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A Study of the Distribution, Pathogenicity, and Native Microbiome of Three US Strains of *Phasmarhabditis* (*P. californica*, *P. hermaphrodita*, and *P. papillosa*)

A Dissertation submitted in partial satisfaction
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by

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Dedication

I would like to dedicate this dissertation to my mentors and my loved ones. I hope all of this work shows you just how much you have helped me develop as a person and a scientist.

ABSTRACT OF THE DISSERTATION

The Distribution, Pathogenicity, and Native Microbiome of Three US Strains of *Phasmarhabditis* (*P. californica*, *P. hermaphrodita*, and *P. papillosa*)

by

Jacob Alexander Schurkman

Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, June 2022
Dr. Adler Dillman, Chairperson

Phasmarhabditis hermaphrodita, a gastropod specific facultative parasite, has been used as a successful biological control agent across Europe under the brand name Nemaslug®. However, *Phasmarhabditis* nematodes have not been permitted for use in the United States since that they had not been found locally within the country. We surveyed nurseries and garden centers throughout California and identified local populations of *Phasmarhabditis* including *Phasmarhabditis californica*, *P. hermaphrodita*, *Phasmarhabditis papillosa*, and another close relative to *P. papillosa*. We also described the hosts which the nematodes were found in. *P. hermaphrodita* and *P. californica* seem to share an environmental niche in Northern and Central California while *P. papillosa* was only found in Southern California. We also tested the pathogenicity of the discovered local *Phasmarhabditis* strains against the invasive snail *Theba pisana*. The assays were performed to assess the efficacy of the strains as biological control agents. All tested strains caused significant mortality against adult *T. pisana* at five times the Nemaslug® recommended dose (150 IJs/cm^2). Upon further assessment using only *P. californica*, it was found that the strain was not capable of causing an economically significant amount

of mortality to *T. pisana* at the recommended rate (30 IJs/cm^2) or at three times the recommended rate (90 IJs/cm^2). Lastly, we decided to assess the native microbiome of local *Phasmarhabditis* isolates discovered during our surveys. The microbiome of the gastropod specific parasites is not well studied. Our surveys presented great opportunities for us to explore their microbiomes across a large geographic region in their natural habitats. The microbiome assessments were performed via 16S sequencing of *Phasmarhabditis* nematodes freshly identified from gastropod cadavers. We found that their microbial community was influenced by nematode species, location, and gastropod host from which the nematode was collected. The predominant bacteria of the isolates included *Shewanella*, *Clostridium perfringens*, Aeromonadaceae, Pseudomanodaceae, and *Acinetobacter*, with some nematode species having more frequent associations with certain bacterial species than others. These discoveries on the local strains of *Phasmarhabditis* support the possibility of these nematodes as a biological control option within the United States.

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(EPN) Entomopathogenic nematodes

(IJ) Infective Juvenile

(DAE) Days after exposure

CHAPTER 1

Invasive Gastropods and Gastropod Parasitic Nematodes as a Method of Biological Control

An introduction to gastropods

Terrestrial gastropods are land-dwelling snails and slugs in the class Gastropoda (Phylum: Mollusca). Most terrestrial gastropods consume both live and dead plant matter. Thus, they serve a vital role in natural ecosystems by breaking down plant matter and fertilizing the soil. Terrestrial gastropods are often adored by members of the natural ecosystem in which they play a pivotal role in. However, they are often abhorred by agricultural workers because they can destroy crops and leave plants looking undesirable, costing farmers time and money.

Invasive Gastropods and their effects on various organisms and ecosystems

On the west coast of the United States, invasive snails and slugs are among many of the serious pests in agriculture and horticulture. It has been reported that the state of California has about 279 species of terrestrial gastropods within the state, and about 242 of these species are endemic (Roth and Sadeghian, 2003). Some of the most pestiferous of these snails and slugs are invasive to the west coast of the United States. These invasive species include *Deroceras reticulatum*, *Arion hortensis*, *Tandonia budapestensis*, *Cornu aspersum*, *Oxychilus cellarius*, and members of the family *succineidae* (McDonnell, 2009). Invasive gastropods damage nurseries, home gardens,

landscapes, and crops. For example, the invasive snail *C. aspersum* can reduce some California citrus fruit crop yields by 40-50% and occasionally up to 90-100% in years of high rainfall (Pappas and Carman, 1961; Fisher and Orth, 1985; Sakovich, 2002). The plant damage caused by gastropod pests is not always physical damage caused by the snail or slug radulae, it can also be caused by the gastropod vectoring disease into the plant while feeding on tissue. *Cornu aspersum* commonly feeds on the rind of citrus fruits, causing small amounts of damage to the fruits that are often overlooked by pickers and packing house sorters. This damage done to the rind is a great entry point for post-harvest decay organisms like *Pennicilium digitatum* (Sakovich, 2002) that damage the fruit so that it must be thrown away and is no longer profitable for the producer. Terrestrial gastropods have also been found to serve as a host and vector for pathogens like *Alternaria brassicicola*, members of the family *Peronosporaceae*, and other plant pathogenic fungi (Wester et al., 1964; Hasan and Vago, 1966; Turchetti and Chelazzi, 1984). Some species of gastropods can harbor human pathogens as well. It has been postulated that some slugs and snails have been partially responsible for widespread spinach and other salad crop recalls due to the contamination of these plants with *Campylobacter spp*. & *Escherichia. coli* in the feces of sampled gastropods (Sproston et al., 2006; Raloff, 2007). Multiple terrestrial gastropods have also been found to carry *Angiostrongylus cantonensis*, a human parasitic nematode that causes eosinophilic meningitis (Lindo et al., 2004; Teem et al., 2013; Iwanowicz et al., 2015).

While terrestrial gastropods can be problematic for human agriculture and health, they can also be incredibly detrimental to sensitive ecosystems. Multiple wetlands and

marshes are threatened by invasive gastropod species because the invasives thrive in these environments. The invasive gastropods are capable of quick propagation and can reach quite high populations. These large populations result in a lack of resources for other organisms that cannot compete for the resources which the invasive gastropods are consuming (Cowie, 1998; Silliman et al., 2005). Similarly, Hawaii has also had issues with its native snail species disappearing due to the introduction of the carnivorous snail *Achatina fulica* (Cowie, 1998).

Molluscicides as a method to kill pestiferous gastropods

The most common methods of terrestrial gastropod pest control and insect pest control utilizes molluscicides. This includes setting out baits composed of oxidizing or non-oxidizing compounds that upset the balance of water inside of the gastropod's body, or that upset the gastropod's metabolism. This can send the gastropod into organ failure and/or inhibit the gastropod's normal bodily functions. While molluscicides can be successful at eradicating gastropod pests, they do not always work with high efficiency, and therefore allow for the possibility of the gastropods to develop resistance (Walton et al., 1958; Briggs and Henderson, 1987; Port et al., 2000). For example, gastropods do not always ingest enough of some metaldehyde baits to cause significant mortality (Crowell, 1967). These baits are also less effective when applied in moist weather conditions (Crowell, 1967). Other molluscicides composed of copper compounds have also been found to result in insignificant gastropod mortality (Prystupa et al., 1987). Molluscicides are also a non-targeted method of pest control. This means that molluscicides can affect

and even kill other organisms that were not intended to be targeted. For instance, some molluscicides have been found to harm birds, mammals, and invertebrate's health, sometimes causing death (South, 1992; Gurr et al., 2000).

EPNs and biological control

An alternative method to using pesticides or molluscicides to kill insects and gastropods is using biological control agents, in which pest populations are controlled using natural predators. This method of killing pests has been successful (Gurr et al., 2000). Some advantages of using biological control agents instead of chemical agents include a lower chance of resistance development, less ground water pollution, reduced non-target effects, sustainability, and cost effectiveness. One successful example of biological control is the use of entomopathogenic nematodes (EPN's). EPN's are small obligate or sometimes facultative parasitic nematodes which are solely pathogenic to insects (Kaya and Gaugler, 1993). Each species of EPN has a mutualistic relationship with a specific genus of bacteria which aids in killing the host. EPNs belonging to the family *Steinernematidae* associate with bacteria of the genus *Xenorhabdus*, and EPNs belonging to the family *Heterorhabditidae* associate with bacteria of the genus *Photorhabdus*. To kill their hosts, EPNs invade their insect host and release a pathogenic bacteria carried within thegut and mouth cavity of the nematode (Kaya and Gaugler, 1993). EPN's have found success as a biocontrol agent throughout the world and are sold commercially (Kaya and Gaugler, 1993; Grewal and Georgis, 1997).

Phasmarhabditis and biological control

Another nematode that has been utilized for biological control is *Phasmarhabditis hermaphrodita*. In 1987, *P. hermaphrodita* was isolated from *D. reticulatum* where the nematodes were actively reproducing (Glen et al., 1996; Rae et al., 2007). From this discovery, the patented Nemaslug® product was developed in 1994 which utilizes *P. hermaphrodita* as a method of gastropod biocontrol. *Phasmarhabditis hermaphrodita* is a protandrous autogamous hermaphroditic facultative parasitic nematode (Wilson and Grewal, 2005). This means they develop male reproductive organs before female reproductive organs, they are capable of self-fertilization, they can have both male and female reproductive organs, and they are only parasitic when a suitable host presents itself. *Phasmarhabditis hermaphrodita* has been found to parasitize multiple species of invasive gastropods including *Deroceras reticulatum*, *Arion distinctus*, *Tandonia budapestensis*, and many more (Wilson et al., 1993; Wilson and Grewal, 2005). *Phasmarhabditis hermaphrodita* has proven itself to be effective both in field and laboratory assays where it has killed invasive gastropods and allowed for increased crop yields in cabbage and asparagus (Wilson et al., 1993; Ester et al., 2003; Rae et al., 2007). *Phasmarhabditis hermaphrodita* has also been effective in allowing increased wheat crop yields in which it decreased gastropod feeding but did not effectively eradicate gastropods (Wilson et al., 1994a; Glen et al., 2000). The species has been commercialized and sold successfully in Europe as Nemaslug® (BASF Corp., Germany). The strain of *P. hermaphrodita* from Nemaslug® is non-lethal to non-target species including *Lumbricus terrestris*, *Eisenia fetida*, UK strains of *L. terrestris*, *Eisenia hortensis*, *E. fetida*, *Eisenia*

andrei, *Dendrodrilus rubidus*, and *Arthurdendyus triangulates* (Grewal and Grewal, 2003; DeNardo et al., 2004). *Phasmarhabditis hermaphrodita* has also been shown to be non-lethal to several species of insects including *Pterostichus melanarius*, *Tenebrio molitor*, *Zophobas morio* and *Galleria mellonella* (Wilson et al., 1993, 1994b). The *P. hermaphrodita* isolate was also tested for lethality in the snails *Ponentina ponentina*, and *Oxychilus helveticus* where no virulence was recorded (Iglesias et al., 2003). Due to the selectivity of this nematode for some of the more damaging invasive snail species, *P. hermaphrodita* seems to be an exceptional choice for a biocontrol agent.

Methodology used by Phasmarhabditis to kill their gastropod hosts

While EPN's are known to utilize specific mutualistic bacteria to kill their hosts, the mechanism in which *Phasmarhabditis* nematodes kill their hosts is largely unexplored. It is known that *P. hermaphrodita* infects slugs in the area beneath the mantle called the dorsal integumental pouch. The nematodes then move through a small canal into the shell cavity where they reproduce and the juveniles develop. Eventually, as fluid fills up the mantle cavity during infection, a characteristic symptom of a swollen mantle occurs. After onset of the infection, slug mortality occurs within seven to twenty-one days (Wilson et al., 1993; Tan and Grewal, 2001a). Upon slug mortality, the nematode then spreads and reproduces in the slug cadaver (Wilson et al., 1993). The rationality for mortality of the gastropod host is not particularly identified within *Phasmarhabditis*.

BASF rears Nemaslug® (*P. hermaphrodita*) on monoxenic cultures of the bacteria *Moraxella osloensis*. They rear *P. hermaphrodita* this way because *M. osloensis* produces an endotoxin capable of killing *Deroceras reticulatum*, the grey field slug when injected into the mantle of the slug (Tan and Grewal, 2001b). In order for slug death to occur, it seemed as though *M. osloensis* must be vectored into the slug, and *P. hermaphrodita* serves as the vector source (Wilson et al., 1995a). However, when reared in vivo in slugs, *P. hermaphrodita* did not retain *M. osloensis* and was found to be associated with various bacterial species that did not influence its virulence (Rae et al., 2010).

Locality of Phasmarhabditis throughout the world

Phasmarhabditis hermaphrodita is native to Europe where it was originally found and therefore has seen use as a biocontrol agent there (Schneider, 1859; Morand et al., 2004). *P. hermaphrodita* has also been found in Iran, Chile, Egypt, and more recently, the United States (France and M. Gerding, 2000; Karimi et al., 2003; Genena et al., 2011; Tandingan De Ley et al., 2014). However, even though the presence of *P. hermaphrodita* has been verified in these areas, the use of Nemaslug® is restricted in some of these countries, including the United States. The spread of *Phasmarhabditis* throughout the world remains largely unexplored. Also, while the virulence of *P. hermaphrodita* has been heavily researched, the virulence of other species remains largely unexplored. Multiple species including *Phasmarhabditis papillosa*, *Phasmarhabditis neopapillosa*, and *Phasmarhabditis bohemica* have not yet been extensively researched through

virulence assays to test their viability as a biocontrol agent (Rae et al., 2007; Nermut et al., 2017).

Further exploration of *Phasmarhabditis* nematodes is vital to understand how the genus of nematodes may be used as a biocontrol agent. Three different species of *Phasmarhabditis* were discovered at local plant nurseries during a survey completed in California in 2014. The species identified included *P. hermaphrodita*, *P. papillosa*, and a newly described species *P. californica* (Tandingan De Ley et al., 2014, 2016).

Exploring the pathogenicity of Phasmarhabditis

Multiple virulence assays have been performed involving *P. hermaphrodita*. These virulence assays assessed many of the snail and slug species which *P. hermaphrodita* was capable of infecting and killing. However, multiple virulence assays have yet to be done comparing the efficacy of the *Phasmarhabditis* species which were found during previous surveys in California (*P. hermaphrodita, P. papillosa, P. californica*). *Phasmarhabditis hermaphrodita* may not be the most efficient species of *Phasmarhabditis* to be used as a biocontrol agent. Comparative virulence assays testing multiple *Phasmarhabditis* species has the potential to reveal a more efficient species of *Phasmarhabditis*. The more pathogenic species may serve as a more effective method of biocontrol than *P. hermaphrodita* which is sold commercially as Nemaslug®.

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CHAPTER 2

Distribution of *Phasmarhabditis* (Nematode: Rhabditidae) and Their Gastropod Hosts in California Plant Nurseries and Garden Centers

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Abstract

Three species of *Phasmarhabditis* were recovered from 75 nurseries and garden centers in 28 counties in California during fall and winter 2012-2021. A total of 18 mollusk species were recovered, most of them invasive. Nematodes were identified by sequencing the D2-D3 expansion segments of the large subunit (LSU or 28S) rRNA. Based on these surveys, *P. californica* was the most widespread species (37 isolates, 53.6% recovery); followed by *P. hermaphrodita* (26 isolates; 37.7% recovery); *P. papillosa* and a closely related *P. papillosa* isolate (6 isolates; 8.7% recovery). Nematode isolates were mainly collected from four invasive slugs (*Deroceras reticulatum*, *D. laeve*, *Arion hortensis* agg, *Ambigolimax valentianus*) and snails (*Oxychilus* sp and *Discus* sp). Results suggest that *P. californica* and *P. hermaphrodita* share an ecological niche in Northern, Central, Coastal, and Southern California, north of Los Angeles County.

Introduction

The United States harbors a significant diversity of invasive species (Pimentel et al., 2005). They serve as a threat to the country's natural biodiversity since the introduction of invasive species is one of the leading causes of global biodiversity decline (Mckinney and Lockwood, 1999; Clavero and Garcia-Berthou, 2005; Butchart et al., 2010; Gladstone and Bordeau, 2020). While the distribution of numerous invasives have been tracked, some taxonomic groups have been largely neglected, notably terrestrial gastropod species, many species of which are of agricultural and horticultural interest (Barker, 2002; Pyšek et al., 2008; Lowry et al., 2012; Gladstone and Bordeau, 2020).

