or thiodicarb (Bailey, 2002). The chemicals are, however, harmful to certain non-target organisms, such as earthworms, soil arthropods and carabid beetles (Martin & Forrest, 1969; Homeida & Cooke, 1982; Bieri *et al.*, 1989; Kennedy, 1990; Fletcher *et al.*, 1991; Fletcher *et al.*, 1993; Cardoso *et al.*, 2015), and, when overused, can accumulate in the environment, or possibly lead to resistance (Fisher & Orth, 1975; Castle *et al.*, 2017). Increased pressure has been exerted by the regulatory sector and by water suppliers to limit the agricultural use of metaldehyde, due to concerns about its presence in water bodies and drinking water (Castle *et al.*, 2017; Ross, 2019).



Figure 1.2. Molluscicide pellets sold at a nursery.

Iron (Ferric) phosphate has been suggested as a preferred alternative to metaldehyde, with it being suitable for organic systems, although it is perceived to cost more per kilogram (AHDB, 2017; Castle *et al.*, 2017). Initiatives in the United Kingdom have tried to overcome the cost issue, by bridging the price gap between metaldehyde and Iron (Ferric phosphate) (Castle *et al.*, 2017). However, research suggests that high doses of Iron (Ferric) phosphate have negative effects on the earthworm, *Lumbricus terrestris* Linnaeus (Opisthopora: Lumbricidae) (Langan & Shaw, 2006). A study by Edwards *et al.* (2009) also found that

molluscicides that contain Iron (Ferric) phosphate, or the chelating agents ethylenediaminetetraacetic acid (EDTA) or ethylene diamine succinic acid (EDDS), might be hazardous to domestic animals, earthworms and humans and require further investigation (Edwards *et al.*, 2009).

CULTURAL CONTROL AND AGRONOMIC PRACTICES

Conventional cultural control practices include proper soil cultivation, the minimisation of weeds and the establishment of beetle banks (Barker, 2002a; Ross, 2019). The quality and preparation of the seedbed is important for the control of slug populations in crops (Ross, 2019). Ploughing is effective in reducing slug populations, with even the minimum amount of tillage leading to a reduction in the amount of damage caused by pest slugs. By minimising the amount of weed growth in crops, the food and shelter of the slugs is reduced, leading to decreased slug populations. Certain species of carabid beetles are known predators of slugs. However, in conservation agriculture, as proposed by the FAO (2008), minimum soil disturbance, or no till, is one of the main driving factors to promote agricultural soil health, although it might lead to an increase in slug and snail problems.

PHYSICAL BARRIERS

The use of physical barriers is an alternative control option. Electrified fencing on copper barriers is an effective way of deterring terrestrial slugs without killing them, but, because of the high costs involved, the method is more suited to small-scale use (Laznik *et al.*, 2011). Another possible deterrent is the use of antifeedants, in the form of secondary metabolites in such plant tissues as alcohols, alkaloids and aldehydes (Dodds, 1996).

BIOLOGICAL CONTROL

Terrestrial molluscs have many natural enemies, including certain species of birds, mammals, centipedes, millipedes, insects, flatworms, other gastropods, mites, reptiles, spiders, nematodes, ciliophorans, and microsporidia, as well as some bacterial and non-bacterial diseases (Barker, 2004). In South Africa, some vine growers make use of the novel strategy of deploying ducks as a method of biological control over helcid gastropods (Sanderson & Sirgel, 2002; Ross, 2019). The use of predatory gastropods to control the species has also been attempted, with low success rates, however (Sanderson & Sirgel, 2002).

To date, the most successful method of biological control across Europe, has been the use of the mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider) Andr ssy. Other nematode families that have been reported as being parasitic to molluscs include Mermithidae, Diplogasteridae, Rhabditidae, Alloionematidae, Angiostomatidae, Agfidae, Alaninematidae and Cosmocercidae (Barker, 2004; Pieterse *et al.*, 2017a).

The *Phasmarhabditis* Andr ssy (Rhabditida: Rhabditidae) genus currently contains 13 nominal species, with a rise in species descriptions in the past decade (Fig. 1.3): *P. hermaphrodita* (Hooper *et al.*, 1999), *Phasmarhabditis neopapillosa* (Mengert *in* Osche) Andr ssy (Hooper *et al.*, 1999), *Phasmarhabditis papillosa* (Schneider) Andr ssy (Tandingan De Ley *et al.*, 2016), *Phasmarhabditis tawfiki* Azzam (Azzam, 2003), *Phasmarhabditis huizhouensis* Huang, Ye, Ren & Zhao (Huang *et al.*, 2015), *Phasmarhabditis apuliae* Nermut', P  a & Mr  ek (Nermut' *et al.*, 2016), *Phasmarhabditis bonaquaense* Nermut', P  a, Mekete & Mr  ek (Nermut' *et al.*, 2017a), *Phasmarhabditis californica* Tandingan De Ley, Holovachov, McDonnell, Bert, Paine & De Ley (Tandingan De Ley *et al.*, 2016), *Phasmarhabditis bohémica* Nermut', P  a, Mekete & Mr  ek (Nermut' *et al.*, 2017b), *Phasmarhabditis meridionalis* Ivanova & Spiridinov (Ivanova & Spiridinov, 2017), *Phasmarhabditis safricana* Ross, Pieterse, Malan & Ivanova (Ross *et al.*, 2018), *Phasmarhabditis circassica* Ivanova, Geraskina & Spiridinov (Ivanova *et al.*, 2019), and *Phasmarhabditis clausiliiae* Ivanova, Geraskina & Spiridinov (Ivanova *et al.*, 2019). They are facultative mollusc-parasitic, soil nematodes, with the exception of *P. huizhouensis* from rotten leaves, found on all continents, except for Antarctica (Huang *et al.*, 2015).

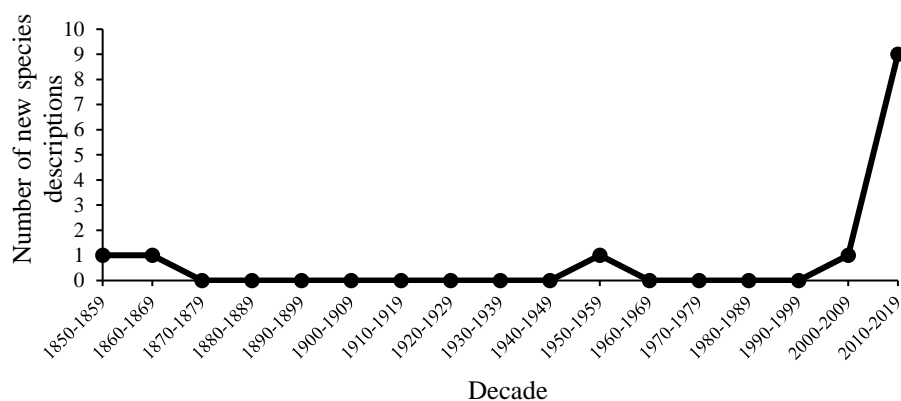


Figure 1.3. The number of new *Phasmarhabditis* species that have been described every decade since the first description of *Phasmarhabditis hermaphrodita* in 1859, until September 2019.

Phasmarhabditis hermaphrodita

Phasmarhabditis hermaphrodita is a facultative parasite that can reproduce either on rich organic material, such as slug faeces (Grewal *et al.*, 2003), or form parasitic or necromenic symbioses with certain mollusc species (Maupas, 1900; Mengert, 1953; Wilson *et al.*, 1993a; Grewal *et al.*, 2003).

Phasmarhabditis hermaphrodita was first isolated in Germany in 1859, from decaying terrestrial molluscs, and described as *Pelodytes hermaphroditus* Schneider (Schneider, 1859). Maupas (1900) later found the infective larvae of *P. hermaphrodita* in the intestine of the slug *Arion ater* (Linnaeus) in Normandy and called it *Rhabditis caussaneli*. Not considering the nematode to be a parasite, the author believed that it remained inside the slug as an infective larva until the slug died, after which it would feed and reproduce on the cadaver. In 1987, during a search for biological control agents for slugs in the United Kingdom, *P. hermaphrodita* was found actively reproducing in the slug *D. reticulatum*. Further research concluded that *P. hermaphrodita* was capable of infecting and killing a number of terrestrial mollusc species (Wilson *et al.*, 1993a).

The terrestrial mollusc families, Agriolimacidae, Arionidae, Milacidae, Limacidae and Vagnulidae (Wilson *et al.*, 1993a; Iglesias & Speiser, 2001; Speiser *et al.*, 2001; Grewal *et al.*, 2003) (Table 1.2), are susceptible to infection by *P. hermaphrodita*, which causes mortality in the aforementioned families. As it is a mollusc-specific parasite, studies have proven that *P. hermaphrodita* is not lethal to the earthworm, *Eisenia fetida* (Savigny) (Haplotaxida: Lumbricidae), or to the insect species *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), *Zophobas morio* Fabricius (Coleoptera: Tenebrionidae) and *Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae) (Wilson *et al.*, 1993b, 1994a; DeNardo *et al.*, 2004; Rae *et al.*, 2005).

Under laboratory conditions, the infective juveniles (IJs) of *P. hermaphrodita* invade *D. reticulatum* within 8-16 h of exposure (Tan & Grewal, 2001). The IJs enter the slug through a dorsal integumental pouch posterior to the mantle and move into the shell cavity, where the IJs develop into self-fertilizing hermaphrodites, and reproduce. Depending on the temperature and nematode density, susceptible hosts soon stop feeding and die within 4-21 days of infection. Once the host slug dies, the nematodes reproduce and feed on the cadaver, until food sources become scarce, after which IJs are formed, which move back into the soil in search of new slug hosts (Wilson *et al.*, 1993a; Tan & Grewal, 2001). *Phasmarhabditis hermaphrodita* is currently

being mass-produced and sold commercially throughout Europe by BASF (formally Becker Underwood) and Dudutech, under the tradenames, Nemaslug[®] and SlugTech[®], respectively (Rae *et al.*, 2007; Ross, 2019).

The techniques for the *in vitro* production of *P. hermaphrodita* are similar to those used for the commercial production of entomopathogenic nematodes (EPNs) (Morand *et al.*, 2004). In order to produce high yields of IJs consistently and to eliminate the risk of other pathogenic bacteria in the medium or product, it is necessary to form a monoxenic nematode-bacteria combination that can be grown in liquid medium (Poinar & Hansen, 1986; Wilson *et al.*, 1995a). In BASF's system, the nematodes are mass-produced in liquid medium in a monoxenic association with the bacteria species *Moraxella osloensis* (Bøvre & Henriksen) Bøvre (Pseudomonadales: Moraxellaceae) in large-scale fermenters (Rae *et al.*, 2007). After undergoing several generations, IJs are formed, which are then harvested to be sold. The IJ larvae yield depends on the growth medium and conditions in the fermenters, with yields of more than 100,000 IJs ml⁻¹ having been achieved (Glen *et al.*, 1994). The IJs are then harvested through centrifugation, and repeatedly washed with water (Young *et al.*, 2002). Once harvested, the nematodes are mixed with an inert carrier, of which the water content has been modified to a level where it causes the nematodes to become dehydrated and immobile, with them then being stored in trays sealed with polythene (Glen *et al.*, 1994). The IJs can survive in the carrier for as long as 6 months (Grewal, 2001).

Table 1.2. The susceptibility of mollusc species to *Phasmarhabditis hermaphrodita*.

Species	Susceptible	Reference
<u>Snails</u>		
Achatinidae		
<i>Achatina fulica</i> Bowdich	No	Williams & Rae, 2015
Clausiliidae		
<i>Clausilia bidentata</i> (Strøm)	No	Wilson <i>et al.</i> , 2000
Endodontidae		
<i>Discus rotundatus</i> (Müller)	No	Wilson <i>et al.</i> , 2000
Helicidae		
<i>Cepaea hortensis</i> (Müller)	Yes	Wilson <i>et al.</i> , 2000
<i>Cepaea nemoralis</i> (Linnaeus)	No	Wilson <i>et al.</i> , 2000
<i>Cerņuella virgata</i> (Da Costa)	Yes	Coupland, 1995
<i>Cochlicella acuta</i> (Müller)	Yes	Coupland, 1995
<i>Cornu aspersum</i> (Müller)	Juveniles only	Glen <i>et al.</i> , 1996
<i>Monacha cantiana</i> (Montagu)	Yes	Wilson <i>et al.</i> , 2000
<i>Theba pisana</i> (Müller)	Yes	Coupland, 1995
Lymnaeidae		
<i>Lymnaea stagnalis</i> (Linnaeus)	Yes	Wilson <i>et al.</i> , 1993c; Morley & Morrit, 2006
Physidae		
<i>Physa fontinalis</i> (Linnaeus)	No	Morley & Morrit, 2006
Pomatiasidae		
<i>Pomatias elegans</i> (Müller)	No	Wilson <i>et al.</i> , 2000
Zonitidae		
<i>Oxychilus helveticus</i> (Blum)	No	Wilson <i>et al.</i> , 2000
<u>Slugs</u>		
Arionidae		
<i>Arion ater</i> (Linnaeus)	Juveniles only	Wilson <i>et al.</i> , 1993a
<i>Arion distinctus</i> Mabile	Yes	Wilson <i>et al.</i> , 1993a; Iglesias & Speiser, 2001
<i>Arion hortensis</i> Férussac	No	Grewal <i>et al.</i> , 2003
<i>Arion intermedius</i> Normand	Yes	Wilson <i>et al.</i> , 1993a
<i>Arion lusitanicus</i> Mabile	Juveniles only	Speiser <i>et al.</i> , 2001; Grimm, 2002;
<i>Arion silvaticus</i> Lohmander	Yes	Wilson <i>et al.</i> , 1993a
<i>Arion subfuscus</i> Draparnaud	No	Grewal <i>et al.</i> , 2003
Limacidae		
<i>Deroceras laeve</i> (Müller)	Yes	Grewal <i>et al.</i> , 2003
<i>Deroceras invadens</i> (Reise, Hutchinson Schunack & Schlitt)	Yes	Wilson <i>et al.</i> , 1993a
<i>Deroceras reticulatum</i> (Müller)	Yes	Wilson <i>et al.</i> , 1993a
<i>Limax maximus</i> (Linnaeus)	No	Grewal <i>et al.</i> , 2003
Milacidae		
<i>Tandonia budapestensis</i> (Hazay)	Yes	Wilson <i>et al.</i> , 1993a
<i>Tandonia sowerbyi</i> (Férussac)	Yes	Wilson <i>et al.</i> , 1993a
Vagnulidae		
<i>Leidyula floridana</i> (Leidy)	Yes	Grewal <i>et al.</i> , 2003

Commercial products with *P. hermaphrodita* are available for use in the garden market as packages of 12 million or 30 million nematodes, or for commercial use in packs with 30 million or 250 million nematodes (Rae *et al.*, 2007) (Fig. 1.3). The product is applied by suspending it in water and by spraying it onto the moist soil with a watering can or hydraulic spraying equipment, at a recommended application rate of 3×10^9 IJs ha⁻¹ (Glen *et al.*, 1994).



Figure 1.4. *Phasmarhabditis hermaphrodita* in its commercial biocontrol product form from BASF under the tradename Nemaslug®.

Phasmarhabditis hermaphrodita has been successfully used in oilseed rape (Wilson *et al.*, 1995b; Speiser & Andermatt, 1996), winter wheat (Wilson *et al.*, 1994b, 1994c), Brussel sprouts (Ester *et al.*, 2003a), strawberries (Glen *et al.*, 2000), asparagus (Ester *et al.*, 2003b), cabbages (Grubisis *et al.*, 2003), hostas (Grewal *et al.*, 2001) and glasshouse orchids (Ester *et al.*, 2003c) to reduce slug damage. However, *P. hermaphrodita* has been reported as not providing protection against slug damage, but the ineffectiveness of the product might be due to the application methods employed, with it requiring further investigation (Wilson *et al.*, 1995b; Glen *et al.*, 2000; Iglesias *et al.*, 2001; Tan & Grewal, 2001; Iglesias *et al.*, 2003). Nevertheless, *P. hermaphrodita* is widely used in Europe, and by 2005, it was estimated that approximately 500 ha was being treated with *P. hermaphrodita* (Rae *et al.*, 2007).

Phasmarhabditis hermaphrodita has, to date, not yet been isolated in South African slugs, meaning that, due to laws prohibiting the introduction of exotic animals into the country, it can neither be imported into, nor be used in, South Africa.

Other mollusc-parasitic nematodes in South Africa

Two surveys of nematodes associated with terrestrial molluscs have previously been conducted in the Western Cape province of South Africa, with four phasmarhabditids thus far found in South Africa (Table 1.3) (Ross *et al.*, 2012; Pieterse *et al.*, 2017b). A total of 13 different nematode species were identified from slug hosts in the Western Cape, ten of which were previously undescribed (Ross *et al.*, 2012; Pieterse *et al.*, 2017b). The nematodes isolated in the first survey included *Agfa flexilis* Dujardin, *Caenorhabditis elegans* (Maupas), *Panagrolaimus* sp., *Rhabditis* sp., *Phasmarhabditis* sp. (SA1), *Phasmarhabditis safricana* Ross, Pieterse, Malan & Ivanova and *Angiostoma margaretae* Ross, Malan & Ivanova, with the latter two species described as a result of this particular survey (Ross *et al.*, 2011, 2012, 2018). The nematode species found in the second survey included *A. flexilis*, *A. margaretae*, *Angiostoma* sp. (SA1), *C. elegans*, a mermithid (SA1), *Phasmarhabditis* sp. (SA3) and *Phasmarhabditis papillosa* Schneider (Andrássy)(Pieterse *et al.*, 2017b,c). This was the first record of *P. papillosa* in South Africa, with previous surveys reporting the nematode in Germany and the USA (Mengert, 1953; Tandingan De Ley, 2016). The mollusc-nematode fauna in South Africa is relatively understudied, with previous work focusing on the Western Cape. Therefore, it is important that surveys focus on other provinces in South Africa, such as KwaZulu-Natal where the weather is warm and humid, thus optimal for molluscs, as well as across the entire African continent. This is important for the discovery of potential candidates and the subsequent development of a biological control agent of molluscs in South Africa (Ross *et al.*, 2012; Pieterse *et al.*, 2017b).

Table 1.3. Slug-nematode associations found in previous surveys in South Africa.

Slug species	Native/ Introduced	Nematode species	Reference
<i>Ariopelta capensis</i> Krauss	Native	<i>Rhabditis</i> sp.	(Ross <i>et al.</i> , 2012)
<i>Ariostalis nebulosa</i> Sirgel	Native	<i>Phasmarhabditis</i> sp. SA1	(Ross <i>et al.</i> , 2012)
<i>Chlamydephorus gibbonsi</i> Binney	Native	-	(Pieterse <i>et al.</i> , 2017b; Ross <i>et al.</i> , 2012)
<i>Deroceras panormitanum</i> (Lesson & Pollonera)	Introduced	<i>Angiostoma margaretae</i> Ross, Malan & Ivanova	(Ross <i>et al.</i> , 2011)
		<i>Angiostoma</i> sp. (SA1)	(Pieterse <i>et al.</i> , 2017b)
		<i>Angiostoma</i> spp.	(Pieterse <i>et al.</i> , 2017b)
		<i>Caenorhabditis elegans</i> (Maupas)	(Pieterse <i>et al.</i> , 2017b)
		<i>Mermithid</i> sp.	(Pieterse <i>et al.</i> , 2017b)
		<i>Phasmarhabditis</i> sp. (SA3)	(Pieterse <i>et al.</i> , 2017b)
		-	(Ross <i>et al.</i> , 2012)
<i>Deroceras reticulatum</i> (Müller)	Introduced	<i>A. margaretae</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>C. elegans</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>Phasmarhabditis safricana</i>	(Ross <i>et al.</i> , 2012, 2018)
		<i>Phasmarhabditis papillosa</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>Rhabditis</i> sp.	(Ross <i>et al.</i> , 2012)
<i>Laevicaulis alte</i> (Férussac)	Native	-	(Ross <i>et al.</i> , 2012)
<i>Ambigolimax valentianus</i> (Férussac)	Introduced	<i>Agfa flexilis</i> Dujardin	(Pieterse <i>et al.</i> , 2017b; Ross <i>et al.</i> , 2012)
		<i>A. margaretae</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>C. elegans</i>	(Ross <i>et al.</i> , 2012; Pieterse <i>et al.</i> , 2017b)
		<i>Panagrolaimus</i> sp.	(Ross <i>et al.</i> , 2012)
<i>Limax flavus</i> Linnaeus	Introduced	<i>A. flexilis</i>	(Ross <i>et al.</i> , 2012)
		<i>Angiostoma</i> spp.	(Pieterse <i>et al.</i> , 2017b)
		<i>C. elegans</i>	(Pieterse <i>et al.</i> , 2017b; Ross <i>et al.</i> , 2012)
		<i>Panagrolaimus</i> sp.	(Ross <i>et al.</i> , 2012)
<i>Milax gagates</i> Draparnaud	Introduced	<i>A. margaretae</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>Angiostoma</i> sp.	(Ross <i>et al.</i> , 2012)
		<i>Angiostoma</i> sp. (SA1)	(Pieterse <i>et al.</i> , 2017b)
		<i>C. elegans</i>	(Pieterse <i>et al.</i> , 2017b)
		<i>Panagrolaimus</i> sp.	(Ross <i>et al.</i> , 2012)
<i>Oopelta flavescens</i> Collinge	Native	-	(Pieterse <i>et al.</i> , 2017b)
<i>Oopelta granulosa</i> Collinge	Native	-	(Ross <i>et al.</i> , 2012)
<i>Oopelta polypunctata</i> Collinge	Native	<i>A. margaretae</i>	(Pieterse <i>et al.</i> , 2017b)
		-	(Ross <i>et al.</i> , 2012)
<i>Testacella maugei</i> Férussac	Introduced	-	(Ross <i>et al.</i> , 2012)

Entomopathogenic nematodes

It is possible to expand the culturing knowledge of *P. hermaphrodita* by reviewing the work done on EPNs (Morand *et al.*, 2004). EPNs are used for the biological control of insect pests, and their desirability as biocontrol agents is based on their virulence and ease of culture (Gaugler & Han, 2002). The most commonly studied species, which are members of the genera *Heterorhabditis* and *Steinernema*, are used to control various insect pests (Grewal *et al.*, 2005). The insects are killed as a result of the interaction between the nematodes and their symbiotic bacteria (*Xenorhabdus* spp. for *Steinernema* and *Photorhabdus* spp. for *Heterorhabditis*), with which they have an obligate mutualism (Poinar & Thomas, 1966; Poinar *et al.*, 1977). Without the symbiotic bacteria, EPNs are ineffective as biocontrol agents, and their reproduction is negatively affected (Poinar & Thomas, 1966; Poinar *et al.*, 1977; Han & Ehlers, 1998). The bacteria serves as a substrate for the nematodes to reproduce and grow, while also producing broad-spectrum antimicrobial agents that prevent the colonisation of other micro-organisms (Frost & Clarke, 2002). EPNs assist in suppressing the immune system of the host, and they serve as a vector for the bacteria, which they transport into the haemocoel of the insect, resulting in the death of the insect within 24-48 h of infection. The EPNs then feed on the bacteria concerned, as well as on the bioconverted insect tissue, completing one to three generations inside the host, before exiting and searching for a new host (Strauch & Ehlers, 1998; Lewis & Clarke, 2012; Stock, 2015). To date, over 116 species of EPNs have been identified worldwide, with 11 species having been developed for commercialisation (Kaya and Koppenhofer, 1999).

MASS PRODUCTION OF EPNS

EPNs are mass produced using *in vivo*, *in vitro* solid or *in vitro* liquid culturing methods (Ehlers & Shapiro-Ilan, 2005). The chosen production method depends on the resources, expertise, cost, time and the amount of product required (El-Sadawy, 2011). Culturing using *in vivo* or *in vitro* solid methods is usually preferred by smaller organisations as it requires less capital investment. *In vitro* liquid culturing is mostly the method of choice for large companies in the developed countries, such as BASF, as it involves a high level of capital investment and running costs, sophisticated engineering and the close monitoring of processes (Ehlers & Shapiro-Ilan, 2005; Lacey & Georgis, 2012).

In vivo

The simplest method for the production of EPNs is through *in vivo* culturing using living insects. The larvae of the greater wax moth, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae), and mealworms, *T. molitor*, are both very susceptible to EPN infection and contain ample nutrients for the production of EPNs (Půža *et al.*, 2016). The larvae of the wax moth are generally used for the rearing of EPNs, with yields of between 0.5×10^5 and 4×10^5 IJs per larva being achieved (Van Zyl & Malan, 2015; Devi & Nath, 2017). This *in vivo* production method, despite being affordable and simple, is labour-intensive and only cost-effective on a small scale, making it inappropriate for large-scale production (Shapiro-Ilan *et al.*, 2012). Similarly, growing slug-parasitic nematodes on freeze-killed slugs would also be labour-intensive, as the slugs would need to be collected by hand.

In vitro

A solid culture method was developed in 1981, in terms of which the EPNs were cultured on polyether polyurethane sponge crumbs that had been impregnated with emulsified pig's kidneys and beef fat and inoculated with a symbiotic bacterium. Yields of between 6×10^5 and 10×10^5 IJs were achieved (Bedding, 1981). In South Africa, low-cost solid *in vitro* culturing methods were tested by Ramakuwela *et al.* (2016) for the production of *Steinernema innovationi* (Cimen, Lee, Hatting & Stock). The highest concentration of IJs obtained was 156,000 IJs/g after four weeks incubation in a larval puree of *Musca domestica* Linnaeus (Diptera: Muscidae) and 3% canola oil, absorbed in sponge cubes in Erlenmeyer flasks (Ramakuwela *et al.*, 2016).

In 1990, a liquid fermentation technique was developed, with which densities of 50×10^{12} IJs/month were produced, which was significantly higher than were the 10×10^{12} IJs/month produced using the solid culture method (Friedman, 1990). This method has been used to produce the steinernematids *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding, *Steinernema feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding, *Steinernema glaseri* (Steiner) Wouts, Mráček, Gerdin & Bedding, *Steinernema riobrave* Cabanillas, Poinar & Raulston and *Steinernema scapterisci* Nguyen & Smart at a scale of 80 000 L, and also the heterorhabditids *Heterorhabditis megidis* Poinar, Jackson & Klein, *Heterorhabditis bacteriophora* Poinar and *Heterorhabditis indica* Poinar, Karunaka & David at scales of 300-2,000 L (Friedman, 1990; Devi & Nath, 2017).

Liquid culture technology was commercialised for the first time in 1992 by Biosys, Palo Alto, California, using the nematode *S. carpocapsae*, upscaled to a fermenter of 80 000 L (Ehlers & Shapiro-Ilan, 2005). Typically, the EPN growth medium consists of a carbon source, such as glycerol or glucose, a protein source, of plant or animal origin, yeast extract, and lipids of plant or animal origin (Pace *et al.*, 1986; Friedman *et al.*, 1989; Han *et al.*, 1993; Surrey & Davies, 1996; Ehlers *et al.*, 1998). Increasing EPN yields can be achieved by altering the liquid medium (Ehlers, 2001), as different species have different medium requirements.

Using internal-loop bioreactors consistently produces higher IJ concentrations than does the use of bioreactors with airlift, flat-blade impellers and bubble columns (Ehlers & Shapiro-Ilan, 2005). Before adding the IJs, the medium is inoculated with the symbiotic bacterium associated with the EPN species, at a volume of 0.5-1% of the total volume, with it then being preincubated for a period of 24-36 h. The nematodes are then added, at a volume of 5-10% of the total culture volume (Ehlers & Shapiro-Ilan, 2005).

The temperature used for culturing depends on the medium and on the EPN species used, and it should always be determined after the optimum growth temperature of the bacterial symbiont has been defined (Ehlers *et al.*, 2000).

Conclusion

In conclusion, *P. hermaphrodita* is the only mollusc-parasitic nematode that has been developed as a commercial biocontrol product, which is currently sold across Europe by BASF and Dudutech, based on its natural distribution. It can however neither be sold, nor used, in South Africa as it has not yet been isolated, and it is considered to be an exotic species. Therefore, a local mollusc-parasitic nematode would be required for commercial development in order to adhere to current regulations. Once a suitable nematode has been found, it must be described and its life cycle analysed, before being established in monoxenic cultures with a bacterial isolate that is pathogenic to slugs. The protocol then needs to be optimised and upscaled to produce the nematode in industrial-scale fermenters.

Aims and objectives

The overall aim of the current study was to search for local nematode isolates, and investigate their biocontrol potential, and where nematodes fail, investigate suitable environmentally friendly alternative control methods.

The specific objectives of this study were to:

1. Conduct a survey of mollusc-parasitic nematodes in KwaZulu-Natal, so as to gain a better knowledge of the mollusc-parasitic nematode population of South Africa, and to find a nematode with biocontrol potential;
2. Analyse the life cycles of local nematode isolates, as well control representatives from Europe (e.g. *P. hermaphrodita*), so as to gain a better understanding of the nematodes, and their potential culture methods;
3. Describe any nematode species found during the survey or through collaborative work across the African continent;
4. Successfully mass-culture the chosen nematode in liquid medium, by means of *in vitro* technology;
5. Investigate other control methods to be used in conjunction with nematodes in an integrated control programme.

The “Instructions for Authors” for the journal *Nematology* have been followed in the writing of the current dissertation. Each chapter is written in the form of a separate paper, which necessarily entails some repetition.

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Chapter 2

Survey of nematodes associated with slugs in the KwaZulu-Natal province of South Africa

Summary

A survey of nematodes associated with slugs was conducted in the KwaZulu-Natal province of South Africa. A total of 300 slugs were collected from nine different sites, including gardens, nurseries and agricultural land. Of the slugs collected, 239 were invasive species from Europe and 61 are indigenous to Africa. The slug species collected were *Ambigolimax valentianus*, *Chlamydephorus gibbonsi*, *Deroceras invadens*, *Deroceras reticulatum*, *Elisolimax flavescens*, *Laevicaulis alte*, *Milax gagates* and *Urocyclus kirkii*. Dissection of the slugs revealed that five of the nine sites had slugs with internal nematodes, and that 19 of the 300 slugs collected were infected with nematodes. *Caenorhabditis elegans*, which was the only nematode species isolated, was cultured in liquid monoxenic cultures, together with the bacterial species *Kluyvera* sp., which has previously shown to be pathogenic to slugs. The nematode-bacterial combinations pathogenicity was tested on the invasive slug *D. invadens*, demonstrating that *C. elegans* was not capable of causing mortality in the slugs, even in association with pathogenic bacterium.

Keywords - *Caenorhabditis elegans*; *Deroceras invadens*, *Kluyvera*; monoxenic culture; pathogenicity; KwaZulu-Natal

Introduction

An estimated 34 non-native terrestrial mollusc species are currently found in South Africa. The majority of the species originate from Europe, mainly because of South Africa's colonial history, as well as the favourable climate conditions of the Cape area (Herbert, 2010). Some of the species have become highly successful in the country, leading to their status as major agricultural pests (Swart *et al.*, 1976; Loubser, 1982; De Jager & Daneel, 2002; Sanderson & Sirgel, 2002; Herbert, 2010; Tribe & Lubbe, 2010). The above has prompted research into finding a suitable nematode parasite for the control of invasive molluscs (Pieterse, 2016).

Surveys and research conducted on all continents, except Antarctica, have identified eight nematode families that associate with terrestrial molluscs as definitive hosts. They are Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (Pieterse *et al.*, 2016). However, the only nematode species that has been successfully developed for the biological control of slugs is *Phasmarhabditis hermaphrodita* (Schneider) Andr assy. It is sold under the trade name Nemaslug[®] and is widely used in Europe, with it being pathogenic to a wide range of molluscs (slugs and snails) (Rae *et al.*, 2007). However, *P. hermaphrodita* has, to date, not been isolated in South Africa, which means that it can neither be imported, nor used, in South Africa, as it is considered to be an exotic species (Agricultural Pests Amendment Act, No. 18 of 1989). Surveys of nematodes associated with molluscs in South Africa are, thus, of vital importance for the potential biocontrol development of a local nematode species to control invasive molluscs.

Two surveys of nematodes associated with terrestrial molluscs have previously been conducted in the Western Cape province of South Africa (Ross *et al.*, 2012; Pieterse *et al.*, 2017). The first was conducted in 2009, with 521 slugs being dissected, and the second being conducted between 2012 and 2015, with 3290 slugs being dissected. Thirteen different slug species were found from these two surveys combined, and seven of which were indigenous and six invasive. A total of 13 different nematode species were identified from slug hosts in the Western Cape, ten of which were previously undescribed (Ross *et al.*, 2012; Pieterse *et al.*, 2017). The nematodes isolated in the first survey included *Agfa flexilis* Dujardin, *Caenorhabditis elegans* (Maupas), *Panagrolaimus* sp., *Rhabditis* sp., *Phasmarhabditis* sp. (SA1), *Phasmarhabditis safricana* Ross, Pieterse, Malan & Ivanova and *Angiostoma margaretae* Ross, Malan & Ivanova, with the latter two species described as a result of this survey (Ross *et al.*, 2011, 2012, 2018). The nematode species found in the second survey

included *A. flexilis*, *A. margaretae*, *Angiostoma* sp. (SA1), *C. elegans*, a mermithid (SA1), *Phasmarhabditis* sp. (SA3) and *Phasmarhabditis papillosa* Schneider (Andrássy) (Pieterse *et al.*, 2017). This was the first record of *P. papillosa* in South Africa, with previous surveys reporting the nematode in Germany and the USA (Mengert, 1953; Tandingan De Ley, 2016). To date, the only mollusc-nematode surveys in South Africa have focused on the Western Cape province, with the other province being overlooked despite their varied mollusc fauna.

The purpose of the current study was to conduct a survey of nematodes associated with molluscs in KwaZulu-Natal, a sub-tropical area on the east coast of South Africa, known to be home to a diverse range of mollusc species. The second aim of the study was to test the pathogenicity of nematodes found during the survey, when grown on a bacterium known to cause mortality in slugs.

Methodology

COLLECTIONS

Terrestrial slugs were manually collected from nine different sites, including farms, gardens and nurseries in the KwaZulu-Natal province of South Africa, between 7 February 2017 and 29 November 2017, with the help of local growers, and with the permission of the landowners concerned (Fig. 2.1). The aim was to include a variety of sites, rather than a large number of sites. Plastic containers (145 mm × 205 mm × 80 mm) with perforated lids, and lined with moistened paper towels, were used to store and transport the slugs. The location and date of collection were noted on each container, with approx. 20 slugs per container. The slugs were fed carrot slices, which were replaced with new ones daily, and boxes were cleaned weekly.

DISSECTION

Once in the laboratory, the slugs were washed with 0.9% saline solution to remove any external nematodes, and dissected, using a dissecting microscope (Leica MZ7s), so as to determine visually the presence of internal nematodes. The nematodes that were found were placed on White traps (Dutky *et al.*, 1964) to encourage reproduction, and then fixed in 70% ethanol for molecular analysis, or heat-killed with hot (85 °C) triethanolamine-formalin TAF (7 ml 37% formaldehyde, 2 ml triethanolamine, 91 ml distilled water) (Courtney *et al.*, 1955), for the purpose of morphological analysis.