Many invasive terrestrial gastropods in the United States are present on the west coast, especially in California, Oregon, Washington, and Hawaii, where most gastropod surveys have been conducted. For example, in California, it is estimated that there are approximately 279 species of terrestrial gastropods, 37 of which are invasive (Roth and Sadeghian, 2003). These gastropods were likely introduced via the horticultural trade when gastropods residing on plant products were delivered to western states (Cowie et al., 2008; Bergey et al., 2014).

Some of these introduced gastropods are considered among the most pestiferous slugs and snails. These species include *Deroceras reticulatum* (Müller 1774), *Arion hortensis* (Ferrusac 1819), and *Cornu aspersum* (Müller 1774) (Mc Donnell et al., 2009). For example, *C. aspersum* can reduce some California citrus fruit crop yields by 40–50% and occasionally up to 90– 100% in years of high rainfall (Pappas and Carman, 1961; Sakovich, 2002). Terrestrial gastropods do not only cause direct physical damage to plants, but they can also spread disease. They have been found to serve as vectors for pathogens like *Alternaria brassicicola* (Saccardo 1880), and members of the family Peronosporaceae, and other plant pathogenic fungi (Wester et al., 1964; Hasan and Vago, 1966; Turchetti and Chelazzi, 1984). Some gastropod species have also been found to harbor human pathogens. It has been postulated that some slugs and snails have been partially responsible for spinach and other salad crop recalls due to the discovery of *Campylobacter* spp. and *Escherichia coli* (Migula 1895) in the feces of sampled gastropods (Sproston et al., 2006; Raloff, 2007). Multiple terrestrial gastropods have also

been found to carry *Angiostrongylus cantonensis* (Chen 1935), the causative agent for eosinophilic meningitis (Lindo et al., 2004; Teem et al., 2013; Iwanowicz et al., 2015).

Invasive terrestrial gastropods can also be detrimental to sensitive ecosystems. Multiple wetlands and marshes are threatened by invasive gastropod species because they thrive in these environments with a lack of natural predators (Cowie, 1998; Silliman et al., 2005). The invasive gastropods are capable of quick propagation and can reach large populations within a relatively short period of time. These large invasive populations result in a lack of resources for other endemic organisms which cannot compete with the invasives (Cowie, 1998; Silliman et al., 2005). Additionally, some native snail species have disappeared due to the introduction of the carnivorous snail *Euglandia rosea* (Ferrusac, 1821) (Cowie, 1998; Silliman et al., 2005). Horticultural and agricultural trade across the world brings danger to endemic organisms. To prevent invasive gastropods from being introduced through horticultural and agricultural trade, effective methods of pest control must be utilized.

The most common method of gastropod pest control is the use of molluscicides. One of the most widely used molluscicides is metaldehyde formulated as pelleted baits. These baits attract gastropods and upon ingestion are rapidly hydrolyzed to acetaldehyde, which causes the animal to produce excess mucus, dehydrate, and ultimately die (Triebskorn et al., 1998; Castle et al., 2017). However, these baits have variable efficacy due to a range of factors including weather conditions, different levels of attractiveness, and failure of a gastropod to consume enough bait (Crowell, 1967). Also, metaldehyde baits, along with most other molluscicides, are not targeted methods of pest control.

Metaldehyde baits can harm a variety of different organisms including dogs, humans, and other organisms upon consumption (Castle et al., 2017). For example, ranking below chocolate ingestion, metaldehyde poisoning is the second most common cause of poisoning in canines (Cope et al., 2006). In mammals, metaldehyde is an irritant to the skin, eyes, mucous membranes, throat, and respiratory tract (Castle et al., 2017). The active ingredient may be leached at points of application and found in downstream river catchments at a level that can cause harm to non-target populations away from the application site (Gillman et al., 2012).

Parasitic nematodes within the genus *Phasmarhabditis* can be effective biological control agents against pestiferous gastropods with more targeted results compared to molluscicides (Rae et al., 2007). There are currently 16 nominal species of *Phasmarhabditis* worldwide (Wilson et al., 1993; Azzam, 2003; Huang et al., 2015; Nermut et al., 2016a,b; Tandingan De Ley et al., 2016; Ivanova and Spiridonov, 2017; Nermut et al., 2017; Pieterse et al., 2017; Ross et al., 2018; Pieterse, 2020; Zhang and Liu, 2020; Ivanova and Spiridonov, 2021). All species tested for their biological control potential have been shown to specifically target and kill gastropods, providing protection to a variety of crops (Wilson et al., 1993; Rae et al., 2007; Mc Donnell et al., 2018b, Mc Donnell et al., 2020; Nermut et al., 2020; Tandingan De Ley et al., 2020). *Phasmarhabditis hermaphrodita* is the most well-known and well-studied member of the genus. It is a facultative parasite that feeds on bacteria and can live saprobically or necromenically on gastropods or their feces (Tan and Grewal, 2001). *P. hermaphrodita* has seen success as a biological control agent across Europe as the commercially

available product Nemaslug®. The species has undergone nontarget testing with various species of earthworms, as well as native, non-pest European slugs and snails (Wilson et al., 2000; Grewal et al., 2003; Rae et al., 2005; Nardo et al., 2010). It did not cause mortality in any of the non-target species tested, suggesting that it is a safer alternative in Europe to traditional molluscicides, which are lethal to many organisms other than gastropods. Until recently *Phasmarhabditis* had not been isolated within the United States. Therefore, due to agricultural policies, such as the National Environmental Policy Act (Montgomery, 2011), the commercialized Eurasian strain was not approved for use within the United States and as of 2021, it is still not commercially available in the United States. To find a viable biological control agent for gastropods in the United States, gastropod-nematode surveys within the country were performed to find a local species of nematode capable of causing mortality in slugs and snails. Three different surveys from 2000 to 2010 were performed to search for a gastropod biological control agent in the United States (Grewal et al., 2000; Kaya and Mitani, 2000; Ross et al., 2010). Most of the surveys looked for the presence of *Phasmarhabditis*, but they also searched for a variety of other nematode species found within gastropods and assessed their virulence. None of the surveys recovered any *Phasmarhabditis* species or other candidates for a gastropod biological control agent. However, over the past 8 years three species of *Phasmarhabditis* have been confirmed in California and one has been found in Oregon (Tandingan De Ley et al., 2014, 2016; Mc Donnell et al., 2018a). Thus, these local populations of *Phasmarhabditis* species should be the focus of future gastropod biological control research in the United States.

The first series of surveys which lead to the discovery of three *Phasmarhabditis* species in the United States in 2014 were conducted from 2012 to 2017 in California nurseries and garden centers. They were performed to search for potential biocontrol agents of invasive snails or slugs and to determine the distribution of parasitic nematodes including *Phasmarhabditis*. The species identified were *P. hermaphrodita*, *P. papillosa*, and a newly described species *P. californica* (Tandingan De Ley et al., 2014, 2016). As the next step, we evaluated the potential use of the local strains as biological control agents against invasive pestiferous gastropods in California (Tandingan De Ley et al., 2020).

Additional surveys were performed in 2018–2021 to determine the presence and distribution of gastropods and their associated *Phasmarhabditis* species, and to determine if the genus is widely established throughout the state. This series of extensive gastropod-nematode surveys is the first in the state of California. Such surveys have the potential to identify previously unknown nematode-gastropod relationships or identify new species of nematodes with biocontrol potential. These types of discoveries have been seen in other surveys performed across the globe (Ross et al., 2012; Tandingan De Ley et al., 2014; Mc Donnell et al., 2018a; Brophy et al., 2020). In this survey, we aimed to determine the presence and distribution of *Phasmarhabditis* nematodes and the diversity of gastropods in nurseries and garden centers throughout California.

Materials and methods

Collection and maintenance of gastropods

We conducted gastropod surveys in 75 nurseries and garden centers during fall and winter months between 2012 and 2021 throughout California, covering at least 2 nurseries in each of the 28 counties surveyed. For ease of reference, the state was divided into three geographical areas: Northern California, Central California, and Southern California (Table 2.1 and Supplementary Fig. 2.1). During the course of these surveys, 6,590 gastropod specimens were collected and brought back to the Insectary and Quarantine Facility and Departments of Entomology and Nematology at UC Riverside under CDFA Permits 2942 (2012–2018) and 3449 (2018–2022).

Gastropods were collected from nurseries and garden centers for a total of 1 person-hour per visit. For example, if 2 people were sampling, each person's collection time would be 30 min. The gastropods were removed and collected from underneath potted plants, foliage, or plant trays on the ground using clean metal spatulas, and then immediately stored in plastic containers lined with moistened paper towels and covered with punctured lids (to maintain aeration). These containers were placed inside a cooler and at the end of each sampling day, the collected gastropods were sorted into 540 ml deli containers lined with a moistened paper towel and contained organic carrot pieces for food. The gastropods were sorted phenotypically by species, and the deli containers were labeled accordingly, and kept in coolers. Gastropods from different nurseries were kept in separate deli containers. The deli containers were cleaned every other day and were provided with a new moist paper towel and fresh organic carrot pieces. After each survey

trip was completed, the gastropods were examined again to ensure they were identified correctly. In order to accurately identify gastropods, we used the methods described in Mc Donnell et al. (2009). We also had years of experience identifying California gastropods based off of the guide and received verification of our identifications by collaborating with gastropod expert Rory McDonnell. Once the gastropods were sorted correctly in the lab, relevant information was recorded and summarized, tracking the dates of collection, as well as the life history of the gastropods (e.g., when they were killed, viewed for infection, or whether they were infected). The gastropods were kept in the lab at room temperature with continued fresh changes of paper towel and organic carrot discs every other day. Each gastropod that died was given an accession number and immediately transferred to plated 1.1% plain agar (1 L: 10 g agar, 900 ml H2O) in order to obtain nematodes in seed culture, as described in Tandingan De Ley et al. (2014). To encourage better growth of nematodes, we modified the method and used nematode growth medium [NGM; 1 L: 3 g NaCl, 20 g Agar, 2.5 g Peptone, 975 ml deionized H2O, 10 ml Uracil (2 g/L) were added to a liter of deionized water, autoclaved, and let cool, to which were added 25 ml filtered KPO4, 1 ml filtered MgSO4, 1 ml CaCl2, and 1 ml Cholesterol 5 mg/ml)]. As surveys progressed in 2018, and in the interest of time, speed, and laboratory space, we modified our nematode recovery method, following the protocol of Wilson et al. (2016), i.e., decapitating slugs in batches Q18 and immediately placing them on NGM. This shortened our gastropod maintenance period, likely with the same outcome because gastropods infected with *Phasmarhabditis* were assumed to have harbored the nematode at the collection site. However, if

Phasmarhabditis was transmitted within the laboratory, it is likely that the transmission only occurred across conspecifics collected at the same collection site since these gastropods were kept in the same container.

The gastropod-nematode surveys conducted between 2012– 2017 and 2018–2021 were analyzed separately due to differences of collection time and survey methods. During the 2018–2021 survey, non-*Phasmarhabditis* nematodes were identified from host gastropods whereas this was not done in the 2012–2017 survey as a search for biocontrol candidates was targeted at finding *Phasmarhabditis* spp. and determining their distribution n California nurseries and garden centers. Each of the surveys also covered different counties throughout California, where the 2012–2017 survey often covered more nurseries and garden centers within each county, sometimes surveying the same nurseries multiple times. The 2018–2021 survey only surveyed each nursery once, and mostly covered two nurseries or garden centers per county (Table 2.1 and Supplementary Fig. 2.1). While the methodology of collecting gastropods remained the same throughout each of the surveys, the separate analyses of the two allows for the assessment of gastropod diversity and abundance across time and allows for results to be interpreted upon each method.

Nematode recovery and molecular analyses

At least 5 individual nematodes that emerged from slug cadavers were picked from seed culture plates and grown on individual NGM plates, kept at 17C. These plates of uniparental strains were labeled as single nematode isolations and were designated a

unique accession number. Preliminary examination was done through a stereomicroscope, using morphological traits e.g., the presence of large phasmids and vulval body position, to identify suspected *Phasmarhabditis*. After suspects were identified, at least 2 individual nematodes from each single nematode isolation were prepared for PCR and DNA sequencing of the ribosomal RNA (D2-D3 domains of the large subunit or LSU), as described in Tandingan De Ley et al. (2014). When necessary, the small subunit (SSU) was also sequenced following the same protocols. Contigs were assembled and compared by BLAST with published sequences in GenBank using CodonCode Aligner (CodonCode Corp., 58 Beech Street, Dedham, MA, United States) to verify their identity or determine if sequences were unique.

Results

Gastropod Survey

A total of 18 different gastropod species were recovered from all surveys. Sixteen of the 18 species recovered were invasive species, representing 99.8% of the total individuals collected (Fig. 2.1). These include: *Arion hortensis* (Ferrusac 1819), A*rion distinctus* (Mabille 1869), *Arion rufus* (Linnaeus 1758), *Arion subfuscus* (Draparnaud 1805), *Cornu aspersum* (Müller 1774), *Deroceras laeve* (Müller 1774), *Deroceras reticulatum* (Müller 1774), *Deroceras invadens* (Reise et al., 2011), *Discus* spp., *Ambigolimax valentianus* (Ferussac 1821), *Sucinnea* spp., *Oxychilus* spp., *Milax gagates* (Lessona and Pollonera 1882), *Boettgerilla pallens* (Simroth 1912), *Cochlicopa lubrica* (Müller 1774), *Rumina decollata* (Linnaeus 1758), *Prophysaon andersoni* (Cockerell

1890), and *Limacus flavus* (Linnaeus 1758) (Figures 2.1– 2.3). Both surveys from 2012 to 2017 and 2018 to 2021 recovered far more slug species (12) than snail species (6) (Fig. 2.1). The two surveys, although completed over different years and with some differences between the counties visited and the nematodes which were chosen to be identified, were approximately congruent with a few notable disparities. The earlier survey obtained a greater number of *D. reticulatum* specimens in Southern California nurseries compared to the later survey (28.12% vs. 6.17%). *Discus* spp. were recorded during the later survey but were not collected during 2012–2017 (Fig. 2.1). Also, the earlier gastropod surveys yielded a larger abundance of *D. invadens* across all areas of California. Each of the surveys also demonstrated that *A. valentianus* was the predominant gastropod species in nurseries. However, the second most common species collected during the 2012–2017 survey was *D. reticulatum*, while *D. laeve* was the second most common species during the 2018– 2021 campaign. In general, more gastropod individuals were found at nurseries in Northern California than in other areas of California and fewer gastropod species were recovered in Southern California, indicating a possible decrease in gastropod abundance in a southward direction throughout the state (Figures 2.1–2.3).

Phasmarhabditis survey

A total of 69 *Phasmarhabditis* isolates were collected from all surveys. *Phasmarhabditis californica* was the most widespread species (37 isolates, 53.6% of all *Phasmarhabditis* recovered); followed by *P. hermaphrodita* (26 isolates; 37.7% recovery); *P. papillosa* and a *P. papillosa* closely related isolate (6 isolates; 8.7%

recovery) (Table 2.2). The sequence of the D2-D3 expansion segment of 28S rDNA of this isolate was uploaded to Genbank (accession ID OL455007). Isolates were recovered from 5 invasive slug species: *D. reticulatum* (54%), *D. laeve* (25%), *A. hortensis* agg (5.7%), *A. valentianus* (8.7%) and two snails, *Oxychilus* spp. (5.8%) and *Discus* spp. (1.4%) (Table 2.2). Interestingly, isolates of *Phasmarhabditis* were mostly collected from *D. reticulatum* (53.6%), which was not the most abundant gastropod species found throughout the state. Only 8.7% of the isolates were collected from the most common gastropod, *A. valentianus* (Figures 2.1–2.3). However, about 78% of all isolates were collected from gastropod species within the genus *Deroceras* (Table 2.2).