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION

For morphological observation, the nematodes that were heat-killed in TAF were mounted on temporary microscope slides, covered with a cover slip and studied with a compound microscope (Leica DM200, Leica Microsystems, Wetzlar, Germany), fitted with a digital camera (Ross *et al.*, 2016). The morphology of the nematodes was then studied by observing the length and tail shape of the nematodes, and by checking for the presence of males.

For molecular analysis, the nematodes from the 70% ethanol were removed, and their DNA extracted by picking into a mix of 25 μ l Chelex beads and 5 μ l Proteinase K in 0.2-ml PCR tubes (Ross *et al.*, 2010). The tubes were placed in a thermocycler at 65 °C for 1 h, and then at 95 °C for 10 min, after which they were centrifuged at 8000 rpm for 5 min, with the supernatant then being transferred to a clean tube, which was kept at -20 °C (Ross *et al.*, 2010).

The samples were then subjected to a polymerase chain reaction (PCR) of the small subunit (SSU) rRNA gene (Ross *et al.*, 2010). The PCR cycling parameters involved a primary denaturation stage at 94 °C for 5 min, then 35 cycles of the three temperatures, 94 °C for 60 s, 55 °C for 90 s and 72 °C for 2 min, followed by a final cycle of 72 °C for 10 min (Ross *et al.*, 2010). Sequence traces were inspected and assembled using CLC Main Workbench 7.6.4 (<https://www.qiagenbioinformatics.com/>), and then compared to those on the database of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) by using the BLASTN tool (Altschul *et al.*, 1990).

ORIGIN OF BACTERIA

Kluyvera sp. (Genbank accession number KX531097) was isolated by Pieterse *et al.* (2016) with its ability to cause mortality in slugs proven, making it an ideal choice for the present study. *Kluyvera* sp. were revived from 15% glycerol cultures stored at -80 °C, by adding 200 μ l to 30 ml Luria Broth (LB) (10 g NaCl, 10 g Tryptone, 5 g yeast extract per litre, autoclaved at 121 °C for 20 min) in a 250-ml Erlenmeyer flask, and then left on an orbital shaker (140 rpm) at 28 °C for 48 h.

MONOXENIC CULTURES

To grow nematodes that were only associated with one bacterial species, the nematodes had to first be sterilised of all bacteria. This was done by using a method adapted from Lunau *et al.* (1993). Infective juveniles (IJs) of *C. elegans* were added to a frozen and defrosted slug on a

9.5-cm Petri dish lined with moistened Whatman[®] No. 1 filter paper. The dish was sealed with parafilm and incubated at 18 °C for 48 h, at which stage adult hermaphrodites with eggs were first observed. The Petri dish was then washed with 0.9 % saline solution and passed through a 32 µm sieve to remove all life stages, apart from that of the adult hermaphrodites. The hermaphrodites were then added to Eppendorf tubes, centrifuged and aspirated to 300 µl. The nematodes were then crushed, using a sterilised plastic grinding rod, for 30 sec, after which 700 µl of a NaOH/bleach mix (0.2 g NaOH, 2.25 ml bleach in 7.75 ml sterile water) was added to the tubes. The NaOH/bleach mix was then left to work on the nematode debris and eggs for 8 min, with the tubes being occasionally shaken. The mixture in the tubes was then washed by means of centrifuging the tubes, removing the top liquid layer and adding sterilised distilled water. The washing process was repeated three times. The remaining eggs were then pipetted into 200 µl sterile tryptone soy broth in a sterile 24-well culture plate (Nunc[™]), which was then sealed with parafilm and left at 25 °C for 48 h. Where there were no signs of bacterial contamination after 48 h, the nematodes involved were used for liquid culturing.

LIQUID CULTURES

After 48 h growth of the *Kluyvera* sp. in the LB, 1.2 ml (4%) of the bacteria was added to 30 ml liquid culture medium (9 g pig kidney, 17.4 g yeast extract, 8.6 g egg yolk powder, 52.6 g sunflower oil, in 1 L distilled water, autoclaved at 121 °C for 20 min) in a 250-ml Erlenmeyer flask, which was again incubated on an orbital shaker (140 rpm) at 28 °C for 48 h. After the bacteria had grown for 48 h in the medium, the sterile J1s of *C. elegans* were added. The flask was then incubated at 18 °C on an orbital shaker (140 rpm) for 14 days. After two weeks, the contents of the flask were washed several times with sterile distilled water through a 32 µm sieve to separate and remove all adult hermaphrodite stages, and then through a 25 µm sieve to remove any remaining medium and life stages that were smaller than the IJ. The above was then used as inoculum for the pathogenicity test.

PATHOGENICITY TEST

A total of 120 *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Agriolimacidae) slugs were used for the pathogenicity experiment. This species was chosen as it is a known slug pest in Europe and was the only species available in large numbers at the time of collection. The slugs were each kept in their own Petri dish (100 mm × 15 mm) lined with Whatman[®] No. 1 filter paper. Twenty Petri dishes were then moistened with 800 µl of distilled water containing 2000 IJs of *C. elegans*, monoxenically cultured with *Kluyvera* sp. The control dishes were

moistened with 800 μ l of distilled water only. A total of 20 tested and 20 control slugs were provided with a carrot slice as a food source, with it being replaced every 2 days. The Petri dishes were kept at 18 °C for a period of 14 days. The plates were observed daily, and any dead slugs were dissected to determine whether nematode infection had occurred. The experiment was repeated three times with fresh slugs, using a total of 120 slugs.

Results

SLUGS COLLECTED

A total of 300 terrestrial slugs were collected and dissected, consisting of seven species. Three of the species were introduced from Europe, with four being indigenous to Africa. Of the 300 slugs that were collected, 239 were invasive species. Only 61 of the slugs collected were endemic to southern Africa, including *Chlamydephorus gibbonsi* Binney, *Elisolimax flavescens* (Keferstein) (Fig. 2.2a) and *Laevicaulis alte* Férussac (Fig. 2.2c) (Table 2.2). *Caenorhabditis elegans* was only found in *L. alte* of the endemic species, while in the introduced species, it was only associated with the *Deroceras* species, including *D. panormitanum*, *D. reticulatum* (Fig. 2.2b), and *D. invadens*.

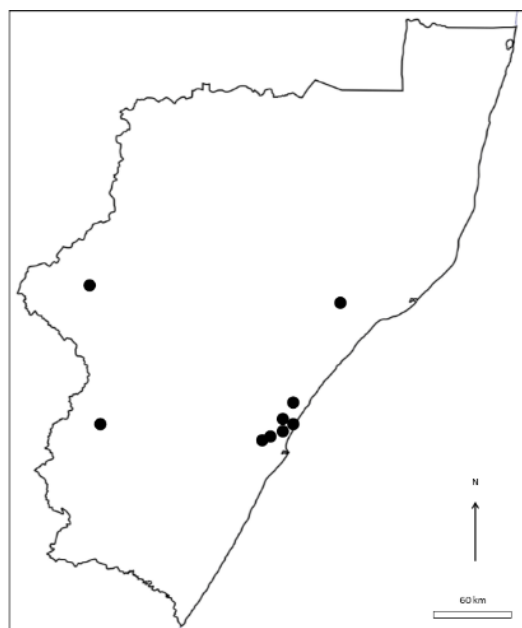


Figure 2.1. Map of the collection sites in the KwaZulu-Natal province.

Table 2.1. Number of species of slugs collected in KwaZulu-Natal.

Family	Species	Indigenous/ Introduced	Number collected
Agriolimacidae	<i>Deroceras invadens</i> Reise, Hutchinson, Schunack & Schlitt	Introduced	205
	<i>Deroceras reticulatum</i> (Müller)	Introduced	10
Chlamydephoridae	<i>Chlamydephorus gibbonsi</i> Binney	Indigenous	5
Limacidae	<i>Ambigolimax valentianus</i> Férussac	Introduced	24
Urocyclidae	<i>Elisolimax flavescens</i> (Keferstein)	Indigenous	40
	<i>Urocyclus kirkii</i> Gray	Indigenous	11
Veronicellidae	<i>Laevicaulis alte</i> Férussac	Indigenous	5

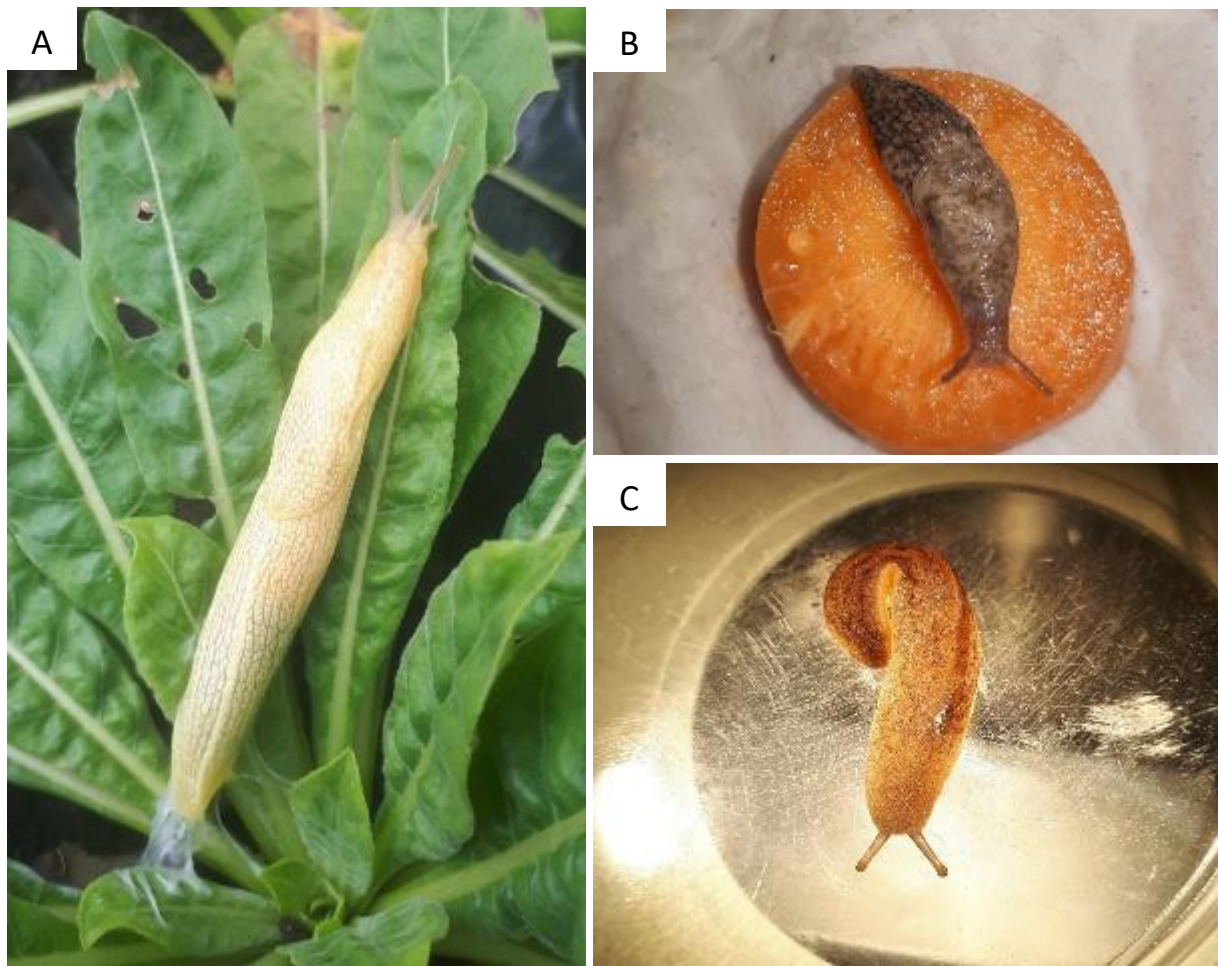


Figure 2.2. A: *Ambigolimax flavescens* (endemic species), B: *Deroceras reticulatum* (invasive species), and C: *Laevicaulis alte* (endemic species) collected in KwaZulu-Natal.

NEMATODES ISOLATED

Five of the nine sites that were surveyed were found to have nematodes present (56%). Only 19 of the 300 slugs that were collected and dissected were infected with nematodes (6.3%). The only nematode species that was isolated was *Caenorhabditis elegans* (Table 2.2; Fig. 2.3).

Table 2.2. Nematodes isolated from slugs collected from KwaZulu-Natal, including host and location data.

Location	Coordinates	Habitat	Slug species	Nematode parasite
Underberg	29°45'02.9"S 29°30'52.0"E	Farm	<i>D. invadens</i>	<i>C. elegans</i>
			<i>A. valentianus</i>	-
Winterton	28°45'59.7"S 29°26'46.5"E	Farm	<i>D. invadens</i>	-
Durban	29°49'36.6"S 30°55'47.5"E	Nursery	<i>D. invadens</i>	-
			<i>Laevicaulis alte</i>	-
Durban	29°46'17.4"S 31°03'07.0"E	Nursery	<i>Elisolimax flavescens</i>	-
			<i>D. invadens</i>	-
Durban	29°42'10.3"S 31°02'08.4"E	Nursery	<i>E. flavescens</i>	-
			<i>D. invadens</i>	-
Durban	29°45'37.7"S 31°01'55.0"E	Nursery	<i>E. flavescens</i>	-
			<i>D. invadens</i>	<i>C. elegans</i>
			<i>A. valentianus</i>	-
			<i>Deroceras reticulatum</i>	<i>C. elegans</i>
			<i>L. alte</i>	-
			<i>Urocyclus kirkii</i>	-
			<i>Chlamydephorus gibbonsi</i>	-
Eshowe	28°53'07.7"S 31°28'19.3"E	Garden	<i>E. flavescens</i>	<i>C. elegans</i>
Durban	29°49'46.7"S 30°52'47.5"E	Garden	<i>E. flavescens</i>	<i>C. elegans</i>
			<i>U. kirkii</i>	-
Tongaat	29°32'39.8"S 31°08'11.4"E	Garden	<i>E. flavescens</i>	<i>C. elegans</i>



Figure 2.3. Light microscope photo of *Caenorhabditis elegans* adult hermaphrodite isolated during the survey of KwaZulu-Natal.

PATHOGENICITY

Pathogenicity studies showed that no significant difference was found in the number of deaths when the slugs were treated with *C. elegans* monoxenically grown with *Kluyvera* sp., compared to untreated slugs. *Caenorhabditis elegans* was found inside all dead slugs that were treated, but not in the dead control slugs.

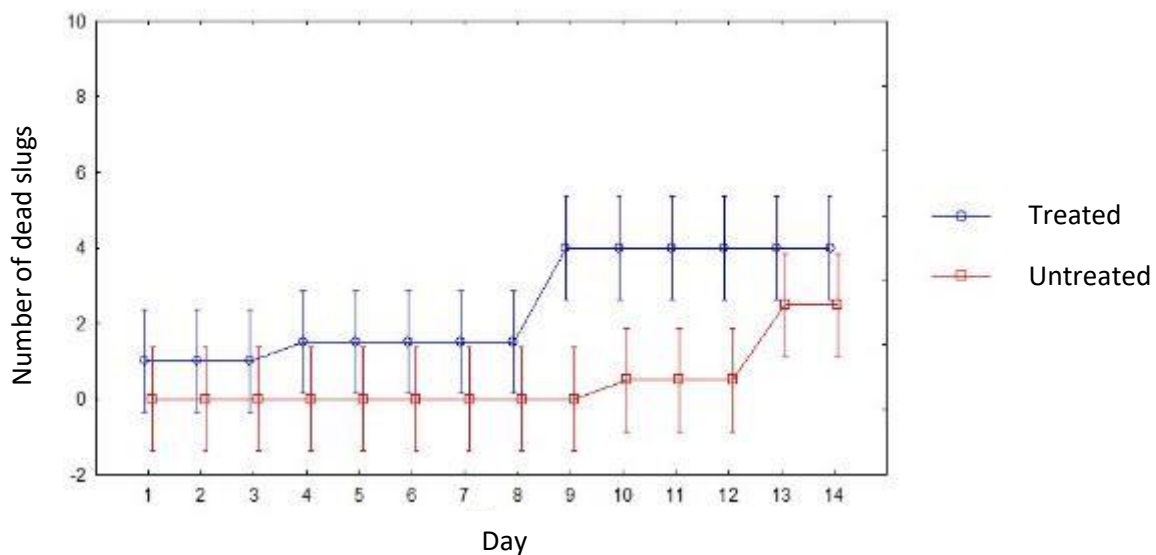


Figure 2.4. Graph displaying mortality of *Deroceras invadens* with untreated control vs. treatment with monoxenic cultures of *Caenorhabditis elegans*.

Discussion

This is the first mollusc-nematode survey ever conducted in KwaZulu-Natal. Four slug species were found, including *C. gibbonsi*, endemic to the Transkei and the KwaZulu-Natal province; *E. flavescens*, endemic to central and southern Africa; *Urocyclus kirkii* endemic to central and southern Africa; and *L. alte*, originating in the Afro-Asian regions (Herbert & Kilburn, 2004). Three introduced slug species were found, including *D. invadens*, a well-known pest species found in many parts of the world; *D. reticulatum*, a synanthropic species initially from Europe and *A. valentianus*, a European species that has been introduced in many countries in Africa, Oceania, North and South America and Asia (Waldén, 1961; Wiktor, 2000; Herbert & Kilburn, 2004; Reise *et al.*, 2011).

Of the 300 slugs that were collected, 239 were introduced species and 61 (20%) were indigenous to Africa. A possible reason for the large number of invasive species is due to many of the sample sites being disturbed sites, with a visible human impact. Further sampling in pristine areas might have delivered more indigenous species than the above. A low level of endemism was also found by Ross *et al.* (2012) and Pieterse *et al.* (2017), who found 6% and 7% native slugs, respectively

During this survey, five of the nine sites had nematodes present (56%), with only 19 of the 300 slugs collected, having been infected with nematodes (6%). The results are similar to those of Ross *et al.* (2012) and Pieterse *et al.* (2017), where 6% and 8% infectivity was recorded, respectively.

The only nematode found during the mollusc-nematode survey of KwaZulu-Natal was *C. elegans*. *Caenorhabditis elegans* was also found in surveys of the Western Cape province of South Africa, however it is highly unusual for this to be the only nematode isolated (Ross *et al.*, 2012; Pieterse *et al.*, 2017). *Caenorhabditis elegans* is an androdioecious nematode that is mostly isolated from rotting fruit, compost, stems, and some invertebrates (Schulenburg & Félix, 2017). Its original substrate in nature seems to be microbe-rich rotting plant material (Schulenburg & Félix, 2017). Commonly forming phoretic or necromenic relationships with its invertebrate hosts, it is often found in slug intestines (Kiontke & Sudhaus, 2006). It possesses the ability to enter and exit the slug host, possibly as a means of transport, or to escape unfavourable environmental conditions (Petersen *et al.*, 2015). It might also use the microbe-rich intestines of slugs as a suitable habitat for proliferation. The interaction between

Caenorhabditis species and slugs could hint at a possible mutualistic, commensal or parasitic relationship (Petersen *et al.*, 2015; Schulenburg & Félix, 2017).

Despite the high prevalence of *C. elegans*, pathogenicity studies demonstrated that *C. elegans* is not capable of causing mortality in *D. invadens*, even when it is grown with bacteria that is capable of causing mortality in slugs (Pieterse *et al.*, 2016). A possible explanation for the above is that the nematodes do not enter the body of the slug, or perhaps they recovered from the IJ stage, and expelled the bacteria before entering the slug's body. Petersen *et al.* (2015) found that *C. elegans* is capable of entering and exiting its slug host, possibly to escape adverse environmental conditions (Petersen *et al.*, 2015). The Petri dishes, lined with moist filter paper and containing a slice of carrot, as used in the pathogenicity test in this study, possibly created a suitable enough environment for *C. elegans*, so that the nematodes did not need to enter the slug's body.

The isolation of *C. elegans* in the present survey, as well as in the other two previous surveys conducted in South Africa, indicates that it is widespread within the country, has a closer relationship with molluscs than previously thought, and is well-established in many different habitats (Ross *et al.*, 2012; Pieterse *et al.*, 2017). There are a number of possible reasons for *C. elegans* widespread occurrence. Hodgkin and Barnes (1991) found that the number of sperm produced, and the reproductive mode of the wild-type hermaphrodites of *C. elegans*, are optimised to enable rapid population growth (Hodgkin & Barnes, 1991). *Caenorhabditis elegans* is also capable of using slugs, isopods and chilopods as vectors, possibly to escape unfavourable environments, with it also having the ability to nictate to facilitate its contact and transport by associated animals or vectors (Kiontke & Sudhaus, 2006; Kruitbos & Wilson, 2010; Petersen *et al.*, 2015). The findings are also supported by population genetic studies, which suggest that *C. elegans* is capable of migrating over long distances, and even between continents (Koch *et al.*, 2000; Sivarsundar & Hey, 2003; Barrière & Félix, 2005; Haber *et al.*, 2005; Petersen *et al.*, 2015).

The mollusc-nematode fauna in South Africa is relatively understudied, with previous work focusing on the Western Cape, and this study concentrating on KwaZulu-Natal. It is recommended that future work focuses on surveying the other provinces in South Africa, as well as across the African continent

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Chapter 3

Distribution and feeding behaviour of *Chlamydephorus gibbonsi* in the Western Cape province of South Africa

(format adapted from the following published paper:

Pieterse, A., Malan, A.P., Sirgel, W.F. & Ross, J.L. (2017). Distribution and feeding behaviour of *Chlamydephorus gibbonsi* in the Western Cape province of South Africa. *Journal of Conchology* 42(6), 523-529.)

Summary

The purpose of this study was to investigate the distribution and feeding behaviour of the South African predatory slug, *Chlamydephorus gibbonsi* Binney, 1879 (Mollusca: Gastropoda), which is endemic to the eastern region, but has now spread to the Western Cape province (WCP). A total of 210 *C. gibbonsi* specimens were collected from the WCP between January 2012 and December 2015. Slug numbers were found to steadily increase from nine specimens collected in 2012, to 111 specimens collected in 2015, indicating establishment of *C. gibbonsi* in its new habitat. Of the sample sites studied, 13.7% were found to be positive for *C. gibbonsi*, including sites in George, Knysna, Swellendam, Hermanus and Stellenbosch. The habitats of positive sample sites were all commercial nurseries. Feeding behaviour of *C. gibbonsi* is described using an earthworm of the genus *Amyntas*.

Keywords - Chlamydephoridae; Distribution; Feeding; Invasion; Earthworms

Introduction

Terrestrial pulmonate molluscs (both snails and slugs) are commonly herbivores feeding on algae, fungi and plant material, hence their interest as economically significant pests. However, occasionally species that are primarily herbivorous will feed on living invertebrates, or decaying flesh of dead animals, and under extreme conditions, can resort to cannibalism (South, 1992). Obligate carnivory occurs in several slug families e.g. Chlamydephoridae, Papillodermidae, Plutoniidae, Rathouisiidae, Testacellidae, as well as some snail species (Herbert, 2000).

The families Rhytididae, Streptaxidae and Chlamydephoridae are all carnivorous terrestrial molluscs indigenous to South Africa, the latter of the three the sole slug representative. Chlamydephoridae are typically narrow, firm and dry to the touch and have a characteristic postero-dorsally positioned pneumostome. Additionally, the dorsal surface is marked with well-defined grooves radiating from the pneumostome. An anatomical review of the family was conducted by Watson (1915), and taxonomy and biogeography was examined by Forcart (1967), van Bruggen (1969, 1978) and Herbert (1997). Like many carnivorous slugs Chlamydephoridae are secretive, well camouflaged, and are subterranean, so little is known of their behaviour and distribution.

Chlamydephoridae are represented by nine species in South Africa, and five are from the Eastern region, with *Chlamydephorus gibbonsi* Binney, 1879, *Chlamydephorus burnupi* (Smith, 1892) and *Chlamydephorus dimidius* (Watson, 1915) thought to be endemic to the area. *Chlamydephorus gibbonsi* occurs in a wide range of habitats, spanning open thornveld to indigenous forests. Initial distribution was thought to range from eastern Zimbabwe, through to the Eastern Cape of South Africa (Herbert, 1997). However, Herbert & Kilburn (2004) noted that *C. gibbonsi* had been found in KwaZulu-Natal at the Ngome Forest, Lake Sibaya, Colenso and Pietermaritzburg, and in the Eastern Cape from Transkei and East London. Herbert (1997) also found a single outlying specimen in the Western Cape, as did Ross *et al.* (2012), indicating that the species has invaded the WCP. To date, very little is known of the distribution or the invasive behaviour of *C. gibbonsi*.

The feeding habits of chlamydephorids are poorly understood, and their proposed prey of earthworms and other molluscs, is based on distinctive anatomical features such as dagger-like teeth and a short digestive system. Herbert (2000) reported observations of *C. dimidius* feeding on snails, and *C. burnupi*, *Chlamydephorus bruggeni* (Forcart, 1967) and *Chlamydephorus*

sexangulus (Watson, 1915) have been reported feeding on pill millipedes (Herbert 2000). However, the feeding habits of *C. gibbonsi* have yet to be fully elucidated. Watson (1915) proposed that they feed on earthworms, but gave no details of observations, and Herbert (2000) supported this proposal based on the fact that the slug has subterranean habitats.

During fieldwork in George (33°59'27"S 22°30'54"E), a specimen of *C. gibbonsi* was observed feeding on an earthworm (*Amyntas* sp.). The two invertebrates had been placed in the same sample box, and although the killing process had not been observed, the earthworm was alive when collected. This observation confirmed the proposal of Watson (1915) and Herbert (2000), but provided little information regarding the killing process. Therefore, additional samples of *C. gibbonsi* were brought back to the laboratory in order to explore this behaviour further. This study details observations on the distribution and feeding behaviour of *C. gibbonsi* in the Western Cape of South Africa.

Materials and methods

Fifty-one sample sites around the Western Cape were examined for slugs between January 2012 and December 2015. Habitats included domestic gardens, nurseries, agricultural land, forest areas and road side verges. A total of one hour was spent at each location, focusing on as many different vegetation and microhabitat types as possible. Collected slugs were stored in sealed containers at one hundred percent humidity, and transported to the laboratory where they were identified using morphological analysis and dissection of genitalia.

Under laboratory conditions, single specimens of *C. gibbonsi* were placed in plastic containers lined with moist paper towels. Slugs were kept in isolation for three days to allow for starvation. Individual earthworms (*Amyntas* sp.) were then added to the experimental arena. Feeding behaviour was recorded and photographed.

Results

A total of 2876 slugs were collected in the WCP between 2012 and 2015 and of these, 7.3% were *C. gibbonsi*. Numbers of *C. gibbonsi* were found to steadily increase, from nine specimens collected in 2012, to 111 specimens collected in 2015 (Fig. 3.1). Seven sample sites, all commercial nurseries, were positive for *C. gibbonsi*: three sites in George (33°59'27"S, 22°30'54"E; 33°59'40"S, 22°32'19"E; 33°59'38"S, 22°23'35"E) and single sites in Knysna (34°01'57"S, 22°59'22"E), Swellendam (34°02'21"S, 20°32'49"E), Hermanus (34°24'41"S, 19°12'01"E) and Stellenbosch (33°54'24"S, 18°50'38"E).

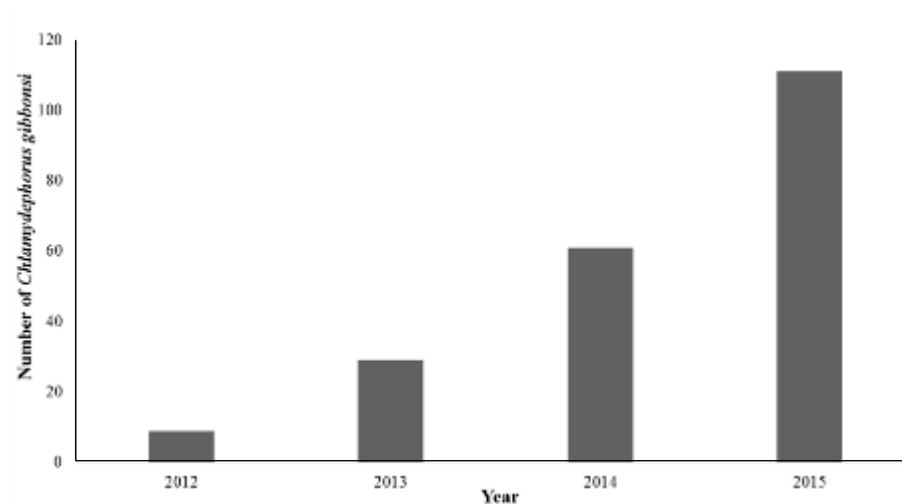


Figure 3.1. Number of *Chlamydephorus gibbonsi* specimens collected in the Western Cape between 2012 and 2015.

On encountering prey *C. gibbonsi* spends an extended time exploring the surface of the earthworm with its inferior tentacles (Fig. 3.2A). It then slowly evaginates the anterior part of the buccal chamber through the mouth (Fig. 3.2B). By doing so, the odontophore covered by the radula is carried forward, but initially does not protrude from the buccal cavity. The odontophore is then rapidly thrust forward to collide with the prey, followed by a rapid retraction in a piston-like action. During this action, radular teeth are lodged into the body wall of the prey, resulting in the prey being partly drawn into the slug's mouth. Simultaneously, the evaginated part of the buccal cavity is also invaginated.

The finer details of the effective capturing and swallowing process can be understood by examining the morphology of the concerned structures. The process of hauling the prey into the alimentary system of the slug is permitted by adaptations of the radula, including numerous transverse rows of teeth borne on a radular membrane, which extend over the dorsal and anterior surface of the odontophore, and is U-shaped in cross section. The central tooth of the radula, which is situated at the deepest point of the 'U', and has a few lateral teeth on either side, are small and under developed. Following each transverse row laterally, the teeth gradually enlarge, and by approximately the tenth lateral tooth, are considered fully developed (Fig. 3.3).

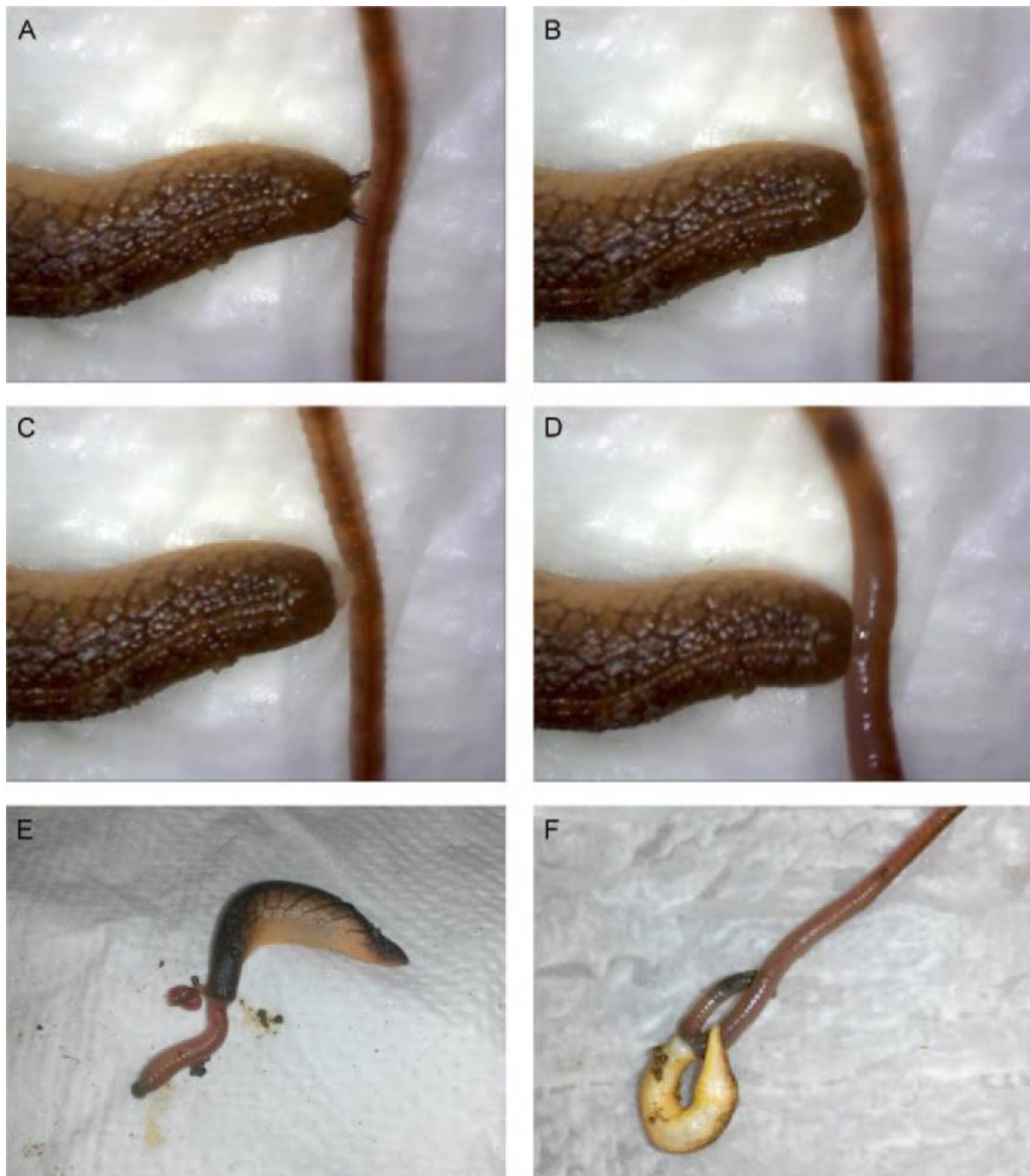


Figure 3.2. *Chlamydephorus gibbonsi* capturing and killing the *Amynthus* sp. A, *Chlamydephorus gibbonsi* exploring the surface of the worm with its inferior tentacles. B, Evagination of the anterior section of the buccal cavity. C, D, Ejection of odontophore and hooking the hapless annelid on long radular teeth. E, F, Feeding and swallowing of the worm through a combination of suction and movement of the odontophore.

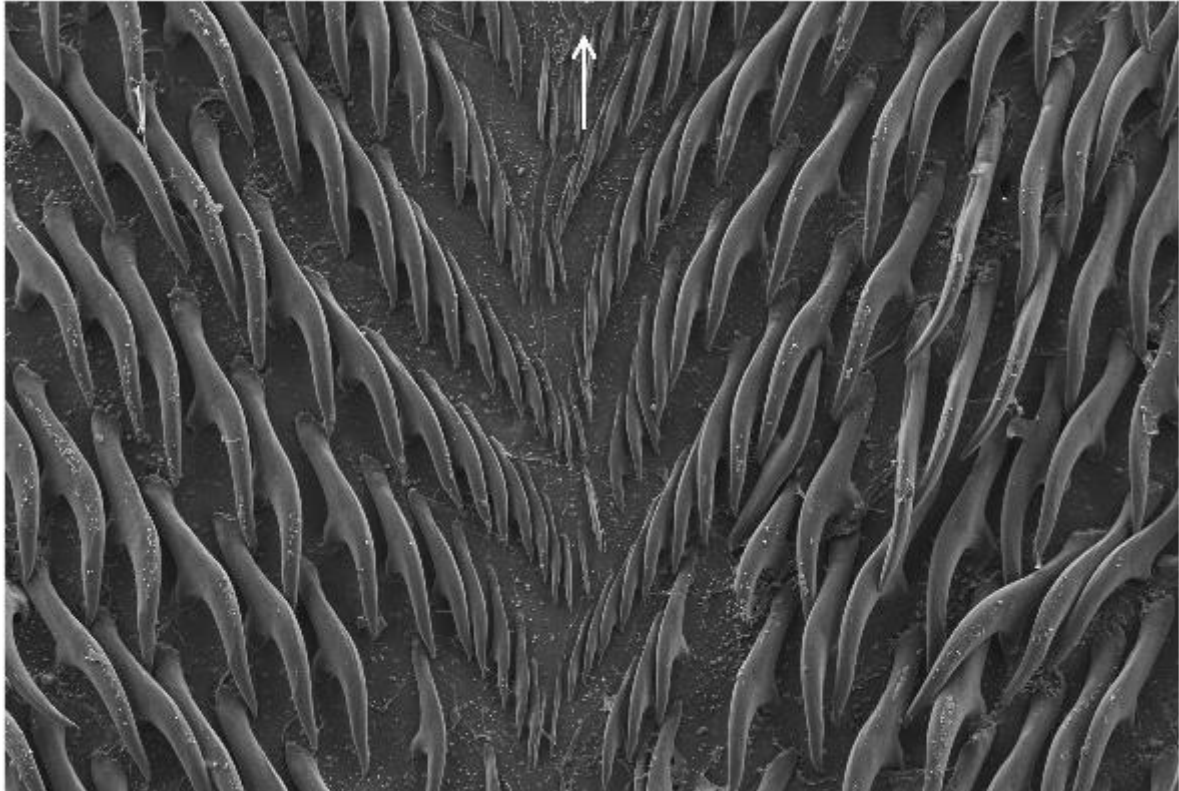


Figure 3.3. Radula of *Chlamydephorus gibbonsi* (magnification approximately 101x), showing the under developed centrally located teeth, as well as the more lateral, more developed teeth. Arrow indicating the direction of the mouth opening.

Each fully developed lateral tooth has the shape of a long curved thorn, and consists of a cusp and a basal plate, which are carried on the radular membrane. The cusp consists of an apical and basal portion. The sharply pointed apical portion (Fig. 3.4A) is slightly flattened on its ventral side, and extends posteriorly within the buccal bulb. At its base, it passes over into the cylindrical basal portion, which gradually, but strongly, enlarges towards its base, especially in the antero-posterior plane (Fig. 3.4B).

Near its base, this portion is also strongly curved ventrally to form an angle of approximately ninety degrees, where it transmits into the basal plate. The latter is an elongated disc-shaped structure implanted, and longitudinally orientated, on the radular membrane. It is important to note that this base plate is deeply concave as seen from its ventral side (Fig. 3.4C). The concavity gives the impression that it could function like an articulation socket. At the same time, the elongated basal plate would enable the cusp of each tooth to maintain its posterior orientation, even when traction is exerted on it during the process of inhauling of the prey. The

anterior margin of the radular membrane is fused to the ventral and lateral walls of the buccal cavity ventrally, and laterally to the anterior margin of the odontophore.

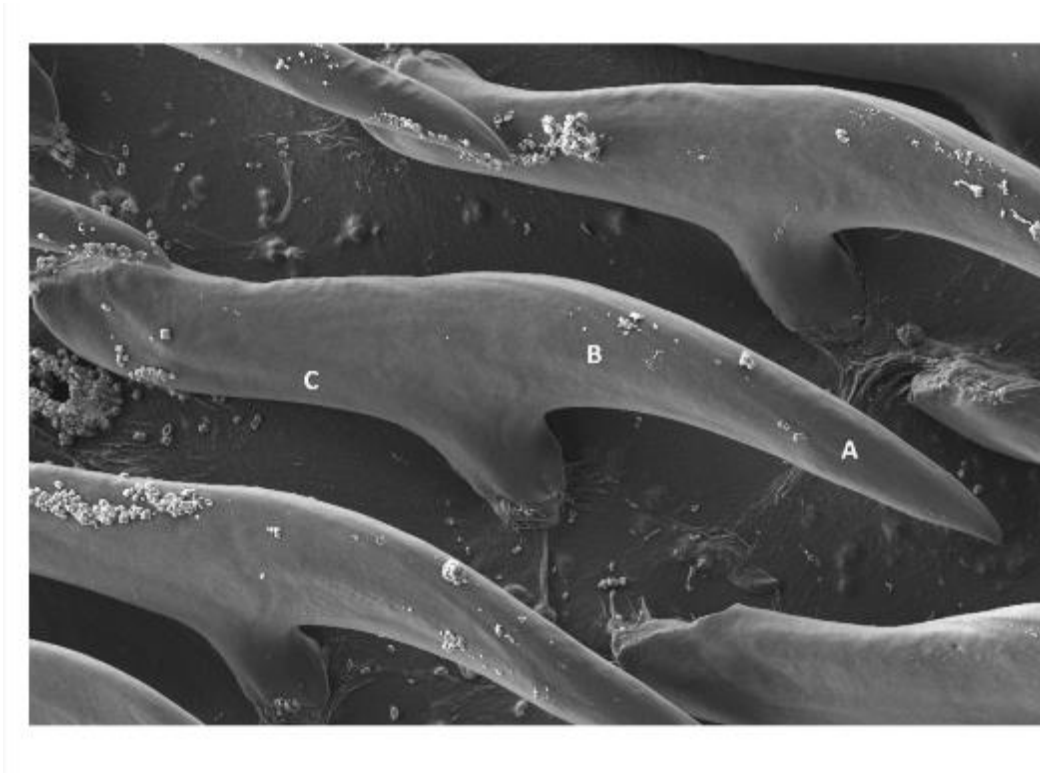


Figure 3.4. Medio-dorsal view of a well-developed lateral tooth of *Chlamydephorus gibbonsi* (magnification approximately 450x) displaying; A, the apical portion with a sharp tip, orientated posteriorly; B, basal portion; and C, the deeply concave basal plate implanted on the radular membrane.

Bearing the above mentioned morphology of the radula in mind, the capturing and hauling of the prey can further be elaborated on. During the final stages, when the odontophore is rapidly thrust towards the prey, it is clear that the inextensible radular membrane has to slide over the surface of the odontophore, and this will carry the teeth of at least the anterior part of the radula anteriorly, and glide over the anterior edge of the odontophore. As each of these curved teeth transgress the anterior edge of the odontophore, they naturally splay and flip over to then point anteriorly and laterally. This “flipping-over” action of each tooth could be facilitated by the concavity on the ventral side of the basal plate of each tooth. On impact with the surface of the prey, these now anteriorly directed sharp teeth thrust into the body wall of the prey. The odontophore then rapidly retracts and the teeth crossing the anterior edge of the odontophore flip back to restore their original, posteriorly directed position. The teeth that penetrated the prey perform a grabbing action, before returning to their original orientation,

thus drawing the prey into the buccal cavity of the slug. Teeth situated more anteriorly in the radula will hook onto the prey as they are retracted over the anterior edge of the odontophore, thus strengthening the hauling process (Fig. 3.2C, D). As mentioned above, the anterior section of the buccal cavity is simultaneously invaginated during the retraction of the odontophore. This exerts suction on the prey, thus aiding its intake. The swallowing process of the prey (Fig. 3.2E, F) involves the radula repeatedly moving anteriorly and posteriorly within the buccal cavity. With each anterior movement, the teeth will be withdrawn from the prey only to hook further onto its body, when the next posterior movement commences. The entire capturing and swallowing process takes approximately one hour.

Discussion

Interest in the science and management of biological invasions has expanded in recent years due to the sharp increase in the introduction of invasive species in virtually all major habitats on Earth (Mack *et al.*, 2000). An organism is considered invasive when it successfully establishes and thrives outside its natural range. Successful invasions arise from the transportation of alien species from one location to another, by activities of man, either intentionally or unintentionally, resulting in the establishment of these species in a new geographical region (Perrings *et al.*, 2010). Invasions are occurring at an extraordinary rate due to the expansion of trade and the economical ease of travel. The introduction of alien species can have severe consequences on agricultural and horticultural industries, as well as having a direct impact on the economy, natural environments, biodiversity and human health (Mack *et al.*, 2000).

The observations of this study indicate that *C. gibbonsi* has successfully invaded the WCP and is now widespread, as far west as Stellenbosch. Previous studies by Herbert (1997) and Ross *et al.*, (2012) found only a single specimen of *C. gibbonsi* in the Western Cape, however, this study demonstrates that slug numbers have increased in recent years. In order to regulate this process, it is important to have an increased understanding of the causes and mechanisms of the spread of these species. Reports from around the world suggest that the horticultural industry acts as a vector for invasive species (Cowie *et al.*, 2008). This can be confirmed in this instance, with positive sample sites being commercial nurseries. We propose that the slug has been spread to the WCP through transportation of plant material.

We herewith confirm that *C. gibbonsi* feeds on earthworms and this process is documented in figure 3.2. This procedure is very similar to the feeding habits of the carnivorous family

Testacellidae, which also prey on earthworms, as well as other molluscs (Webb, 1893; Quick, 1960). Crampton (1975) provides a comprehensive study of the feeding process of *Testacella*, detailing a two phased process involving an initial stage of seizing the worm and drawing the first part of the body through the mouth; and then a second phase whereby the remainder of the worm is ingested. This process was previously observed by Stokes (1958) and further described and photographed by Liberto *et al.* (2011), who observed that feeding generally takes up to one hour.

The introduction of *C. gibbonsi* to the WCP may have an impact on earthworm abundance, now that it has been confirmed that they play a role in the diet of *C. gibbonsi*. Future work should monitor the impact of *C. gibbonsi* on earthworm populations, as earthworms are important to the soil biota (Lavelle *et al.* 1997), nutrient dynamics (Schmidt & Curry, 1999), decomposition processes (Bonkowski *et al.*, 2001), soil microorganisms (Clapperton *et al.*, 2001), microarthropods (McLean & Parkinson, 1998), seed germination (Grant, 1983) and plant growth (Brussaard, 1999). Furthermore, they also play a substantial part of the diet of aboveground invertebrate (Guillemain, Loreau & Daufresne, 1997) and vertebrate predators (Ferrari & Weber, 1995).

Acknowledgements

This work was financially supported by the Protein Research Foundation (Trust 5802/94 Project P07/20/179/14), and the National Research Foundation of South Africa (NRF-THRIP TP14062571871). The authors would also like to acknowledge the University of Aberdeen and Stellenbosch University.

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Chapter 4

***Phasmarhabditis* sp. (KEN1) (Nematoda: Rhabditidae), a parasite of the slug, *Polytoxon robustum*, from Kenya**

Summary

A new species within the *Phasmarhabditis* genus has been isolated from the slug, *Polytoxon robustum*, from Nairobi, Kenya. The nematode was isolated based on collaborative work, and identified using morphological, morphometrical, molecular and phylogenetic analyses. *Phasmarhabditis* sp. (KEN1) is characterised by an infective juvenile, currently with the longest body length in the genus, measuring 1232 (1107-1336) μm . Also by the presence of males with a bursa bearing nine bilateral pairs of genital bursal papillae, and one pair of papilliform phasmids flanking the tail, cephalate paired spicules, with an arc length of 71 (57-81) μm , as well as by females with a vulva located at midbody region and a conoid tail shape, with two phasmids located at *ca* 40% of tail length. The molecular phylogeny of the new species, as inferred from its SSU (small subunit) rRNA gene, places *Phasmarhabditis* sp. (KEN1) generically close to undescribed phasmarhabditids from South Africa, suggesting an African grouping, while the D2-D3 (large ribosomal subunit) and ITS region analyses relate *Phasmarhabditis* sp. (KEN1) with the Vietnamese *Phasmarhabditis meridionalis*, but only under weak bootstrap support, and only in the absence of D2D3 sequences for the aforementioned African phasmarhabditid group. The lack of such sequence data highlights the importance of encouraging any new species description to be supported by sequences for the small subunit (SSU) rRNA gene, the D2-D3 rRNA gene, the ITS1, 5.8S, ITS2 rRNA gene and the mitochondrial cytochrome *c* oxidase subunit I (*mtCOI*) gene. This is the fifth phasmarhabditid found on the African continent, and the third new species from Africa. This new species bringing the total complement of the genus to 14 species.

Keywords – description; gastropods; morphology; morphometrics; new species; phylogeny; taxonomy; Africa

Introduction

This year marks the 160th anniversary since the establishment of the genus *Phasmarhabditis* Andrassy, which was originally founded with the description of *Phasmarhabditis hermaphrodita* (Schneider) Andrassy (*Pelodytes hermaphroditus*) (Schneider, 1859). The above was followed by the description of *Phasmarhabditis papillosa* Schneider Andrassy in 1866, and of *Phasmarhabditis neopapillosa* (Mengert in Osche) Andrassy in 1954 (Schneider, 1866; Osche, 1954). However, it was over 60 years later that the genus boom occurred, with a notable nine new species added to the genus since 2015. To date, there are 13 nominal species in the genus *Phasmarhabditis*, including: *P. hermaphrodita*; *P. neopapillosa*; *P. papillosa*; *P. tawfiki* Azzam; *P. huizhouensis* Huang, Ye, Ren & Zhao; *P. apuliae* Nermut', Půža & Mráček; *P. bonaquaense* Nermut', Půža, Mekete & Mráček; *P. californica* Tandingan De Ley, Holovachov, Mc Donnell, Bert, Paine & De Ley; *P. bohémica* Nermut', Půža, Mekete & Mráček; *P. meridionalis* Ivanova & Spiridonov; *P. safricana* Ross, Pieterse, Malan & Ivanova; *P. circassica* Ivanova, Geraskina & Spiridinov; and *P. clausiliae* Ivanova, Geraskina & Spiridinov. Members of the *Phasmarhabditis* genus are soil-dwelling, facultative mollusc-parasitic nematodes, with the exception of *P. huizhouensis*, which has been isolated from rotten leaves (Huang *et al.*, 2015).

A major reason for the recent growth of *Phasmarhabditis* relates to the worldwide search for nematode candidates to develop as biological control agents for molluscs. In 1993, Wilson *et al.* (1993) noted that *P. hermaphrodita* was capable of parasitising a range of slug species, and, in the following year, the nematode was launched as a commercial product under the tradename Nemaslug[®] by BASF (formally Becker Underwood). It is also produced by Dudutech, who sell it under the trade name SlugTech[®]. Nemaslug[®], is currently sold in 15 European countries and contains the free-living, non-feeding, third-stage infective juvenile (IJ), which is also known as the dauer larva. The larvae concerned have the ability to infect slugs through the dorsal integumental pouch, and then to enter the shell cavity below the mantle. Once the nematodes are inside the slug, they recover to the feeding third juvenile stage and develop into adults, which, subsequently, reproduce, causing a swelling of the mantle. Death of the slug usually occurs within 4 to 21 days after exposure (Wilson *et al.*, 1993; Tan & Grewal, 2001). The nematodes then reproduce on the slug cadaver and, on depletion of the food source, enter the IJ stage, which leaves the cadaver in search of a new host (Wilson & Rae, 2015). The commercial strain of *P. hermaphrodita* is a monoxenic combination with the bacterium, *Moraxella osloensis* Bovre & Henriksen, although the relationship is not symbiotic,

but one that has been artificially created. *Phasmarhabditis hermaphrodita* can be successfully used to control a range of terrestrial molluscs, including members of the families Agriolimacidae, Arionidae, Milacidae, Limacidae and Vaginulidae (Rae *et al.*, 2007). The success of the product, along with its restricted use in non-native territories, has inspired researchers around the world to survey slug-parasitic nematodes, and to investigate their biocontrol potential, in the quest to launch the next commercial bio-molluscicide product (Ross, 2019).

With reference to Sudhaus (2011), the name *Phasmarhabditis* was regarded as a junior synonym of *Pellioiditis* (Dougherty) Timm, with the type species being *Pellioiditis pellio* (Schneider) Timm. However, such nomenclature has been rejected by Nermut *et al.* (2016a) and Ross *et al.* (2018), based on phylogenetic studies, and, therefore, the current paper describes the new species as being in the same genus referred to as *Phasmarhabditis*. The species within *Phasmarhabditis* are usually distinguished based on their morphometrics, the characteristics of the female tail, the spicule size, the presence or absence of males, and the bursal papillae, and are supported by molecular characterisation and phylogenetic analyses. The *Phasmarhabditis* genus has a worldwide continental distribution, with the exception of the Antarctica (France & Gerding, 2000; Azzam, 2003; Karimi *et al.*, 2003; Genena *et al.*, 2011; Ross *et al.*, 2012; Wilson *et al.*, 2012; Tandingan De Ley *et al.*, 2014; Ivanova & Spiridonov, 2017; Pieterse *et al.*, 2017; Waki, 2017; Ross *et al.*, 2018; Ivanova, Geraskina & Spiridonov, 2019), and three of the species have been found on the African continent, including *P. papillosa* (South Africa), *P. safricana* (South Africa) and *P. tawfiki* (Egypt) (Azzam, 2003; Ross *et al.*, 2018).

During a collaborative survey of the nematodes associated with terrestrial slugs in Kenya, a nematode belonging to *Phasmarhabditis* was isolated from the slug, *Polytoxon robustum* (Simroth) (Gastropoda: Urocyclidae), from Nairobi. The objective of the current study was to use the biology, morphology, morphometrics and molecular methods to describe a new species in the genus, as *Phasmarhabditis* sp. (KEN1) and to add to the body of knowledge concerning the occurrence and diversity of this diverse group of nematodes.

Materials and methods

COLLECTION OF SLUGS

The slug *P. robustum* was collected from a garden in Nairobi, Kenya. The slug was rinsed with 0.9% saline solution to remove any external nematodes, after which it was dissected, and

the internal nematodes were recovered. The nematodes were then maintained *in vivo* on defrosted, freeze-killed slugs, on a moistened Whatman No. 1 filter paper in a Petri dish.

IDENTIFICATION OF NEMATODES

Thirty nematodes of each life stage were observed for morphological identification and morphometric analysis. Ex-sheathed IJs were used for morphometric and morphological analysis. After being killed and fixed with hot (85°C) TAF (2% triethanolamine, 8% formalin in distilled water) (Courtney *et al.*, 1955), the nematodes were mounted on a glass microscope slide with a paraffin wax ring, which was covered with a cover slip. The slides were then viewed, and the measurements were taken with a compound microscope (Leica DM200, Leica Microsystems), fitted with a digital camera and using the Leica Application Suite V3.5.0 software, with live measurement capability. The bursa of the males were observed by transferring the males that were fixed in TAF to a drop of lactophenol with 0.002% acid fuchsin on a microscope slide for staining. After 20 min, the males were transferred to a clear drop of lactophenol, where their bursa was cut off, using the edge of a syringe needle, with the rest of the nematode being discarded. A cover slip was then added and gently pressed to position the bursa for ventral viewing (Nguyen *et al.*, 2004), then again gently pressed and moved around to separate the gubernaculum and spicules from the rest of the bursa for observation (Malan *et al.*, 2016).

SCANNING ELECTRON MICROSCOPY (SEM)

For SEM photos, the males, females and IJs were fixed in TAF, and left for 3 days, after which each nematode was washed three times in 0.05M cacodylate buffer for 15 min, and then dehydrated in a graded ethanol series (70%, 80%, 90%, 100%, and then again at 100%). The nematodes were then critical point dried using liquid carbon dioxide, mounted on SEM stubs and sputter coated with 20 nm gold/palladium (66/33%). The samples were then viewed, using an FEI Qanta 200 ESEM (Düren), operating at 10 kV in high-vacuum mode (Malan *et al.*, 2016).

MOLECULAR ANALYSES

After being individually picked, the live nematodes were placed in 200- μ l PCR tubes with a mix of Chelex[®] and Proteinase K, for the purpose of DNA extraction (Ross *et al.*, 2010). The samples were then subjected to a polymerase chain reaction (PCR) of the small subunit (SSU) rRNA gene (Ross *et al.*, 2010), the D2-D3 large subunit (LSU) rRNA gene (Nguyen, 2007;

Ivanova & Spiridonov, 2010) and the Internal Transcriber Spacer (ITS = ITS1, 5.8S, ITS2) rRNA region (Vrain *et al.*, 1992; Nadler *et al.*, 2005). The sequence traces were then inspected and assembled using CLC Main Workbench 7.6.4 (CLC Bio, Aarhus, Denmark), after which they were uploaded to the GenBank database (<http://www.ncbi.nlm.nih.gov/>), at the National Centre for Biotechnology Information (NCBI).

PHYLOGENETIC ANALYSES

Phylogenetic analyses of *Phasmarhabditis* sp. (KEN1) were conducted using the SSU, D2-D3 genes and ITS region, along with additional sequences downloaded from GenBank, giving a total of 31 sequences with SSU, 32 of D2-D3, and 23 of ITS sequences for the analyses. Representatives were chosen of the genera *Agfa* Dougherty, *Angiostoma* Dujardin and *Phasmarhabditis*, along with the genera *Oscheius* and *Pellioiditis* as outgroups. The sequences were aligned manually, using BioEdit Sequence Alignment Editor (Hall, 1999), and regions of ambiguous alignment were removed, leaving 406, 160 and 231 unambiguously aligned positions for SSU, D2-D3 and ITS, respectively. Maximum likelihood (ML), distance and maximum parsimony (MP) analyses were chosen for phylogenetic analyses, using the software packages PHYML (Guindon & Gascuel, 2003) and PHYLIP (Felsenstein, 2007). Sequences were analysed using Modeltest, with a time-reversible (GTR) model, and an eight-category gamma correction, with the fraction of the invariant sites being generated from ML analysis. Bootstrap support included 1000 replicates, with the figures 65% and above being included in the tree.

Results

***Phasmarhabditis* sp. (KEN1) n. sp.**

MEASUREMENTS

See Tables 4.1-4.4.

Table 4.1. Morphometrics of *Phasmarhabditis* sp. (KEN1). Measurements are in μm and in the form: mean \pm s.d. (range).

Character	Male		Female	Infective juvenile
	Holotype	Paratype		
n	-	20	20	20
L	1992	1989 \pm 77 (1835-2034)	2151 \pm 178 (1918-2340)	1232 \pm 105 (1107-1336)
a	23.1	23.1 \pm 1.2 (20.9-24.2)	22.1 \pm 1.3 (20.0-24.1)	27.6 \pm 2.0 (25.3-30.5)
b	8.1	8.7 \pm 1.4 (7.9-11.5)	8.3 \pm 0.5 (7.6-8.9)	6.4 \pm 0.5 (5.7-7.2)
c	31.9	39.7 \pm 7.8 (31.9-49.7)	12.9 \pm 1.0 (11.6-14.2)	6.9 \pm 0.4 (6.3-7.5)
c'	1.2	1.1 \pm 0.2 (0.7-1.3)	3.9 \pm 0.3 (3.4-4.3)	6.6 \pm 0.5 (5.8-7.3)
V	-	-	50.5 \pm 1.0 (48.8-51.9)	-
Body diam.	86	86 \pm 2.0 (83-89)	98 \pm 10.1 (85-117)	45 \pm 4.8 (38-50)
Stoma length	21	25 \pm 6.8 (20-38)	21 \pm 1.5 (19-23)	-
Stoma diam.	7.1	6.2 \pm 2.0 (4.2-9.2)	6.9 \pm 0.9 (6.0-8.5)	-
Excretory pore	254	248 \pm 25.0 (201-276)	241 \pm 11.5 (225-253)	178 \pm 9.5 (170-192)
Pharynx length	246	233 \pm 36.3 (160-256)	259 \pm 9.4 (248-271)	192 \pm 11.5 (183-214)
Corpus length	142	136 \pm 18.1 (99-146)	149 \pm 3.0 (144-153)	-
Metacorpall expansion	26	27 \pm 0.8 (26-28)	29 \pm 1.0 (27-30)	-
Basal bulb diam.	37	37 \pm 0.7 (36-38)	41 \pm 2.5 (37-45)	19 \pm 1.8 (17-22)
Nerve ring	170	171 \pm 17.0 (142-194)	185 \pm 6.7 (177-194)	148 \pm 11.3 (138-168)
Anal body diam.	52	49 \pm 2.4 (45-52)	44 \pm 3.6 (38-49)	27 \pm 3.3 (25-34)
Tail	55	52 \pm 10.5 (37-63)	168 \pm 16.6 (136-186)	179 \pm 11.9 (165-196)
Spicule length	73	71 \pm 10.2 (57-81)	-	-
Gubernaculum length	31	32 \pm 4.1 (25-36)	-	-

Table 4.2. Morphometric comparison of *Phasmarhabditis* species males grown *in vivo*. Measurements are in μm and in the form: mean \pm s.d. (range).

Character	<i>Phasmarhabditis</i> sp. (KEN1)	<i>P.</i> <i>safricana</i>	<i>P.</i> <i>papillosa</i> SA	<i>P.</i> <i>circassica</i>	<i>P.</i> <i>clausiliae</i>	<i>P.</i> <i>apuliae</i> BAR	<i>P.</i> <i>bohemica</i> CH1	<i>P.</i> <i>bonaquaense</i> NDV	<i>P.</i> <i>neopapillosa</i> UK	<i>P.</i> <i>meridionalis</i>	<i>P.</i> <i>tawfiki</i>
n	20	14	25	10	10	9	15	20	20	9	10
L	1989 \pm 77 (1835-2034)	1477 \pm 139 (1220-1702)	1529 \pm 79 (1390-1679)	1200 \pm 236 (916-1607)	1183 \pm 221 (905-1540)	2096 \pm 128 (1898-2363)	1683 \pm 91 (1515-1818)	1829 \pm 224 (1414-2121)	1585 \pm 90 (1432-1771)	1317 \pm 140 (1159-1526)	1337 \pm 159 (980-1535)
a	23.1 \pm 1.2 (20.9-24.2)	22.6 \pm 2.0 (20.7-29.9)	25.3 \pm 2.0 (20.6-28.3)	24.4 \pm 2.7 (20.6-29.2)	23.6 \pm 3.6 (16.8-28.6)	21.8 \pm 3.1 (18.8-29.3)	19.2 \pm 2.2 (15.4-22.5)	20.8 \pm 3.9 (14.0-27.7)	18.9 \pm 1.3 (16.7-21.3)	22.1 \pm 2.0 (19.2-25.8)	17.9 \pm 1.7 (14.8-19.9)
b	8.7 \pm 1.4 (7.9-11.5)	7.6 \pm 0.8 (6.8-9.6)	6.9 \pm 0.6 (5.9-8.1)	6.3 \pm 0.6 (4.9-6.9)	6.2 \pm 1.1 (4.6-7.9)	8.3 \pm 0.6 (7.4-9.7)	7.7 \pm 0.6 (6.8-8.9)	7.7 \pm 1.3 (5.9-10.5)	6.5 \pm 0.6 (5.9-7.6)	7.1 \pm 0.5 (6.5-7.6)	5.8 \pm 0.9 (4.8-7.6)
c	39.7 \pm 7.8 (31.9-49.7)	38.7 \pm 7.8 (29.4-56.7)	29.9 \pm 2.1 (25.8-35.4)	37.2 \pm 5.1 (29.2-45.9)	45.1 \pm 9.6 (28.3-61.6)	37.2 \pm 2.9 (32.4-40.5)	42.5 \pm 4.1 (35.2-51.1)	36.1 \pm 5.7 (25.8-45.2)	32.2 \pm 2.7 (27.5-33.7)	41.5 \pm 3.9 (34.2-46.8)	27.5 \pm 4.6 (18.2-34.9)
c'	1.1 \pm 0.2 (0.7-1.3)	1.0 \pm 0.2 (0.8-1.3)	1.3 \pm 0.1 (1.1-1.5)	1.1 \pm 0.1 (0.9-1.4)	0.9 \pm 0.2 (0.7-1.2)	1.4 \pm 0.1 (1.2-1.6)	1.2 \pm 0.1 (1.0-1.5)	1.4 \pm 0.1 (1.2-1.6)	1.1 \pm 0.1 (0.9-1.2)	-	-
Body diam.	86 \pm 2.0 (83-89)	65 \pm 5.0 (55-74)	61 \pm 4.7 (53-74)	49 \pm 7.0 (40-64)	52 \pm 15.0 (35-87)	96 \pm 8.4 (80-101)	89 \pm 9.9 (80-101)	90 \pm 10.9 (70-101)	84 \pm 7.8 (78-94)	60 \pm 7.0 (45-67)	81 \pm 9.1 (65-100)
Stoma length	25 \pm 6.8 (20-38)	20 \pm 1.5 (18-24)	20 \pm 1.1 (18-22)	20 \pm 1 (18-22)	17 \pm 1 (16-18)	16 \pm 0.7 (16-18)	16 \pm 1.0 (14-18)	20 \pm 1.6 (18-22)	19 \pm 0.8 (17-20)	-	21 \pm 2.1 (17-25)
Stoma diam.	6.2 \pm 2.0 (4.2-9.2)	4.2 \pm 0.4 (4.0-5.0)	5.4 \pm 0.5 (4.6-6.8)	4.0 \pm 1.0 (3.0-6.0)	5.0 \pm 1.0 (4.0-7.0)	6.2 \pm 1.2 (4.8-8.0)	5.0 \pm 0.5 (4.8-6.4)	5.8 \pm 0.7 (4.8-6.4)	-	-	-
Excretory pore	248 \pm 25.0 (201-276)	174 \pm 17.0 (140-190)	205 \pm 13.1 (178-226)	163 \pm 22.0 (140-192)	157 \pm 12.0 (144-175)	223 \pm 8.8 (203-234)	191 \pm 8.0 (183-207)	196 \pm 12.2 (164-211)	196 \pm 10.6 (185-220)	176 \pm 13.0 (164-190)	191 \pm 36.3 (113-250)
Pharynx length	233 \pm 36.3 (160-256)	195 \pm 13.0 (169-210)	224 \pm 14.2 (201-257)	191 \pm 29.0 (147-244)	193 \pm 25.0 (152-233)	235 \pm 6.1 (226-246)	202 \pm 10.5 (185-227)	220 \pm 18.4 (176-252)	246 \pm 11.0 (223-261)	185 \pm 11.0 (174-208)	-
Corpus length	136 \pm 18.1 (99-146)	116 \pm 10.0 (100-130)	130 \pm 8.0 (118-148)	110 \pm 20.0 (87-152)	111 \pm 12.0 (90-131)	-	-	-	119 \pm 4.5 (114-128)	-	114 \pm 23.6 (80-160)
Metacarpal expansion	27 \pm 0.8 (26-28)	22 \pm 2.0 (20-25)	30 \pm 2.7 (24-34)	24 \pm 3.0 (19-29)	22 \pm 3.0 (19-29)	-	-	-	-	-	-
Basal bulb	37 \pm 0.7 (36-38)	32 \pm 2.0 (28-36)	38 \pm 2.6 (34-44)	25 \pm 4.0 (15-30)	29 \pm 4.0 (24-35)	37 \pm 3.2 (31-39)	33 \pm 2.4 (27-35)	24 \pm 2.8 (27-35)	-	-	-
Nerve ring	171 \pm 17.0 (142-194)	142 \pm 12.0 (113-160)	165 \pm 10.2 (149-184)	138 \pm 21.0 (108-167)	133 \pm 17.0 (103-158)	181 \pm 4.6 (176-191)	160 \pm 9.0 (145-176)	172 \pm 13.9 (140-195)	164 \pm 8.8 (151-177)	141 \pm 7.0 (135-154)	152 \pm 27.0 (113-200)
Anal body diam.	49 \pm 2.4 (45-52)	39 \pm 5.0 (30-48)	41 \pm 2.3 (37-45)	30 \pm 3.0 (25-32)	29 \pm 4.0 (26-38)	40 \pm 3.6 (35-47)	33 \pm 3.1 (27-35)	38 \pm 2.7 (31-43)	-	-	-
Tail	52 \pm 10.5 (37-63)	39 \pm 6.0 (30-51)	51 \pm 3.2 (46-59)	32 \pm 4.0 (27-40)	27 \pm 4.0 (23-33)	57 \pm 2.7 (51-59)	40 \pm 3.3 (35-47)	51 \pm 3.0 (47-55)	49 \pm 2.8 (45-54)	32 \pm 5.0 (25-38)	49 \pm 3.9 (44-54)
Spicule length	71 \pm 10.2 (57-81)	61 \pm 8.0 (53-83)	62 \pm 3.4 (55-69)	58 \pm 5.0 (47-66)	54 \pm 8.0 (46-67)	81 \pm 4.3 (73-87)	74 \pm 5.0 (66-82)	77 \pm 4.8 (68-86)	68 \pm 2.8 (60-71)	76 \pm 4.0 (71-83)	63 \pm 8.2 (54-75)
Gubernaculum length	32 \pm 4.1 (25-36)	32 \pm 3.0 (25-34)	31 \pm 2.2 (27-34)	26 \pm 3.0 (20-40)	28 \pm 5.0 (23-35)	44 \pm 5.4 (35-49)	35 \pm 3.6 (27-39)	36 \pm 3.3 (29-40)	33 \pm 1.7 (31-37)	43 \pm 2.0 (40-46)	36 \pm 3.2 (33-40)

Table 4.3. Morphometric comparison of *Phasmarhabditis* species females grown *in vivo*. Measurements are in μm and in the form: mean \pm s.d. (range).