Phasmarhabditis isolates were collected and identified from Northern, Central, and Southern California. They were found in about 46% of all California counties surveyed. Results suggest that *P. californica* and *P. hermaphrodita* share an ecological niche throughout Northern CA and Central CA, whereas *P. papillosa* is mostly present by itself in Southern California. This could be due to a founders effect or climate conditions. However, an unidentified close relative of *P. papillosa* was found in Monterey County (Central California) (Fig. 2.4). Other nematode species were also recovered and identified from the surveys performed between 2018 and 2021. However, the non-*Phasmarhabditis* isolates are not representative of nematode diversity throughout California nurseries. This is because *Phasmarhabditis* was targeted, and only a select few nematodes from gastropod cadavers or seed cultures which did not morphologically resemble *Phasmarhabditis* were identified using 28S D2-D3 rDNA sequencing. Criteria for nematodes to be selected when they did not resemble *Phasmarhabditis* were not

completely randomized. Nematodes which were not commonly observed (i.e. not a species of *Caenorhabditis*) were always selected for identification. Across locations, the most abundant non-*Phasmarhabditis* species identified was *Caenorhabditis elegans*, followed by *C. remanei* and *Rhabditophanes* spp. (Table 2.3). Other nematode species which are not typically considered to be associated with gastropods were also discovered. For example, *Cruzia americana*, a known opossum parasite, was discovered in a collected gastropod host (Li, 2019; Table 2.3). Some gastropod species that did not yield any associated *Phasmarhabditis* were found to have a variety of other associated nematode species (Table 2.4). A. valentianus had the most diverse nematode associations that included *A. dentiferum*, *Bursilla* spp., *C. elegans*, *C. remanei*, *C. tonkinensis*, and *Rhabditophanes* spp. (Table 2.4). However, this may well be the result of the larger sample size we obtained of *A. valentianus* compared to the other gastropod species. All nematode species identified can be found in Supplementary Table 2.1, as well as the host species they were discovered in. The locations in which all non-*Phasmarhabditis* nematodes were identified can be found in Supplementary Figure 2.2.

Discussion

This was the first extensive gastropod and nematode survey performed throughout California. The surveys from 2012 to 2017 and 2018 to 2020 combined covered a total of 28 counties and resulted in the collection of 18 different gastropod species from 6,590 specimens. A total of 69 *Phasmarhabditis* isolates were collected. The most common gastropod species recovered was *A. valentianus*. According to this survey, invasive slug

and snail species are more common than native gastropod species in California nurseries. This is possibly due to the heavy floricultural traffic in nurseries which can transport multiple pests from other geographic areas. Gastropod abundance decreased as we moved southward through California (Figures 2.1–2.3). This could be due to the desert and chapparal climates which occur in most of the southern sections of California, while Northern California climates include more precipitation. Slugs are prone to desiccation; therefore, lower survival rates in these climate conditions are plausible (Sternberg, 2000).

Phasmarhabditis species were found throughout all three geographic areas of California (Northern, Central, and Southern). Three species were identified throughout the state, *P. hermaphrodita*, *P. californica*, and *P. papillosa*. Also, one isolate was recovered in Monterey County which seems to be a close relative to or a variant of *P. papillosa* (Fig. 2.4); with morphological characteristics diagnostic to the species. However, based on genetic analyses of the D2-D3 expansion segments of rRNA, it varies by 2 transitions, 1 ambiguity, 1 transversion, and 2 insertions/deletions (Thymine instead of Cytosine in nucleotide positions 46 and 67; C/T instead of Cytosine in position 140, Adenine instead of a Cytosine in position 261, and 2 indels on positions 346 and 347, respectively). Surveys in Oregon showed the same 3 *Phasmarhabditis* species, and interestingly, *P. hermaphrodita* was recovered from a slug in a *Brassica* field in Salem, OR, suggesting it may also be present in the wider agricultural environment (Howe et al., 2020). In California, our surveys focused solely on nurseries and garden centers because (1) horticulture is one of the most valuable agricultural industries in the state (2) slugs and snails are major pests in the industry and (3) transportation of plants to and from

retail nurseries presumably causes gastropods to be moved around the state over great distances than would otherwise be possible. The nurseries themselves could therefore be focal locations for exposure of invasive slug and snail species to a greater diversity of gastropod associated nematodes than is likely to occur in production fields or greenhouses. Based on the Oregon finding, it is likely that these gastropod-infecting nematodes may have also found their way as hitchhikers into agricultural and horticultural fields and backyard gardens in California. However, that has yet to be determined as these production areas were not covered in our surveys.

Additionally, recent studies based on mitochondrial DNA COI gene phylogenies, showed that *Phasmarhabditis hermaphrodita* U.S. isolates and strains (including isolates from the CA 2012 to 2017 survey and OR surveys) had haplotypes that were nearly identical to *P. hermaphrodita* collected in the United Kingdom for commercialization of Nemaslug®. They were placed together in an intraspecific monophyletic clade with the Nemaslug® strain (Howe et al., 2020). We can hypothesize that *P. hermaphrodita* found in the United States likely came from areas where Nemaslug® was used in Europe. It is also possible that the product has been used illegally in the country. Invasion of California and Oregon probably came about from agricultural trade with interstate movement of infected soil and/or slugs/snails. The invasive slugs from Europe and some of the specimens found in this survey likely came with these same nematodes of near-identical haplotypes. It is also possible that the nematodes came to California on slugs many years ago before the commercialization of Nemaslug®, and they have stayed in the region by infecting *Deroceras* slugs and other suitable host pest slugs which are now

established in California. In the study by Howe et al. (2020), available *P. californica* strains at that time were also studied. As with *P. hermaphrodita*, all *P. californica* haplotypes (CA, United States; United Kingdom; and New Zealand belonged to one single, strongly supported clade. Interestingly, *P. californica* shares the same geographical niche and host gastropod species as *P. hermaphrodita* from Northern to Southern California. However, *P. papillosa* seems to only inhabit areas of Southern California (Fig. 2.4).

Some gastropod species were more commonly infected with *Phasmarhabditis* than others. The gastropod host *A. valentianus*, which was the most frequently found gastropod, only accounted for about 8.5% of the *Phasmarhabditis* isolates collected. *A. valentianus* may have a more developed immune response to parasitic nematodes compared to other slugs, however, this has yet to be determined. The majority of *Phasmarhabditis* nematodes collected from the survey were collected from *D. reticulatum*. The host *D. reticulatum* accounted for about 55% of all *Phasmarhabditis* nematodes collected. In total, the genus *Deroceras* accounted for about 74.1% of the identified *Phasmarhabditis* nematodes (Table 2.2). *D. reticulatum* is a common slug pest across Europe, especially in areas near Ireland and the United Kingdom where Nemaslug® was originally discovered (Kerney, 1999). This serves as additional evidence that an infected invasive species of gastropod from Europe likely brought *Phasmarhabditis* to the United States where the relationship between the gastropod hosts remained.

Multiple gastropods collected throughout the survey were infected and/or associated with a diverse array of nematodes (other than *Phasmarhabditis*) (Supplementary Table 2.1 and Tables 2.3, 2.4). *C. elegans* and *C. remanei* were the most prominent nematodes found within gastropod hosts. These nematodes are not uncommon in gastropods, and though the interaction between *C. remanei* and gastropods has not been thoroughly explored, *C. elegans* is thought to have a phoretic association with gastropods (Caswell-Chen et al., 2005; Ross et al., 2012; Petersen et al., 2015; Rae, 2017; Sudhaus, 2018). *C. elegans* is also known to have phoretic associations with some species of earthworms and arthropods (Kiontke and Sudhaus, 2006; Brophy et al., 2020). Other interesting nematode species were also discovered during the California surveys including *Cosmocercoides tonkinensis*, which is not commonly associated with gastropod hosts (Supplementary Table 2.1 and Tables 2.3, 2.4; Sudhaus, 2018). *C. tonkinensis* has only been described in reptiles (Tran et al., 2015). However, for another member of the genus, *Cosmocercoides dukae*, mollusks are a known host (Anderson, 1960). A survey done in 2014 which identified *P. hermaphrodita* in California also recovered species other than *Phasmarhabditis* within gastropod hosts. Some of these species included *Alloionema appendiculatum* (a common parasite of slugs), *C. elegans*, *C. briggsae*, a new species *A. similis*, and species of *Oscheius* (Tandingan De Ley et al., 2014; Holovachov et al., 2016). Our non-*Phasmarhabditis* results share in some of these genus recoveries, except for *Alloionema* spp. (Table 2.3). The absence of this genus throughout both surveys spanning from 2012 to 2021 is unexpected and intriguing since it was discovered in past surveys in similar locations (Laznik et al., 2010).

The occurrence of *P. hermaphrodita* and other species of the genus in North America has regulatory implications for potential biocontrol strategies against non-native slug and snail species that are pests of agriculture on this continent. Since the nematode occurs throughout the state, its use in a similar manner to Nemaslug® may be a feasible option. Its use could potentially save the California specialty crop industry about 64 million dollars, and is therefore worth exploring as a biological control option (Supplementary Table 2.2). The recovery of *Phasmarhabditis* from local plant nurseries and garden centers throughout California was not entirely surprising as these are considered transport hubs for non-native gastropod species (Bergey et al., 2014). It is not known if *Phasmarhabditis* exists in the natural environment throughout California where horticultural practices do not take place. In order to better understand the presence of *Phasmarhabditis* in the state, further surveillance is required in horticultural and agricultural field production areas, as well as natural ecosystems. Also, additional non-target and target host experiments with *Phasmarhabditis* are required to have a deeper understanding of how these potential biological control agents will affect the local ecosystem where they would likely be introduced. Additionally, host experimentation should be performed in mesocosms or other field-like conditions to determine efficacy.

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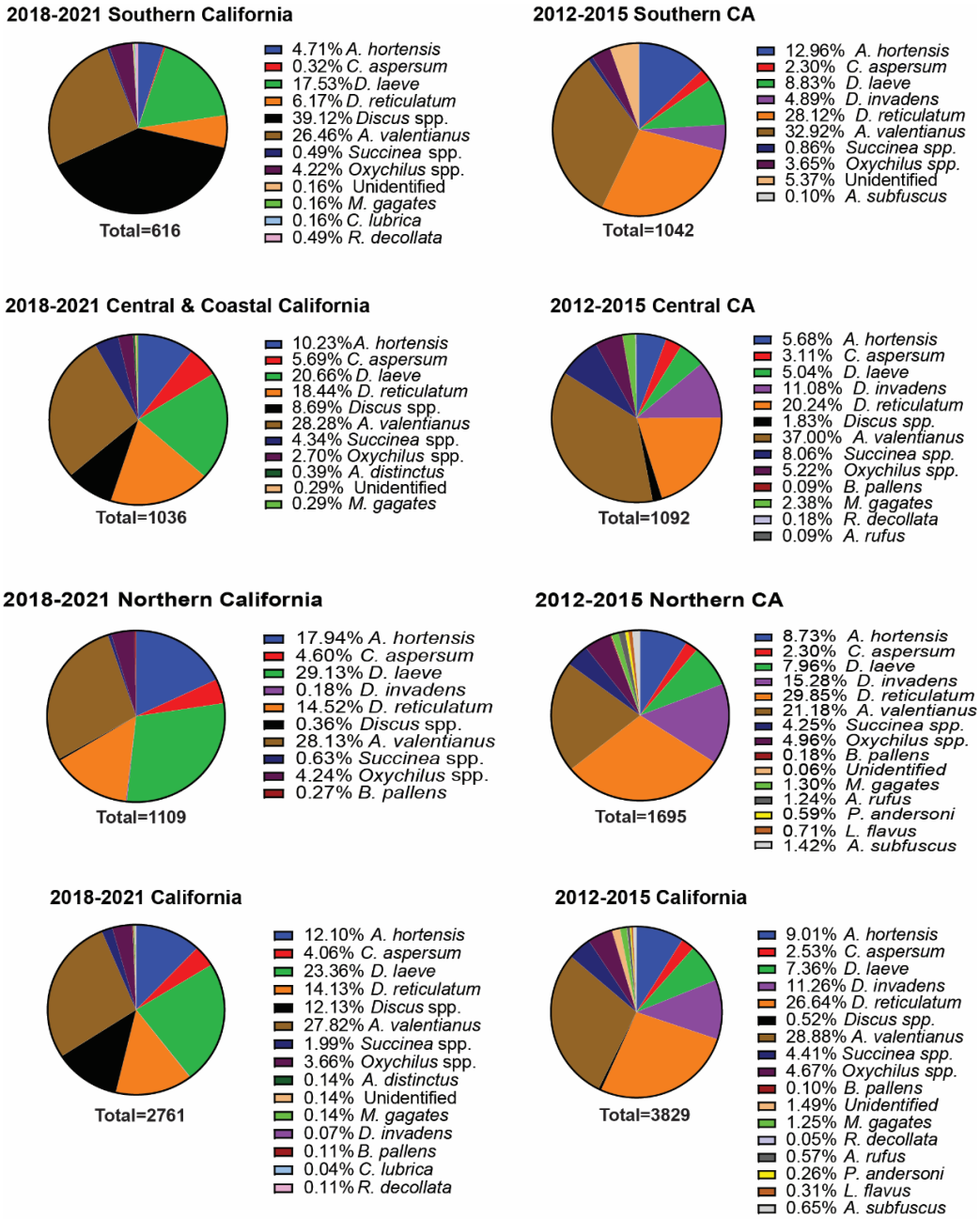

Figure 2.1 Percent recovery of terrestrial gastropods from different geographical regions of California during the 2012–2017 and 2018–2021 surveys. Surveys were performed during late fall or winter. Survey methods included 1 person hour searching for gastropods throughout each nursery. Collected gastropods were sorted by species and were taken back to the laboratory for later verification of species identity

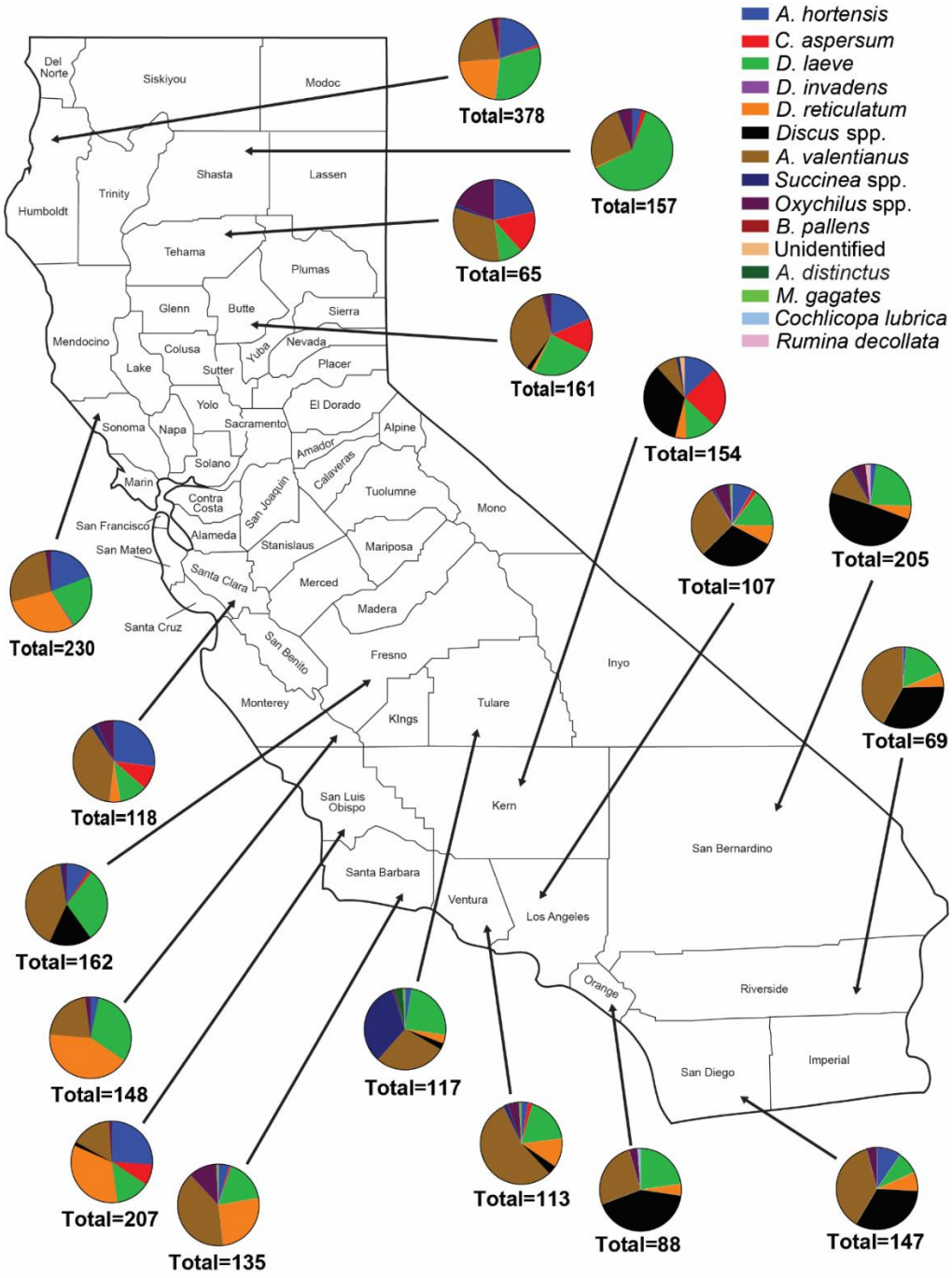

Figure 2.2 Abundance and species richness of terrestrial gastropods collected in each California county surveyed between 2018 and 2021. Surveys were performed during late fall or winter. Survey methods included 1 person hour searching for gastropods throughout each nursery. Collected gastropods were sorted by species and were taken back to the laboratory for later verification of species identity.

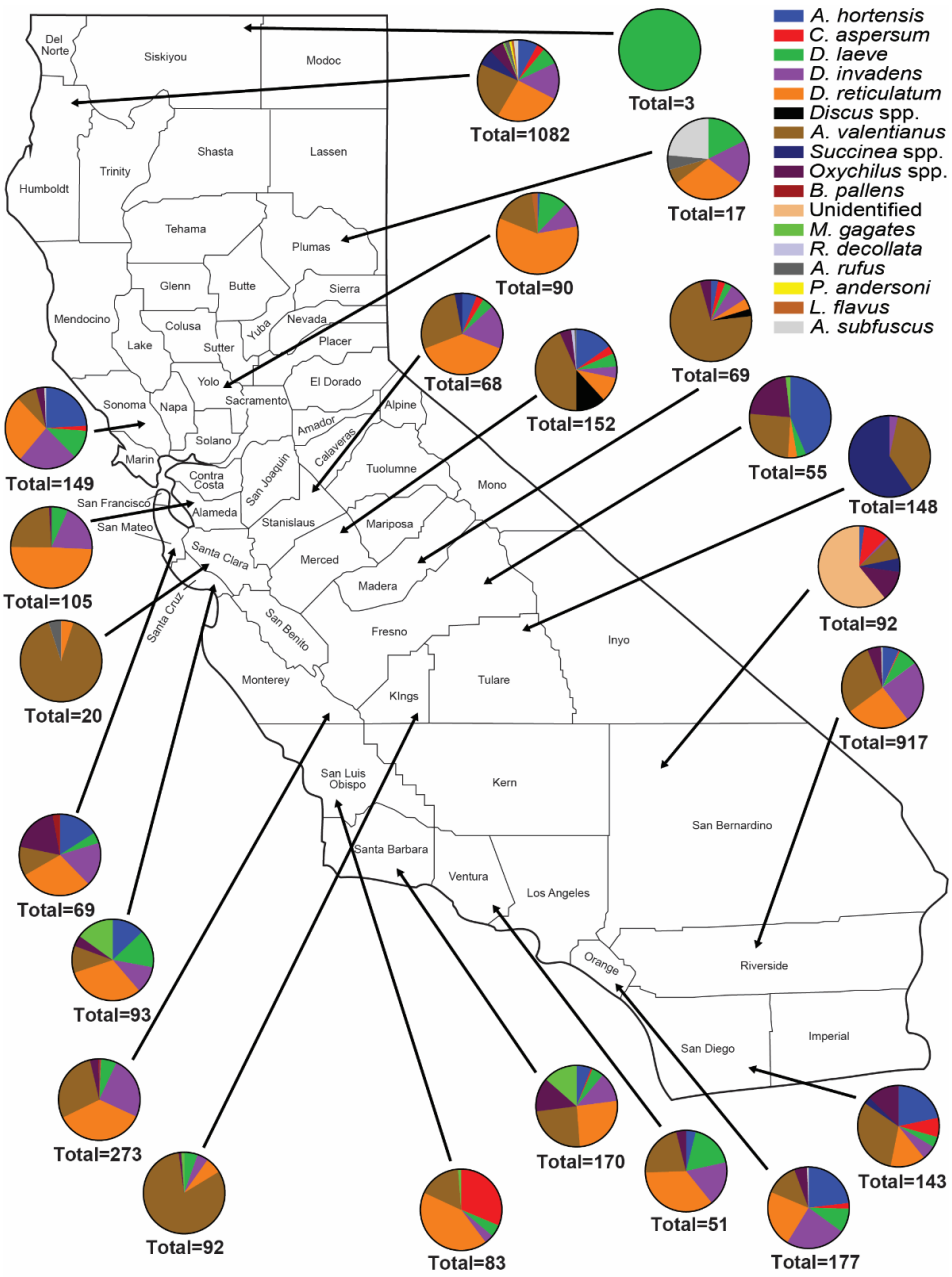

Figure 2.3 Abundance and species richness of terrestrial gastropods collected in nurseries in each California county surveyed between 2012 and 2017. Surveys were performed during late fall or winter. Survey methods included 1 human hour searching for gastropods throughout each nursery. Collected gastropods were organized by species and were taken back to the laboratory for later verification of species identity.

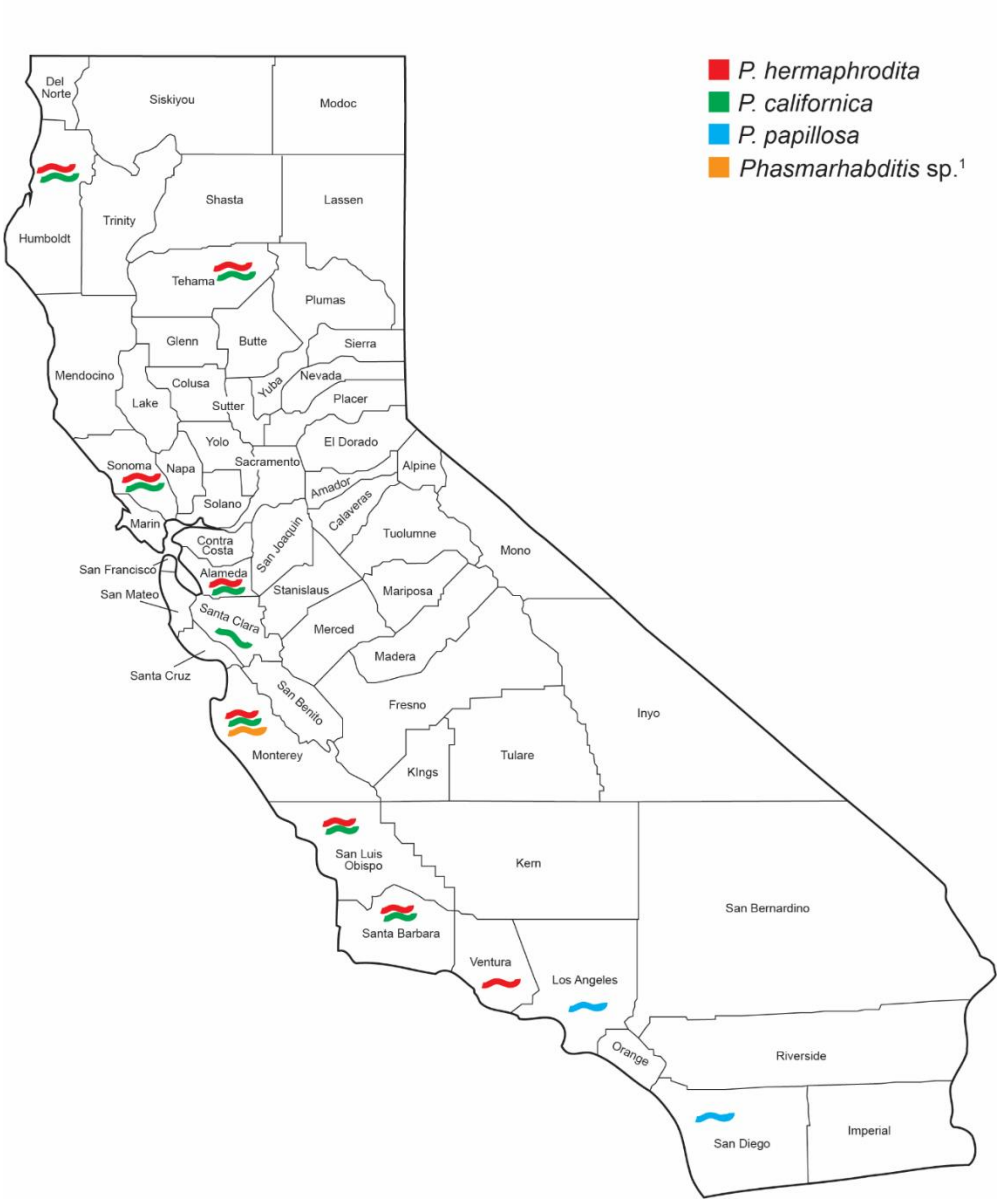

Figure 2.4 Phasmarhabditis species recovery and distribution among 28 California counties surveyed between 2012 and 2021. Species were identified by sequencing the D2–D3 expansion segments of the large subunit (LSU or 28S) ribosomal RNA and contigs compared by BLAST with published sequences in GenBank.

List of Tables

Table 2.1 Shows the California counties which were surveyed for gastropods and *Phasmarhabditis*

2012-2015	2018-2021
Alameda	Butte
Fresno	Fresno
Humboldt	Humboldt
Kings	Kern
Madera	Los Angeles
Merced	Monterey
Monterey	Orange
Orange	Riverside
Plumas	San Bernardino
Riverside	San Diego
San Bernardino	San Luis Obispo
San Diego	Santa Barbara
San Luis Obispo	Santa Clara
San Mateo	Shasta
Santa Barbara	Sonoma
Santa Clara	Tehama
Santa Cruz	Tulare
Siskiyou	Ventura
Sonoma	
Stanislaus	
Tulare	
Ventura	
Yolo	

Table 2.2 *Phasmarhabditis* species including hosts, sampling locations and morphological/genetic characterization from surveys performed between 2012 and 2021.

Nematode Species	**County**	**Host**	**Number of Gastropods Found with *Phasmarhabditis***
P. hermaphrodita			
	Alameda	*Deroceras reticulatum*	1
	Humboldt	*Ambigolimax valentianus*	1
	Humboldt	*Deroceras reticulatum*	2
	Monterey	*Deroceras laeve*	1
	Monterey	*Deroceras reticulatum*	6
	San Luis Obispo	*Deroceras laeve*	1
	San Luis Obispo	*Deroceras reticulatum*	10
	Santa Barbara	*Oxychilus sp.*	1
	Sonoma	*Deroceras laeve*	1
	Tehama	*Arion hortensis*	1
	Tulare	*Deroceras laeve*	1
P. californica			
	Alameda	*Deroceras reticulatum*	1
	Humboldt	*Arion hortensis*	1
	Humboldt	*Ambigolimax valentianus*	1
	Humboldt	*Deroceras laeve*	5
	Humboldt	*Deroceras reticulatum*	3
	Humboldt	*Oxychilus draparnaudi*	1
	Kern	*Discus sp.*	1
	Monterey	*Ambigolimax valentianus*	1
	Monterey	*Deroceras laeve*	1
	Monterey	*Deroceras reticulatum*	2
	Santa Clara	*Ambigolimax valentianus*	1
	Santa Clara	*Arion hortensis*	1
	San Luis Obispo	*Arion hortensis*	1
	San Luis Obispo	*Deroceras reticulatum*	2
	Santa Barbara	*Deroceras laeve*	5
	Santa Barbara	*Oxychilus draparnaudi*	2
	Sonoma	*Deroceras reticulatum*	3
	Tehama	*Deroceras reticulatum*	4
	Ventura	*Deroceras laeve*	1

P. papillosa			
	Los Angeles	*Ambigolimax valentianus*	2
	Los Angeles	*Deroceras laeve*	1
	Los Angeles	*Deroceras reticulatum*	1
	San Diego	*Deroceras reticulatum*	1
Phasmarhabditis spp.			
	Monterey	*Deroceras reticulatum*	1

Table 2.3 Shows all recovered nematodes other than *Phasmarhabditis* during the California gastropod survey between 2018 and 2021

Southern California		**Central California**		**Northern California**		**California**	
Species	**# Found**	**Species**	**# Found**	**Species**	**# Found**	**Species**	**# Found**
Angiostoma dentiferum	0	*Angiostoma dentiferum*	3	*Angiostoma dentiferum*	0	*Angiostoma dentiferum*	3
Bursilla spp.	1	*Bursilla spp.*	0	*Bursilla spp.*	0	*Bursilla spp.*	1
Caenorhabditis elegans	27	*Caenorhabditis elegans*	90	*Caenorhabditis elegans*	97	*Caenorhabditis elegans*	214
Caenorhabditis remanei	0	*Caenorhabditis remanei*	20	*Caenorhabditis remanei*	26	*Caenorhabditis remanei*	46
Choriorhabditis cristata	0	*Choriorhabditis cristata*	0	*Choriorhabditis cristata*	2	*Choriorhabditis cristata*	2
Cosmocercoides pulcher	0	*Cosmocercoides pulcher*	1	*Cosmocercoides pulcher*	0	*Cosmocercoides pulcher*	1
Cosmocercoides tonkinensis	11	*Cosmocercoides tonkinensis*	5	*Cosmocercoides tonkinensis*	0	*Cosmocercoides tonkinensis*	16
Cuzia americana	1	*Cuzia americana*	0	*Cuzia americana*	0	*Cuzia americana*	1
Oscheius tipulae	2	*Oscheius tipulae*	0	*Oscheius tipulae*	4	*Oscheius tipulae*	6
Rhabditophanes spp.	3	*Rhabditophanes spp.*	16	*Rhabditophanes spp.*	10	*Rhabditophanes spp.*	29

Table 2.4 Shows the species of nematodes (other than *Phasmarhabditis*) present in the cadavers of host gastropods found throughout gastropod surveys performed between 2018 and 2021.

Arion hortensis	***Ambigolimax valentianus***	***Cornu aspersum***	***Deroceras laeve***	***Deroceras reticulatum***
Caenorhabditis elegans	*Angiostoma dentiferum*	*Caenorhabditis elegans*	*Caenorhabditis elegans*	*Caenorhabditis elegans*
Caenorhabditis remanei	*Bursilla spp.*	*Caenorhabditis remanei*	*Caenorhabditis remanei*	*Caenorhabditis remanei*
Oscheius tipulae	*Caenorhabditis elegans*	*Rhabditophanes spp.*	*Cosmocercoides pulcher*	*Choriorhabditis cristata*
Rhabditophanes spp.	*Caenorhabditis remanei*		*Cosmocercoides tonkinensis*	*Cosmocercoides tonkinensis*
	Cosmocercoides tonkinensis		*Cruzia americana*	*Rhabditophanes spp.*
	Oscheius tipulae		*Rhabditophanes spp.*	
	Rhabditophanes spp.			
Discus spp.	***Milax gagates***	***Oxychilus spp.***	***Succinea spp.***	
Caenorhabditis elegans	*Caenorhabditis elegans*	*Caenorhabditis elegans*	*Caenorhabditis elegans*	
Caenorhabditis remanei		*Caenorhabditis remanei*	*Choriorhabditis cristata*	
Oscheius tipulae		*Rhabditophanes spp.*		
Rhabditophanes spp.				