Character	<i>Phasmarhabditis</i> sp. (KEN1)	<i>P. safricana</i>	<i>P. papillosa</i> (SA)	<i>P. hermaphrodita</i> (UK)	<i>P. circassica</i>	<i>P. clausiliae</i>	<i>P. apuliae</i> (BAR)	<i>P. bohemica</i> (CH1)	<i>P. bonaquaense</i> (NDV)	<i>P. neopapillosa</i> (UK)	<i>P. meridionalis</i>	<i>P. tawfikii</i>
n	20	11	25	20	13	14	15	15	20	20	8	20
L	2151 \pm 178 (1918-2340)	1598 \pm 300 (1160-2071)	1923 \pm 171 (1622-2380)	1799 \pm 279 (1509-2372)	1830 \pm 291 (1522-2510)	1276 \pm 197 (1050-1730)	2623 \pm 163 (2262-2848)	2079 \pm 126 (1777-2222)	2349 \pm 186 (1878-2626)	2227 \pm 190 (1817-2449)	1612 \pm 294 (1057-1931)	1716 \pm 347 (1150-2380)
a	22.1 \pm 1.3 (20.0-24.1)	15.6 \pm 1.4 (13.2-17.8)	18.3 \pm 2.0 (14.2-22.2)	19.5 \pm 3.1 (13.6-28.9)	21.7 \pm 1.8 (18.9-25.1)	22.0 \pm 2.7 (16.2-25.4)	17.3 \pm 1.9 (15.4-22.3)	14.4 \pm 1.3 (10.6-15.7)	17.4 \pm 2.3 (13.3-21.7)	16.0 \pm 1.8 (14.6-16.2)	17.8 \pm 3.1 (14.3-24.6)	17.3 \pm 2.5 (12.0-20.0)
b	8.3 \pm 0.5 (7.6-8.9)	7.9 \pm 0.8 (6.7-9.3)	7.4 \pm 0.7 (6.5-9.5)	7.2 \pm 1.1 (5.9-9.3)	7.5 \pm 0.5 (6.7-8.6)	6.0 \pm 0.7 (5.1-7.2)	9.2 \pm 0.8 (8.0-10.9)	8.2 \pm 0.6 (7.3-9.5)	8.8 \pm 0.8 (6.9-10.5)	7.7 \pm 0.5 (7.2-8.4)	7.6 \pm 1.0 (5.6-8.5)	6.5 \pm 1.0 (4.9-8.7)
c	12.9 \pm 1.0 (11.6-14.2)	26.6 \pm 5.6 (18.3-37.4)	17.9 \pm 1.9 (13.9-21.0)	15.8 \pm 2.8 (13.2-24.0)	22.7 \pm 3.4 (16.8-27.5)	13.7 \pm 2.5 (10.3-18.4)	17.5 \pm 2.3 (14.1-21.3)	20.3 \pm 3.6 (14.2-27.6)	28.2 \pm 5.0 (20.0-38.0)	14.2 \pm 1.2 (12.1-16.9)	24.2 \pm 2.3 (19.0-32.9)	11.1 \pm 3.3 (5.9-15.6)
c'	3.9 \pm 0.3 (3.4-4.3)	1.7 \pm 0.2 (1.4-2.0)	2.0 \pm 0.2 (1.6-2.4)	3.0 \pm 0.3 (2.4-3.6)	2.4 \pm 0.4 (1.8-3.0)	3.2 \pm 0.8 (2.2-5.3)	3.4 \pm 0.4 (2.7-3.8)	2.3 \pm 0.4 (1.7-2.9)	1.2 \pm 0.1 (1.0-1.3)	3.9 \pm 0.5 (3.3-5.0)	-	-
V	50.5 \pm 1.0 (48.8-51.9)	51.5 \pm 0.9 (51.1-54.0)	49.3 \pm 4.2 (40.2-56.0)	51.0 \pm 2.6 (48.0-60.0)	53.1 \pm 0.0 (49.3-56.3)	53.9 \pm 0.0 (47.4-59.3)	48.8 \pm 4.3 (44.0-58.3)	50.3 \pm 5.2 (38.9-62.5)	54.0 \pm 6.0 (45.6-66.7)	51.0 \pm 1.1 (48.0-52.0)	52.5 \pm 1.8 (50.3-56.1)	-
Body diam.	98 \pm 10.1 (85-117)	104 \pm 26.0 (65-155)	107 \pm 17.4 (79-146)	94 \pm 15.8 (71-118)	85 \pm 16.0 (68-124)	59 \pm 12.0 (42-80)	153 \pm 17.4 (111-171)	145 \pm 10.9 (141-181)	136 \pm 11.6 (121-161)	141 \pm 19.2 (101-174)	94 \pm 27.0 (43-134)	96 \pm 13.2 (70-110)
Stoma length	21 \pm 1.5 (19-23)	20 \pm 3.2 (12-23)	23 \pm 4.1 (20-34)	19 \pm 0.8 (17-20)	23 \pm 2.0 (21-27)	19 \pm 2.0 (17-24)	21.5 \pm 0.8 (21-22)	17 \pm 1.2 (16-19)	20 \pm 1.4 (18-22)	21 \pm 1.2 (19-24)	-	20 \pm 1.7 (17-24)
Stoma diam.	6.9 \pm 0.9 (6.0-8.5)	5.4 \pm 1.2 (4.0-8.0)	5.5 \pm 1.1 (3.0-7.2)	-	6.0 \pm 1.0 (5.0-7.0)	6.0 \pm 1.0 (5.0-8.0)	7.0 \pm 0.8 (6.4-8.0)	5.3 \pm 0.7 (4.8-6.4)	6.5 \pm 0.4 (6.4-8.0)	-	-	-
Excretory pore	241 \pm 11.5 (225-253)	178 \pm 31.0 (140-235)	230 \pm 16.1 (200-287)	212 \pm 11.0 (192-231)	211 \pm 26.0 (168-278)	171 \pm 27.0 (145-235)	249 \pm 11.6 (222-258)	204 \pm 6.6 (196-215)	231 \pm 12.9 (203-246)	216 \pm 10.6 (199-231)	172 \pm 23.0 (142-210)	223 \pm 24.2 (165-270)
Pharynx length	259 \pm 9.4 (248-271)	202 \pm 33.0 (147-259)	260 \pm 18.9 (232-302)	251 \pm 10.4 (235-270)	243 \pm 30.0 (210-320)	215 \pm 30.0 (180-282)	250 \pm 9.2 (230-265)	237 \pm 9.8 (217-256)	249 \pm 12.7 (228-276)	290 \pm 8.0 (277-303)	212 \pm 16.0 (185-230)	245 \pm 27.7 (178-270)
Corpus length	149 \pm 3.0 (144-153)	120 \pm 20.0 (86-150)	148 \pm 15.8 (108-183)	115 \pm 5.6 (109-126)	146 \pm 29.0 (123-230)	125 \pm 17.0 (106-159)	-	-	-	144 \pm 10.7 (126-168)	-	126 \pm 18.4 (100-156)
Metacarpal expansion	29 \pm 1.0 (27-30)	32 \pm 1.6 (29-34)	35 \pm 4.1 (29-43)	-	30 \pm 3.0 (27-35)	24 \pm 3.0 (20-30)	-	-	-	-	-	-
Basal bulb diam.	41 \pm 2.5 (37-45)	36 \pm 5.0 (28-43)	45 \pm 4.4 (36-57)	-	34 \pm 2.0 (30-39)	27 \pm 3.0 (20-30)	39 \pm 2.0 (35-43)	39 \pm 2.7 (35-47)	40 \pm 2.6 (35-43)	-	-	-
Nerve ring	185 \pm 6.7 (177-194)	145 \pm 22.0 (106-183)	185 \pm 15.8 (163-217)	166 \pm 7.4 (154-177)	170 \pm 24.0 (140-238)	155 \pm 22.0 (126-195)	216 \pm 4.3 (207-222)	184 \pm 7.9 (168-196)	202 \pm 11.6 (188-215)	188 \pm 11.3 (168-205)	154 \pm 16.0 (133-180)	168 \pm 37.6 (135-200)
Anal body diam.	44 \pm 3.6 (38-49)	37 \pm 7.0 (28-50)	54 \pm 4.9 (46-69)	-	37 \pm 7.0 (20-45)	30 \pm 6.0 (20-40)	45 \pm 4.0 (39-51)	46 \pm 2.7 (43-51)	72 \pm 8.3 (59-86)	-	-	-
Tail	168 \pm 16.6 (136-186)	61 \pm 13.0 (46-87)	108 \pm 11.7 (87-143)	114 \pm 7.8 (99-129)	82 \pm 21.0 (54-125)	94 \pm 13.0 (79-111)	152 \pm 16.7 (125-187)	105 \pm 16.0 (78-137)	85 \pm 12.9 (67-110)	157 \pm 15.3 (141-174)	68 \pm 17.0 (44-97)	128 \pm 22.7 (85-140)

Table 4.4. Morphometric comparison of *Phasmarhabditis* species IJs grown *in vivo*. Measurements are in μm and in the form: mean \pm s.d. (range).

Character	<i>Phasmarhabditis</i> sp. (KEN1)	<i>P. safricana</i>	<i>P. papillosa</i> SA	<i>P. circassica</i>	<i>P. clausiliiae</i>	<i>P. apuliae</i> BAR	<i>P. bohemica</i> CHI	<i>P. bonaquaense</i> NDV	<i>P. neopapillosa</i> UK	<i>P. meridionalis</i>	<i>P. tawfiki</i>
n	20	4	25	12	17	20	16	20	20	9	20
L	1232 \pm 105 (1107-1336)	543 \pm 71 (497-648)	900 \pm 63 (813-1042)	896 \pm 43 (813-982)	742 \pm 60 (620-805)	812 \pm 53 (707-888)	553 \pm 46 (474-636)	902 \pm 77 (808-1050)	1010 \pm 26 (955-1063)	839 \pm 45 (770-912)	966 \pm 109 (750-1140)
a	27.6 \pm 2.0 (25.3-30.5)	24.1 \pm 1.7 (22.0-25.9)	28.0 \pm 1.6 (25.5-32.9)	24.6 \pm 1.7 (22.2-27.3)	24.4 \pm 1.6 (21.7-27.0)	29.2 \pm 5.6 (23.3-42.0)	22.2 \pm 4.4 (15.7-29.0)	30.7 \pm 4.0 (21.7-38.4)	23.8 \pm 0.7 (22.4-24.8)	24.5 \pm 1.2 (23.0-25.8)	23.8 \pm 2.3 (18.8-27.5)
b	6.4 \pm 0.5 (5.7-7.2)	4.7 \pm 0.8 (4.0-5.9)	5.4 \pm 0.4 (4.9-6.3)	5.6 \pm 0.3 (5.3-6.0)	4.8 \pm 0.3 (4.2-5.4)	5.1 \pm 0.4 (4.5-5.7)	4.3 \pm 0.4 (3.7-5.1)	5.3 \pm 0.6 (4.6-6.7)	5.6 \pm 0.2 (5.4-6.0)	5.4 \pm 0.2 (5.3-5.7)	4.9 \pm 0.7 (4.2-6.6)
c	6.9 \pm 0.4 (6.3-7.5)	9.8 \pm 0.9 (8.7-9.8)	7.0 \pm 0.4 (6.5-8.4)	7.8 \pm 0.6 (7.2-8.6)	7.2 \pm 0.8 (5.9-8.6)	6.9 \pm 0.5 (6.1-7.9)	7.0 \pm 0.9 (5.5-9.3)	11.2 \pm 1.5 (8.3-13.8)	7.2 \pm 0.4 (6.2-7.8)	7.4 \pm 0.5 (7.0-8.0)	7.0 \pm 1.3 (5.0-8.8)
c'	6.6 \pm 0.5 (5.8-7.3)	9.8 \pm 0.9 (8.7-9.8)	6.4 \pm 0.5 (4.9-7.1)	5.9 \pm 1.2 (4.4-8.3)	5.8 \pm 0.6 (4.6-6.8)	6.9 \pm 0.9 (5.6-8.5)	5.3 \pm 1.0 (3.8-7.7)	4.5 \pm 0.5 (3.8-5.3)	5.8 \pm 0.5 (4.8-6.8)	-	-
Body diam.	45 \pm 4.8 (38-50)	23 \pm 2.0 (20-25)	32 \pm 2.0 (29-36)	37 \pm 3.0 (32-43)	31 \pm 3.0 (27-34)	29 \pm 3.7 (20-30)	26 \pm 5.0 (20-30)	30 \pm 3.4 (24-39)	42 \pm 1.2 (40-44)	34 \pm 2.0 (32-38)	40 \pm 3.8 (30-45)
Excretory pore	178 \pm 9.5 (170-192)	111 \pm 8.0 (105-116)	-	137 \pm 6.0 (128-146)	121 \pm 10.0 (107-137)	148 \pm 7.8 (138-159)	105 \pm 5.7 (92-119)	151 \pm 10.3 (129-172)	154 \pm 4.6 (148-159)	125 \pm 4.0 (120-130)	173.8 \pm 21.3 (143-200)
Nerve ring	148 \pm 11.3 (138-168)	80 \pm 15.0 (65-95)	115 \pm 9.5 (102-145)	114 \pm 6.0 (105-130)	105 \pm 7.0 (95-119)	120 \pm 7.8 (111-135)	84 \pm 7.8 (72-103)	123 \pm 7.9 (110-137)	119 \pm 4.1 (111-123)	114 \pm 6.0 (103-122)	137 \pm 17.8 (100-196)
Pharynx length	192 \pm 11.5 (183-214)	117 \pm 9.0 (110-129)	167 \pm 14.0 (147-207)	161 \pm 8.0 (147-169)	154 \pm 7.0 (140-167)	141 \pm 5.4 (133-153)	108 \pm 6.0 (98-119)	149 \pm 8.0 (134-162)	180 \pm 7.3 (168-187)	156 \pm 5.0 (149-164)	181 \pm 28.5 (154-255)
Basal bulb diam.	19 \pm 1.8 (17-22)	10 \pm 2.0 (8-12)	15 \pm 1.2 (13-18)	13 \pm 3.0 (11-22)	13 \pm 1.0 (13-14)	16 \pm 1.0 (14-18)	10 \pm 1.1 (8-13)	18 \pm 1.8 (14-21)	-	-	-
Anal body diam.	27 \pm 3.3 (25-34)	-	20 \pm 1.9 (18-25)	20 \pm 3.0 (15-25)	18 \pm 1.0 (15-20)	17 \pm 1.9 (16-20)	15 \pm 2.2 (12-20)	18 \pm 1.8 (16-21)	-	-	-
Tail	179 \pm 11.9 (165-196)	57 \pm 4.0 (52-60)	128 \pm 6.8 (113-148)	117 \pm 13.0 (90-134)	104 \pm 11.0 (82-123)	118 \pm 7.4 (109-132)	80 \pm 7.7 (58-93)	82 \pm 7.8 (72-102)	141 \pm 7.7 (131-163)	116 \pm 13.0 (100-135)	144 \pm 29.3 (105-180)

DESCRIPTION

Male

Common in *in vivo* cultures. Body shorter and smaller than that of the female. Body straight, with a slight posterior curve when heat-killed. Body smooth, with slight transverse striation only visible with SEM. Lateral field with three ridges and four lines. Body tapering to blunt anterior end, with lips continuous with body. Six lips divided into pairs, each with one prominent inner labial papilla and one cephalic papilla, situated posterior to labial papilla. Stoma triangular, cylindrical and cuticularised, with a length of 25 (20-38) μm and a diameter of 6.2 (4.2-9.2) μm . Pharynx with cylindrical procorpus, slightly swollen metacarpus, a thin isthmus surrounded by nerve ring near the anterior end of a swollen basal bulb. Excretory pore inconspicuous and located posterior to basal bulb. Spicules, gubernaculum and peloderan, proximally open bursa making up copulatory apparatus. Bursa bearing nine bilateral pairs of genital bursal papillae, and one pair of papilliform phasmids flanking the tail. Pairs 1-5 of the rays of the genital papillae located anterior to cloacal aperture, and pairs 6-9 post-cloacal, with the last three pairs of rays grouped together. Each ray with a single papilla, with the first ray located beyond the bursal rim, and its papillae located on the tip of rays. Fifth and ninth papillae located dorsally, and the other six ventrally. A precloacal papilla located on the anterior cloacal lip, pair of cone-shaped sublateral papillae present posterior to cloacal opening, and only visible with SEM. Spicules cephalate, paired and separate, with an arc length of 71 (57-81) μm . Spicule blades slightly curved, and gradually tapering to a pointed terminus. Gubernaculum short and *ca* 50% of spicule length. Tail short and blunt, flanked by pair of papilliform phasmids. See Figure 4.1C, G, H and Figure 4.2.

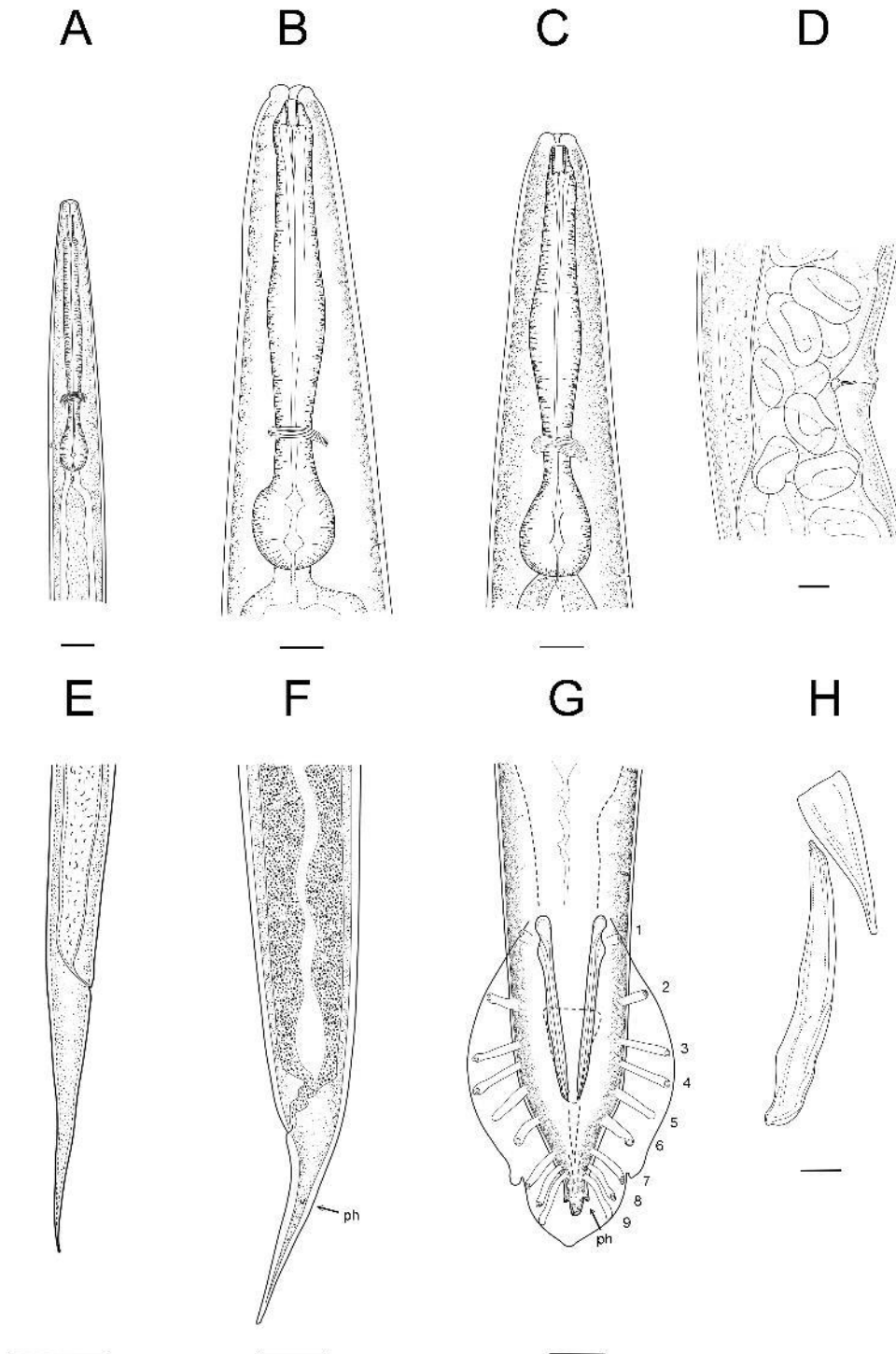


Figure 4.1. Line drawings of *Phasmarhabditis* sp. (KEN1). A: Infective juvenile anterior region; B: Female anterior; C: Male anterior; D: Female vulval region; E: IJ tail region, lateral view; F: Female tail region, lateral view; G: Male tail region, ventral view; H: Male gubernaculum (top) and spicule (bottom). (Scale bars: A, E, F = 50 μm ; B, C, D, G = 20 μm ; H = 10 μm).

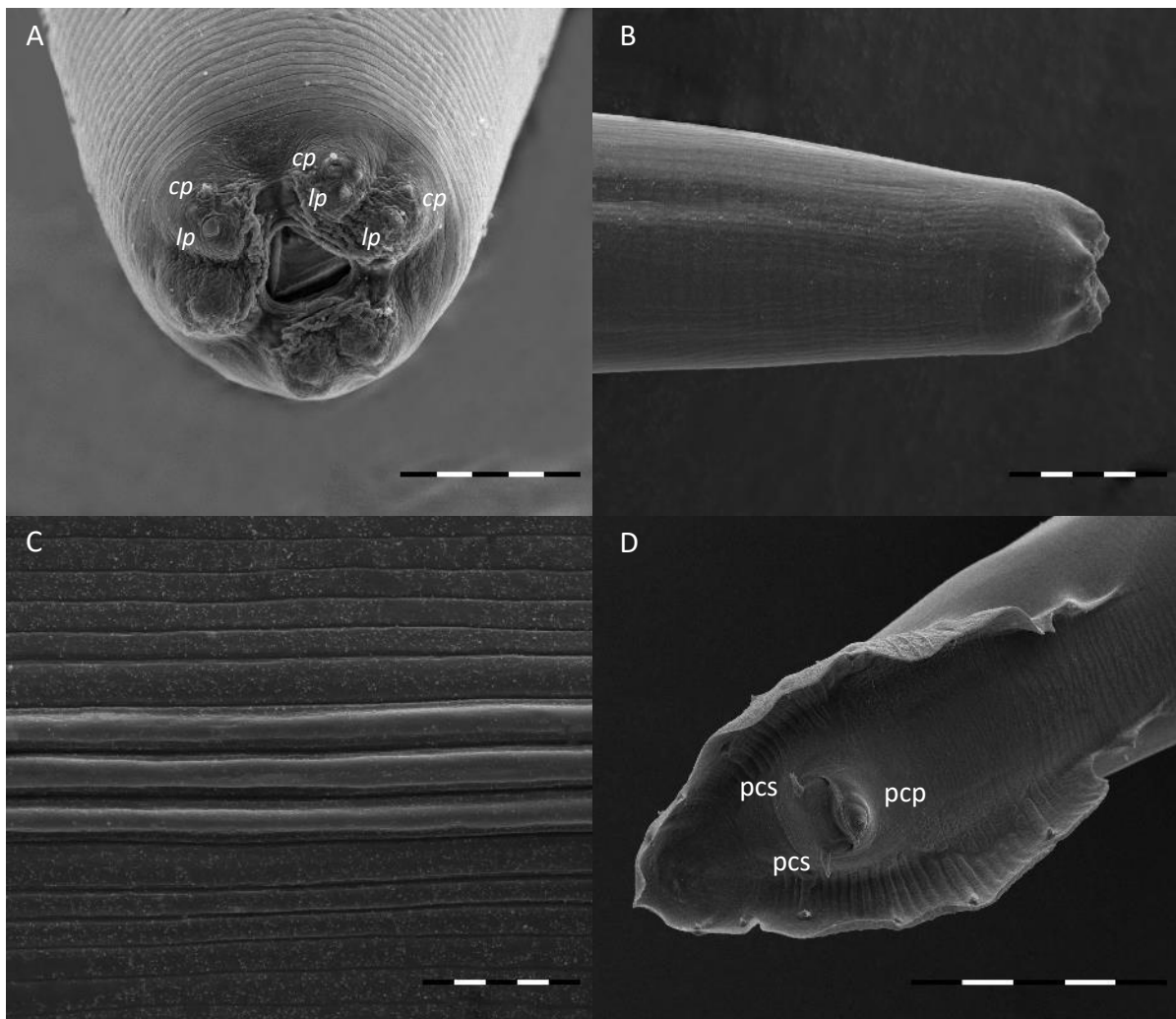


Figure 4.2. *Phasmarhabditis* sp. (KEN1) male. A: *En face* view; B: Anterior end; C: Lateral field; D: Male caudal region with open bursa and nine pairs of papillae. Scale bars: A = 10 μm ; B = 20 μm ; C = 5 μm ; D = 50 μm . Abbreviations: cp = cephalic papilla, lp = labial papilla, pcs = post-cloacal sensilla, pcp = precloacal papilla.

Female

Body 1.9-2.3 mm long and slightly bent in the middle when heat-killed, with transverse striations visible with SEM. Lateral field developed, with three ridges and two incisions. Body tapers to a blunt anterior end. Lip region protruding slightly from body, with six lips grouped into pairs. Each lip with one prominent inner labial papilla, and one slightly less prominent outer labial papilla. Mouth triangular and cylindrical. Stoma moderately cuticularised, with a length of 21 (19-23) μm and a diameter of 6.9 (6.0-8.5) μm . Procorpus of pharynx cylindrical, metacarpus slightly swollen, isthmus narrow and surrounded in the middle by nerve ring. Basal bulb large, with excretory pore situated opposite middle of the basal bulb. Excretory duct well cuticularised. Reproductive system didelphic, amphidelphic and reflexed. In mature females, gonads filled with round oocytes, eggs often hatching inside female body. Vulva located at midbody region (51%), a closed transverse slit with protruding lips. Anus an arcuate slit. Tail conoid shape, gradually tapering to a sharp tip, with two phasmids located at *ca* 40% of tail length. See Figure 4.1B, D, F and Figure 4.3.

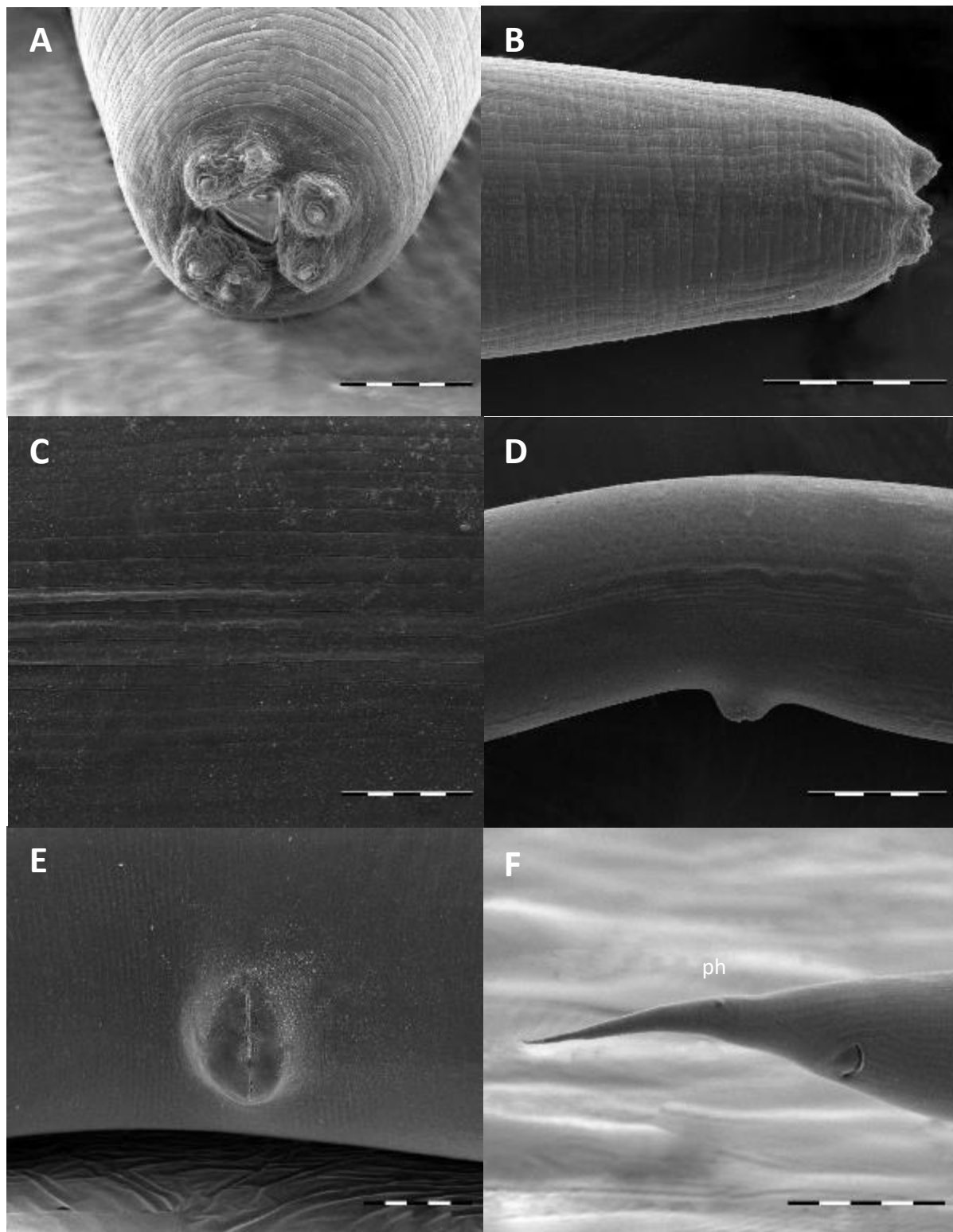


Figure 4.3. *Phasmarhabditis* sp. (KEN1) female. A: *En face* view; B: Anterior end; C: Lateral field; D: Midbody region with vulva; E: Vulval region; F: Tail region. Scale bars: A = 10 μm ; B = 20 μm ; C = 10 μm ; D = 50 μm ; E = 20 μm ; F = 50 μm .

Infective juvenile

Body shorter and slenderer than that of adult stages, with a body length of 1.1-1.3 mm. Straight when heat-killed. Cuticle with clear transversal and longitudinal striations. Longitudinal striations ending near head region. Head rounded, not offset from body contour. Flat lip region with six lips, grouped in pairs. Mouth aperture closed. Amphidial apertures small and closed, situated on the side of the head. Weakly sclerotised excretory pore located across from basal bulb. Isthmus long and thin, surrounded by nerve ring 148 (138-168) μm from anterior end. Anus crescent-shaped transverse slit. Tail long and tapering to a sharp point. Hyaline region undeveloped and phasmids not observed. See Figure 4.1A, E and Figure 4.4.

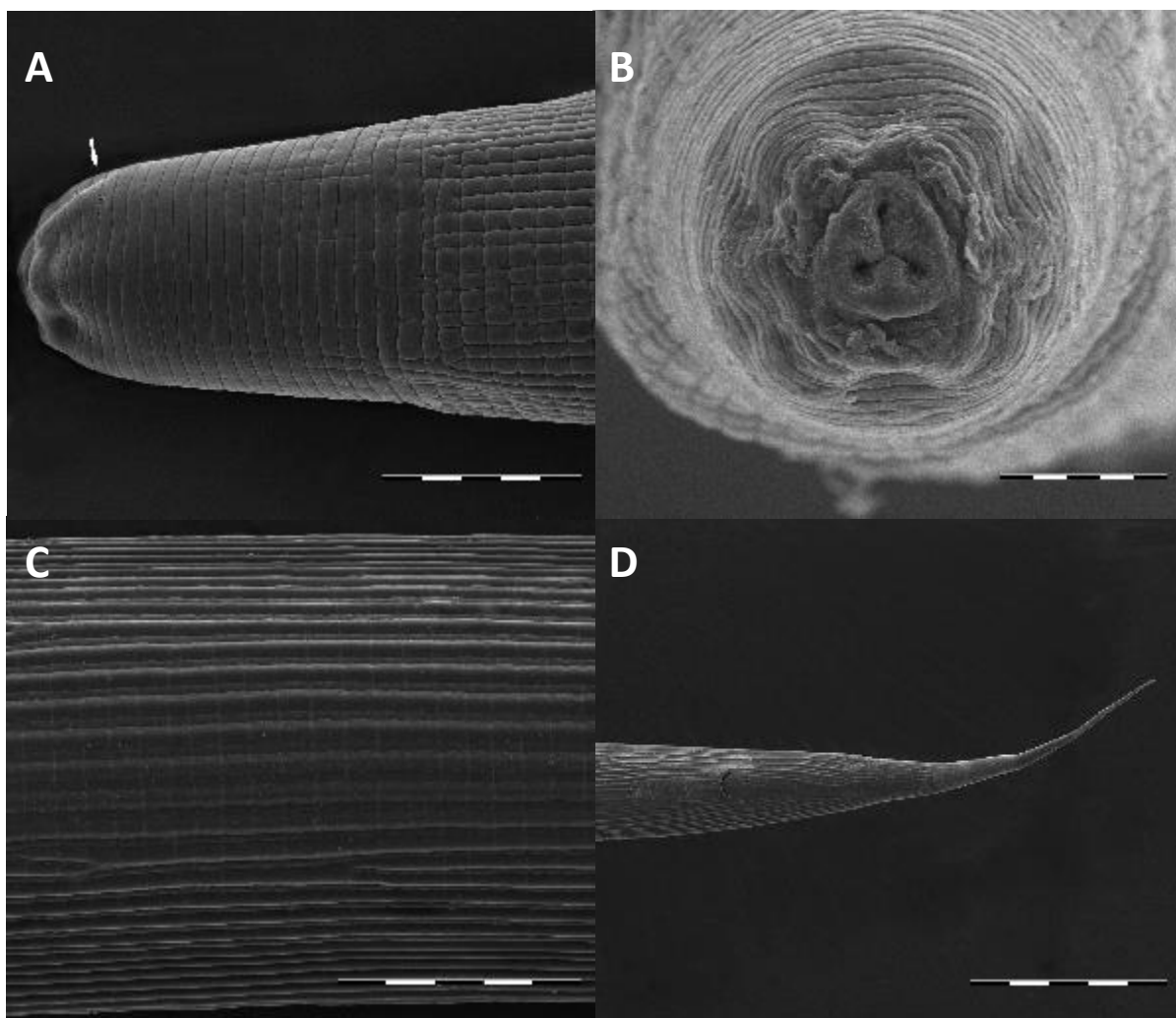


Figure 4.4. *Phasmarhabditis* sp. (KEN1) infective juvenile (ensheathed). A: Anterior end with amphid visible; B: *En face* view; C: Lateral field with sheath; D: Tail region. Scale bars: A = 10 μm ; B = 5 μm ; C = 20 μm ; D = 50 μm .

TYPE LOCALITY AND HABITAT

Type specimens are nematodes isolated from the slug *Polytoxon robustum*, which was collected from Nairobi, Kenya, (1°13'40.5"S, 36°50'56.4"E) between April and May 2018.

TYPE MATERIAL

The holotype male and paratypes males, females and infective juveniles were deposited in the National Museums of Kenya repository.

DIAGNOSIS AND RELATIONSHIPS

Phasmarhabditis sp. (KEN1) is characterised by the morphometrics and morphology of the female, male and IJ (Table 4.1). The male is characterised by a spicule length of 71 (57-81) μm , a gubernaculum length of 32 (25-36) μm , and a bursa with nine pairs of papillae. The female is characterised by a body length of 2151 (1918-2340) μm , a body diameter of 98 (85-117) μm , and a conical, tapering tail. The IJ is characterised by the longest body length in the genus, measuring 1232 (1107-1336) μm , a body diameter of 45 (38-50) μm , and a long tail, measuring 179 (165-196) μm .

Table 4.1 summarises the morphometric data of the males, females and IJs of *Phasmarhabditis* sp. (KEN1), respectively, with it being used as a reference for the following comparison of the new species with other known *Phasmarhabditis* species. As the food source and bacterial species that *Phasmarhabditis* species feed on affect their body size, *Phasmarhabditis* sp. (KEN1) was only morphometrically compared with other *Phasmarhabditis* species grown *in vivo*.