Supplementary Material

Table S2.1 Shows all nematode species identified and the gastropods they were discovered in during the 2018-2021 survey.

Nematode/ Gastropod Species	**Northern California**		**Nematode/ Gastropod Species**	**Central California**		**Nematode/ Gastropod Species**	**Southern California**		**Total**	
Angiostoma dentiferum	x		***Angiostoma dentiferum***	3		***Angiostoma dentiferum***	x		***Angiostoma dentiferum***	3
x	x		*Ambigolimax valentianus*	x		x	x		*Ambigolimax valentianus*	
Bursilla sp.	x		***Bursilla spp.***	x		***Bursilla spp.***	1		***Bursilla spp.***	1
x	x		x	x		*Ambigolimax valentianus*			*Ambigolimax valentianus*	
C. elegans	97		***C. elegans***	90		***C. elegans***	27		***C. elegans***	214
Arion hortensis			*Arion hortensis*			*Arion hortensis*			*Arion hortensis*	
Ambigolimax valentianus			*Ambigolimax valentianus*			*Ambigolimax valentianus*			*Ambigolimax valentianus*	
Cornu aspersum			*Cornu aspersum*			*Deroceras reticulatum*			*Cornu aspersum*	
Deroceras laeve			*Deroceras laeve*			*Discus spp.*			*Deroceras laeve*	
Deroceras reticulatum			*Deroceras reticulatum*			*Succinea spp.*			*Deroceras reticulatum*	

Milax Gagates			*Discus spp.*			x			*Discus spp.*	
Oxychilus spp.			*Succinea spp.*			x			*Milax gagates*	
C. remanei	26		***C. remanei***	20		***C. remanei***	x		*Oxychilus spp.*	
Arion hortensis			*Ambigolimax valentianus*			x			*Succinea spp.*	
Ambigolimax valentianus			*Deroceras laeve*			x			***C. remanei***	46
Cornu aspersum			*Deroceras reticulatum*			x			*Arion hortensis*	
Deroceras laeve			*Discus spp.*			x			*Ambigolimax valentianus*	
Deroceras reticulatum			*Oxychilus spp.*			x			*Cornu aspersum*	
Oxychilus spp.			x			x			*Deroceras laeve*	
Choriorhabditis cristata	2		***Choriorhabditis cristata***	5		***Choriorhabditis cristata***	x		*Deroceras reticulatum*	
Deroceras reticulatum			x			x			*Discus spp.*	
Succinea spp.			x			x			*Oxychilus spp.*	
Cosmocercoides pulcher	x		***Cosmocercoides pulcher***	x		***Cosmocercoides pulcher***	x		***Choriorhabditis cristata***	2
x			*Deroceras laeve*			x			*Deroceras reticulatum*	
Cosmocercoides tonkinensis	x		***Cosmocercoides tonkinensis***	x		***Cosmocercoides tonkinensis***	11		*Succinea spp.*	

x			*Deroceras laeve*			*Ambigolimax valentianus*			***Cosmocercoides pulcher***	1
x			x			*Deroceras laeve*			*Derocer as laeve*	
x			x			*Deroceras reticulatum*			***Cosmocercoides tonkinensis***	16
Cruzia americana	x		***Cruzia americana***	x		***Cruzia americana***	1		*Ambigolimax valentianus*	
x			x			*Deroceras laeve*			*Derocer as laeve*	
Oscheius tipulae	4		***Oscheius tipulae***	x		***Oscheius tipulae***	2		*Derocer as reticulatum*	
Arion hortensis			x			*Discus spp.*			***Cruzia americana***	1
Ambigolimax valentianus			x			x			*Derocer as laeve*	
Rhabditophanes	10		***Rhabditophanes***	16		***Rhabditophanes***	3		***Oscheius tipulae***	6
Ambigolimax valentianus			*Arion hortensis*			*Ambigolimax valentianus*			*Arion hortensis*	
Cornu aspersum			*Ambigolimax valentianus*			*Discus spp.*			*Ambigolimax valentianus*	
Deroceras laeve			*Cornu aspersum*			*Oxychilus spp.*			*Discus spp.*	
Oxychilus spp.			*Deroceras laeve*			x			***Rhabditophanes***	29
x			*Deroceras reticulatum*			x			*Arion hortensis*	
									Ambigolimax	

									valentianus	
									Cornu aspersum	
									Deroceras laeve	
									Deroceras reticulatum	
									Discus spp.	
									Oxychilus spp.	

Table S2.2 Shows the estimated return of investment (ROI) of using *Phasmarhabditis* on California specialty crops which are frequently affected by gastropod pests. These crops include those found in nurseries, greenhouses, floriculture, and sod industries. Data assumes a mean damage reduction of 50.38% based on Rae *et al*., 2007. ROI chart was made based off chart made and presented by Irma Tandingan De Ley, 2017 (unpublished). Sourced information comes from the 2012 Census of Agriculture for Specialty Crops vol. 2 part 8 (published February 2015): https://agcensus.usda.gov/Publications/2012/Online_Resources/Specialty_Crops/SCROPS.pdf

	California Specialty Crops	
Agriculture	**Number of Farms**	**Sales**
Nurseries, Greenhouses, Floriculture, and Sod	3,890,000	$2,547,307,000
Assumed 5% Loss to Gastropod Damage		$127,365,350
Potential Gain from *Phasmarhabditis* Use		$64,166,663

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CHAPTER 3

Size and Dose Dependence of *Phasmarhabditis* Isolates (*P. hermaphrodita, P. californica, P. papillosa*) on the Mortality of Adult Invasive White Garden Snails (*Theba pisana*)

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Abstract

Theba pisana is an invasive snail pest which has established itself in San Diego County and some areas of Los Angeles County, California. The snail has grown to large populations in some areas and mitigation is becoming necessary to stop the spread of the species. In a previous study, three US strains of *Phasmarhabditis* species (*P. californica, P. papillosa,* and *P. hermaphrodita*) effectively killed juvenile (0.25 gram each, 4-6 mm wide) *T. pisana* in laboratory conditions at 5 times (150 IJs/cm^2) the recommended dose of what is used for Nemaslug®. Based on laboratory assays, we demonstrated that the same three US strains of *Phasmarhabditis* can effectively kill larger adult *T. pisana* (0.4-1.2 gram, 11.5-15mm wide) within two weeks at the same dose. All tested *Phasmarhabditis* strains were more efficient at killing *T. pisana* than the compared molluscicide Sluggo Plus®. Results further showed that the most virulent strain, *P. californica* did not effectively kill *T. pisana* at lower doses of 30 IJs/cm^2 and 90 IJs/cm^2. Additional research is needed to develop the most efficient means of application of *Phasmarhabditis* to control *T. pisana* in the field.

Introduction

Terrestrial snails and slugs belong to the class Gastropoda (Phylum: Mollusca). They play important roles throughout a variety of ecosystems where they act as detritivores and plant feeders, inherently breaking down plant materials and fertilizing the soils they occupy (Jennings and Barkham, 1979; Prather et al., 2013). While many terrestrial gastropods are thought of as pestiferous nuisances which invade agricultural

spaces and damage produce, multiple native species only occupy specific niches where they serve critical roles as detritivores such as banana slugs *Ariolimax* Mörch 1859 or other slugs (Gervais et al., 1998). However, many terrestrial gastropods are invasive agricultural pests, threatening native biodiversity (Mckinney and Lockwood, 1999; Clavero and Garcia-Berthou, 2005; Butchart et al., 2010; Gladstone and Bordeau, 2020). In California, it is estimated that there are about 279 species of terrestrial gastropods, however 37 of those species are invasive (Roth and Sadeghian, 2003). These invasive species are the gastropod pests which are typically found throughout the agricultural industry (Roth and Sadeghian, 2003). They are hypothesized to have arrived in California via horticultural trade when infested produce products were delivered for trade (Cowie et al., 2008; Bergey et al., 2014). Some of these invasive gastropod species can cause agricultural damage with significantly reduced crop yields (Pappas and Carman, 1961; Fisher and Orth, 1985; Sakovich, 2002).

Gastropods are also capable of spreading plant and human diseases. Terrestrial gastropods have been found to harbor *Alternaria brassicicola*, the causative agent of black leaf spot, and other pathogenic fungi (Wester et al., 1964; Hasan and Vago, 1966; Turchetti and Chelazzi, 1984). They are also thought to be partially responsible for salad crop recalls after both *Campylobacter spp.* and *Escherechia coli* were reported in the feces of some gastropods (Sproston et al., 2006; Raloff, 2007). Multiple gastropod species have also been found to harbor the human parasite *Angiostrongylus cantonensis*, the causative agent for rat lung worm disease (Kim et al., 2014).

Theba pisana (Müller 1774) is an invasive gastropod species also known as the Italian white snail. It has been introduced to various countries across the globe (Däumer et al., 2012). The snails have become an important pest throughout most of the countries they have invaded where extremely dense populations occur (Baker, 1988; Baker and Vogelzang, 1988). They are known to be active during the wet periods of the year when they feed on leaves and stems of plants. During the hot and dry periods, they aggregate in large clusters where they crawl up large stalks and aestivate (Baker, 1988; Baker and Vogelzang, 1988). They can cause damage to a variety of different plants like ornamental flowers, vegetables, citrus, almond and olive trees, and grapevines (Avidov and Harpaz, 1969; Swart et al., 1976; Godan, 1983; Baker, 1988). They have also been found to cause damage to farming machinery by clogging equipment which takes up large clusters of snails on plant material and to livestock by causing livestock to reject hay heavily infested with *T. pisana* (Durr, 1946; Joubert C.J. and Walter S.S., 1951; Godan, 1983; Baker and Vogelzang, 1988). In California, *T. pisana* is considered a pest with limited distribution that is known to cause economic or environmental detriment, and is mostly present in southern sections of California near the location it was first isolated in La Jolla, San Diego CA (Chace E.P., 1915). Rating the pest allows the state to take specific governmental actions against the pest upon finding it within the state. *T. pisana* has been noted in Los Angeles County, and was recently reported in Half Moon Bay, San Mateo County, CA (https://agwm.smcgov.org/white-garden-snail). The snails cause major aesthetic disturbances on public and private properties and sometimes render facilities unusable (Fig. 3.1) (De Ley et al., 2020). Although the snails are currently restricted to

certain spotty areas of distribution, this displays significant potential to damage natural ecosystems or agriculture, human health, or commerce; and is suggested to be of top national quarantine importance in the US (Cowie et al., 2009).

Mitigation of the Italian white snail in California is necessary to protect local biodiversity and public health. Methods to control populations of *T. pisana* have included metaldehyde baits, sprayable molluscicides, burning, barricading/trapping, and hand picking (Basinger A.J., 1927; Pilsbry H.A., 1939; Flint M.L., 2011; Deisler J.E. et al., 2015). While some of these methods have proven effective (Radwan et al., 1992; Abdelgaleil, 2010), they are not sufficiently targeted to the Italian white snail and therefore may also be toxic to native mollusks (Flint M.L., 2011) and a variety of other organisms (Castle et al., 2017). Molluscicide use also provides the possibility of developed resistance over a prolonged period of exposure (Dai et al., 2015). It has been discovered that some snails have already developed resistance to metaldehyde baits, one of the more commonly used methods of gastropod pest control (Salmijah et al., 2000). They can also contaminate groundwater as they leach into the soil (Castaneda and Bhuiyan S.I., 1996).

The use of biological control agents to manage gastropod pests is considered safer and more environmentally sustainable relative to molluscicides. Biological control is a more targeted methodof integrated pest management (IPM). *Phasmarhabditis hermaphrodita* (Schneider 1859) is the most well-known example of a successful biological control agent against pestiferous gastropods. The nematode was discovered in Europe where it has seen extensive commercial and home use under the brand

Nemaslug® (BASF Agricultural Solutions, United Kingdom). *P. hermaphrodita* is an effective biological control agent in a variety of different environments including fruit, vegetable, and ornamental crops across many European locations (Wilson et al., 1993b; Rae et al., 2007; Mc Donnell et al., 2018, 2020; de Ley et al., 2020). It is a parasitic nematode that infects gastropods. *P. hermaphrodita* was found safe to various species of earthworms, as well as native, non-pest European slugs and snails, though additional non-target testing is needed (Wilson et al., 2000; Grewal et al., 2003; DeNardo et al., 2004; Rae et al., 2005).

Recently, three *Phasmarhabditis* species were discovered in California including *P. californica*, *P. hermaphrodita*, and *P. papillosa* (Tandingan De Ley et al., 2014, 2016a, 2016b). Pathogenicity assays have been performed using each of these local strains and it was found that they caused significant mortality in *Deroceras reticulatum* in laboratory and field simulated conditions (Mc Donnell et al., 2020; Schurkman et al., 2022a). The US strains have also been tested against small (4-6mm) young *T. pisana* in controlled lab conditions where all strains caused 100% mortality within 5 days. The strains' efficacies were compared to the molluscicide Sluggo Plus® (Monterey Lawn and Garden, Fresno CA, USA) and the nematodes were shown to be equally as effective at causing mortality in *T. pisana*. However, larger *T. pisana* can exist in large populations throughout the year (Cowie, 1984a) (Fig. 3.1). *T. pisana* are generally considered adults once their shell size surpasses 10mm (Johnson, 1980). Their maturity can also be assessed based off of the time of year and size differences within a population (Cowie, 1984b). In the past, larger gastropods were found to be more resistant to *Phasmarhabditis*

(Glen et al., 1996; Speiser et al., 2001). Therefore, in order to assess the efficacy of the US *Phasmarhabditis* strains against *T. pisana*, their lethality should be determined when applied to both large adults and small juvenile snails.

Another important aspect of evaluating the efficacy of a biological control agent is the minimum effective dose. The recommended dose when applying Nemaslug® is 30 infective juveniles (IJs)/cm^{2}of soil substrate. When US *Phasmarhabditis* strains were originally tested against *T. pisana*, a 5-fold dose of 150 IJs/cm^2 was used (de Ley et al., 2020). This was done only to show that the US strains were capable of killing the snails. A lower dose of nematodes is preferable in both economic and production terms. A decreased and more economically sound and effective dosage of US *Phasmarhabditis* species should be determined against *T. pisana*.

We tested the efficacy of three US strains of *P. californica* (ITD726), *P. hermaphrodita* (ITD272), and *P. papillosa* (ITD510) and Sluggo Plus® against larger adult *T. pisana* (11.5-15mm). Based on the comparative efficacy of these 3 species, we further tested *P. californica* at lower doses of 30 IJs/cm^2 (Nemaslug ®-recommended dose) and 90 IJs/cm^2 against the larger-sized and heavier *T. pisana*.

Materials and Methods

Field collection of T. pisana snails

Multiple locations within San Diego County, California previously identified to have large populations of invasive *T. pisana* (personal communication with C. Wilen, UCANR) Area IPM Advisor). The snails were collected under CDFA permit 3449 from

an empty non-cultivated grassy field near a commercial lot, adjacent to multiple commercial buildings in Carlsbad, California (33.1289523, -117.2489610).

Size dependence assay on Theba pisana *exposed to Phasmarhabditis* spp.

Three *Phasmarhabditis* species applied at 5x the recommended dose (150 IJs/cm^2) and a recommended dose of Sluggo Plus® were tested against *T. pisana* held within test arenas consisting of a container (33.5cm L x 11.5cm H x 18.5cm W) filled with 3 layers of (a) pea gravel (350mL) at the bottom, (b) a fabric barrier (Dewitt 3' x 100' 6 Year Weed-Barrier Landscape Fabric) that fitted the tray and (c) 600g of autoclaved soil (75% SunGro Sunshine No. 4 mix and 25% UC soil mix 3) (Matkin and P. A. Chandler, 1957). Six hundred milliliters of deionized water was added to each arena to adjust the soil moisture. Two 6-week-old periwinkle (*Vinca minor* L.) were planted 3cm to the left and right of the arena's center as a possible substrate for aestivation. A 16.5cm^2 area in the middle of the arena was enclosed with a copper wire to prevent snail escape and to limit the area of application (Fig. 3.2A). Additionally, the lid with multiple holes was placed on top of the arena. *T. pisana* used in this study ranged from 11.5-15mm wide and 0.4-1.2 grams.

Dose dependence assay on Theba pisana exposed to Phasmarhabditis californica

Arenas used to assay *T. pisana* against the recommended dose of Nemaslug ® (30 IJs/cm^2) and 3x the recommended dose (90 IJs/cm^2), as well as the recommended dose of

Sluggo Plus® consisted of a 1436.5cm^3 (13cm x 13cm x 8.5cm) container filled with 100g of the same autoclaved soil described above (Matkin and P. A. Chandler, 1957). One hundred milliliters of deionized water was added to each arena to adjust the soil moisture. Two 1 month old periwinkle (*Vinca minor* L.) were planted about 4cm from the edge (Fig. 3.2B). A lid with multiple holes was placed on top of the arena to prevent snail escape. This smaller arena was used due to limited space within the laboratory temperature-controlled incubator. The snails used in this study were within the same size range as previously described, however their weights ranged from 0.5-1.3 gram.

Nematode preparation

The IJs used for inoculation were prepared using a modified white trap method (Kaya and Stock S. P., 1997) using frozen *Ambigolimax valentianus* Ferussac, 1822 inoculated with mixed stages of each *Phasmarhabditis* species. *Ambigolimax valentianus* within the white traps were inoculated with xenic cultures of *P. californica* (ITD726), *P. hermaphrodita* (ITD272), and *P. papillosa* (ITD510). Infective juveniles were the only stage of nematode used throughout all experiments. All IJs were collected from the modified white traps and stored in tissue culture flasks. IJs were quantified within a tissue culture flask by counting their number in a 10µL drop of water and repeating this measurement 5 times to calculating the average IJs/10µL. The necessary volume of IJs was pipetted into individual conical tubes and the final volume was adjusted to 10mL using double distilled water prior to application to a test arena.

The higher recommended dose of 4.88kg/m^2 of iron phosphate (Sluggo Plus®, active ingredients (a.i.) are: 0.97% iron phosphate and 0.07% Spinosad (a mixture of spinosyn A and spinosyn D)) was used as the control molluscicide. A no-nematode, snail-only treatment was also added for comparison. Sluggo Plus® was chosen for use instead of the more popular metaldehyde baits because of its recent increase in popularity which came about due to metaldehyde bait's known potential for non-target effects.

Experimental setup

Ten pre-weighed snails of the mentioned weight range were divided into 3 different groups of light, medium, and heavy corresponding to 3 replicates for each trial to avoid size bias. To arrange the snails by weight, all of them were weighed individually and sorted by weight. Snails were then assigned to light, medium, or heavy categories based on their sorting. The snails were introduced on the soil around the *V. minor* plants. After snail introduction, the nematode inoculum was applied evenly to the arena using an auto pipettor. The number of dead snails were recorded daily for 2 weeks, with a snail determined to be dead if it did not move for >24 hours and the snail did not respond to prodding with a toothpick. A toothpick was placed next to each putatively dead snail to mark its location and determine whether movement occurred. Additionally, dead snails usually had withdrawn foot muscles without the presence of dried mucus (epiphragm) that is typically produced to prevent desiccation during aestivation. Also, all dead snails had the presence of mixed stage nematodes present within their shell and externally, and throughout the body (Fig. 3.3). All experimental trials had 3 replicates and were repeated

thrice. The 1st series of assays to compare the lethality of all 3 *Phasmarhabditis* spp. at 5-fold the recommended dose were performed inside a diurnal growth incubator with alternating temperatures of 20ºC and 15ºC for a 12-hour day/night cycle. The 2nd series to assess the lethality of *P. californica* at the Nemaslug ® -recommended dose and 3 –fold this dose was performed on a benchtop in the laboratory at room temperature (~23ºC). The test arena was altered due to limited space within the incubator. All arenas were covered with a fabric barrier (Dewitt 3' x 100' 6 Year Weed-Barrier Landscape Fabric) to prevent excess light exposure.

All statistical analyses were performed with GraphPad Prism 9, utilizing Mantel-Cox log-rank analyses to compare each treatment to each other.

Results

Lethality of Phasmarhabditis papillosa, P. californica, and P. hermaphrodita at 5-fold the recommended dose (150 IJs/cm^2) against Theba pisana

Application of *P. papillosa, P. californica*, and *P. hermaphrodita,* at 5 times the recommended dose (150 IJs/cm^2) resulted in 86-97% mortality after 2 weeks which was significantly greater mortality compared to both untreated control and the commercial molluscicide Sluggo Plus® ($p < 0.0001$ for all treatments compared to control and Sluggo Plus®) (Fig. 3.4). There were no statistical differences among *Phasmarhabditis* treatments ($p > 0.05$). *P. californica* caused the highest mean mortality of 97% 14 days after exposure (DAE), followed by *P. papillosa* (91%) and *P. hermaphrodita* (86%) (Fig. 3.4). Sluggo Plus® also caused significant mean mortality of 28% compared to the

untreated control, 14 DAE ($p < 0.0001$), however it caused significantly less mortality compared to all three US *Phasmarhabditis* spp. ($p < 0.05$) (Fig. 3.4).

Lethality of Phasmarhabditis californica at the recommended (30 IJs/cm^2) and 3-fold (90 IJs/cm^2) dose against Theba pisana

Treatment of *P. californica* at the Nemaslug ®-recommended dose (30IJs/cm^2) resulted in only 3.3% mortality and was not different compared to the untreated control ($p > 0.9999$) 14 DAE (Fig. 3.5). However, at 3 times the recommended dose (90 IJs/cm^2) snail mortality increased to 8.9% and was significantly different than the untreated control ($p < 0.0039$) (Fig. 3.5). Interestingly, treatment with Sluggo Plus® in the *P. californica* dosage assay did not cause significant mortality compared to the untreated control ($p = 0.0815$), whereas it caused significant mortality in the previous comparative assay of 3 *Phasmarhabditis* spp. (Fig. 3.4 and 3.5). While the 3 times dose caused significant mortality of *T. pisana* compared to the snail only/untreated control, the mortality rate was low enough to disregard the dose for potential use.

Discussion

The three US strains of *Phasmarhabditis* spp. (*P. californica*, *P. hermaphrodita*, and *P. papillosa*) caused mortality in both large adult and small juvenile *T. pisana*. All three caused significantly higher mortality in adult *T. pisana* compared to the chemical molluscicide Sluggo Plus® and the control with no nematodes when applied at 5 times the recommended dose of 150 IJs/cm^2 (Fig. 3.4). Compared to our previous findings on

the efficacy of these *Phasmarhabditis* spp. to smaller-sized (2-6mm) juvenile *T. pisana*, it takes about 9 more days for the same level of mortality to be observed in larger adult *T. pisana* (Tandingan De Ley et al., 2020). This result shows a size-dependent response to *Phasmarhabditis* exposure, similar to Glen et al. (1996) and Speiser et al. (2001), but adult snails were not capable of successfully fending off nematode infection. Our results agree with previous findings on naturally occurring *Phasmarhabditis* in France, where strains of *P. hermaphrodita* isolated from *T. pisana* and *Trochoidea elegans* (Gmelin, 1791) caused similar mortality rates (100% mortality 6 DAE) when applied to snails less than 6mm and snails larger than 10mm (Coupland, 1995). However, previous work also found that the smaller snails died at a faster rate than the larger snails (Coupland, 1995).

Multiple studies have shown that *Phasmarhabditis'* efficacy as a biological control agent is dependent on the size or life stage of the gastropod host. Tested species of gastropod neonates have been found to be more susceptible to *Phasmarhabditis* infection and mortality, and adults have increased resistance or immunity to *Phasmarhabditis* infection (Speiser et al., 2001; Grimm, 2002; Grewal et al., 2003; Grannell et al., 2021). *Phasmarhabditis* resistance also seems to be species specific. For example, *D. reticulatum* seems to be susceptible to multiple *Phasmarhabditis* species at various sizes throughout all life stages (Tan and Grewal, 2001a; Mc Donnell et al., 2020; Schurkman et al., 2022a). Multiple other slug and snail species have been tested for susceptibility to *Phasmarhabditis* as well, and there are varying mortality results for each gastropod species (Wilson et al., 1993b; Grewal et al., 2003; Rae et al., 2007).

The lower dosage (30 IJs/cm^2 and 90 IJs/cm^2) assay showed that a more economically sound (less product and thus cheaper to purchase) dose of *P. californica* and likely other species of *Phasmarhabditis* may not be effective against *T. pisana*. This is because *P. californica* was the most virulent of the three tested species in the 5X lethality assay and it was incapable of causing sufficient mortality at lower doses (Fig. 3.5). The best result using lower doses only caused a mortality rate of about 9% after 14 days (Fig. 3.5). This is in congruence with previous studies done with *P. californica* on the brown garden snail *Cornu aspersum*. Other researchers found that *P. californica* was not capable of causing significant mortality to the snail 21 DAE at the recommended rate (30 IJs/cm^2) (60). However, *P. californica* has been found to kill *D. reticulatum* at a lower, more economically feasible dose. One study found that *P. californica* was able to cause significant mortality at about 45IJs/cm^2 and 90 IJs/cm^2 (Mc Donnell et al., 2020). However, it took significantly longer to cause mortality in the slugs at lower doses.

The Sluggo Plus® control in the lower dose assay did not cause similar mortality to what was observed in the 5X pathogenicity assay even though the same concentration of Sluggo Plus® was provided. In the lower dose assay, Sluggo Plus caused a mortality rate of only about 3% whereas in the assay that used a 5X dose of US *Phasmarhabditis* strains, the same Sluggo Plus® caused a mortality rate of about 28% (Figures 3.4 and 3.5). This discrepancy could have occurred for a variety of reasons. There were differences between each of the assays, specifically the arena design. The lower dose assay was not performed in an incubator; therefore, there were no day/night-controlled temperature cycles. Instead, the arenas were consistently at room temperature which

fluctuated between about 21-23C. The lower dose assay also had no copper barriers in use due to the size of the arenas. It is possible that the copper barriers in the 5X pathogenicity assay forced an increased exposure of treatment to the snails, or perhaps even decreased the snail's health itself. It has been found that copper carbonate is toxic to some aquatic and semiaquatic snails (Nebeker et al., 1986; Hoang and Rand, 2009; Besser et al., 2016). Copper carbonate forms when copper is exposed to moisture. Therefore, it is possible that the snails in assays that used copper barriers were exposed to copper carbonate, which helped increase observed mortality rates. Exposure to copper carbonate could have come from direct contact of the copper barrier, or from copper leaching into the soil. However, experiments assessing copper carbonate's toxicity to gastropods are performed in liquid exposure (Nebeker et al., 1986; Hoang and Rand, 2009; Besser et al., 2016). The toxicity of solid copper carbonate on snails has not been evaluated and therefore no immediate conclusions about the use of copper barriers can be made. However, copper hydroxide, a common occurrent in some soil fertilizers, has been shown to be toxic to some terrestrial snails, including *T. pisana* (Eshra, 2014) . While copper barriers would not have led to exposure to copper hydroxide, exposure to multiple copper compounds in both solid and liquid forms should be explored in the future.

T. pisana are known to climb up tall structures or plants in the spring or summertime when temperatures begin to increase. They do this to aestivate and protect themselves from desiccation (Cowie, 1985). *Phasmarhabditis* nematodes are not likely to climb up tall structures or plants, as they are susceptible to desiccation. However, *T. pisana* often lay eggs on moist soil and during the late spring young snails emerge and

begin to climb. This means that there is a period when the snail progeny is disproportionately located on the soil surface. Soil application of *Phasmarhabditis* as a biological control agent is therefore likely more beneficial when applied during periods of snail emergence or during time periods when snails are laying eggs. *Phasmarhabditis* could also be sprayed on plants as needed for immediate application, however the nematodes would not persist in such applications. Further research is needed to identify methods of *Phasmarhabditis* application to target *T. pisana*. However, our data suggest that *Phasmarhabditis* may be a feasible option for the mitigation of *T. pisana* due to its specificity to gastropods and safety to other non-target species.

Figures

Figure 3.1 A gutter cluttered with *Theba pisana* behind a local business in Oceanside, CA (A). A utility box covered with aestevating *T. pisana* beside a local business in Oceanside, CA (B). Clusters of aestivating *T. pisana* on a tree beside a sidewalk in Oceanside, CA (C).

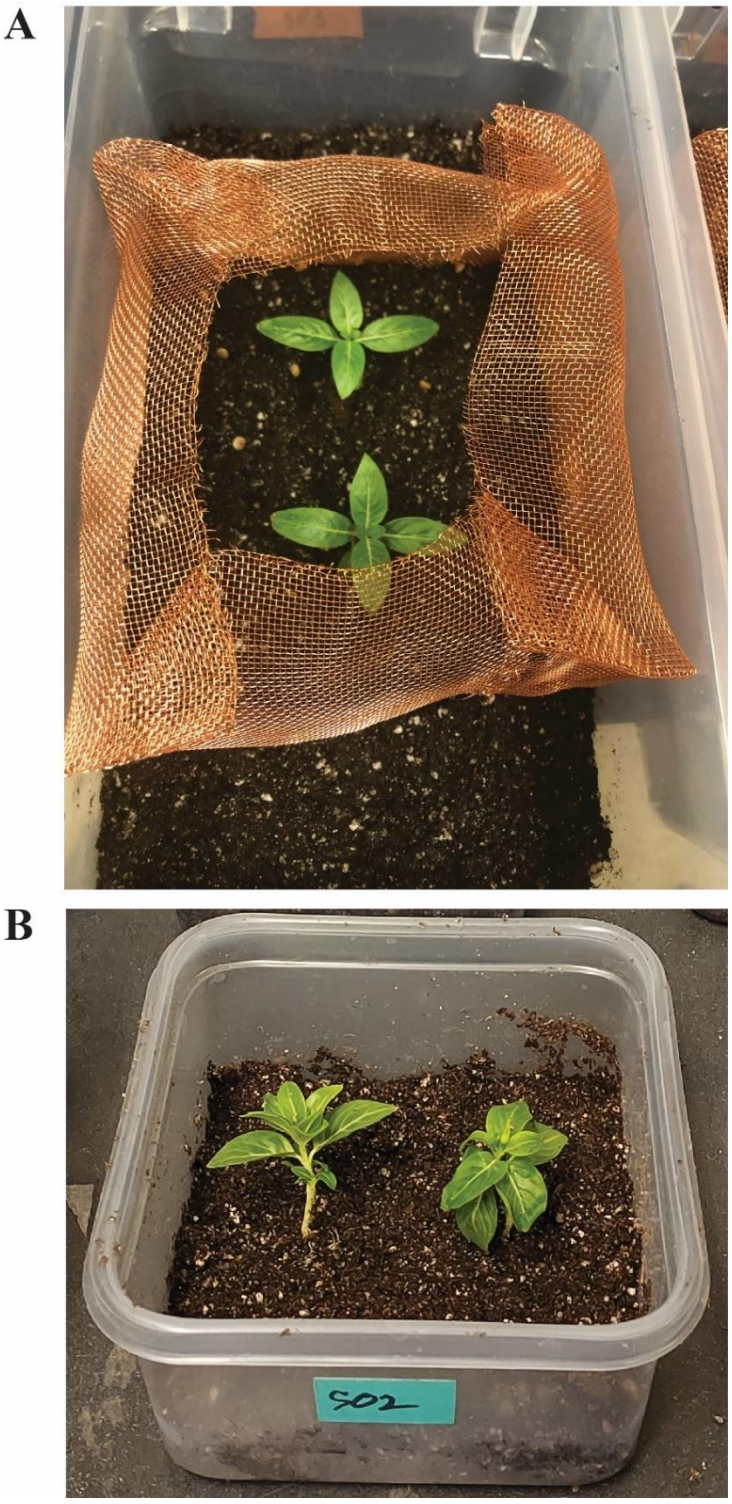

Figure 3.2 Treatment arena for determining the lethality of three US strains of *Phasmarhabditis californica* (ITD726), *P. papillosa* (ITD510), and *P. hermaphrodita* (ITD272) against (11.5-15mm/0.4-1.2 gram) *Theba pisana* at 5 times recommended dose of 150IJs/cm^2 (A) and for the dosage dependence of *P. californica* lethality against (11.5-15mm/0.5-1.3 gram) *T. pisana* at 30 IJs/cm^2 (Nemaslug® recommended dose) and 90 IJs/cm^2 (B).