The males of *Phasmarhabditis* sp. (KEN1) can be distinguished from the males of *P. safricana* by the former's longer body length and larger body diameter (Table 4.2). The female of *Phasmarhabditis* sp. (KEN1) can be distinguished from the female of *P. safricana* by its longer, thinner body and longer tail (Table 4.3). The female of *Phasmarhabditis* sp. (KEN1) has a vulva, with a transverse slit with protruding (Fig. 4.1D; 4.3D) lips, whereas the vulva of *P. safricana* consists of a wide transverse slit, with flat lips. The most important difference is that the female tail of *Phasmarhabditis* sp. (KEN1) is conoid (Fig. 4.1F; Fig. 4.3F) and gradually tapers, whereas the tail of *P. safricana* is dome-shaped, with a spike. The IJs of *Phasmarhabditis* sp. (KEN1) are significantly larger than are those of *P. safricana*, with a body length of 1232 (1107-1336) *vs* 543 (497-648) μm and a body diameter of 45 (38-50) *vs* 23 (20-25) μm (Table 4.4).

The males of *Phasmarhabditis* sp. (KEN1) have longer and larger bodies than do those of *P. papillosa* (SA), with a body length of 1989 (1835-2034) vs 1529 (1360-1679) μm , and a body diameter of 86 (83-89) vs 61 (53-74) μm (Table 4.2). The females of *Phasmarhabditis* sp. (KEN1) are similar in size to the females of the South African strain of *P. papillosa*, but the former can be distinguished based on the tail shape. *Phasmarhabditis* sp. (KEN1) females have a conoid, gradually tapering tail with a length of 168 (136-186) μm , whereas *P. papillosa* (SA) females have a cupola-shaped tail, with a length of 108 (87-143) μm , that constricts into a thin tip (Pieterse *et al.*, 2017). The IJs of *Phasmarhabditis* sp. (KEN1) are significantly larger than are those of *P. papillosa* (SA), with a body length of 1232 (1107-1336) vs 900 (813-1042) μm (Table 4.4).

Males are commonly found in cultures of *Phasmarhabditis* sp. (KEN1), but they are rarely found in *P. hermaphrodita*. The females of *Phasmarhabditis* sp. (KEN1) and the UK strain of *P. hermaphrodita* are similar in shape and size. Both species have a conical tail shape, with *Phasmarhabditis* sp. (KEN1) having a longer tail length of 168 (136-186) vs 108 (87-143) μm (Table 4.3). The females can also be distinguished by the vulva, as *Phasmarhabditis* sp. (KEN1) has a transverse slit, with visibly protruding lips, whereas the vulva of *P. hermaphrodita* has only a transverse slit (Hooper *et al.*, 1999).

The males of *Phasmarhabditis* sp. (KEN1) are significantly larger than are those of *P. circassica*, with a body length of 1989 (1835-2034) vs 1200 (916-1607), and a body diameter of 86 (83-89) vs 49 (40-64) μm (Table 4.2). *Phasmarhabditis* sp. (KEN1) females can be distinguished from those of *P. circassica* by their longer tail, with a length of 168 (136-186) vs 82 (54-125) μm (Table 4.3). The IJs of *Phasmarhabditis* sp. (KEN1) are significantly larger than are those of *P. circassica*, with the latter having a characteristic cuticular cap present on its head, which is not present in *Phasmarhabditis* sp. (KEN1) (Ivanova *et al.*, 2019). The females, males and IJs of *Phasmarhabditis* sp. (KEN1) are significantly larger, with longer tails, than are those of *P. clausiliae* (Tables 4.2-4.4). The IJs of *P. clausiliae* also have a cuticular cap, which is similar to that of *P. circassica*, which distinguishes it from the IJs of *Phasmarhabditis* sp. (KEN1) (Ivanova *et al.*, 2019).

The males are similar in size and morphology to those of *S. apuliae* (Nermet' *et al.*, 2016a) (Table 4.2, 4.3). The females of *P. apuliae* (strain BAR) have a larger body size than has *Phasmarhabditis* sp. (KEN1), with a body length of 2623 (2262-2848) vs 2151 (1918-2340) μm , and a much larger body diameter, of 153 (111-171) vs 98 (85-117) μm (Table 4.3). However, the IJs of *P. apuliae* are significantly smaller than are those of *Phasmarhabditis* sp. (KEN1), with a length of 812 (707-888) vs 1232 (1107-1336) μm . The females of

Phasmarhabditis sp. (KEN1) and *P. bohémica* (strain CH1) are morphometrically similar, except for their body diameter. The females of *Phasmarhabditis* sp. (KEN1) have a smaller body diameter of 98 (85-117) μm , whereas *P. bohémica* females are more robust, with a body diameter of 145 (141-181) μm (Table 4.3). The males and IJs of *Phasmarhabditis* sp. (KEN1) have significantly longer body lengths than do those of *P. bohémica*, with *Phasmarhabditis* sp. (KEN1) IJs being almost double as long as *P. bohémica* IJs (Tables 4.2 and 4.4). The IJs of *P. apuliae* and *P. bohémica* have large, open amphidial openings in the head region, which are small and almost inconspicuous in *Phasmarhabditis* sp. (KEN1) (Nermut' *et al.*, 2016a, 2017).

The males of *Phasmarhabditis* sp. (KEN1) and *P. bonaquaense* have similar morphometrics and morphology. The IJs of *Phasmarhabditis* sp. (KEN1) have a significantly longer body length and tail than do the IJs of *P. bonaquaense* (Table 4.4). The females of *Phasmarhabditis* sp. (KEN1) can be distinguished from those of *P. bonaquaense* (strain NDV) by their smaller body diameter of 98 (85-117) μm and a conical tail, as the *P. bonaquaense* females have a body diameter of 136 (121-161) μm and a cupola-shaped tail, with a long filiform terminus (Nermut' *et al.*, 2016b) (Table 4.3).

The females of *Phasmarhabditis* sp. (KEN1) can be distinguished from the females of *P. neopapillosa* (UK strain) by their thinner bodies, with a body diameter of 98 (85-117) vs 141 (101-174) μm . The males are morphometrically similar, except for a longer male body length in *Phasmarhabditis* sp. (KEN1), of 1989 (1835-2034) vs 1585 (1432-1771) μm (Hooper *et al.*, 1999). The IJs of *Phasmarhabditis* sp. (KEN1) have a longer body and tail than do those of *P. neopapillosa* (Table 4.4). The females of *Phasmarhabditis* sp. (KEN1) have a significantly longer body length and tail than do the females of *P. meridionalis*, with a body length measuring 2151 (1918-2340) vs 1612 (1057-1931) μm , and a tail length of 168 (136-186) vs 68 (44-97) μm . The tail shape of the females in *Phasmarhabditis* sp. (KEN1) and *P. meridionalis* also differs, with the latter having a cupola-shaped tail with filamentous terminus (Ivanova & Spiridonov, 2017). The IJs of *Phasmarhabditis* sp. (KEN1) are significantly longer, and have longer tails, than do the IJs of *P. meridionalis* (Table 4.4). The females, males and IJs of *Phasmarhabditis* sp. (KEN1) are larger in body size than are those of *P. tawfikii* (Tables 4.2-4.4), but with similar morphology (Azzam, 2003).

MOLECULAR DIFFERENTIATION AND PHYLOGENETIC RELATIONSHIPS

Sequences obtained for *Phasmarhabditis* sp. (KEN1) were deposited in the NCBI GenBank, under MN626643 for SSU rRNA gene, MN626640 for D2-D3 (LSU rRNA gene) and

MN626639 for ITS (ITS1, 5.8S, ITS2 rRNA). Phylogenetic analyses were conducted using the SSU, D2-D3 genes and ITS region of nematode taxa, representing the genera *Agfa*, *Angiostoma* and *Phasmarhabditis*, with *Pellioiditis* and *Oscheius* spp. as the outgroups. Figures 4.5, 4.6 and 4.7 show a representative ML tree, although bootstrap support illustrates each method of analysis.

Phylogenetic analysis with ML, distance and MP methods of the SSU placed *Phasmarhabditis* sp. (KEN1) with *Phasmarhabditis* sp. SA1 and *Phasmarhabditis* sp. SA3 (66/67/69), with the aforementioned nematodes forming a sister group to *P. hermaphrodita*, *P. neopapillosa*, *P. bohémica*, *P. meridionalis*, *P. huizhouensis*, *P. apuliae*, *P. safricana*, *P. papillosa*, *P. californica*, *P. bonaquaense*, *Phasmarhabditis* sp. EM434, *A. gandavense*, *A. limacis*, *A. margaretae* and *A. norvegicum* (Fig. 4.5).

Phylogenetic analysis of the D2-D3 gene grouped *Phasmarhabditis* sp. (KEN1) with *P. meridionalis*, under weak bootstrap support. The nematodes, along with *A. margaretae*, *A. milacis*, *A. norvegicum*, *A. gandavense*, *A. flexilis*, *A. tauricus*, *A. kimmeriense*, *Phasmarhabditis* sp. EM434, *P. apuliae*, *P. safricana*, *P. papillosa*, *P. bonaquaense*, *P. californica*, *P. bohémica* and *P. huizhouensis* formed a sister group to *P. hermaphrodita* and *A. limacis*, with *A. dentiferum* being in the basal position (Fig. 4.6).

Phylogenetic analysis of the ITS region grouped *Phasmarhabditis* sp. (KEN1) with *P. meridionalis* and *A. dentiferum* with weak bootstrap support. The nematodes, along with *A. norvegicum*, *A. margaretae*, *A. gandavense*, *A. kimmeriense* and *A. flexilis*, form a sister group to *P. apuliae*, *P. bonaquaense*, *P. papillosa* and *P. californica*, with *P. hermaphrodita* and *P. neopapillosa* in basal position (Fig. 4.7).

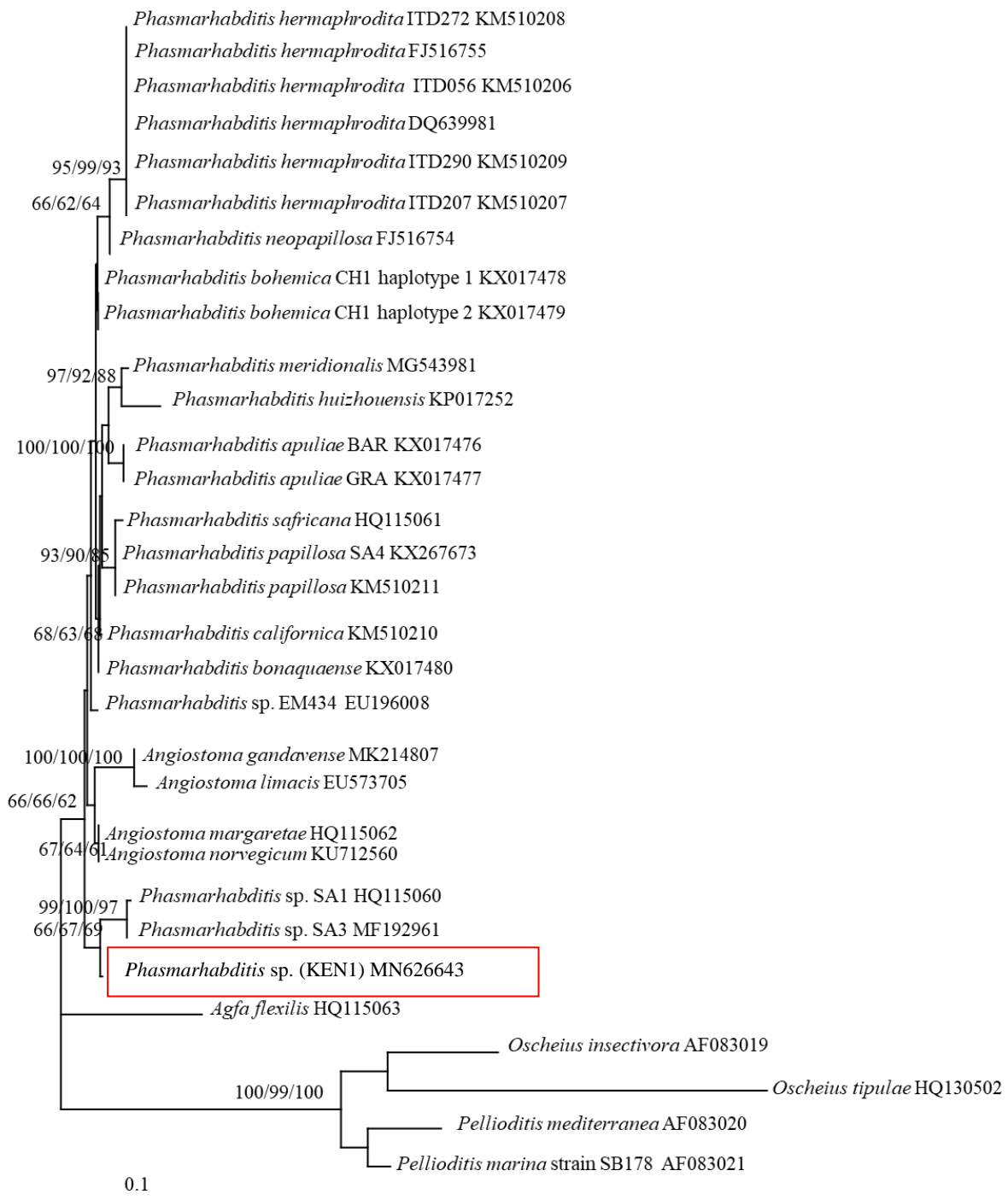


Figure 4.5. Phylogenetic relationships of *Phasmarhabditis* sp. (KEN1), along with other related *Phasmarhabditis*, *Agfa* and *Angiostoma* species, based on analysis of the 18S small subunit (SSU) rDNA gene, as inferred from the use of ML, distance, and MP methods. *Oscheius tipulae*, *O. insectivore*, *Pellioditis marina* and *P. mediterranea* were used as outgroup taxa. Bootstrap values (65% and above) are assigned next to the relevant nodes.

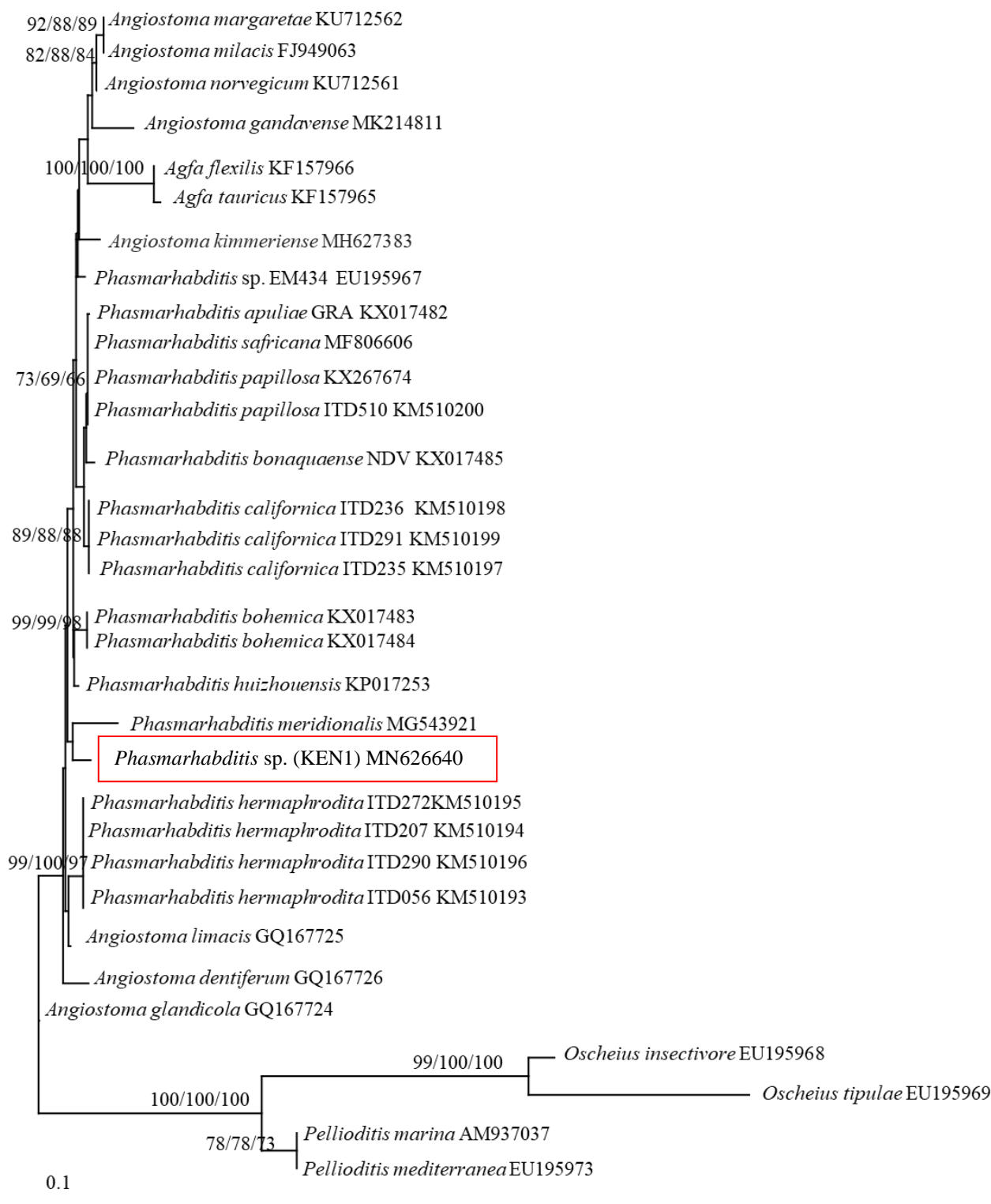


Figure 4.6. Phylogenetic relationships of *Phasmarhabditis* sp. (KEN1), along with other related *Phasmarhabditis*, *Agfa* and *Angiostoma* species, based on analysis of the D2-D3 (LSU) rRNA gene, as inferred from the use of ML, distance, and MP methods. *Oscheius tipulae*, *O. insectivore*, *Pellioiditis marina* and *P. mediterranea* were used as outgroup taxa. Bootstrap values (65% and above) are assigned next to the relevant nodes.



Figure 4.7. Phylogenetic relationships of *Phasmarhabditis* sp. (KEN1), along with other related *Phasmarhabditis*, *Agfa* and *Angiostoma* species, based on analysis of the ITS region, as inferred from the use of ML, distance, and MP methods. *Oscheius tipulae*, *Pellioiditis marina* and *P. mediterranea* were used as outgroup taxa. Bootstrap values (65% and above) are assigned next to the relevant nodes.

Discussion

To date, there are thirteen nominal species of *Phasmarhabditis*: *P. hermaphrodita*, *P. neopapillosa*, *P. papillosa*, *P. huizhouensis*, *P. tawfiki*, *P. californica*, *P. bonaquaense*, *P. apuliae*, *P. bohémica*, *P. meridionalis*, *P. safricana*, *P. circassica* and *P. clausiliiae*, with *Phasmarhabditis* sp. (KEN1) bringing up the total complement to 14 species.

Phasmarhabditis species are often morphologically and genetically similar, making them difficult to identify accurately (Nermut' *et al.*, 2016a; Tandingan De Ley *et al.*, 2016; Ivanova & Spiridonov, 2017); however, it is hoped that, in future, sequences of the *Cox1* gene will help facilitate the identification of *Phasmarhabditis* species. Species in the genus are usually distinguished based on morphometrics, the female's tail shape, the absence or presence of males, the bursal papillae, the spicule size, the morphology of the IJ and molecular and phylogenetic analysis. Body size can also be greatly influenced by growth conditions (Wilson *et al.*, 1995; Nermut' *et al.*, 2014), with Hooper *et al.* (1999) demonstrating the impact of *in vivo* and *in vitro* culturing on the body size of *P. hermaphrodita*.

Phasmarhabditis sp. (KEN1) can be characterised by the conical shape of the female's tail, the presence of males, the exceptional body length of the IJ (1232 (1107-1336) μm) and the molecular characteristics of the SSU, D2-D3 and ITS rRNA.

The phylogenetic analyses of the SSU, D2D3 genes and ITS region show that certain members of *Phasmarhabditis*, *Angiostoma* and *Agfa* are difficult to differentiate molecularly, thus supporting the conclusions of Ross *et al.* (2010), who demonstrated that the genera *Agfa*, *Angiostoma* and *Phasmarhabditis* are molecularly conserved, yet morphologically diverse, with them having potentially evolved to adapt to their parasitic lifestyle. Nermut' *et al.* (2016a) proposed the possibility of certain *Angiostoma* species being misidentified, due to their phylogenetic positioning, but the results of the current paper, along with the findings of Ivanova, Geraskina & Spiridonov (2019), reject the hypothesis, with *Phasmarhabditis* sp. (KEN1) being grouped with *P. meridionalis*, along with representatives of the *Angiostoma* and *Agfa* species, as being in the ITS tree (66/65/59), albeit only with weak bootstrap support.

Phasmarhabditis sp. (KEN1) is the third *Phasmarhabditis* species to be described from the African continent, with *P. tawfiki* having been described from Egypt in 2003 (Azzam, 2003), and with *P. safricana* having been described from South Africa in 2018 (Ross *et al.*, 2018). With no molecular data yet being available for *P. tawfiki*, it was not possible, at the present stage, to determine its phylogenetic relationship with *Phasmarhabditis* sp. (KEN1). However, from the phylogenetic analyses of the SSU, D2-D3 and ITS region, *Phasmarhabditis* sp.

(KEN1) did not group together with the South African nematode, *P. safricana*. However, in the SSU phylogenetic analysis, it can be seen that the *Phasmarhabditis* sp. (KEN1) grouped with *Phasmarhabditis* sp. SA1 and *Phasmarhabditis* sp. SA3, which are two undescribed phasmarhabditids isolated from slugs in South Africa, suggesting a possible African grouping. The above grouping, however, occurs in the absence of D2D3 sequences for the aforementioned African phasmarhabditid group. The lack of such sequence data highlights the importance of encouraging any new species description to be supported by sequences for SSU gene, the D2-D3 gene, the ITS region and the mitochondrial cytochrome *c* oxidase subunit I (*mtCOI*) gene.

The current record is the first record of a *Phasmarhabditis* nematode present in Kenya. The nematode was isolated from the local slug, *P. robustum*. This species is widespread in Africa, with it having been found in Rwanda, South Sudan, Uganda, Kenya, Tanzania, Zimbabwe and the Democratic Republic of Congo (Van Goethem, 1977; Rowson *et al.*, 2016). Ross *et al.* (2010, 2012) found that surveys carried out in the USA and South Africa showed higher infection of invasive slugs by *Phasmarhabditis* spp. compared to that of native species (Ross *et al.*, 2010, 2012). However, in Norway, the infection rate of the invasive *Arion vulgaris* Moquin-Tandon, 1855 by *P. hermaphrodita* was similar to that of the local arionid species (Ross *et al.*, 2015).

The discovery of the new species, as well as the number of new and unidentified *Phasmarhabditis* species found in South Africa (Ross *et al.*, 2012; Pieterse *et al.*, 2017; Ross *et al.*, 2018), highlights the importance of conducting further nematode surveys across Africa. To date, the only African countries surveyed are South Africa, Egypt and now Kenya, opening up the possibility that many phasmarhabditids still await discovery.

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Chapter 5

Life cycle of five nematodes associated with terrestrial molluscs:

Phasmarhabditis hermaphrodita, *Phasmarhabditis papillosa*, *Phasmarhabditis bohémica*, *Phasmarhabditis* sp. (KEN1) and *Caenorhabditis* *elegans*.

Abstract

The success of *Phasmarhabditis hermaphrodita* (Schneider) Andrassy (Rhabditida: Rhabditidae), as a biological control agent of molluscs, has led to a worldwide interest in phasmarhabditids. However, little information is available on the life cycle of nematodes within the genus. In the current study, the life cycle of *P. hermaphrodita*, *Phasmarhabditis papillosa*, *Phasmarhabditis bohémica*, and *Phasmarhabditis* sp. (KEN1), along with *Caenorhabditis elegans* due to its widespread distribution in KwaZulu-Natal, are studied using *in vivo* cultures, with the aim of understanding the biology and potential of the aforementioned nematodes to be mass-cultured in liquid media. Infective juveniles (IJs) of each species were added to defrosted slug cadavers and monitored, from IJ recovery through to second-generation egg formation. The results demonstrated that *C. elegans* had the shortest life cycle, and that it was able to form eggs 6 days after IJ recovery, while *P. bohémica* took 8 days, and *P. hermaphrodita*, *P. papillosa* and *Phasmarhabditis* sp. (KEN1) took 10 days to form eggs. Based on the time taken to complete a life cycle, as well as on the reproductive strategy involved, *C. elegans* is considered to be the easiest to mass-culture of the nematodes studied, due to its hermaphroditic reproduction strategy, as well as to the fact that it completes its life cycle 2 days earlier than does *P. bohémica*, and 4 days earlier than do *P. hermaphrodita*, *P. papillosa* and *Phasmarhabditis* sp. (KEN1). However, *P. hermaphrodita* has an advantage over the other *Phasmarhabditis* species, in that it is capable of forming self-fertilising hermaphrodites, whereas both males and females are required for the reproduction of *P. papillosa*, *P. bohémica* and *Phasmarhabditis* sp. (KEN1). The results of the study should contribute to the knowledge of the biology of the genus and of the *in vitro* liquid culture of different species of the genus.

Keywords – infective juveniles; liquid media; reproduction; mass-culture, biocontrol

Introduction

Mollusc-parasitic nematodes (MPN) are comprised of eight families, including Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (Pieterse *et al.*, 2017a). Within the Rhabditidae (Rhabditida), two of the nematode genera that are most commonly isolated from the molluscs are *Phasmarhabditis* and *Caenorhabditis* (Karimi *et al.*, 2003; Ross *et al.*, 2010, 2012; Wilson *et al.*, 2016; Pieterse *et al.*, 2017b).

Phasmarhabditis hermaphrodita (Schneider) Andr ssy is the only MPN to be successfully developed as a biological molluscicide (Rae *et al.*, 2007). The nematode is commercially available from BASF (formally Becker Underwood) and Dudutech, under the trade names, Nemaslug[®] and SlugTech[®], respectively. Like other rhabditid nematodes, *P. hermaphrodita* responds to depleting food supply and to poor environmental conditions with the formation of a metabolically-suppressed, third juvenile stage, which is known as the infective juvenile (IJ), or the dauer juvenile. IJs, which are adapted for long-term survival, actively search out molluscs, entering through natural openings (Wilson *et al.*, 2012). The IJs then recover and develop into self-fertilising hermaphrodites, whereupon they start to reproduce. The host usually dies within 4 to 21 days following infection, after which the nematodes colonise the entire slug cadaver, feeding and reproducing until the food source is depleted. The IJs are then produced, which move back into the soil in search of new hosts (Wilson *et al.*, 1993; Tan & Grewal, 2001). *Phasmarhabditis hermaphrodita* is capable of infecting a number of mollusc species, including the snail species *Monacha cantiana* (Montagu), *Cepaea hortensis* (M ller), *Theba pisana* (M ller), *Cochlicella acuta* (M ller), *Cer­nuella vigata* (Da Costa) and *Lymnaea stagnalis* (L.), as well as the slug species *Deroceras reticulatum* (M ller), *D. panormitanum* (Lessona and Pollonera), *D. laeve* (M ller), *Arion silvaticus* Lohmander, *A. intermedius* Normand, *A. distinctus* Mabile, *Tandonia sowerbyi* (F russac), *T. budapestensis* (Hazay) and *Leidyula floridana* (Leidy) (Rae *et al.*, 2007). It also has a necromenic life cycle (Mengert, 1953), as well as being able to reproduce on such substrates as slug faeces homogenates and bacterial substrates (Tan & Grewal, 2001; MacMillan *et al.*, 2009; Nermut' *et al.*, 2014). Although *P. hermaphrodita* has been the most studied MPN, to date, very little is known about its biology and life cycle. The information could be key, when considering improved mass production conditions.

In addition to *P. hermaphrodita*, there are 12 other described species of phasmarhabditids, several of which have been considered for their potential commercial value, but none of which

has yet had its life cycles described. One such nematode is *Phasmarhabditis papillosa* (Schneider) Andrásy, which was first described in 1866, based on the minimal amount of information regarding the female body length, as well as regarding the basic morphometric data relating to the males and females (Schneider, 1866). Schneider (1871) later reported that the males and females of *P. papillosa* occur in roughly equal numbers, whereas the males are relatively rare in cultures of *P. hermaphrodita* (Andrásy, 1983; Tandingan De Ley *et al.*, 2016). In 2016, *P. papillosa* was re-described after being isolated for the first time in California, USA (Andrásy, 1983; Tandingan De Ley *et al.*, 2016). In 2017, the description was further updated after the nematode was isolated for the first time in South Africa (Pieterse *et al.*, 2017b). In the same study, Pieterse *et al.* (2017c) demonstrated that *P. papillosa* is capable of causing mortality in the European slug species, *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Agriolimacidae) (Pieterse *et al.*, 2017c).

Phasmarhabditis bohémica Nermut, Půža, Mekete & Mráček was described in 2017 from *D. reticulatum* in the Czech Republic (Nermut *et al.*, 2017). The ability of the species to cause mortality in its hosts requires further investigation, as no apparent mortality was caused under natural conditions, but the mass mortality of *D. reticulatum* was observed under laboratory conditions (Nermut *et al.*, 2017). In the description of males, females and IJs of the nematode it was reported that *P. bohémica* is capable of completing its life cycle on decaying animal matter within several days when it is kept at 15°C (Nermut *et al.*, 2017).

Phasmarhabditis sp. (KEN1) was isolated from the slug *Polytoxon robustum* (Simroth) (Urocyclidae), collected in Nairobi, Kenya. Details of the description of the nematode can be found in Chapter 4 and is currently under review for publication.

Caenorhabditis elegans (Maupas), which is a free-living nematode, is believed to have a phoretic relationship with slugs, with no effect on host fitness (Kiontke & Sudhaus, 2006). However, Petersen *et al.* (2015) report that *C. elegans*, which is capable of entering the bodies of slugs, is transported and expelled alive within the faeces, hinting at a possible parasitic relationship. *Caenorhabditis elegans* is popular as a model organism, due to the ease with which it can be grown and maintained under laboratory conditions. It has a life cycle spanning 3 days when grown at 25°C, and an average life span of 2 to 3 weeks. The adult hermaphrodite can produce 300 to 350 offspring when self-fertilising, with even more being produced when males are present, which occur at very low frequencies (Wood, 1998). *Caenorhabditis elegans* reproduces through a form of protandrous hermaphroditism, which is different to hermaphroditism in other species. The gonads of morphological females first produce a certain amount of sperm, then switch over to oogenesis, without reverting to spermatogenesis. As the

hermaphrodites lack male reproductive organs, they use their own sperm to fertilise their own eggs. Transfer of sperm only occurs when the hermaphrodites mate with the males, which occurs rarely (Brenner, 1988; Stewart & Phillips, 2002).

In the current study, the life cycles of the nematodes *P. hermaphrodita*, *P. papillosa*, *P. bohémica*, *Phasmarhabditis* sp. (KEN1) and *C. elegans* from *in vivo* cultures with freeze-killed slug cadavers were studied.

Materials and Methods

SOURCE OF NEMATODES

The following nematode species were used in the study: *P. hermaphrodita*, *P. papillosa*, *P. bohémica*, *Phasmarhabditis* sp. (KEN1) (see Chapter 4) and *Caenorhabditis elegans*. The *P. papillosa* and *C. elegans* that were obtained from a collection of the Department of Conservation Ecology and Entomology at Stellenbosch University were collected during a survey conducted by Pieterse *et al.* (2017b). The *P. bohémica* and *P. hermaphrodita* isolates used in the study was kindly supplied by Dr Vladimir Půža from the Biology Centre CAS, Institute of Entomology ASCR, Laboratory of Entomopathogenic Nematodes, Czech Republic. The *Phasmarhabditis* sp. (KEN1) (see Chapter 4) was obtained in a collaborative study undertaken with Dr Solveig Haukeland from the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi, Kenya, and Dr Jenna Ross (co-supervisor).

NEMATODE PREPARATION

The life cycles of *P. hermaphrodita*, *P. papillosa*, *P. bohémica*, *Phasmarhabditis* sp. (KEN1) and *C. elegans* were studied by adding 100 IJs (rinsed with distilled water) of each specie to 1 g freeze-killed, homogenised *D. invadens*, placed on modified White traps, along with a piece of damp filter paper in a 90-mm-diameter Petri dish. The cultures were sealed with Parafilm[®] and kept in darkness at 20°C. Every 24 h thereafter, for the duration of a complete life cycle (until egg formation for the next generation), one Petri dish of each species was washed with 0.9% saline, and the nematodes were heat-killed (~85°C) with triethanolamine-formalin (TAF) fixative (Courtney *et al.*, 1955). The nematodes were then classified into the different life stages and counted using a compound microscope (Leica DM200, Leica Microsystems), so as to determine the population structure on each day. All nematodes multiplied successfully on the homogenized slug substrate.

NEMATODE BIOLOGICAL OBSERVATIONS AND MEASUREMENTS

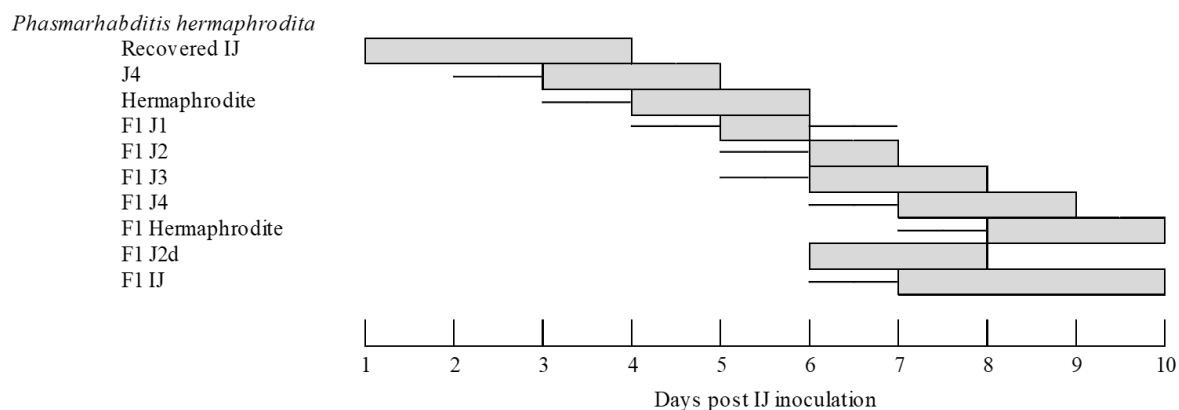
Twenty-five nematodes of each life stage were measured to provide a clear classification of each of the nematode species studied. The nematodes were placed in a drop of water on a microscope slide covered with a coverslip, and killed with gentle heat on a hot plate. They were then measured using a compound microscope (Leica DM200, Leica Microsystems), fitted with a digital camera and with the software Leica Application Suite V3.5.0, with live measurement capability. The body length and width, as well as the life stage of the nematodes, were recorded. The experiment was repeated twice, providing an average measurement of each life stage, based on 50 nematodes.

Results

DEVELOPMENT OF THE DIFFERENT LIFE STAGES

The life cycle development of each of the five nematode species, grown *in vivo*, is illustrated via bar graphs, demonstrating growth from egg hatch (J1) through to new egg formation (Fig. 5.1).

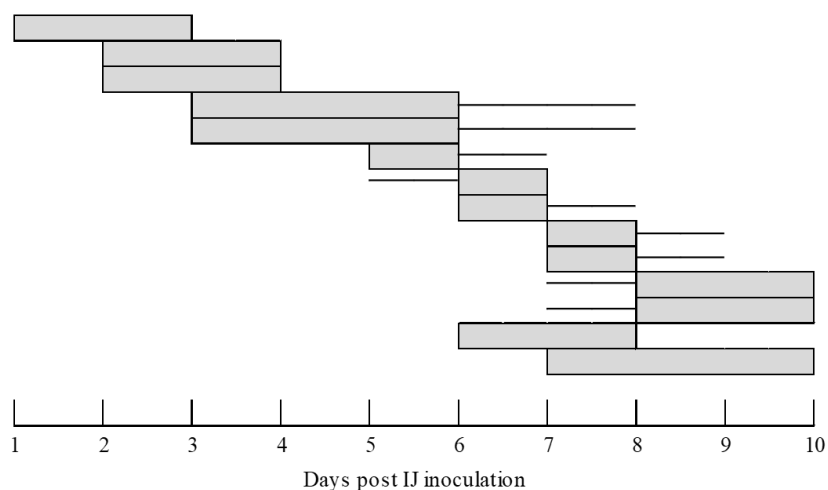
A



B

Phasmarhabditis papillosa

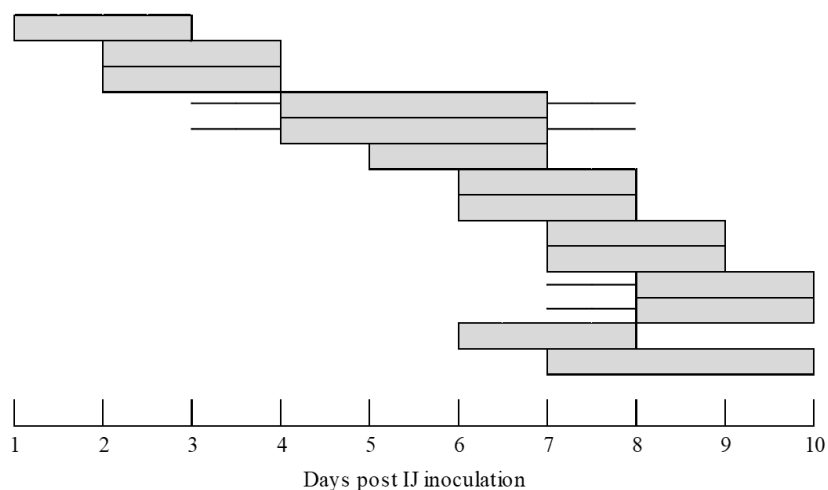
- Recovered IJ
- J4 Male
- J4 Female
- Parental Male
- Parental Female
- F1 J1
- F1 J2
- F1 J3
- F1 J4 Male
- F1 J4 Female
- F1 Male
- F1 Female
- F1 J2d
- F1 IJ



C

Phasmarhabditis sp. (KEN1)

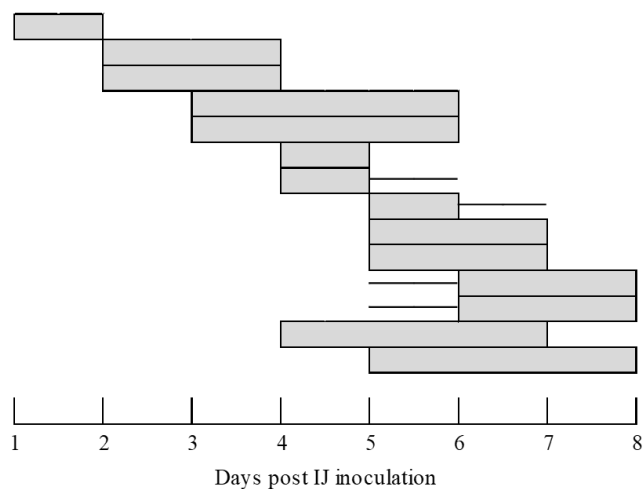
- Recovered IJ
- J4 Male
- J4 Hermaphrodite
- Parental Male
- Parental Hermaphrodite
- F1 J1
- F1 J2
- F1 J3
- F1 J4 Male
- F1 J4 Hermaphrodite
- F1 Male
- F1 Hermaphrodite
- F1 J2d
- F1 IJ



D

Phasmarhabditis bohémica

- Recovered IJ
- J4 Male
- J4 Hermaphrodite
- Parental Male
- Parental Hermaphrodite
- F1 J1
- F1 J2
- F1 J3
- F1 J4 Male
- F1 J4 Hermaphrodite
- F1 Male
- F1 Hermaphrodite
- F1 J2d
- F1 IJ



E

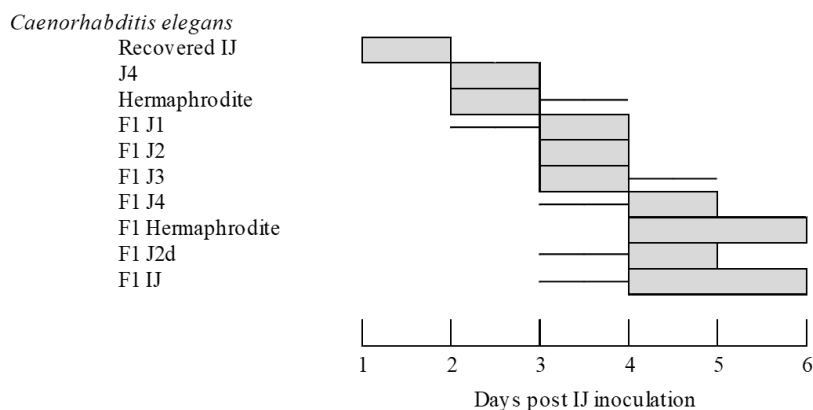


Figure 5.1. Life span of the parental nematodes and F1 generation of A: *Phasmarhabditis hermaphrodita*, B: *P. papillosa*, C: *P. bohémica*, D: *Phasmarhabditis* sp. (KEN1) and E: *Caenorhabditis elegans*, when grown *in vivo*. The lines indicate when the population of each stage was less than 10% of the maximum.

In populations of *P. hermaphrodita*, the recovery of IJs occurred 1 day after inoculation (DAI), with the males and hermaphrodites developing by the second DAI, with some nematodes reaching adulthood. On the third DAI, most of the population consisted of adult males and hermaphrodites, with eggs starting to hatch, and first juveniles (J1s) starting to appear. By the fourth DAI, the development of the second and third juvenile (J2 and J3) phases was observed. New IJs had developed by the fifth DAI. Adult hermaphrodites and males also started developing at the fifth DAI, reaching maturity only by the sixth DAI, with them showing fertilised eggs only by the end of the eighth DAI. In some hermaphrodites, *endotokia matricida* was observed after the eighth DAI.

In the populations of *P. papillosa*, the IJs started recovering at the first DAI, and the males and females were distinguishable by the second DAI. By the third DAI, the males and females reached adulthood, by which time female egg development had also started. By the fourth DAI, the J1s of the next generation had started to appear, with them having developed into J2s by the fifth DAI, and into J3s by the sixth DAI. The males and females started developing again on the seventh DAI, while some juveniles developed into IJs at such a point. Mating started when the males and females reached adulthood, from the eighth DAI onwards, with fertilised eggs developing by the end of the tenth DAI. *Endotokia matricida* was observed at the tenth DAI in some females.

In the populations of *P. bohémica*, the IJs started recovering on the first DAI, with the majority of adult males and females forming after the third DAI. By the fourth DAI, the first J1 larvae

of the second generation had started to appear and develop into J2 and J3, or into J2d. By the fifth DAI, the majority of the nematodes in the population consisted of J4 males, J4 females, J3 larvae, J2d larvae, or IJs. On the sixth DAI, many of the males and females had reached maturity, with them having already developed their reproductive organs. By the eighth DAI, the females contained fertilised and developed eggs, which were ready for release, or which hatched out inside the body of the adult female nematode.

In the *Phasmarhabditis* sp. (KEN1) populations, the IJs had started to recover by the end of the first DAI, with them starting to show male and female characteristics by the second DAI. The first-generation males and females were fully developed by day 4, and started producing J1 larvae by day 5. By the sixth DAI, most of the second-generation larvae had developed into J2, J3 or J2d stages, with, by the seventh DAI, J4 males, J4 females and IJs having formed. On the eighth DAI, the males and females had completed their development, and they were ready for mating, and, by the tenth DAI, the females had fertilised and developed their eggs, that were either released, or which hatched out inside the adult female's body.

The *C. elegans* adult hermaphrodites had already started to form by the second DAI. By the third DAI, the adult hermaphrodites had already produced offspring, with the juveniles of all stages being present (J1, J2, J3, J4, J2d). By the fourth DAI, the IJs had formed again, and the adult hermaphrodites had developed. The adult hermaphrodites then developed eggs on the sixth DAI. The presence of males was not observed.

DIFFERENCE IN BODY LENGTH

The comparison of the mean body length and width of the different life stages of each of the five species is shown in Table 5.1. The IJs of *P. hermaphrodita* are longer and thinner than are those of *P. papillosa* and *P. bohémica*, with a body length of 922 μm and a body width of 30 μm , compared to a body length and width of 796 μm and 31 μm in *P. papillosa*, and of 737 μm and 36 μm in *P. bohémica*. The IJs of *P. hermaphrodita* are, however, generally shorter and thinner than are those of the undescribed *Phasmarhabditis* sp. (KEN1), which has the largest IJ body size, measuring 1134 μm in length and 41 μm in width. The IJs of each of the four *Phasmarhabditis* species are much larger than are those of *C. elegans*, whose IJs measure 659 μm in length and 24 μm in width.

Table 5.1. Mean body length and width (μm) of the different life stages of *Phasmarhabditis hermaphrodita*, *P. papillosa*, *P. bohémica*, *Phasmarhabditis* sp. (KEN1) and *Caenorhabditis elegans*.

Species	Generation	Stage	Day	Length	Width
<i>P. hermaphrodita</i>	F1	J1	5	322 (312-340)	18 (17-18)
	F1	J2	6	398 (371-429)	22 (19-25)
	F1	J3	6	479 (442-501)	31.4 (29-33)
	F1	J4	7	913 (835-1040)	56 (44-63)
	F1	Hermaphrodite	8	2038 (1812-2201)	121 (108-136)
	F1	J2d	6	682 (540-825)	34 (29-39)
	F1	IJ	7	922 (835-1051)	30 (28-34)
<i>P. papillosa</i>	F1	J1	5	346 (283-422)	17 (12-23)
	F1	J2	6	475 (426-518)	24 (18-27)
	F1	J3	6	588 (538-628)	28 (24-32)
	F1	J4M	7	942 (847-1018)	45 (33-60)
	F1	J4F	7	1170 (1065-1264)	60 (53-67)
	F1	Male	8	1381 (1241-1484)	67 (61-73)
	F1	Female	8	1889 (1772-2012)	95 (90-101)
	F1	J2d	6	620 (541-697)	35 (29-39)
	F1	IJ	7	796 (749-852)	31 (26-36)
<i>P. bohémica</i>	F1	J1	4	292 (260-320)	17 (14-20)
	F1	J2	4	366 (339-411)	23 (21-25)
	F1	J3	5	408 (380-442)	24 (18-28)
	F1	J4M	5	563 (508-643)	33 (28-41)
	F1	J4H	5	854 (797-912)	57 (55-58)
	F1	Male	6	1627 (1520-1765)	94 (91-96)
	F1	Female	6	2247 (1997-2584)	133 (128-139)
	F1	J2d	4	542 (513-569)	37 (26-51)
	F1	IJ	5	737 (520-869)	36 (29-47)
<i>Phasmarhabditis</i> sp. (KEN1)	F1	J1	5	336 (328-350)	20 (16-25)
	F1	J2	6	466 (443-487)	30 (29-30)
	F1	J3	6	635 (510-725)	33 (29-38)
	F1	J4M	7	1126 (1008-1229)	73 (56-80)
	F1	J4F	7	1113 (1070-1203)	74 (60-88)
	F1	Male	8	1908 (1809-2041)	87 (77-96)
	F1	Female	8	2191 (1884-2335)	103 (89-117)
	F1	J2d	6	883 (862-905)	46 (44-47)
	F1	IJ	7	1134 (999-1301)	41 (32-56)
	<i>C. elegans</i>	F1	J1	3	240 (206-265)
F1		J2	3	358 (309-393)	18 (15-22)
F1		J3	3	529 (492-553)	25 (24-27)
F1		J4	4	771 (715-815)	35 (28-44)
F1		Hermaphrodite	4	1172 (1003-1331)	69 (51-96)
F1		J2d	4	456 (423-496)	21 (18-26)
F1		IJ	4	659 (582-866)	24 (18-30)

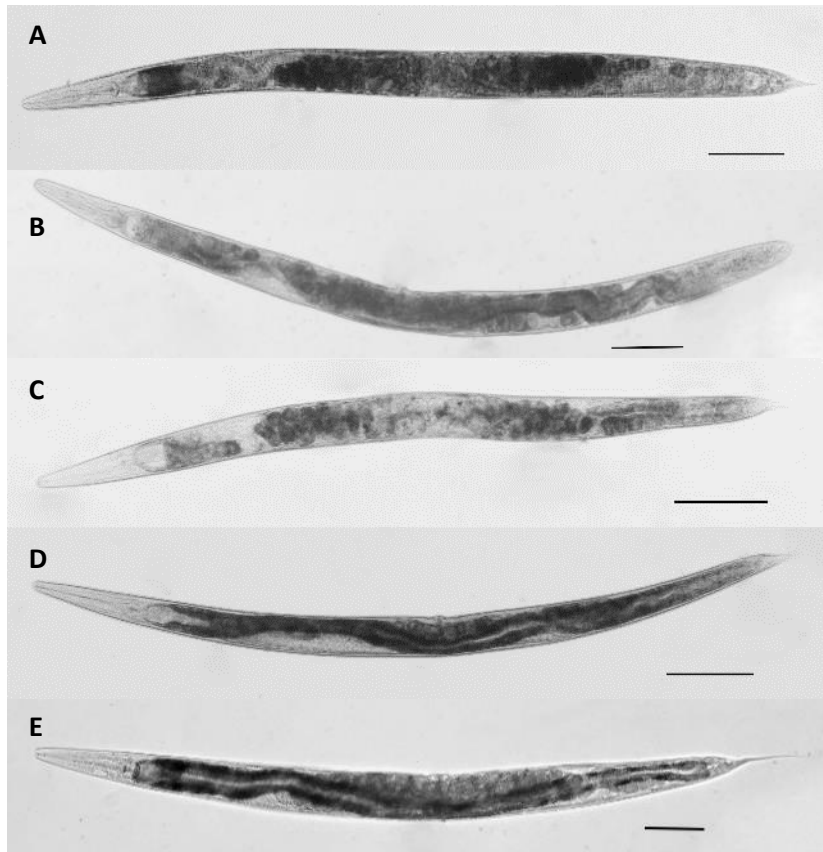


Figure 5.2. Images of A: Hermaphrodite of *Phasmarhabditis hermaphrodita*; B: Female *P. papillosa*; C: Female *P. bohemica*; D: Female *Phasmarhabditis* sp. (KEN1); and E: Hermaphrodite of *C. elegans* (Scale bars: A-D = 200 μm ; E = 100 μm).

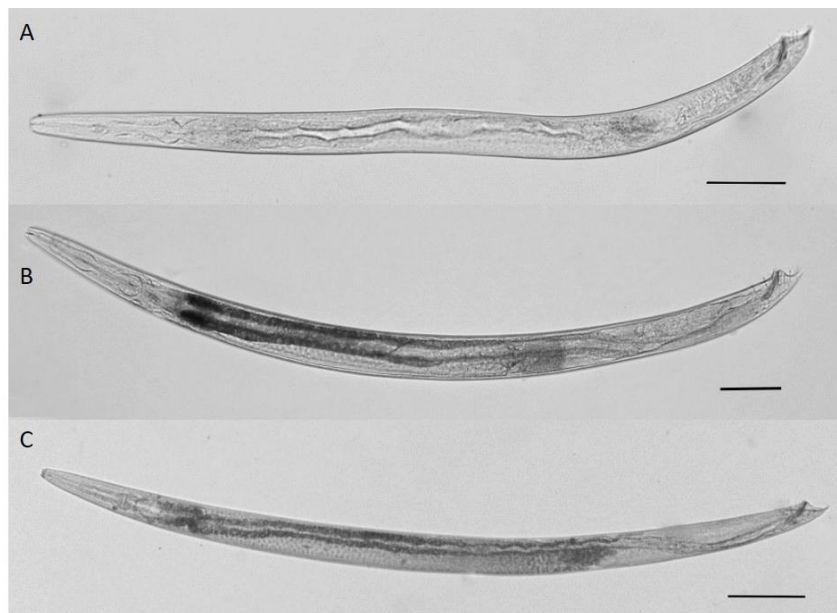


Figure 5.3. Images of males of A: *Phasmarhabditis papillosa*; B: *P. bohemica*; and C: *Phasmarhabditis* sp. (KEN1) (Scale bars: A = 100 μm ; B = 100 μm ; C = 200 μm).

The adult hermaphrodites of *P. hermaphrodita* are longer (2038 μm) and thicker (121 μm) than are the females of *P. papillosa* (1889 μm length, 95 μm width), but both are smaller than the adult females of *Phasmarhabditis* sp. (KEN1), which measure 2191 μm in length and 103 μm in width. The adult females of *P. bohémica* were the largest of the *Phasmarhabditis* species, measuring 2247 μm in length and 133 μm in width. The adult hermaphrodites of *C. elegans* were smaller than were those of the other *Phasmarhabditis* species, measuring 1171 μm in length and 69 μm in width.

Of the three species that had males present, *P. papillosa* had the smallest males, measuring 1381 μm in length and 67 μm in width. The males of *P. bohémica* were larger, measuring 1627 μm in length and 94 μm in width, with the males of *Phasmarhabditis* sp. (KEN1) being the largest, with a length of 1908 μm and a width of 87 μm .



Figure 5.4. Infective juveniles of A: *Phasmarhabditis hermaphrodita*; B: *P. papillosa*; C: *P. bohémica*; D: *Phasmarhabditis* sp. (KEN1); and E: *C. elegans* (Scale bars: A-C = 100 μm ; D = 200 μm ; E = 100 μm).

Development into adulthood took approximately 48 h longer for *P. hermaphrodita*, *P. papillosa* and *Phasmarhabditis* sp. (KEN1) (day 8) compared to *P. bohémica*, which developed into adult males and females by day 6. All the life stages of *C. elegans* were markedly smaller than were those of *P. papillosa* and *P. hermaphrodita*. Adult *C. elegans* developed into the adult stage two days earlier (on day 4) than did *P. bohémica*, and 4 days earlier than did the *P. hermaphrodita*, *P. papillosa* and *Phasmarhabditis* sp. (KEN1).

The most important difference between the five species is the mode of reproduction. *Phasmarhabditis papillosa*, *P. bohemica* and *Phasmarhabditis* sp. (KEN1) formed males and females that required copulation to reproduce, whereas *P. hermaphrodita* and *C. elegans* only formed self-fertilising hermaphrodites, meaning that each nematode could reproduce on its own.

Discussion

Of the many nematode species associated with terrestrial molluscs, *P. hermaphrodita* is the only species, to date, to have been successfully formulated into a commercial biological molluscicide (Rae *et al.*, 2007). Its success as a biocontrol agent can be attributed to its wide host range, to its capacity to form self-fertilising hermaphrodites, as well as to its ability to cause mortality to its mollusc hosts within 4 to 21 days after infection (Rae *et al.*, 2007). The success of the product, paired with the fact that the regulation in many countries restricts the sale of *P. hermaphrodita* where the nematode is not indigenous, has led to an increase in the amount of research being done in the area. Over the last five years, a boom has occurred in the newly described *Phasmarhabditis* species, bringing the total complement of the genus to thirteen: *P. hermaphrodita*; *P. neopapillosa* (Mengert *in* Osche) Andr ssy; *P. papillosa*; *P. tawfiki* Azzam; *P. huizhouensis* Huang, Ye, Ren & Zhao; *P. apuliae* Nermu , P za & Mr cek; *P. bonaquaense* Nermu , P za, Mekete & Mr cek; *P. californica* Tandingan De Ley, Holovachov, McDonnel, Bert, Paine & De Ley; *P. bohemica* Nermu , P za, Mekete & Mr cek; *P. meridionalis* Ivanova & Spiridonov; *P. safricana* Ross, Pieterse, Malan & Ivanova; *P. circassica* Ivanova, Geraskina & Spiridinov; and *P. clausiliidae* Ivanova, Geraskina & Spiridinov. Aside from the work conducted on *P. hermaphrodita*, very little work has yet been published on the mass culture and virulence of other nematodes within the *Phasmarhabditis* genus (Pieterse *et al.*, 2017c).

The potential of a nematode candidate to be considered for biocontrol development depends on its virulence, as well as on the ease with which it can be mass-cultured. An important part of nematode mass production is having an understanding of, and the ability to manage, the nematode population dynamics. The current study provides new insights into the life cycles of the six nematodes associated with slugs: *P. hermaphrodita*, *P. papillosa*, *P. bohemica*, *Phasmarhabditis* sp. (KEN1) and *C. elegans*. Of the aforementioned nematodes, *C. elegans* was found to be the easiest to mass-culture in the liquid media in the study, due to its hermaphroditic reproduction strategy, and to its ability to complete its life cycle within 6 days, being 2 days earlier than *P. bohemica*, and 4 days earlier than *P. hermaphrodita*, *P. papillosa*

and *Phasmarhabditis* sp. (KEN1). Therefore, *C. elegans* could make an ideal candidate for commercial development. Petersen *et al.* (2015) found that *C. elegans* can be transported in the slug's intestine without causing obvious harm to the slug itself, but they also concluded that, as a parasitic relationship might be possible, further investigation is required (Petersen *et al.*, 2015). In several slug-nematode surveys conducted in South Africa, *C. elegans* was found to be the nematode most commonly isolated from the slug hosts (Ross *et al.*, 2012; Pieterse *et al.*, 2017b). The widespread distribution and high prevalence of *C. elegans* in South Africa warrants further investigation, in relation to its association with its slug hosts.

In the current study, the commercially exploited nematode, *P. hermaphrodita*, took 10 days to complete its life cycle, with it taking 4 days longer in the case of *C. elegans*. However, *P. hermaphrodita* has an advantage over *C. elegans*, with its proven ability to parasitise a range of mollusc species (Rae *et al.*, 2007). *Phasmarhabditis hermaphrodita* also has a benefit over the other phasmarhabditids in the present study, in that it forms self-fertilising hermaphrodites. The above means that the reproduction of *P. hermaphrodita* might be easier in liquid cultures, compared to that of *P. papillosa*, *P. bohémica* and *Phasmarhabditis* sp. (KEN1). However, it should be noted that *P. bohémica* has an advantage over the other *Phasmarhabditis* species, in that it develops into the adult stage 2 days earlier, but, due to the inability of *P. bohémica* to produce self-fertilising hermaphrodites, it can be regarded as a more complex biocontrol candidate. The above means that the culturing concerned would take longer, and that the conditions within the flasks or fermenters would have to be altered to allow copulation between the males and females. When mass-culturing nematodes, the goal is to produce the largest amount of IJs in the least amount of time. Even a day or two difference in the life cycle length of the nematodes will therefore have a significant effect on production efficiency.

Phasmarhabditis papillosa was one of the three phasmarhabditids that took the longest to complete its life cycle (10 days). The result is that, despite the promising pathogenicity results noted by Pieterse *et al.* (2017c), of *P. papillosa* being able to control *D. invadens*, based on the life cycle results of the current study, it would not, necessarily, make a better biocontrol candidate, compared to *P. hermaphrodita*.

So as to optimise the conditions in liquid cultures for the development of nematode species, it is crucial to understand their life cycles, and to have a basic knowledge of the different life stages concerned. The results of the study regarding the life cycle and the body size of the different species can be used for future improvements on the culturing of the nematodes and on the optimising of yields for possible commercial production. To enable the recommending of nematode species for, or their eliminating from, consideration for development as possible

biocontrol agents, the pathogenicity and the life cycles of the other species in the *Phasmarhabditis* genus should also be investigated.

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Chapter 6

***In vitro* liquid culture of nematodes associated with slugs**

Summary

The only commercially available nematode for the biological control of molluscs is *Phasmarhabditis hermaphrodita*, grown *in vitro* with *Moraxella osloensis*. To-date this nematode cannot be used in South Africa due to regulations. Therefore, there is an interest to develop a locally isolated nematode and develop as a biocontrol candidate. However, to do this, one of the first processes is to identify potential nematode candidates through surveys (Chapter 2) and reviewing current collections, and then the next process is to identify suitable bacterial candidates for *in vitro* development. In this study, ten bacterial isolates were investigated, including five bacterial candidates isolated from slugs, namely *Pseudomonas* sp. (2), *Pseudomonas* sp. (3), *Pseudomonas* sp. (4), *Aeromonas* sp. and *Buttiauxella* sp., along with two previously isolated slug-associated bacteria (*Pseudomonas* sp. (1) and *Kluyvera* sp.), and three bacterial species associated with entomopathogenic nematodes (*Photorhabdus heterorhabditis*, *P. luminescens* subsp. *noenieputensis* and *Xenorhabdus khoisanae*). These bacteria were tested for their ability to cause mortality to *Deroceras invadens*, as well as support nematode growth. The nematodes used in this study included *Phasmarhabditis papillosa* and *Caenorhabditis elegans*, isolated in South Africa, along with *Phasmarhabditis* sp. (KEN1) from Kenya, and *Phasmarhabditis bohemica*. Initial mortality studies demonstrated that the bacteria *Kluyvera* sp., *Aeromonas* sp. and *Pseudomonas* sp. (3) caused 100% mortality when they were injected into the haemocoel of *D. invadens*. However, in growth studies, *Pseudomonas* sp. (4) was the most preferred bacterium, leading to recovery and reproduction in all nematode species, with the exception of *Phasmarhabditis* sp. (KEN1), in which it caused the death of the infective juveniles (IJs). In flask studies, *P. bohemica* showed exceptional growth with *Pseudomonas* sp. (1), and so was chosen for further investigation. The effect of inoculating flasks with 1%, 3% or 5% *Pseudomonas* sp. (1) bacteria, as well as with 1000, 2000 or 3000 IJs/ml of *P. bohemica*, was evaluated by assessing the total number of nematodes and IJs, and the proportion of IJs in the flasks every 2 days for 14 days. The results indicated that a 1% bacteria inoculation led to higher total nematode and IJ yield, with flasks with the highest IJ inoculum (3000 IJs/ml) having a positive effect on the total number of nematodes and IJs in cultures of *P. bohemica*.

Key words - *Phasmarhabditis*, mass production, bacteria, infective juveniles, pathogenicity

Introduction

Terrestrial slugs (Mollusca: Gastropoda) are a major pest of many crops in South Africa and are mostly controlled through the use of chemical molluscicides. One possible method for the control of molluscs is biocontrol using mollusc-parasitic nematodes (MPNs). MPNs include nematodes from the families Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (Pieterse *et al.*, 2017). *Phasmarhabditis hermaphrodita* (Schneider) Andr ssy (Rhabditida: Rhabditidae), which is the only MPN that has been successfully developed into a biocontrol product to-date, is currently being mass-produced and sold commercially throughout Europe by BASF (formally Becker Underwood) and Dudutech, under the tradenames, Nemaslug[®] and SlugTech[®], respectively (Rae *et al.*, 2007; Ross, 2019). It is capable of parasitising various mollusc species from the families Agriolimacidae, Limacidae, Arionidae, Vagnulidae and Milacidae (Wilson *et al.*, 1993; Iglesias & Speiser, 2001; Speiser *et al.*, 2001; Grewal *et al.*, 2003) (see Table 1.2 from Chapter 1). The nematode is sold as a water-dispersible formulation with the active ingredient being the infective juvenile (IJ) stage which, when dissolved in water and applied, moves around in the soil in search of slug hosts, causing death 4-21 days after infection (Wilson *et al.*, 1993; Tan & Grewal, 2001).

Phasmarhabditis hermaphrodita has been mass-produced using both *in vivo* and *in vitro* methods, although the former production involves the field collection, or rearing, of mollusc hosts in the laboratory, and it is not economically viable. The majority of research has, therefore, focused on the *in vitro* production of the nematode, either in xenic cultures, with a mix of unknown bacteria, or in monoxenic cultures, using only one known bacterial species (Wilson *et al.*, 1995a). The use of monoxenic cultures, however, is known to offer more predictable results, with it being more effective in producing a high number of IJs with consistent pathogenicity (Wilson *et al.*, 1995a; Ehlers & Shapiro-Ilan, 2005).

In the search for a bacterial species for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) tested the pathogenicity of nine bacterial isolates by injecting 10 μ l of each into the haemocoel of *Deroceras reticulatum* M ller (Agriolimacidae) slugs. Of the bacterial isolates tested, only *Aeromonas hydrophila* (Chester) Stanier (Proteobacteria: Aeromonadaceae) and *Pseudomonas fluorescens* (Fl gge) Migula (Proteobacteria: Pseudomonadaceae) caused significant mortality in the slugs. However, as *A. hydrophila* did not support the growth of *P. hermaphrodita*, it could not, therefore, be considered as a monoxenic

bacterial candidate. Bacterial isolates that supported the growth of *P. hermaphrodita* were then used in monoxenic foam chip cultures and/or liquid cultures, in combination with the nematode, after which the pathogenicity of the nematode was tested on *D. reticulatum* in a soil-based bioassay system. The results indicated that *P. hermaphrodita* caused significant mortality in slugs, producing the highest yields of IJs when grown together with the bacterial isolate *Moraxella osloensis* (Bøvre & Henriksen) Bøvre (Proteobacteria: Moraxellaceae). The above-mentioned bacterial species was, therefore, chosen for the commercial production of *P. hermaphrodita* (Wilson *et al.*, 1995b).

The BASF *P. hermaphrodita* product is produced in large-scale fermenters using *in vitro* liquid culturing, similar to that which is used for the commercial production of entomopathogenic nematodes (EPNs), and in a monoxenic association with the bacterium species *M. osloensis* (Morand *et al.*, 2004; Rae *et al.*, 2007). Establishing a monoxenic nematode-bacteria combination that can be grown in a liquid medium is essential for the consistent production of high IJ yields, and to eliminate the risk posed by the presence of the other pathogenic bacteria in the medium (Poinar Jr & Hansen, 1986; Wilson *et al.*, 1995a). The IJ life stage is produced when stressful environmental factors occur, or when the food sources become depleted (Ross, 2010). Depending on the growth medium concerned and the prevailing conditions in the fermenters, different levels of IJ concentrations are produced, with yields of over 100 000 IJs/ml having been achieved (Glen *et al.*, 1994).

The discovery of the potential of nematodes as the biocontrol agents of insect and mollusc pests has led to wide-scale interest in methods for their mass production. There is also a vast body of research on the mass-production techniques used for EPNs which can be translated to the mass-culturing of MPNs. Depending on the costs, time, resources, expertise and amount of product required, EPNs are mass-produced using *in vivo*, *in vitro* solid or *in vitro* liquid culturing methods (Ehlers & Shapiro-Ilan, 2005; El-Sadawy, 2011; Abd-Elgawad *et al.*, 2017). Although *in vivo* and *in vitro* solid culturing methods are labour-intensive, they require relatively low capital investment, whereas the *in vitro* liquid culturing is the method of choice for large companies in the developed countries, as the method requires sophisticated engineering, a high level of capital investment and running cost, and close monitoring (Ehlers & Shapiro-Ilan, 2005; Lacey & Georgis, 2012).

The objectives of the current study were to isolate, identify, culture and test the pathogenicity of bacterial isolates capable of supporting the *in vitro* culture of mollusc-associated nematode

species. In addition, the study also focused on optimising the *in vitro* liquid culture method of successful nematode-bacterial candidates, by means of testing the effect of bacteria inoculum and IJ inoculum density on the total nematode yield, on the IJ yield, and on the proportion of IJs present after 14 days.

Materials and Methods

SOURCE OF NEMATODES

The following nematode species were used for this study: *Phasmarhabditis bohemica* Nermut, Půža, Mekete & Mráček (Nematoda: Rhabditidae), *Phasmarhabditis papillosa* (Schneider) Andrassy (Nematoda: Rhabditidae), *Phasmarhabditis* sp. (KEN1) (see Chapter 4) and *Caenorhabditis elegans* (Maupas) (Nematoda: Rhabditidae). The *P. papillosa* and *C. elegans* were obtained from a collection of the Department of Conservation Ecology and Entomology at Stellenbosch University and were initially collected during a survey by Pieterse *et al.* (2017). The *P. bohemica* isolate used in the study was kindly supplied by Dr Vladimir Půža from the Biology Centre CAS, Institute of Entomology ASCR, Laboratory of Entomopathogenic Nematodes, Czech Republic. The *Phasmarhabditis* sp. (KEN1) (see Chapter 4) was obtained in a collaborative study with Dr Solveig Haukeland from the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi, Kenya, and Dr Jenna Ross (co-supervisor).

ISOLATION AND SOURCE OF BACTERIAL ISOLATES

A total of ten bacterial isolates were used in this study. Five bacterial candidates were isolated by dissecting *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Mollusca: Agriolimacidae), swabbing the intestine and body cavity, and streaking bacteria onto nutrient agar plates (3 g beef extract, 5 g Tryptone, 8 g NaCl, 15 g agar in 1 L of water, autoclaved for 20 min at 121°C), which were incubated at 25°C for 48 h. The plates were then visually inspected and different bacterial colonies were subcultured onto fresh plates, until only a single species remained on each plate. Two additional slug-associated bacterial isolates, which were obtained from a collection of the Department of Conservation Ecology and Entomology at Stellenbosch University, were collected during a survey conducted by Pieterse (2016). Three bacterial species associated with EPNs, which were also included in the study, were obtained from the collection of the Department of Conservation Ecology and Entomology at Stellenbosch University.

IDENTIFICATION OF BACTERIA

Bacterial isolates isolated from dissected *D. invadens* in this study were identified by extracting the total genomic DNA using a Zymo Research fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). The DNA of the 16S rRNA gene was then amplified, using the primer pair 8F and 1512R (Felske *et al.*, 1997). The amplified products were then sequenced by the Central Analytical Facilities (CAF) at Stellenbosch University. Sequence traces were then inspected and assembled using the software CLC Main Workbench 7.6.4 (CLC Bio, Aarhus, Denmark, <http://www.clcbio/products/clc-main-workbench/>) and analysed using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were then uploaded to the GenBank database (<http://www.ncbi.nlm.nih.gov/>), at the National Centre for Biotechnology Information (NCBI).

BACTERIA PATHOGENICITY

Bacterial isolates were streaked onto nutrient agar plates (3 g beef extract, 5 g Tryptone, 8 g NaCl, 15 g agar in 1 L water, autoclaved at 121°C for 20 min) and grown at 25°C for 24 h. The plates were then washed with 5 ml autoclaved 0.9% saline solution under sterile conditions, with 10 µl of each bacterial isolate being injected directly into the haemocoel of ten *D. invadens* specimens. The control slugs were injected with 10 µl sterile 0.9% saline solution. After having been treated per bacterial isolate, ten slugs were placed in plastic containers (145 mm × 205 mm × 80 mm) lined with moist tissue paper and covered with perforated lids. The boxes were kept at 18°C for 5 days, and the slugs were provided with carrot discs as food source that were replaced daily. After 5 days, the number of dead slugs was counted. The experiment was repeated with a fresh batch of slugs.