Figure 3.3 A dead adult *Theba pisana* snail with mixed stages of *Phasmarhabditis californica* (ITD726) within the shell cavity.

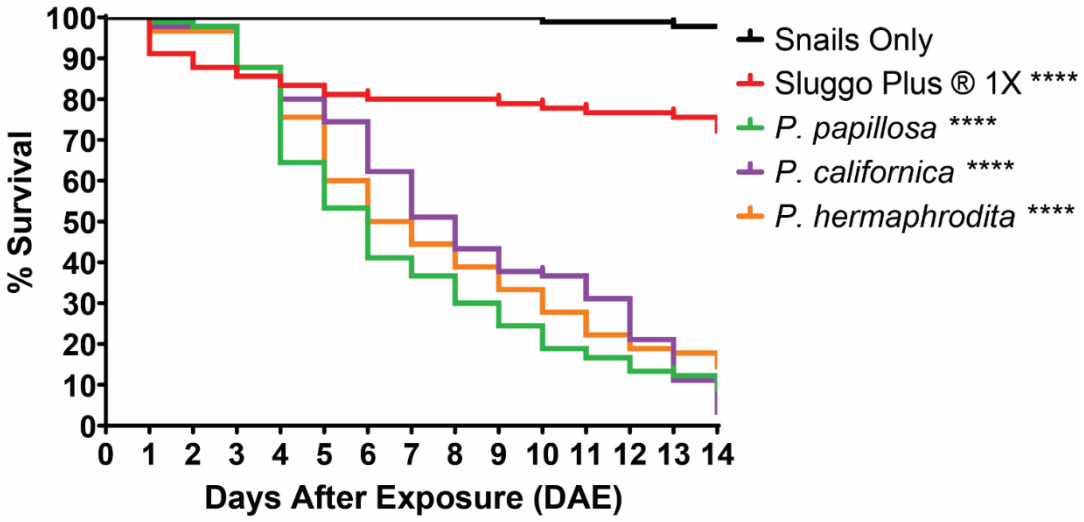

Figure 3.4 Kaplan Meier graph showing the percent survival of large adult *Theba pisana* over 14 days after exposure to 5 times the Nemaslug ®-recommended dose (150 IJs/cm^2) of three US strains of *Phasmarhabditis californica* (ITD726), *P. papillosa* (ITD510), and *P. hermaphrodita* (ITD271), and Sluggo Plus®. The snails only control included a treatment with no application of *Phasmarhabditis*. **** indicates a p value less than 0.0001 compared to untreated control. Statistical analyses were performed by doing Mantel-Cox log rank analyses comparing each treatment to each other.

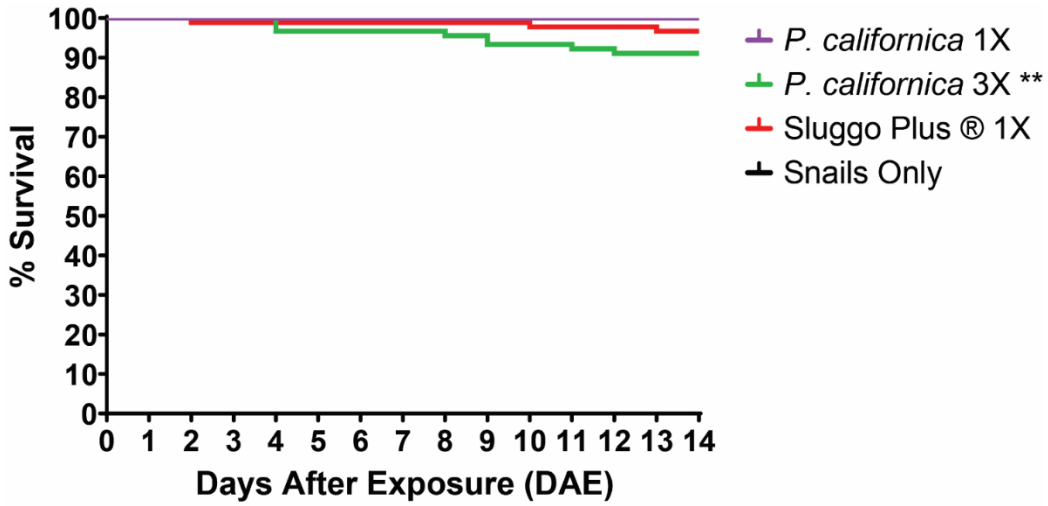

Figure 3.5 Kaplan Meier graph showing the percent survival of adult *Theba pisana* over 14 days after exposure to the Nemaslug ®-recommended dose (30 IJs/cm^2) and three times the recommended dose (90 IJs/cm^2) of *Phasmarhabditis californica* (ITD726) and Sluggo Plus®. ** indicates a p value less than 0.01 compared to the untreated (snails only) control. Statistical analyses were performed by doing Mantel-Cox log rank analyses comparing each treatment to each other.

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CHAPTER 4

The Native Microbiome of *Phasmarhabditis* Isolates Across Central and Southern California

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Abstract

Nematodes in the genus *Phasmarhabditis* can infect and kill slugs and snails, which are important agricultural pests. The mechanism leading to host death is unknown but may involve contributions from nematode-associated bacteria. The native microbiome of *Phasmarhabditis* is unexplored; previous *Phasmarhabditis* microbiome studies has focused on lab grown or commercially reared nematodes, and in order to obtain a deeper understanding of the parasite and its host interactions, it is crucial to understand the natural microbial communities associated with this organism in the wild. We sampled *Phasmarhabditis* directly from their natural habitats in Central and Southern California and identified their native microbiome via 16S amplicon sequencing. We found that the *Phasmarhabditis* microbial community was influenced by the nematode species, location, and gastropod host from which the nematode was collected. The predominant bacteria of the *Phasmarhabditis* isolates collected included *Shewanella*, *Clostridium perfringens*, Aeromonadaceae, Pseudomanodaceae, and *Acinetobacter*, with some nematode species having more frequent associations with certain bacterial species than others. More work on the natural microbiome of *Phasmarhabditis* is needed to determine the role of bacteria in nematode virulence to snails.

Introduction

Nematodes are one of the most ecologically diverse groups of organisms on Earth. They exist on every continent, surviving in all climates where decomposition occurs (Ingham, 1994; Bongers and Bongers, 1998; De Ley, 2006; Schafer, 2016). Some

exist as free-living organisms like *Caenorhabditis elegans*, and many have evolved to form a variety of parasitic relationships like *Ascaris lumbricoides*, or the entomopathogenic nematode (EPN) *Steinernema feltiae* that has been utilized for biological control against pestiferous insects (Kaya and Gaugler, 1993; Riddle et al., 1997; Marilyn, 2008). EPN's have evolved specific mutualistic relationships with bacterial species in their gut that helps to kill various insects (Grewal and Georgis, 1997). Recent metagenomic analyses have indicated that the commensal microbial community of EPNs, the gut microbiome, is more complex than originally thought, leading to the possibility of a native EPN pathobiome that assists with insect killing (Ogier et al., 2020).

One nematode that has been successfully used as a biological control agent is *Phasmarhabditis hermaphrodita*. All members of the genus are gastropod-specific facultative parasites. *P. hermaphrodita* was discovered in Europe and has been commercialized for biological control in Europe under the name Nemaslug® (Bayer, UK). *Phasmarhabditis* nematodes are effective at killing pestiferous gastropods in laboratory and agricultural settings such as nurseries and a variety of crops (Wilson et al., 1994a, 1994b; Rae et al., 2007; McDonnell et al., 2020; Tandingan De Ley et al., 2020; Schurkman et al., 2022a), but are safe to tested non-gastropod organisms (Grewal and Grewal, 2003; Iglesias et al., 2003; DeNardo et al., 2004; Rae et al., 2005). However, due to the discovery of *Phasmarhabditis* in Europe, its use has not yet been permitted in the United States since invasive species are not permitted for biological control use in the country.

It was originally thought that *Phasmarhabditis* nematodes kill their hosts in a manner similar to EPNs, which employ mutualistic and pathogenic microbes to assist with insect killing. This hypothesis was supported by the discovery that *P. hermaphrodita* cultured with *Moraxella osloensis* was highly pathogenic to the grey field slug *Deroceras reticulatum*, more so than when it was cultured on other bacteria (Wilson et al., 1995a; Tan and Grewal, 2001b). The selection of bacteria to test came from isolates identified in *P. hermaphrodita* Infective juveniles (IJs), dead *D. reticulatum*, and xenic foam chip cultures (Wilson et al., 1993a, 1995a; Poinar and Thomas, 2012). The species identified and tested included *Aeromonas hydrophila*, *Aeromonas* sp., *Flavobacterium breve*, *Flavobacterium odoratum*, *Moraxella osloensis*, *Providencia rettgeri*, *Pseudomonas fluorescens* (isolate no. 140), *Pseudomonas fluorescens* (isolate no. 141), and *Serratia proteamaculans*. When these bacteria were injected into *D. reticulatum*, *A. hydrophila* and *P. fluorescens* (isolate no. 140) caused the most mortality. However, *P. fluorescens* (isolate no. 140) was able to facilitate better growth when culturing *P. hermaphrodita*, which may be indicative of this bacterial species serving as a food source. Nematodes grown on *M. osloensis* exhibited the highest pathogenicity while also allowing for good nematode growth. Another experiment showed that axenic *P. hermaphrodita* did not cause mortality in *D. reticulatum* while those reared on *M. osloensis* did (Tan and Grewal, 2001b). These experiments led to the assumption that *P. hermaphrodita* likely has a natural association with *M. osloensis*, similar to the association of EPNs with pathogenic bacteria (An et al., 2008; Wilson and Rae, 2015). However, the natural

association of other bacteria with *Phasmarhabditis* in the wild and how this association contributes to nematode host virulence remained largely unexplored.

In 2010, it was shown that *P. hermaphrodita* associates with many bacterial species that do not affect its virulence (Rae et al., 2010), in contrast to the existing understanding of *Phasmarhabditis* virulence (Wilson et al., 1995a). Rae *et al*., 2010 suggested that *P. hermaphrodita* does not associate with specific bacteria due to the observation of inconsistent and varied bacterial assemblages with the nematode (Rae et al., 2010). However, the study was unable to identify key microbial species that regularly occur within *Phasmarhabditis* because taxonomic identification was not performed throughout the experiment. Therefore, the study was unable to identify if *M. osloensis* was present. In another study, bacteria were identified from lab grown *Phasmarhabditis*, by allowing nematodes to crawl on LB agar plates and identifying some of the bacterial colonies that subsequently arose (Howe et al., 2020). Eight genera of bacteria were identified that were hypothesized to have come from the lab grown *Phasmarhabditis*. *Pseudomonas* was the only genus found in this most recent study that was also found in 1995 (Wilson et al., 1995a; Howe et al., 2020). These mirror findings related to the native and naturally occurring microbiome of the model organism *Caenorhabditis elegans*, where *Pseudomonas* was also identified (Dirksen et al., 2016), though prolonged *in vitro* growth in the lab raises the possibility of association with microbes not commonly found with *Phasmarhabditis* in the wild.

Describing the natural and infected microbiome of the host could help to distinguish whether microbes present within *Phasmarhabditis* originated from the host or

from another source. Very little microbiome research has been done on *D. reticulatum*, the slug often used in *Phasmarhabditis* studies (Walker et al., 1999). However, gut microbiome metagenomic analyses have been performed on other slug species like *Ambigolimax valentianus* which identified a core microbiome of *Citrobacter*, *Delftia*, *Erwinia*, *Arthrobacter*, *Stenotrophomonas*, *Pseudomonas*, *Rhodococcus*, and *Bacillus*. *Arion ater*'s microbiome was also found to be influenced by the gastropod they were collected from, while the soil microbial community itself could also be influenced by the introduction of the slug (Jackson, 2020; Jackson et al., 2021). A gut metagenomic analyses of the slug *A. ater* has also been performed. The most abundant bacterial genera in the gut of *A. ater* included *Enterobacter*, *Citrobacter*, *Pseudomonas*, and *Escherichia* (Joynson et al., 2017).

All microbiome studies that have taken place involving *Phasmarhabditis* have used lab cultured nematodes, and the microbiome changes upon introduction to a lab environment, especially when the nematodes are grown on monoxenic cultures (Dirksen et al., 2016). Recently, three species of *Phasmarhabditis* were discovered during surveys of California nurseries and gardens (Tandingan De Ley et al., 2014, 2016a). Between 2018 – 2021 additional surveys for *Phasmarhabditis* nematodes were performed (Schurkman et al., 2022b). Nematodes collected in this most recent survey were used to identify the natural microbiome of *Phasmarhabditis* isolates across the Central and Southern California regions. Similarities or differences between *Phasmarhabditis* isolates could help to further the understanding of the role that the microbiome plays in the host parasite relationship between *Phasmarhabditis* nematodes and their hosts.

Materials and Methods

Phasmarhabditis survey collection

Fourteen plant nurseries from Central California and 5 nurseries from Southern California were surveyed for gastropods infected with *Phasmarhabditis* as described in (Schurkman et al., 2022b). In short, 1 person hour was spent searching for gastropods. After 1 person hour, gastropods were sorted into containers by species and taken back to the laboratory at UC Riverside. Gastropods were decapitated and placed on 1% plain agar to create seed cultures in petri dishes (1L: 10g agar, 900ml H_2O) and their bodies were observed for the presence of nematodes under a dissecting microscope. Upon finding a nematode(s) which phenotypically resembled a member of the *Phasmarhabditis* genus (i.e., the significant presence of phasmids), up to 5 individuals were placed on Nematode Growth Medium (NGM; 1L: 3g NaCl, 20g Agar, 2.5g Peptone, 975ml deionized H_2O, 10ml Uracil (2g/L) were added to a liter of deionized water, autoclaved, and let cool, to which were added 25ml filtered KPO_4, 1ml filtered $MgSO_4$, 1ml $CaCl_2$, and 1ml Cholesterol (5mg/ml)) to create single nematode isolation plates with a single nematode on each of the plates. All nematodes on single nematode isolation plates were therefore considered identical species of the same strain since the plates originated from one single nematode. The single nematode isolation plates were stored at 17C. Individual nematodes suspected to be *Phasmarhabditis* were picked from single nematode isolation plates and were prepared for PCR and DNA sequencing of their rDNA on the D2-D3 domains of the LSU, as described in (Tandingan De Ley et al., 2014). Contigs were assembled and compared by BLAST with published sequences on Genbank using CodonCode Aligner

(CodonCode Corp., 58 Beech Street, Dedham, MA, USA) and the nematode identities were verified.

Phasmarhabditis treatment and storage

All nematodes suspected to be a member of the *Phasmarhabditis* genus via microscopy were prepared for microbiome analysis. This preparation was done for each nematode before single nematode isolation plates were created. To prepare, nematodes in seed cultures which phenotypically matched those that were used for single nematode isolation were subjected to a rinse. When possible, at least one nematode from each seed culture was washed in sterile M9 buffer (1L: 3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml 1M $MgSO_4$, H_2O to 1L) thrice and then placed inside of 10µl of sterile H_2O in a 200µl PCR tube which was stored at -20C for future use. These nematodes were labeled as washed nematodes. When possible, at least one nematode was also not subjected to any treatment at all, and the nematode was immediately picked and placed inside of 10µl of sterile H_2O in a 200µl PCR tube which was stored at -20C. These nematodes were labeled as unwashed nematodes. Lastly, 10µl of dead and partially decomposed gastropod tissue was pipetted into 10µl of sterile H_2O in a 200µl PCR and was stored at -20C. The washes were performed to rinse excess material from the cuticle of the nematode. Comparisons to unwashed nematodes and decomposed slug tissue were done in order to observe whether the washes significantly altered the microbiome of *Phasmarhabditis*. Upon finding a *Phasmarhabditis* nematode via 28S sequencing, we used the corresponding nematode(s) which was stored in the -20C freezer for microbiome analysis.