BACTERIA FEEDING PREFERENCE

Each of the ten bacterial isolates detailed earlier in this chapter were streaked onto five Wout's agar plates (9.5-cm-diam.) each and left at 25°C for 24 h (Wouts, 1981). A hundred IJs, in *ca.* 200 µl distilled water, of the four nematode species (*P. bohémica*, *P. papillosa*, *Phasmarhabditis* sp. (KEN1) and *C. elegans*), were added to the plates. The plates were then covered with parafilm and left at 18°C for 5 days. After 5 days, the plates were visually inspected with a light microscope (Leica MZ7s), so as to determine whether the nematodes had survived, recovered and reproduced in association with the different bacterial isolates. The

experiment was repeated three times. The plates were scored based on four categories: Dead (0), Alive (1), Recovered (2), and Reproduced (3). Plates categorised as ‘Dead’ showed 100% dead IJs; those categorised as ‘Alive’ showed that despite the IJs having survived, they neither recovered nor developed; those categorised as ‘Recovered’ showed that the IJs had developed into adults, but had not reproduced; and those categorised as ‘Reproduced’ showed the presence of adult nematodes with next-generation juveniles (J1-J3).

AXENISATION OF NEMATODES

As *P. bohemica* showed good survival, recovery and reproduction among a range of bacterial isolates in the previous section, it was used for the monoxenic cultures. To establish such cultures, the eggs were harvested from adult female nematodes. IJs were added to 9.5-cm- diam. Petri dishes, lined with a piece of moist filter paper, containing freeze-killed *D. invadens*. The Petri dishes were sealed with parafilm and left at 18°C. The IJs of *P. bohemica* were then left for 96 hours to develop into adult males and females, and for the females to form fertilised eggs. The plates were then washed with saline solution and passed through a 212-µm aperture sieve to remove any life stages that were not adult females. The adult females were then added to Eppendorf tubes and lightly crushed for 30 sec with an autoclaved plastic grinding rod. A mixture of bleach and NaOH (2.25 ml bleach, 0.2 g NaOH in 10 ml distilled autoclaved water) was then added to the tubes, with it being left to work on the nematode mix for 8 min. The mixture in the tubes was then centrifuged and washed three times with sterile water, so as to remove all bleach and NaOH solution, until only a pellet with eggs in water remained. The mixture was then pipetted into 200 µl sterile tryptone soy broth (TSB) in 24-well plates, sealed with parafilm and left for 2 days at 25°C to test for bacterial contamination. If, after 2 days, no bacterial growth had occurred, and the nematodes had hatched, the J1 and J2 nematodes were used for liquid media culturing.

ESTABLISHING A LIQUID CULTURE

For the liquid culture experiments, the bacterial isolate *Pseudomonas* sp. (1) (GenBank accession number: KX531096) and *P. bohemica* were used, as they demonstrated a good response in the experiment detailed, in the bacterial feeding preference section. Thirty millilitres of Luria Broth (LB) (10 g NaCl, 10 g Tryptone, 5 g yeast extract/L water, autoclaved at 121°C for 20 min) in a 250 ml Erlenmeyer flask was inoculated with *Pseudomonas* sp. (1) and incubated on an orbital shaker (140 rpm), at 28°C for 48 h. A 250-ml Erlenmeyer flask with 30 ml liquid culture media (LCM) (9 g pig kidney, 17.4 g yeast extract, 8.6 g egg yolk powder,

52.6 g sunflower oil/L water; autoclaved for 20 min at 121°C) was then inoculated with 1.2 ml (4%) of *Pseudomonas* sp. (1) in LB. The flask was then again incubated on an orbital shaker (140 rpm) at 28°C for 48 h. Once the bacteria had grown inside the LCM, the sterile eggs and juvenile stages obtained after the axenisation of the nematodes were added to the flasks. The flasks were then incubated at 18°C on orbital shakers at 140 rpm for 14 days, at which point the majority of the population in the flask were at the IJ stage. This flask was then used as inoculum for the following experiments, with it, hereafter, being referred to as the ‘inoculum flask’.

BACTERIAL INOCULUM CONCENTRATION

To test the impact of bacteria inoculum density, nine 250-ml Erlenmeyer flasks containing 30 ml LCM were inoculated with three different *Pseudomonas* sp. (1) inoculum concentrations: 1%, 3%, or 5%. Three replicate flasks were set up per treatment. IJs from the inoculum flask were then added to the flasks to achieve an initial nematode density of 2000 IJs/ml. All nine flasks were incubated at 18°C on an orbital shaker (140 rpm) in the dark for 14 days. Every 2 days, the total number of nematodes and the total amount of IJs were recorded. The experiment was conducted twice.

NEMATODE INOCULUM CONCENTRATION

To test the impact of nematode inoculum density, nine 250-ml Erlenmeyer flasks containing 30 ml LCM were inoculated with 1.2 ml (4%) *Pseudomonas* sp. (1) in LB, after which they were incubated on an orbital shaker (140 rpm) at 28°C for 48 h. Three treatments were established: 1000 IJs/ml, 2000 IJs/ml, and 3000 IJs/ml, with three replicate flasks per treatment. The flasks were incubated at 18°C on an orbital shaker (140 rpm) in the dark for 14 days. Every 2 days, the total number of nematodes and IJs were recorded. The experiment was conducted twice.

STATISTICAL ANALYSIS

For the bacteria pathogenicity data, a generalised linear model (GLZ) with a Poisson distribution and a log link function was used. The above was confirmed by means of a log-likelihood ratio test, giving a chi-squared value of 2.7844 and $P < 0.001$. The liquid culture data were analysed using a repeated-measures analysis of variance (ANOVA), with the compound symmetry assumption on the correlations over the days concerned.

Results

ISOLATION AND IDENTIFICATION OF BACTERIA

Five bacterial isolates were identified in the current study from the intestine and body cavity of *D. invadens* (Table 6.1). They included three *Pseudomonas* spp., an *Aeromonas* sp. and a *Buttiauxella* sp., and the generated 16S rRNA sequences were submitted to GenBank (Table 6.1). GenBank details of the additional slug-associated bacterial species and EPN-associated bacterial species are indicated in Tables 6.2 and 6.3, respectively.

Table 6.1. The partial 16S rRNA gene accession numbers of five bacterial isolates from *Deroceras invadens* collected from George sample sites with NCBI matches, with identity and coverage of between 98 and 100%.

Bacterial isolate	GenBank accession number	Habitat	NCBI match			
			Species/strain	GenBank accession number	Query coverage (%)	Percentage identity (%)
<i>Pseudomonas</i> sp. (2)	MN611311	Water treatment plant	<i>Pseudomonas</i> sp. H3-5	MN197816	100	99
<i>Pseudomonas</i> sp. (3)	MN611355	Soil	<i>Pseudomonas</i> sp. 36 DCP	MK072848	100	99.78
<i>Pseudomonas</i> sp. (4)	MN611356	Woodland soil	<i>Pseudomonas</i> sp. S3Bt34y	MH463748	100	99.28
<i>Aeromonas</i> sp.	MN611353	Aquatic	<i>Aeromonas salmonicida</i> SHY16-3432	CP038102	100	99.29
<i>Buttiauxella</i> sp.	MN611312	Snails	<i>Buttiauxella</i> sp. P5	DQ223872	98	99.15

Table 6.2. GenBank accession numbers of the two slug-associated bacterial species used in the current study obtained from the collection housed at the Department of Conservation Ecology and Entomology at Stellenbosch University.

Bacterial isolate	GenBank accession number	Reference
<i>Pseudomonas</i> sp. (1)	KX531096	Pieterse, 2016
<i>Kluyvera</i> sp.	KX531097	Pieterse, 2016

Table 6.3. The GenBank accession numbers of the three bacterial species associated with EPNs used in the current study obtained from the collection housed at the Department of Conservation Ecology and Entomology at Stellenbosch University

Bacterial isolate	Strain	GenBank accession number	Reference
<i>Photorhabdus heterorhabditis</i>	SF41 ^T	HQ142626	Ferreira <i>et al.</i> , 2014
<i>P. luminescens</i> subsp. <i>noenieputensis</i>	AM7	JQ424880	Ferreira <i>et al.</i> , 2013a
<i>Xenorhabdus khoisanae</i>	SF87 ^T	HQ142625	Ferreira <i>et al.</i> , 2013b

BACTERIA PATHOGENICITY

All bacterial isolates tested caused significant mortality ($P < 0.001$) in the slug host *D. invadens*, with the exception of *Buttiauxella* sp. in this study. *Pseudomonas* sp. (3), *Kluyvera* sp. and *Aeromonas* sp. all caused 100% mortality 5 days after being injected into the slugs. *Photorhabdus heterorhabditis* caused mortality in 16 slugs and *Pseudomonas* sp. (4), *X. khoisanae* and *Pseudomonas* sp. (1) caused mortality in eight of 20 slugs after 5 days. *Pseudomonas* sp. (2), *P. luminescens* and *Buttiauxella* sp. were the least pathogenic, with *Pseudomonas* sp. (2) and *P. luminescens* only killing four slugs, and *Buttiauxella* sp. killing none (Fig. 6.1).

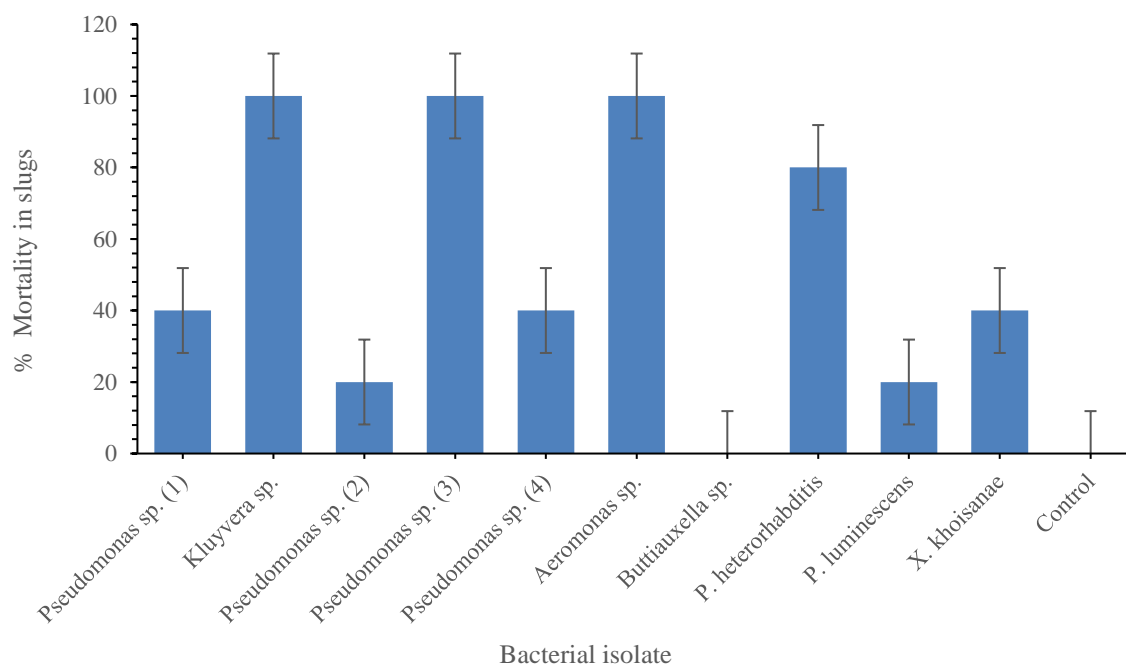


Figure 6.1. Mortality of *Deroceras invadens*, 5 days after being injected with different bacterial isolates.

BACTERIAL FEEDING PREFERENCE

Phasmarhabditis bohemica was able to reproduce when feeding on *Pseudomonas* sp. (1), (2), (3) and (4), *Buttiauxella* sp., as well as *P. luminescens* and *X. khoisanae* from EPNs. Although the IJs recovered and developed into adults, they did not reproduce when feeding on *Kluyvera* sp., and they survived, but remained in their IJ phase, when they were tested with *Aeromonas* sp. and *P. heterorhabditis* (Table. 6.4).

Table 6.4. Ability of different bacterial isolates to support the growth of the nematodes *Phasmarhabditis bohemica*, *Phasmarhabditis papillosa*, *Phasmarhabditis* sp. (KEN1) and *Caenorhabditis elegans*.

Bacterial isolate	Growth support score			
	<i>P. bohemica</i>	<i>P. papillosa</i>	<i>Phasmarhabditis</i> sp. (KEN1)	<i>C. elegans</i>
<i>Aeromonas</i> sp.	1	2	0	1
<i>Buttiauxella</i> sp.	3	1	3	2
<i>Kluyvera</i> sp.	2	2	3	3
<i>Pseudomonas</i> sp. (1)	3	2	3	2
<i>Pseudomonas</i> sp. (2)	3	2	0	2
<i>Pseudomonas</i> sp. (3)	3	2	0	2
<i>Pseudomonas</i> sp. (4)	3	3	0	3
<i>Photorhabdus heterorhabditis</i>	1	0	0	0
<i>Photorhabdus luminescens</i>	3	1	0	3
<i>Xenorhabdus khoisanae</i>	3	2	0	0

Score: 0 = dead; 1 = alive; 2 = recovered; 3 = reproduced.

Phasmarhabditis papillosa only reproduced when tested with *Pseudomonas* sp. (4). When tested with *Pseudomonas* species (1), (2) and (3), as well as with *Kluyvera* sp., *Aeromonas* sp. and *X. khoisanae*, the IJs, despite developing into adult males and females, did not reproduce. When tested with *Buttiauxella* sp. and *P. luminescens*, the *P. papillosa* IJs survived, but did not recover, and, with *P. heterorhabditis*, all the IJs died (Table 6.4).

Phasmarhabditis sp. (KEN1) reproduced when tested with *Pseudomonas* sp. (1), *Kluyvera* sp. and *Buttiauxella* sp., but died when it was tested with any of the other bacterial candidates (Table 6.4).

Caenorhabditis elegans was able to reproduce when feeding on *Kluyvera* sp., *Pseudomonas* sp. (4) and *P. luminescens*. The IJs of *C. elegans* developed into adults but did not reproduce

when tested on *Pseudomonas* sp. (1), (2) and (3), as well as on *Buttiauxella* sp., and they did not recover from the IJ phase when they were tested with *Aeromonas* sp. As with *Phasmarhabditis* sp. (KEN1), *C. elegans* died when it was tested with the EPN bacterial isolates, *P. heterorhabditis* and *X. khoisanae* (Table. 6.4).

BACTERIAL INOCULUM CONCENTRATION

All flasks displayed exponential growth in the combination of *P. bohemica* and *Pseudomonas* sp. (1) with regards to the total number of nematodes between days 2 and 6. This growth slowed down between days 6 and 10, and even more so between days 10 and 14. The flasks that were inoculated with 3% and 5% bacteria seemingly neared a stationary phase in nematode numbers by day 14 (Fig. 6.2a). The flasks containing 1% bacterial inoculum had a significantly higher (ANOVA, $F = 3023$, $df = 30, 180$, $P < 0.001$) total number of nematodes throughout the experimental period, reaching an average count of 63126 nematodes/ml on day 14 (Fig. 6.2A). The flasks inoculated with 5% bacteria had the second highest total amount of nematodes throughout the experiment, reaching an average count of 42340 nematodes/ml on day 14 (Fig 6.2A). The flasks inoculated with 3% bacteria had the lowest total nematode yield throughout the experiment, reaching only 34965 nematodes/ml by day 14 (Figure 6.2A).

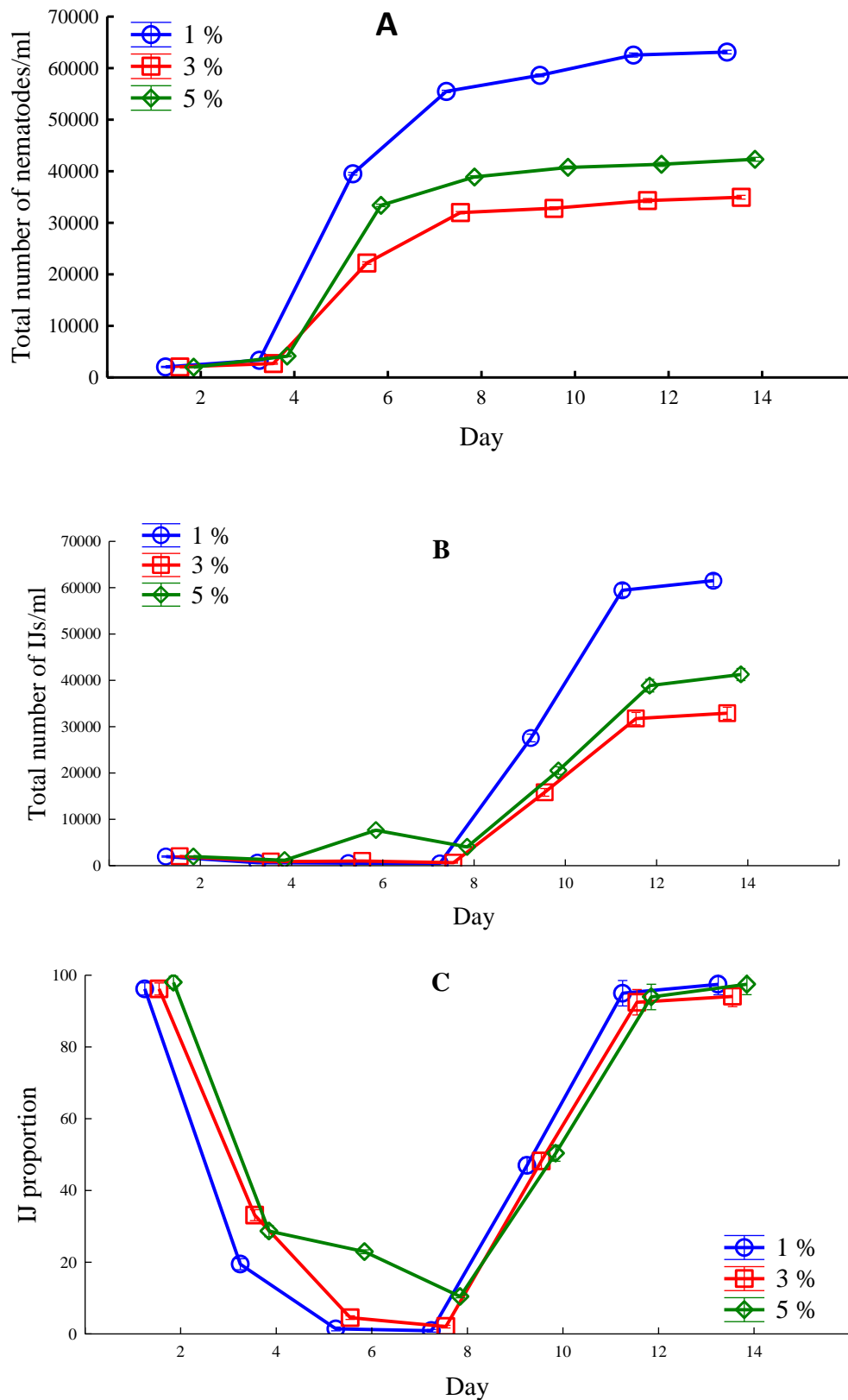


Figure 6.2. Influence of different bacterial densities (1%, 3% and 5% *Pseudomonas* sp. (1)) on (A) total number of nematodes/ml, (B) total number of IJs/ml, and (C) IJ proportion in total nematode population of *Phasmarhabditis bohemica*.

The results show very low IJ numbers from day 2 to day 6, by which time most of the IJs had recovered and developed into other stages. However, by day 8, population numbers reached a point where IJ formation was again induced, causing a sharp increase in IJ numbers for all bacterial concentrations. The formation of IJs then slowed down again for all flasks from day 12 onwards, reaching an almost stationary phase by day 14 (Fig. 6.2B). The flasks inoculated with 1% bacteria had a significantly higher total number of IJs (61515 IJs/ml) ($F = 336.23$; $df = 30, 180$; $P < 0.001$) on day 14 than did those inoculated with 3% or 5% bacteria. The flasks inoculated with 5% had 41,270 IJs/ml on day 14, and the flasks inoculated with 3% had the lowest number of IJs, with a concentration of only 32909 IJs/ml (Fig. 6.2B).

The results show a sharp decrease in the IJ proportion from day 2 to day 4, with it slowing down by day 6, and reaching a low at day 8, as the IJs recovered and the other life stages came to make up a larger portion of the population than before. However, between days 8 and 12, an exponential increase occurred in the IJ proportion of the population, as overcrowding in the flasks induced the formation of the IJ stage. By day 12, the increase in IJ proportion slowed down, again nearing a stationary phase by day 14 (Fig. 6.2C). The flasks inoculated with 1% and 5% had the same proportion of IJs on day 14, both reaching 97% IJs. The proportion was significantly higher ($F = 38.99$; $df = 30, 180$; $P < 0.001$) than it was for the IJ proportion of flasks inoculated with 3% bacteria, which yielded 94% IJs (Fig. 6.2C).

NEMATODE INOCULUM CONCENTRATION

The flasks in the IJ inoculum density experiment showed a low total number of nematodes at day 4, which slowly increased between days 4 and 6, and then showed exponential growth between days 6 and 8. However, after day 8, the increase in the total number of nematodes started to slow down, and to near a stationary phase by day 14 in all the flasks (Fig. 6.3A). The flasks inoculated with 3000 IJs/ml showed a rise in total nematode numbers after day 6, yielding a significantly higher total number of nematodes (45156 nematodes/ml) (ANOVA, $F = 3023$; $df = 30, 180$; $P < 0.001$) by day 14 than did the other inoculum concentrations. Flasks inoculated with 1000 IJs/ml yielded 26839 nematodes/ml, which rate was also significantly higher (ANOVA, $F = 3023$; $df = 30, 180$; $P < 0.001$) than was that of the 24110 nematodes/ml yield of flasks inoculated with 2000 IJs/ml (Fig 6.3A).

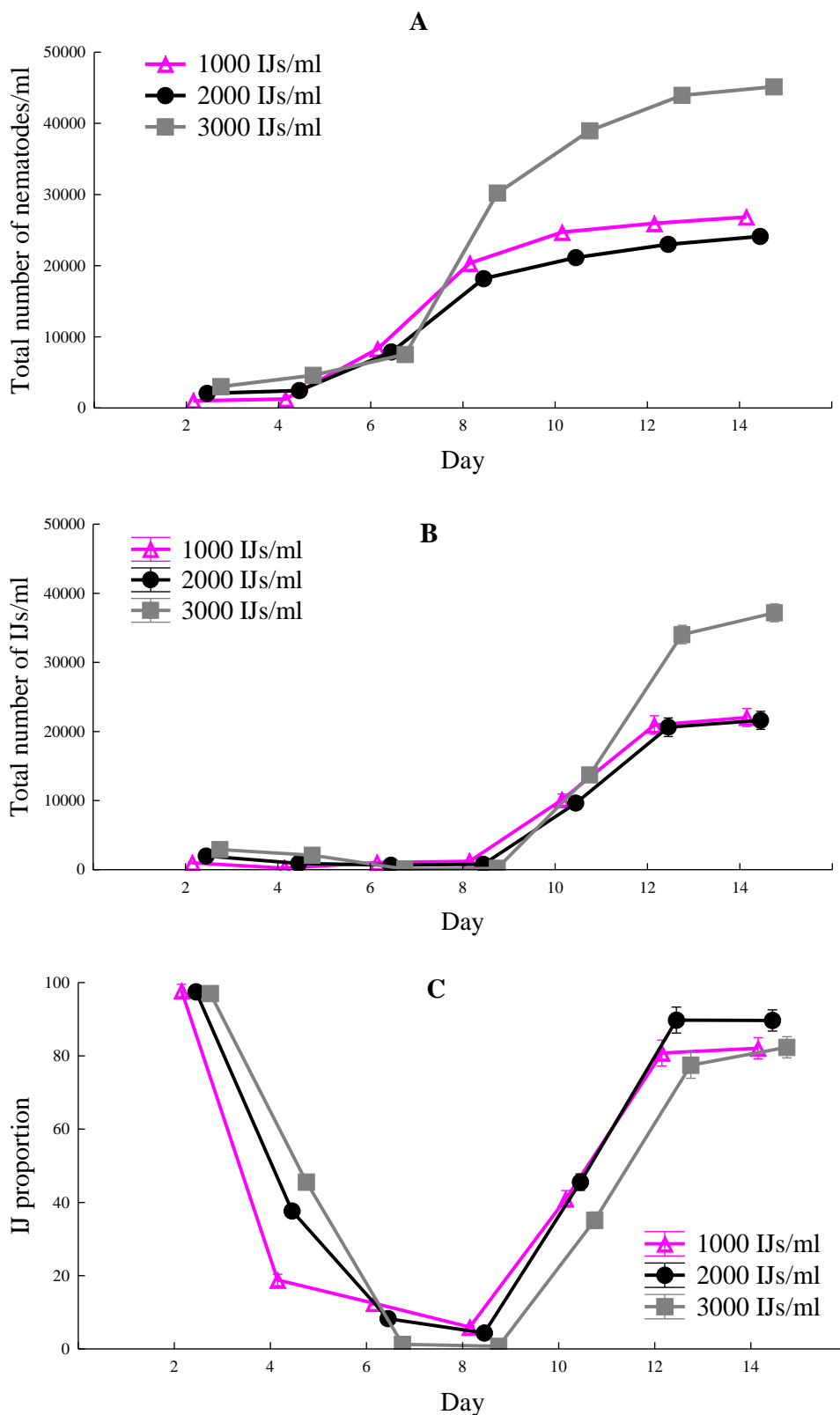


Figure 6.3. Influence of different infective juvenile inoculum densities (1000, 2000 and 3000 IJs/ml) on (a) total number of nematodes/ml, (b) total number of IJs/ml, and (c) IJ proportion in total nematode population of monoxenic cultures containing *Phasmarhabditis bohemica* and *Pseudomonas* sp. (1).

All the flasks showed a slow decrease in the total number of IJs from day 2 onwards, as the IJs recovered and developed into other life stages. The number of IJs reached a low at day 8, after which a sharp increase in numbers was observed in all the flasks between days 8 and 12, as overcrowding in the flasks induced the formation of IJs. The formation of IJs slowed down from day 12 onwards, nearing a plateau by day 14 (Fig. 6.3b). The flasks inoculated with 3000 IJs/ml had significantly higher ($F = 336.23$; $df = 30, 180$; $P < 0.001$) numbers of IJs from day 10 onwards, reaching a high of 37177 IJs/ml by day 14. No significant difference ($P < 0.001$; $df = 30, 180$; $F = 336.23$) was found in the total number of IJs in the flasks inoculated with 1000 IJs/ml (22038 IJs/ml) and in the flasks inoculated with 2000 IJs/ml (21,621 IJs/ml) (Fig. 6.3b).

A rapid decrease in the IJ proportion was observed for all the flasks between days 2 and 6, as the nematodes recovered from the IJ phase and started to reproduce. The IJ proportion reached a low on day 8, after which all the flasks showed a rapid increase in the IJ proportion. By day 12, all the flasks showed a relatively slow rate of increase in the IJ proportion, which neared a stationary phase by day 14 (Fig. 6.3c). On days 10 and 12, significant differences ($F = 38.99$; $df = 30, 180$; $P < 0.001$) were found in the IJ proportions of the three treatments, with the flasks inoculated with 2000 IJs/ml having the highest proportion of IJs, followed by the flasks inoculated with 1000 IJs/ml, with the 3000 IJs/ml flasks having the lowest IJ proportion. However, by day 14, the flasks inoculated with 2000 IJs/ml had a significantly higher ($F = 38.99$; $df = 30, 180$; $P < 0.001$) IJ proportion (90%) than did the other two treatments, which showed no significant difference ($F = 38.99$; $df = 30, 180$; $P < 0.001$) in IJ proportion, with both having an average of 82% IJs each (Fig. 6.3c).

Discussion

In the current study, ten bacterial isolates were tested based on their pathogenicity to the slug host *D. invadens*, as well as their ability to grow with different slug-associated nematode candidates. Of these ten bacterial isolates, five were isolated from the body cavity of *D. invadens*, namely *Pseudomonas* sp. (2), *Pseudomonas* sp. (3), *Pseudomonas* sp. (4), *Aeromonas* sp. and *Buttiauxella* sp. The species, along with two previously identified slug-associated bacterial isolates from Pieterse (2016), and three bacterial species from EPNs, were used in the experiment. The nematode species that were tested were *P. bohemica*, *P. papillosa*, *C. elegans* and *Phasmarhabditis* sp. (KEN1). *Pseudomonas* sp. (4) was the bacterium preferred by most of the nematode species, leading to recovery and reproduction in all the species concerned, except for *Phasmarhabditis* sp. (KEN1), in which it caused mortality. In their search for a

bacterial species suitable for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) tested *A. hydrophila*, finding that it was capable of causing mortality in *D. reticulatum*. Although it was isolated from the intestines of *P. hermaphrodita* IJs, it was not capable of successfully supporting growth in *P. hermaphrodita*. Wilson *et al.* (1995b) also tested two isolates of *Pseudomonas fluorescens* in foam chip cultures, finding it capable of supporting the growth of *P. hermaphrodita*.

The pathogenicity tests of the bacterial species isolated from slugs, and of the three bacterial species associated with EPNs, showed that *Pseudomonas* sp. (3), *Kluyvera* sp. and *Aeromonas* sp. were most pathogenic, causing 100% mortality of slugs. *Buttiauxella* sp. was the least pathogenic of the bacteria tested, as it was found to cause zero mortality. The other bacterial isolates, including the species associated with EPNs, despite causing significant mortality in slugs, were less pathogenic.

In EPN studies, the association between the mutualistic bacteria of EPNs is very strong, and it was always believed that, in the case of *Steinernema*, each nematode species was always associated with its own unique bacterial species (Dreyer *et al.*, 2018). However, it was found that *X. khoisanae* has the ability to switch between nematode species, and even between distantly related clades (Dreyer *et al.*, 2018). Lee and Stock (2010) show that at least 17 host switches of strains of *Xenorhabdus* species occur between *Steinernema* species, and even between species of different clades. In the case of the slug nematode *P. hermaphrodita*, the association between the nematode and the bacteria concerned is relatively weak, and many bacterial species were tested to determine the optimum production and viability that could be attained from being grown together with *P. hermaphrodita* (Wilson *et al.*, 1995b).

While selecting a bacterium for the monoxenic culturing of *P. hermaphrodita*, Wilson *et al.* (1995b) found that, when *M. osloensis* was injected into the body cavity of *D. reticulatum*, it was not pathogenic. However, when *P. hermaphrodita* was grown in a monoxenic liquid culture together with *M. osloensis*, and the pathogenicity of the resulting nematodes was retested, they were pathogenic to *D. reticulatum*. Such was especially the case when the pathogenic bacteria species were injected into the body cavities of the slugs. Wilson *et al.* (1995b) concluded that the interaction between the bacterium and both the nematode and the immune system of the slug host is important, when testing the pathogenicity of different nematode-bacteria combinations.

Again, reflecting on EPN studies, EPNs have shown not to rely solely on the bacteria to kill the host after infection, but that, after recovery, the IJs tend to release venom proteins that are lethal and that actively contribute to the nematode-bacterium complex pathogenicity (Lu *et al.*, 2017). Therefore, the bacteria that were tested for pathogenicity in the current study, by means of being injected into the haemocoel of slugs, need to be tested in combination with a slug-parasitic nematode species, as well. The procedures followed in the present study are similar to those used in work that was done by De Ley *et al.* (2017). The researchers collected 40 bacterial species associated with slugs, identifying them by means of using the 16S RNA gene. Currently, the bacterial species are systematically being screened in bioassays to determine their pathogenicity, along with the three species of *Phasmarhabditis* and the other available rabditid cultures found in North America (De Ley *et al.*, 2017).

Phasmarhabditis bohemica was established in *in vitro* liquid cultures with the bacterial species *Pseudomonas* sp. (1). The culture methods employed were then further refined by means of testing the effect of bacterial inoculum density and the number of IJs added to flasks on the total nematode yield, and the IJ yield and proportion. The results obtained showed that the flasks inoculated with 1% bacteria had significantly higher total nematode and IJ yields than did the flasks inoculated with 3% or 5% bacteria. The flasks inoculated with 1% and 5% bacteria had the same IJ proportions by day 14, which were higher than were obtained with those inoculated with 3% bacteria. The higher total nematode and IJ yields obtained with 1% bacteria inoculation confirm the recommendation by Ehlers (2001) that the inoculum density of the symbiotic bacteria be between 0.5 and 1% of the culture volume when mass-producing EPNs.

The flasks inoculated with 3000 IJs/ml had the highest yield of nematodes and the highest yield of IJs when compared to the yield of flasks inoculated with 1000 IJs/ml or 2000 IJs/ml. The flasks inoculated with 2000 IJs/ml, however, had a significantly higher IJ proportion (90%) than did the flasks inoculated with 1000 or 3000 IJs/ml. The results differ from those of the EPN study in Dunn *et al.* (2019), who found that an inoculum concentration of 1000 IJs/ml gave a higher IJ yield in LCM flasks of *S. jeffreyense* than did a concentration of either 2000 or 3000 IJs/ml. However, the effect of inoculum concentration seems to vary between nematode species, as *Heterorhabditis bacteriophora* tends to produce optimal yields with intermediate inoculum density, whereas *Steinernema carpocapsae* produces higher yields in higher inoculum concentrations, and *Heterorhabditis indica* is unaffected by inoculum concentration (Han, 1996; Ehlers *et al.*, 2000; Shapiro-Ilan & Gaugler, 2002).

The small amount of media (30 ml) used in the current study is a possible reason for the high IJ yields obtained from all flasks by day 14. The finding is similar to the results that were obtained in the EPN study by Dunn *et al.* (2019), who found that 30 ml of liquid culture media produced higher yields of *S. jeffreyense* IJs than flasks with 50 ml of LCM. Dunn *et al.* (2019) postulate that the higher nematode yield in lower media volumes can be accredited to the greater surface-area-to-volume ratio in the flasks, which leads to the enhanced transfer, or distribution, of oxygen. A possible reason for the large proportion of the IJs in the cultures is the subsequent overcrowding in the media, which leads to the accumulation of waste products and ammonia (Shapiro-Ilan *et al.*, 2000; San-Blas *et al.*, 2008). High concentrations of ammonia are produced when nematode populations are overcrowded, which has been proven to induce the emergence of IJs in the EPN *Steinernema feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding (Wright, 2004; San-Blas *et al.*, 2008). Ross (2010) also demonstrates that nematodes secrete a series of small-molecule pheromones when they encounter starvation or overcrowding, which facilitates the communication between the nematodes, and which causes the formation of IJs.

The biocontrol potential of *P. bohemica* has not yet been researched. Although the focus of this chapter was to optimize the growth conditions and production of the nematodes, a pathogenicity test needs to be done on slugs to determine if *P. bohemica* and *Pseudomonas* sp. (1) are capable of causing slug mortality when grown in liquid culture media. In future research, MPNs and bacterial candidates isolated in South Africa, as well as other African countries, should undergo similar testing if they are to be developed for commercial production. This is required to determine the ability of the bacteria to support the growth of the nematodes, and to cause mortality in various slug hosts known to be pestiferous. The best nematode-bacterium combination should, then be grown in *in vitro* liquid cultures, with the pathogenicity of the nematode-bacterium combination retested on slug hosts. If a nematode-bacterium combination is found that grows well and that causes significant mortality in slug hosts, its production should be optimized by testing the effect of different growth conditions on the IJ yield, as was done in the current study.

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Chapter 7

Efficacy of a novel environmentally friendly application method of metaldehyde pellets to control *Cornu aspersum* (Helicidae), the brown garden snail

Summary

The brown garden snail, *Cornu aspersum* (Helicidae), is an international pest that thrives in home gardens and in agricultural environments, such as apple orchards. It is also the only alien species that has been recorded from all the provinces of South Africa. Terrestrial mollusc pests are traditionally controlled through the use of chemical molluscicide pellets, with the active ingredient usually being metaldehyde, methiocarb or thiodicarb. However, when they are overused, the chemicals can be toxic to non-target organisms, or accumulate in the environment. Baitchain, a novel molluscicide formulation consisting of metaldehyde bait pellets arranged on a cord is a new product that is designed to be tied around the base of the plant or the tree's stem or trunk for protection. In this study, Baitchain is tested in a shaded apple orchard, by means of tying the bait-assembly around the base of the trees' trunks, and by means of comparing with the commercial product Sluggit, which is a metaldehyde bait pellet that is applied to the soil surface, to determine their impact on *Cornu aspersum*. Two different concentrations were used of each product; Baitchain 15 and Sluggit 15, at 15 g/kg metaldehyde and Baitchain 40 and Sluggit 40, at 40 g/kg metaldehyde, which were either applied on their own, or in combination. All treatments caused a significant decrease (64-86%) in snail numbers after 28 days. In addition, all treatments applied at 40g/kg metaldehyde caused significant snail mortality. Baitchain 15 applied on its own or in combination with Sluggit 15 also caused significant snail mortality. Sluggit 15 applied on its own to the soil surface showed decreased effectiveness, possibly due to the high rainfall levels experienced during the trial. The increased effectiveness achieved by using Baitchain, even at the lower 15/kg concentration, means that it could be applied alone, or in combination, as part of an integrated pest management strategy for snail control.

Keywords - *Cornu aspersum*; molluscicides; Sluggit; Baitchain

Introduction

In 2010, it was estimated that approximately 34 non-native terrestrial mollusc species were present in South Africa. Of the species, 28 are believed to have become established, with 13 being regarded as invasive species (Herbert, 2010). Twenty-nine of the species originate from Europe, of which nine are from the Mediterranean. The above is mainly a result of the European colonial history of South Africa, but it is also because of the Mediterranean-like, temperate winter rainfall climate of the Cape area (Herbert, 2010). Although many introduced terrestrial gastropods in South Africa have become widely distributed, many have not successfully spread into the natural environments, being mostly restricted to transformed habitats and to monoculture crops (Herbert, 2010).

The brown garden snail, *Cornu aspersum* (Müller, 1774) (Helicidae), is an international pest that thrives in areas of human settlement. It is also the only alien species that has been recorded from all nine provinces of South Africa (Herbert, 2010). During unfavourable seasonal conditions, the snail hibernates in the soil at depths of 10-20 mm, and, when conditions improve, it emerges from the soil for mating, thereafter laying eggs at a depth of 30-40 mm (Basinger, 1931). The snail then moves up into the trees, where it spends most of its time resting on the trunk, feeding mostly at night or during moist periods. The snail feeds on developing foliar buds and young leaves in spring, leading to stunted shoot growth and decreased yield of the crops. During the dry summer months, *C. aspersum* may aestivate, by sealing the shell opening with an epiphragm (Sakovich, 2002). In areas of extreme *C. aspersum* infestation, growers estimate that crop losses can reach up to 25% (Sanderson & Sirgel, 2001).

Terrestrial mollusc pests are primarily controlled through the use of chemical molluscicide pellets, usually containing metaldehyde, methiocarb or thiodicarb (Bailey, 2002). The pellets consist mainly of wheat, bran or barley flour, which serves as an attractant, and which is combined with 2-8% of an active ingredient or toxicant (Bailey, 2002). The molluscs encounter the chemicals by feeding on the pellets, or by coming into dermal contact with them, which causes the chemicals to act as stomach or contact poisons (Henderson & Triebkorn, 2002). However, when overused, the chemicals can be toxic to non-target organisms, or they can accumulate in the environment (Fisher & Orth, 1975; Castle *et al.*, 2017). In addition, increased pressure has been exerted from the regulatory sector, and from water suppliers in Europe, to limit the agricultural use of metaldehyde, due to concerns about its presence in drinking water (Castle *et al.*, 2017) and in small mammals (Ross, 2019). Iron phosphate is another chemical that is effective at controlling slugs, with it being less harmful to non-target organisms (Roberts

et al., 1990; Clark, 1993; Koch *et al.*, 2000). The use of iron phosphate is, however, more expensive than is the use of metaldehyde (Sepasi *et al.*, 2019).

The molluscicidal properties of metaldehyde were discovered in the 1930s in South Africa, at which time it was sold as fuel tablets (Gimingham, 1940). Within four years, it became the most popular bait poison recommended for use against terrestrial gastropod pests in the UK (Gimingham, 1940). In 1996, it was estimated that metaldehyde was used on 55% of the crop areas where chemicals were being used against terrestrial gastropods (Garthwaite & Thomas, 1996).

A recent study reported that the use of a physical barrier, in combination with mineral oil and a snail-repellent paint (Sabzarang) containing copper and iron salts, painted as a barrier around the tree trunk was more effective at reducing the numbers of the citrus white snail, *Helicella candeharica* Pfeiffer (Panpulmonata: Helicidae) in citrus trees than was the surface broadcasting of metaldehyde pellets, or iron phosphate pellets, applied to the soil (Sepasi *et al.*, 2019).

The current study tested the efficacy of a novel formulated molluscicide pellet with the active ingredient, metaldehyde, on a cord forming a continuous chain of bait pellets, which is tied around the base of trees' trunks in an apple orchard, and which differs from the conventional surface broadcast of molluscicide pellets. The efficacy of the method was investigated by testing whether *C. aspersum* was able to cross the baited cord barrier and move up into the trees. The innovative product was also compared with molluscicide pellets applied to the soil, to compare the difference in efficacy between the two application methods. The *C. aspersum* numbers in the treated areas were also noted before and after the experiment, so as to determine whether the treatments involved caused a decrease in the snail numbers concerned.

Materials and Methods

ORIGIN OF THE CHEMICALS

The chemical products tested in the trial were obtained from the manufacturer, Orchard Agrikem, based in Worcester in the Western Cape province of South Africa. The efficacy of two different molluscicide products was tested, namely Baitchain, which is a metaldehyde bait pellet formulated on a cord, which is tied around the base of a tree's trunk as a barrier (Fig. 7.1), and Sluggit, a metaldehyde bait pellet, which is applied to the surface of the soil. The products both consist of a bait to attract molluscs, combined with two different concentrations of the active ingredient metaldehyde.



Figure 7.1. Baitchain applied to the trunk of an apple tree in the orchard, with a *Cornu aspersum* snail.

TEST AREA

The trial was conducted in August 2018, and repeated in August 2019, in an apple orchard (34°2'40.74"S, 19°19'0.51"E) covered with 30% shade cloth, outside Villiersdorp, in the Western Cape province of South Africa. Each of the 28 experimental plots measured 14 × 4 m in size. Each plot consisted of a row of six apple trees, planted 2 m apart, on a strip of bare soil, and flanked by a 1-m wide strip of grass on each side. The plots were separated by a barrier of 2 m (Fig. 7.2). The experimental layout consisted of four randomised blocks, each of which was separated into seven different treatment plots. Before the application of treatments, the number of *C. aspersum* snails on the plots were counted. The snails found in the trees were counted and placed on the soil inside the plot, so as to determine whether they were able to survive moving up the base of the tree and crossing the barrier treatment concerned.

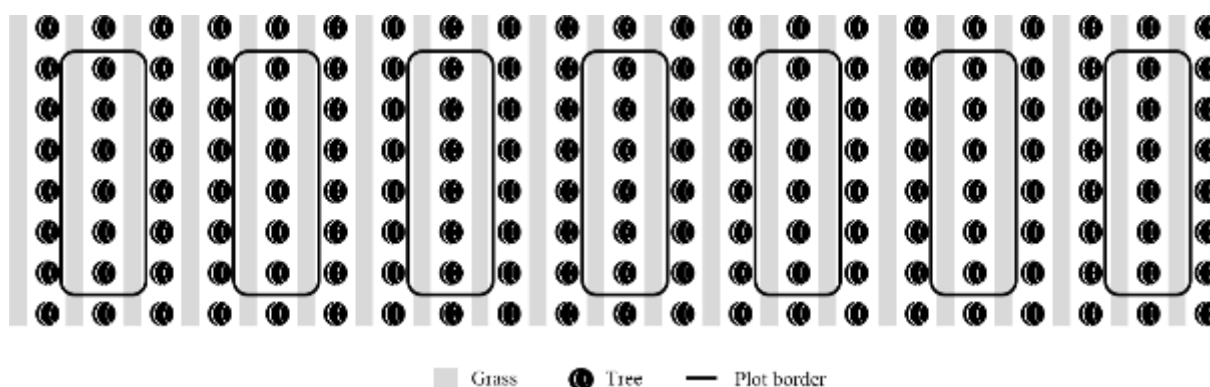


Figure 7.2. Layout of a single block in the orchard, showing seven plots, with one for each treatment.

TREATMENTS

Two different concentrations were used for each product: Baitchain 15 and Sluggit 15, at 15 g/kg (1.5%) metaldehyde, and Baitchain 40 and Sluggit 40, at 40 g/kg (4.0%) metaldehyde. The treatments were: T1 = Baitchain 15; T2 = Sluggit 15; T3 = Baitchain 15 and Sluggit 15; T4 = Baitchain 40; T5 = Sluggit 40; T6 = Baitchain 40 and Sluggit 40; T7 = control.

Sluggit pellets were applied at 8 kg/ha, and Baitchain 15 and Baitchain 40 were applied at 4.79 kg/ha in plots, by tying the product around the base of the trees' trunks, approximately 20 cm above the soil surface. The number of dead snails in the plots were counted and removed every 7 days, for 28 days, after application of the treatments. On day 28, the number of live snails on the plots were also counted.

STATISTICAL ANALYSIS

Repeated-measure analysis of variance (ANOVA) was used to compare the number of live snails in each treatment both before and after the trial. Repeated-measure ANOVA was also used to compare the number of snails killed by each treatment each week during the 28-day trial.

Results

The results of the trial show that all the treatments, compared to the control, caused a significant decrease in the number of live snails after 28 days ($F = 1.754$; $df = 6, 21$; $P < 0.001$). Although the number of live snails per treatment varied before the start of the trial, with the T5 plots having significantly more live snails than the other treatments, by day 28 no significant difference was discernible ($F = 1.754$; $df = 6, 21$; $P < 0.001$) between the numbers of live snails

per treatment, with there being only an overall reduction in the snail numbers concerned (Fig. 7.3).

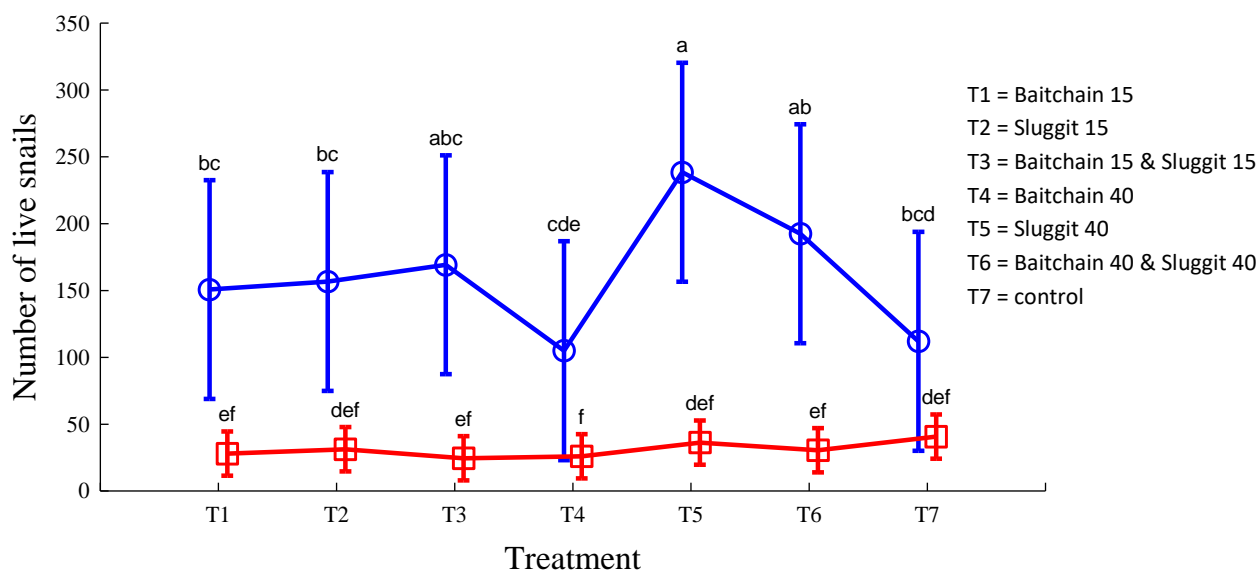


Figure 7.3. The number of live snails counted before (—) and after (—) the field trial for each treatment, with six different concentrations and combinations of Baitchain 15 and Sluggit 15 and Baitchain 40 and Sluggit 40, and an untreated control, after 28 days. The same letters indicate no significant difference ($P > 0.05$) between the different treatments and the number of live snails.

The results of the trial also show that T1 and T2 did not have significantly more snail deaths than did the control, however, when used in combination in T3, significantly more snail deaths occurred than was found in the control on days 14 and 21 ($F = 1.340$; $df = 18, 63$; $P < 0.001$). All three treatments containing the higher dose (40 g/kg) of metaldehyde (T4, T5 and T6) caused significantly more snail deaths than were found to have occurred in the control on days 7, 14, 21 and 28 ($F = 1.340$; $df = 18, 63$; $P < 0.001$). The combination treatment, T6, caused the highest snail mortality levels, which was found to be highest on day 14 ($F = 1.340$; $df = 18, 63$; $P < 0.001$). The above-mentioned number was significantly higher than was the number of dead snails caused by all the other treatments, except in the case of T5 on day 14 ($F = 1.340$; $df = 18, 63$; $P < 0.001$; Fig. 7.4).

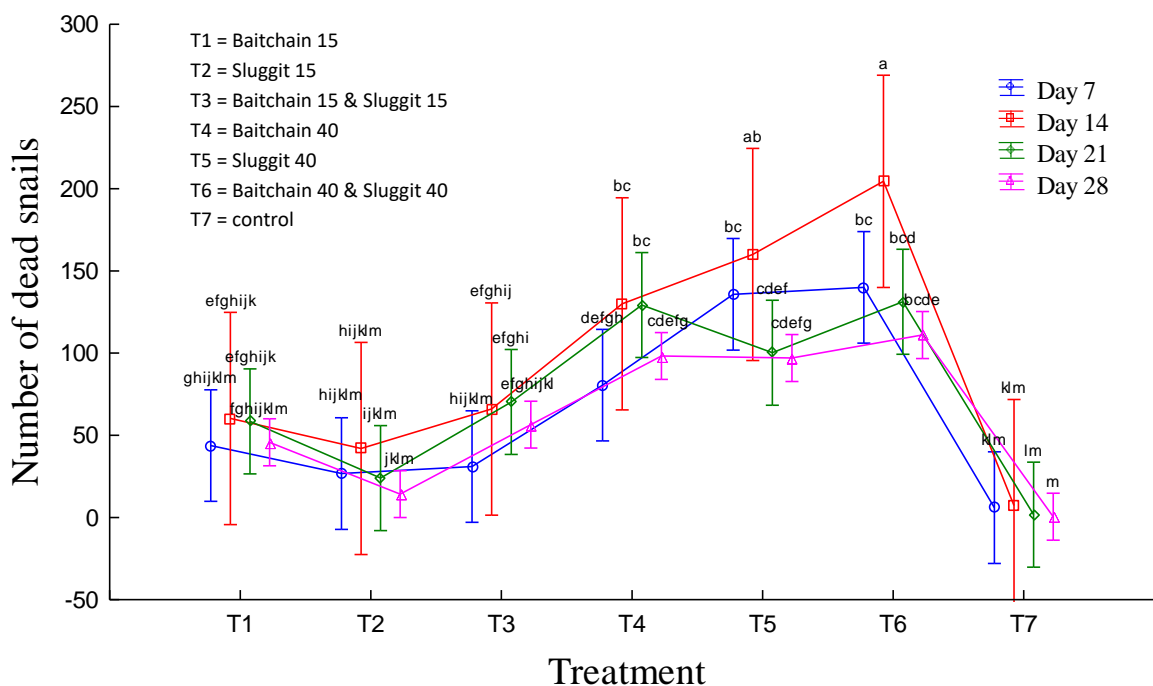


Figure 7.4. The number of dead snails counted for each treatment every 7 days for 28 days, with six different concentrations and combinations of Baitchain 15 and Sluggit 15 and Baitchain 40 and Sluggit 40, and an untreated control. The same letters indicate no significant difference ($P > 0.05$) between the different treatments and the number of dead snails found.

In the case of the T1 treatment, the dead snail numbers were highest on day 14, followed by the numbers that were found dead on day 21, with days 7 and 28 having the lowest number of dead snails. Significantly more dead snails than in the control were found only on day 21 ($F = 1.3399$; $df = 18, 63$; $P < 0.001$; Fig. 7.4). For the T2 treatment, the number of dead snails was highest on day 14, with 168 dead snails being counted on the day, followed by the number that were counted on day 7 (107 snails), with days 21 (96 snails) and 28 (57 snails) having the lowest snail mortality. For the T3 treatment, the highest number of dead snails were recorded on day 21, followed by on day 14, day 28 and day 7, providing 281, 264, 226 and 124 dead snails, respectively. The T4, T5 and combination treatment T6 all had the highest number of dead snails on day 14, with 520, 640 and 818 dead snails being present, respectively (Fig. 7.4).

Discussion

Metaldehyde is usually applied in a compressed pellet form, so as to slow down disintegration, but it has also been applied by means of incorporation into an edible matrix, which is then used to coat an inert granular core, and in emulsified form as a spray (Henderson & Triebkorn, 2002). Another method that has been developed in South Africa is to apply a paste of bran and

metaldehyde around the trunk of vines, so as to prevent the molluscs from moving into the vines (Schwartz & Siebert, 1987). The application method tested in the trial is considered novel as it does not apply the existing bait formulation to the surface of the soil. Instead, it combines the compressed pellet method, which slows down disintegration of the product, with the quick-and-easy application of the bait to the tree's trunk by hand, or with a mechanical applicator mounted to a farm vehicle.

The results of the field trial show that all the treatments at a concentration of 15 g/kg metaldehyde caused a significant reduction in snail numbers. The same treatments, at a metaldehyde concentration of 40 g/kg, also caused a significant decrease in snail numbers. All the treatments using 40g/kg metaldehyde caused significant snail mortality during the trial. The 40 g/kg treatments also caused significantly higher snail mortality than did the 15 g/kg treatments on most days. Baitchain applied at 15g/kg metaldehyde on its own and in combination with Sluggit at 15g/kg also caused significant snail mortality. Sluggit applied on its own at 15g/kg did not cause significant snail mortality but did kill 196 snails in the first season and 232 snails in the second season. A possible reason for the decreased effectiveness of the 15 g/kg pellets when applied to the soil in this study, was the high rainfall experienced in the area in the winter months, when the trial was conducted. A total of 72,6 mm rainfall was recorded during the first season and 20,4 mm in the second season. High rainfall periods might require the pellets to be reapplied more often than at other times of the year.

The effectiveness of the string of pellets, when wrapped around the trunks of the trees, even at a relatively low concentration, means that the method can be used as part of a wider integrated pest management (IPM) strategy, and incorporated into the Ross (2019) mollusc IPM pyramid. The University of California's Statewide IPM Program (UCSIPMP) has defined guidelines for the integrated control of *C. aspersum* in citrus. The guidelines involve the application of a molluscicide early in the season, before the snails move into the trees, in combination with skirt-pruning of the trees, the applying of copper barriers to the trunks, and the releasing of the snail *Rumina decollata* (Linnaeus, 1758), as a biological control measure (UCSIPMP, 1991). However, as *R. decollata* is not present in South Africa, it cannot be used as a biological control method under the terms of the Agricultural Pest Amendment Act, No. 18 of 1989 (South Africa, 1989), which forbids the introduction of exotic species to the country (Ross *et al.*, 2012).

Another biological control strategy is the use of the mollusc-parasitic nematode *Phasmarhabditis hermaphrodita* (Schneider, 1859) Andrassy, 1983 (Rhabditida: Rhabditidae) to control *C. aspersum*. The nematode is currently being sold as a commercial biocontrol

product by BASF and Dudutech, under the tradenames Nemaslug® and Slugtech®, respectively (Rae *et al.*, 2007; Ross, 2019). However, the species has not, as yet, been found in South Africa, which prohibits its sale within the country. The potential of locally isolated mollusc-parasitic nematodes to control invasive molluscs in South Africa is currently being researched (Pieterse, 2016; Pieterse *et al.*, 2017a, b; Ross *et al.*, 2018), and, should a candidate be found capable of infecting *C. aspersum*, it could be used in combination with Baitchain products, as part of an IPM strategy.

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Chapter 8

Conclusion

Terrestrial gastropod molluscs (slugs and snails) (Mollusca: Gastropoda) are primarily controlled through the use of chemicals in the form of molluscicide pellets. Due to growing concern about the environmental and health risks associated with the use of some of the chemicals, alternative control methods are being researched. One of the most successful biocontrol methods available is the use of the mollusc-parasitic nematode, *Phasmarhabditis hermaphrodita* (Schneider) Andr assy, a nematode that has been commercialised by BASF (formally Becker Underwood) and Dudutech, and sold under the trade names, Nemaslug[®] and SlugTech[®], respectively. To date, *P. hermaphrodita* has not been isolated in South Africa, which means that it can neither be commercially used, nor sold, due to the laws that prohibit the introduction of exotic animals to South Africa (in accordance with the amendment of Act 18 of 1989, under the Agricultural Pest Act 36 of 1947). Therefore, the methods of indigenous control require investigation through surveying and isolating local mollusc-parasitic nematodes, and through assessing their potential as biocontrol candidates.

The first part of the current study was focused on surveying the nematodes associated with slugs collected from the KwaZulu-Natal province of South Africa. Previous surveys have focused on the Western Cape, thus the present study is the first to have investigated the mollusc-parasitic nematode populations of KwaZulu-Natal. The majority of the slug species found in the study were invasive, although a number of endemic species were also isolated, being found to contain a high prevalence of *Caenorhabditis elegans* (Maupas). In contrast to the previous surveys, *C. elegans* was the only nematode isolated in KwaZulu-Natal, with 6.7% of the slugs surveyed being infected, thus its association with molluscs warrants further investigation. *Caenorhabditis elegans* commonly forms phoretic, or necromenic, relationships with its invertebrate hosts, with it having often been found in slug intestines. It is capable of entering the body of slugs, possibly as a means of escaping adverse environmental conditions, and of exiting it again through the faeces. The interesting association of slugs with *C. elegans* prompted the conducting of an experiment, in which the pathogenicity of the nematode was tested on the commonly found slug species, *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt (Agriolimacidae), after being grown in *in vitro* liquid cultures in association with *Kluyvera* sp., a bacterium that is known to cause mortality in slugs. A pathogenicity test of 14

days, conducted on 120 *D. invadens* specimens, concluded that *C. elegans* was incapable of causing mortality in the slug species, even when it is associated with a pathogenic bacterium.

One of the slug species collected during the survey was the endemic slug, *Chlamydephorus gibbonsi* Binney. Further research revealed that little information was available about the species, so that the third chapter of the current study was focused on describing the feeding behaviour and distribution of *C. gibbonsi* in the Western Cape. Research from previous mollusc-nematode surveys in South Africa revealed that, although *C. gibbonsi* is endemic to the eastern region of the country, its numbers have increased in the Western Cape, and it has become established in its new habitat. To understand the ecological effects that the above might have on its new habitat, its feeding behaviour was investigated. Observations were made in the laboratory, with scanning electron microscopy photos being taken of the radula and teeth of the snail. A detailed description of the feeding process, during which the slug encounters, attacks and feeds on an *Amyntas* sp. earthworm, was given. The results of the chapter indicate that the introduction of *C. gibbonsi* to the Western Cape might have an impact on earthworm abundance, and that future work should monitor its impact on the earthworm populations concerned.

The fourth chapter describes a new *Phasmarhabditis* species isolated from *Polytoxon robustum* (Simroth, 1896), collected in Kenya. *Phasmarhabditis* sp. (KEN1) is characterised by the females having a conoid tail shape, with two phasmids on the tail, located at *ca* 40% of the tail length, by the presence of males with a bursa, bearing nine bilateral pairs of genital bursal papillae on rays, and one pair of papilliform phasmids flanking the tail, as well as by the infective juvenile (IJ) having the longest body length thus far found in the genus. Phylogenetic analyses placed *Phasmarhabditis* sp. (KEN1) in close proximity to undescribed phasmarhabditids from South Africa, suggesting an African grouping, while the D2-D3 large subunit (LSU) rRNA gene and the ITS (ITS1, 5.8S, ITS2) rRNA region analyses relate *Phasmarhabditis* sp. (KEN1) to the Vietnamese *Phasmarhabditis meridionalis* Ivanona & Spiridinov, but only under weak bootstrap support, and only in the absence of D2-D3 sequences for the aforementioned African phasmarhabditid group. The lack of such sequence data highlights the importance of encouraging any new species description to be supported by sequences for the small subunit (SSU) rRNA gene, the D2-D3 and the ITS region. The species description is only the third new such description of a *Phasmarhabditis* from the African continent, and the fourteenth species in the genus to have been described, once accepted for publication. The biocontrol potential of the new nematode is, as yet, unknown.

The potential of a nematode for development into a biocontrol product depends on its pathogenicity, but also on the ease with which it can be mass-cultured. The mass culturing of a nematode requires a deep understanding of its population dynamics, and of the ability to manage its life cycle. In the fifth chapter, the life cycles of five nematodes associated with molluscs were studied and described. The species chosen were *Phasmarhabditis papillosa* (Schneider) Andr ssy and *C. elegans* (due to its high prevalence in KwaZulu-Natal, as detailed in Chapter 2), isolated in South Africa, along with *Phasmarhabditis* sp. (KEN1) from Kenya (detailed in Chapter 4), and *P. hermaphrodita* and *Phasmarhabditis bohemica* Nermut, P za, Mekete & Mr ček, with both European nematodes having been included in the current study as a reference source for comparing the African phasmarhabditids. The results obtained showed that *C. elegans* was the easiest to mass-culture, due to its hermaphroditic reproductive strategy, and its comparatively short life cycle. *Phasmarhabditis bohemica* had a shorter life cycle than did the other *Phasmarhabditis* species, developing from the IJ stage into adults with fertilised eggs in 8 days, as opposed to in 10 days, as was noted for the other phasmarhabditids in the study. However, *P. hermaphrodita* has an advantage over the other *Phasmarhabditis* species, in that it was the only phasmarhabditid in the study that required neither males nor females for reproduction. The above, therefore, means that, based on the ease of mass culturing, the tested species are ranked in the following order: *C. elegans*, *P. hermaphrodita* and *P. bohemica*, with *P. papillosa* and *Phasmarhabditis* sp. (KEN1) being equally difficult to mass-culture, due to their slow life cycle and reproduction strategy. It is recommended that any phasmarhabditid isolated in the future, and considered for its biocontrol potential, should be subjected to similar testing.

The sixth chapter focused on the *in vitro* culturing of four of the nematodes detailed in Chapter 5: *P. papillosa* and *C. elegans*, isolated in South Africa, *Phasmarhabditis* sp. (KEN1) from Kenya, and *P. bohemica* from Europe, with the latter being included as a reference source for the African phasmarhabditids. The ability of 10 different bacterial isolates to support the growth of the nematodes was tested. They included five bacterial candidates isolated from slugs, namely *Pseudomonas* sp. (2), *Pseudomonas* sp. (3), *Pseudomonas* sp. (4), *Aeromonas* sp. and *Buttiauxella* sp., two previously isolated slug-associated bacteria (*Pseudomonas* sp. (1), *Kluyvera* sp.), and three bacterial species associated with entomopathogenic nematodes (*Photorhabdus heterorhabditis*, *P. luminescens* subsp. *noenieputensis* and *Xenorhabdus khoisanae*). The results obtained demonstrated that *Pseudomonas* sp. (4), a bacterium isolated from the slug *D. invadens*, was the most successful bacteria, and able to support growth in all species, except for in *Phasmarhabditis* sp. (KEN1). Almost all of the nematode species grew

well in association with at least one of the four *Pseudomonas* species, suggesting that the *Pseudomonas* genus is ideal for culturing mollusc-parasitic nematodes. Three bacterial isolates associated with entomopathogenic nematodes were also tested. Surprisingly, two of the isolates were able to support growth in the nematodes, with *P. bohemica* growing well on *Photorhabdus luminescens* and *Xenorhabdus khoisanae*, and *C. elegans* growing on *P. luminescens*. The above situation opens up the possibility that slug-parasitic nematodes could be maintained in laboratory conditions on bacterial isolates associated with EPNs.

The next focus of the sixth chapter was to determine the pathogenicity of the bacterial isolates to the slug host *D. invadens*, through injection into the haemocoel. The most pathogenic (100% mortality) bacterial species were found to be *Kluyvera* sp., *Pseudomonas* sp. (3), and *Aeromonas* sp. In addition, *P. heterorhabditis* was also highly pathogenic, causing 80% slug mortality. However, *Aeromonas* sp. and *P. heterorhabditis* were unable to support successful growth in any of the nematode species, and can, thus, be disregarded. Therefore, *Kluyvera* sp. would be a suitable bacterial candidate for supporting the growth of *C. elegans* and *Phasmarhabditis* sp. (KEN1), while *Pseudomonas* spp. would make ideal bacterial candidates for *P. bohemica*.

Noteworthy however, the previous studies concluded that the pathogenicity of bacterial isolates to slugs differs when they are injected into slugs, and when they are applied in association with a nematode species. The pathogenicity of the bacterial isolates, therefore, requires testing after being grown *in vitro* in association with slug-parasitic nematodes. Future work should, therefore, include the *in vitro* culturing of the nematode/bacterial associations, followed by the conducting of pathogenicity tests on the pestiferous slug species.

The final part of the sixth chapter was focused on growing the nematode *P. bohemica* in association with the bacterial isolate *Pseudomonas* sp. (1) in liquid media cultures, and on testing the effects of bacterial inoculum density and initial IJ inoculum density on the total nematode yield, the IJ yield, and the IJ proportion in the cultures. Results showed that 1% bacteria inoculum led to higher total nematode and IJ yields than 3% or 5%, and 1% and 5% bacteria inoculum yielded the same IJ proportion, but higher than 3% bacteria inoculum. These results are similar to those found for the mass-culturing of EPNs. The results obtained from the initial IJ density experiment showed that a higher initial inoculum concentration of 3000 IJs/ml resulted in higher total nematode and IJ yields compared to an initial concentration of 1000 or 2000 IJs/ml, but that an initial concentration of 2000 IJs/ml resulted in a higher IJ proportion. The effects of IJ inoculum density are, however, variable between species, with the results obtained possibly being applicable only to *P. bohemica*. It is, therefore, recommended that the

effect of IJ inoculum density be tested when optimising new candidates for *in vitro* liquid culturing in future.

The seventh chapter of the thesis focused on testing a novel application method of metaldehyde in an apple orchard. The motivation for the work was the suggestion that the shell of snails has been co-opted to kill parasitic nematodes, thus making nematodes, potentially, an ineffective method for controlling snails. However, the potential harmful effects of metaldehyde, applied as pellets directly to the soil, are well-documented, due to their impact on water resources and non-target organisms, thus suggesting that an alternative, more environmentally aware, product is required. Baitchain is a novel molluscicide application of metaldehyde, with the bait pellets being strung out on a cord, designed to be wrapped around the base of a tree to protect it against mollusc damage, thus meaning that the system avoids applying metaldehyde to the orchard floor. The product was tested along with Sluggit, which is a metaldehyde bait pellet that is applied to the soil, to compare their effectiveness in protecting apple trees from the snail, *Cornu aspersum* (Müller). Both products were tested at two different metaldehyde concentrations, which were applied separately, or in combination. All the treatments used caused a significant reduction in snail numbers after 28 days, and all treatments, except Sluggit, applied at a concentration of 15 g/kg metaldehyde, caused significant mortality. The treatments with a higher concentration of metaldehyde (40 g/kg metaldehyde) were all successful and caused significantly higher levels of snail mortality than did the lower concentrations, as could have been expected. However, the fact that Baitchain, at a relatively low concentration, caused a significant reduction in snail numbers after 28 days, as well as causing significant snail mortality, was an important finding. The novel application method for metaldehyde would tend to have a decreased negative effect on the environment, as it does not come into contact with the soil, where it can affect groundwater, or the soil biota, while still remaining highly effective at limiting snail damage to the trees themselves. It could also be used as part of an integrated pest management system, in conjunction with other control methods.

Future work should be focused on conducting continual surveys of the nematodes associated with molluscs, not only in other provinces of South Africa, but across the African continent. The nematodes should then be identified and described, based on the morphological, morphometrical, molecular (SSU, D2-D3 and ITS) and phylogenetic data concerned, with their life cycles being analysed to test with what degree of ease they can be mass-cultured. Different bacterial candidates should also be isolated and considered for their ability to support the growth of nematode isolates, and their pathogenicity against pestiferous molluscs. The

nematode/bacterial combination should then be grown *in vitro*, with the impact on *in vitro* culturing being noted in terms of the morphometrics of the nematode, with the pathogenicity of the combination then being tested. The optimisation of the nematode/bacterial combination should then be considered, including the inoculum density, the bacterial concentration, the media composition and the temperature. The next step would be upscaling from shaker flasks to desktop fermenters, which is bound to bring about a new set of challenges, before scaling up to commercial level occurs. In addition, the formulation of the nematode/bacterial combination should be considered, along with its shelf life, application method and registration for different crop types.

The development of a nematode into a biocontrol agent for use against molluscs in South Africa can, however, only be successful with the management of an appropriately skilled research team, as well as with the granting of financial support to facilitate the testing and upscaling of the process. South Africa has a wealth of mollusc-parasitic nematodes that have yet to be isolated elsewhere in the world, thus demonstrating the country's unique ecological importance.