DNA Extraction

DNA was extracted from all washed and unwashed nematodes, as well as from the decomposed dead gastropod tissue. The DNA extraction protocol included thawing samples on ice and breaking the individual nematodes into pieces within their PCR tube using a sterile 10µl pipette tip. After breaking the nematodes, the total volume of all samples was brought up to 100µl with sterile PCR grade water. An equal volume of phenol chloroform was then added to each sample. The contents of the small PCR tubes were then transferred to a 1.25ml Eppendorf tube and were mixed via pipettor. The tubes were shaken by hand for 30 seconds and were then centrifuged at 12,000rpm's for 10min at 4C. After centrifugation, the aqueous phase of the solutions was removed and placed in a new 1.25ml Eppendorf tube. The wash with phenol chloroform was repeated once more, and then 400µl of isopropanol stored at -20C was added to the solution. A 1:10 ratio of 3M Sodium Acetate was then added to the solution and was mixed via pipetting up and down. The tubes were then shaken by hand for 30 seconds and 1µl of glycogen stored at -20C was added. The tubes were stored at -20C for 24 hours. After 24 hours, the samples were centrifuged at 13,000rpm's for 30min at 4C to form a pellet. All liquid was then removed from the tubes being careful not to disturb the pellet at the base of the tube. The pellet was carefully washed with 500µl of ethanol which was immediately removed via pipette. The tubes were then stored inside of a 37°C incubator until there was no visible liquid present. The pellet was then resuspended in 50µl of sterile PCR grade water and the DNA concentration was checked using a Qubit 3 Fluorometer (Invitrogen by Thermo Fisher Scientific and life technologies, Waltham, MA USA 02451).

16S rRNA gene library preparation and sequencing

The bacterial 16S rDNA V4 region 515F-806R was amplified according to the earth microbiome project, 16S Illumina protocol (Thompson et al., 2017). Based on the concentrations of our single nematode DNA sample, 1~8µl of the extracted DNA template, 10µl Platinum Hot Start PCR Master Mix (ThermoFisher), and 0.5µl of forward and reverse primers (10µM) were added into the 25µl PCR reaction system, with barcode in the reverse primer. Thermocycler condition was 4°C for 3 min, followed by 30 cycles (94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec), and 72°C for 10 min. PCR products were pooled together and submitted to an Illumina MiSeq platform with 2 × 150 bp read lengths.

Data analysis

Raw reads were processed using the open-source software QIIME2 (Bolyen et al., 2019). Samples that had >1000 reads were remained and denoised using dada2 with default settings. Taxonomic classification was performed using classify-sklearn commend against the 99% Greengenes 13_8 reference set trimmed to 250 bp of the V4 hypervariable region. An amplicon sequence variant (ASV) was defined as a group of sequences with a similarity of 100%. Alpha and beta diversity analyses were calculated in QIIME2 and plotted in GraphPad Prism 9. The heatmap was generated using pheatmap package in R program, samples and species clustered using hclust.

The statistical calculations used in QIIME2 were: Kruskal-Wallis test for alpha diversity comparisons, and permANOVA for beta diversity. Mann-Whitney U tests were performed in GraphPad Prism 9 for taxa comparisons.

Results

Diversity of the microbiome in Phasmarhabditis nematodes

Of the total 146 samples from 3 different nematode species from various gastropod hosts collected during surveys between 2019 and 2020, 26 were amplified and sequenced successfully. These 26 gastropods consisted of only three different species, *D. reticulatum*, *D. laeve*, and *A. valentianus*. In total 475,226 raw reads were obtained from the 26 samples. 397,685 high-quality reads were clustered into 337 ASVs at 100% similarity level. Twenty-two samples with read depth >1000 remained for subsequent analyses. Alpha diversity analysis showed that nematode species may be an important factor associated with the diversity of nematode microbiomes. According to observed features, Shannon index, and Faith's phylogenetic diversity (Faith pd), *P. californica* microbiomes exhibited higher richness than those of *P. hermaphrodita* and *P. papillosa* (Fig. 4.1A). Central California samples had significantly higher observed features and Faith pd index (which measures phylogenetic diversity) than Southern California. However, this may reflect the fact that all nematodes collected from Southern California were *P. papillosa*, which had the lowest diversity of observed features (Fig. 4.1). The host of the nematode was not associated with differences in microbial richness or evenness (Fig. 4.2A). No alpha diversity differences were noted across nematodes that

were washed in M9 thrice, unwashed, or collected from decomposed gastropod tissue (Fig. 4.2B).

The overall microbial community structure showed similar trends with alpha diversity analyses. The PCOA plots based on Bray-Curtis distance between sample microbiomes revealed a separation of bacterial composition depending on *Phasmarhabditis* species, especially between *P. californica* and *P. papillosa*, while the *P. hermaphrodita* microbiome overlapped with the other two species (Fig. 4.3A); these differences were statistically significant when tested using permANOVA (Table 4.1). Geographical location also contributed to differences in the nematode microbiome (Fig. 3B), while treatment by washing with M9 did not (Fig. 4.4B). The gastropod host also showed a slight association with the nematode microbiome (Table 4.1). From these data, we conclude that nematode species and location play an important role in shaping the native *Phasmarhabditis* microbiome.

Taxonomic composition of the nematode microbiome

The species-level bacterial community composition in the nematodes was analyzed using the unsupervised hierarchical cluster analysis. For this analysis, all the samples were divided into four groups (Fig. 4.5). Cluster IV consisted of *P. papillosa* samples from Southern California and exhibited enrichment with species belonging to genus Acinetobacter or family Pseudomonadaceae. Cluster III consisted of *P. californica* from Central California. Cluster II consisted of a mixture of *P. papillosa* from Southern California and *P. hermaphrodita* from Central California, which were all collected from

the same gastropod host, *D. reticulatum*; in these microbiomes, members of genus Shewanella and family Aeromonadaceae were the dominant microbial members. Cluster I microbial samples were dominated by a high proportion of *Clostridium perfringens*, though these samples were collected from multiple nematodes and gastropod hosts from Central California. Among the most abundant species, *Shewanella* sp. was significantly increased in cluster II compared to the other clusters; samples in cluster I had 48-86% of *C. perfringens*, which was not shown in any other clusters; species from family Pseudomonadaceae and genus Acinetobacter were significantly enriched in cluster IV; while family Aeromonadaceae was evenly distributed in all clusters (Fig. 4.6).

Discussion

The parasitic life cycle of *Phasmarhabditis* is currently understood to begin with IJs entering the gastropod host through an opening on the mantle. Once in the mantle, they form a lesion within the cavity and eventually enter the body or shell region. While inside the host, they mature into adults and reproduce. As maturation and reproduction occurs, fluid accumulates within the shell cavity causing a diagnostic swelling in the mantle. As the nematodes multiply, the host eventually dies within 4-21 days and the nematodes spread to the rest of the body feeding on bacteria and gastropod remains. Similar to the EPN life cycle, once resources in the gastropod host environment near depletion, the juveniles enter an IJ stage and wait or search for a new host (Wilson et al., 1993b; Tan and Grewal, 2001a). While the life history of *Phasmarhabditis* seems to be understood, the mode of action by which *Phasmarhabditis* nematodes kill their host is

not. A role for microbes in *Phasmarhabditis* gastropod killing has been hypothesized, but the range of microbes associated with this nematode has not been well described using culture-independent techniques.

This study serves as the first analysis of the native microbiome of *Phasmarhabditis*. It assessed the microbiome of *Phasmarhabditis* in multiple natural habitats and aimed to help identify core microbiomes of *Phasmarhabditis* utilizing 16S metabarcoding analysis. Previous *Phasmarhabditis* microbiome work had been done using nematodes which had been kept in culture, leaving the possibility of the nematode's microbiome being altered by laboratory conditions (Wilson et al., 1993a, 1995b; Rae et al., 2010; Dirksen et al., 2016; Howe et al., 2020). Only one other study has used next gen sequencing techniques, however the study did not incorporate native *Phasmarhabditis* and instead used those from culture (Howe et al., 2020). By understanding what microbes are naturally and commonly associated with *Phasmarhabditis*, our findings may help to identify potential microbial contributors to gastropod killing, similar to microbial contributors to EPN virulence. The findings may also reveal crucial bacterial species needed for *Phasmarhabditis* food consumption, survival, or host-parasite interactions. Our results suggest that the gastropod host, location, and species may affect the microbial diversity within the tested *Phasmarhabditis*.

Our findings are not entirely congruent with previous *Phasmarhabditis* microbiome work. Similar to previous studies, we identified *Acinetobacter* and *Pseudomonas* spp. occurring on *Phasmarhabditis*, however, no previous studies identified predominant bacteria like *Shewanella*, Aeromonadaceae, or *C. perfringens* which were identified in

this study (Wilson et al., 1995a; Rae et al., 2010; Howe et al., 2020). Pseudomonaceae and *Acinetobacter* species were enriched in some clusters of *Phasmarhabditis* nematodes, specifically cluster IV which consisted of *P. papillosa* (Fig. 4.6). *Acinetobacter* and Pseudomonaceae bacteria are commonly found in the soil and have been discovered in multiple gastropod species (Ducklow et al., 1981; Wilson et al., 1993a; Ekperigin, 2007; Villena et al., 2010; Joynson et al., 2017; Howe et al., 2020; Jackson, 2020). It was previously found that unhealthy *Biomphalaria glabrata* snails had a core microbiome predominantly made up of *Acinetobacter* and *Moraxella* spp., however healthy snails had a microbiome predominantly made up of *Pseudomonas* spp. (Ducklow et al., 1981). It is possible that *Phasmarhabditis* and gastropods thrive with *Pseudomonas* spp., and the presence of other species like *Acinetobacter* or *Moraxella* spp. (which is used in Nemaslug®) in *Phasmarhabditis* cause increased pathogenicity. However, this hypothesis is disputed from a finding that showed that rearing *Phasmarhabditis* on *Acinetobacter* had no effect on its virulence (Nermut et al., 2014). The interesting pattern in which only *P. papillosa* (discovered only in Southern California) have both increased Pseudomonaceae and *Acinetobacter* needs more study. The reasoning for this pattern may be due to the bacterial diversity and population at the geographic location of collection, or a species-specific relationship with *P. papillosa*. However, another possibility is that *Phasmarhabditis* uses some of the predominant bacterial species as a major food source, and others as contributors towards virulence, or perhaps some bacteria are used as both food and a driver for pathogenesis. Since *Acinetobacter* and Pseudomonaceae are frequently found in soils and are not commonly known as highly virulent bacteria, it is

possible that these predominant bacteria are used as a food source rather than a source of causing pathogenicity. This hypothesis is further supported by the observation that *Phasmarhabditis* grew exceptionally well on agar cultured with *P. fluorescens* (isolate 141) or *P. fluorescens* PSG strain compared to other bacterial species. The *Pseudomonas* bacteria was still not selected for use in a commercial setting because it was not associated with the highest mortality rates, suggesting that it may serve a role as food for the nematodes rather than a source of virulence (Wilson et al., 1995b, 1995a).

Shewanella has been discovered in multiple gastropod species where it causes increased pathogenicity, however all studies which identified this were performed in aquatic environments (Cai et al., 2006; Wang et al., 2008). The finding of an association of *Shewanella* with *Phasmarhabditis* has not previously been reported. The bacteria were not detected in any *P. californica* isolates (Fig. 4.5). This may have been due to a limited sample size throughout the study, or due to a random association of bacteria with *Phasmarhabditis*, as hypothesized in 2010 (Rae et al., 2010). The occurrence of this predominant species may be indicative of it being used as a source of virulence towards the gastropod host, however further research is needed to assess this possibility.

Multiple gastropod species have been found associated with *Clostridium* bacteria (Charrier et al., 2006; Li, 2012). However, like *Shewanella*, the species *C. perfringens* had not previously been found in *Phasmarhabditis* or other nematodes. *C. perfringens* is most well known as a causative agent of food poisoning in mammals (Labbe, 1991). The species is frequently searched for and reported in foods for the sake of public health. There are over 1 million cases of poisoning from *C. perfringens* each year (Grass et al.,

2013). A previous study demonstrated that *C. perfringens* enterotoxin could cause intestinal illness of mammals, and potentially fish and frogs (Robertson et al., 2010). However, it is not known how this bacterium affects snails and nematodes. *Phasmarhabditis* nematodes may serve as vectors for *C. perfringens*, using the bacteria as a weapon to kill their gastropod host. However, this seems less likely since it has been found that some gastropods can naturally harbor and vector the bacteria. It was previously thought that *C. perfringens* was only capable of reproducing in mammals and other endothermic organisms, and therefore only these organisms could vector the pathogenic bacteria (Robertson et al., 2010). More recently it was found that ectotherms such as gastropods, frogs, and fish can also vector the bacteria and therefore these organisms should be monitored as sources of contamination (Frick et al., 2018). Our finding of this bacteria furthers the claim that ectotherms, specifically gastropod-associated nematodes, can act as vectors. However, it was only discovered in cluster I which consisted of *P. californica* and *P. hermaphrodita*. It is possible that only *P. californica*, and *P. hermaphrodita* use *C. perfringens* as a source of virulence. However, further study is needed.

The most predominant bacterial family found throughout all *Phasmarhabditis* species was Aeromonadaceae. This family was found in similar abundance across all species and served as the only predominant commonality within the genus. The family has not previously been found within *Phasmarhabditis* and is not common in many nematodes, but it has been discovered within multiple gastropod species (Villena et al., 2010; Li et al., 2019). It is possible that *Phasmarhabditis* largely assumes the microbial diversity of

its gastropod host. However, this hypothesis needs further experimentation. Since Aeromonadaceae is not commonly known to be highly pathogenic to a variety of organisms and it was the most predominant species across all *Phasmarhabditis* nematodes, it can be hypothesized that *Phasmarhabditis* species utilize the bacteria as a major food source rather than a source of virulence. *P. hermaphrodita* and other members of the genus are known to be bacterivorous (Tan and Grewal, 2001b). However, their native food preferences are unknown. *P. hermaphrodita* has been found to grow well when reared on monoxenic cultures of *P. fluorescens*, but this does not prove its preferred bacterial food source in a native setting (Wilson et al., 1995b). Further experimentation may draw out explanations for the clustering of Aeromonadaceae observed in clusters III and IV within *P. californica* and *P. papillosa* (Fig. 4.5).

Future work to assess the microbial diversity of *Phasmarhabditis* needs to utilize next generation sequencing technology and nematodes which have not been maintained in culture for a long period of time. Further microbiome work with the species from this study (*P.* californica, *P. hermaphrodita*, *P. papillosa*) should be done in order to obtain more isolates for statistical power in identifying the microbiome of the species. Further study would also lead to the possibility of work with less discrepancies in read counts as we observed. Study of other *Phasmarhabditis* species microbiome should also be assessed in order to determine other species specific microbial patterns. It is likely that the maintenance of nematodes in culture on specific media can influence the microbiota (Dirksen et al., 2016). It was recently found that *C. elegans* native microbiome differs from the previously described microbiome, and its microbiome community has some

consistencies across time at the genus level but can be influenced by various substrates and present bacteria. Interestingly, one of the consistent genera in *C. elegans* is *Pseudomonas*, which was one of the predominant bacteria we identified which may serve as a major food source for *Phasmarhabditis* (Dirksen et al., 2016; Johnke et al., 2020). To understand the natural relationships and mechanisms between *Phasmarhabditis* and their hosts, native isolates must be utilized. Next generation sequencing technologies allow for rapid sequencing and identification of these isolates and their microbiomes upon collection, allowing for easy assessment of the native microbial flora and their potential interactions.

Figures

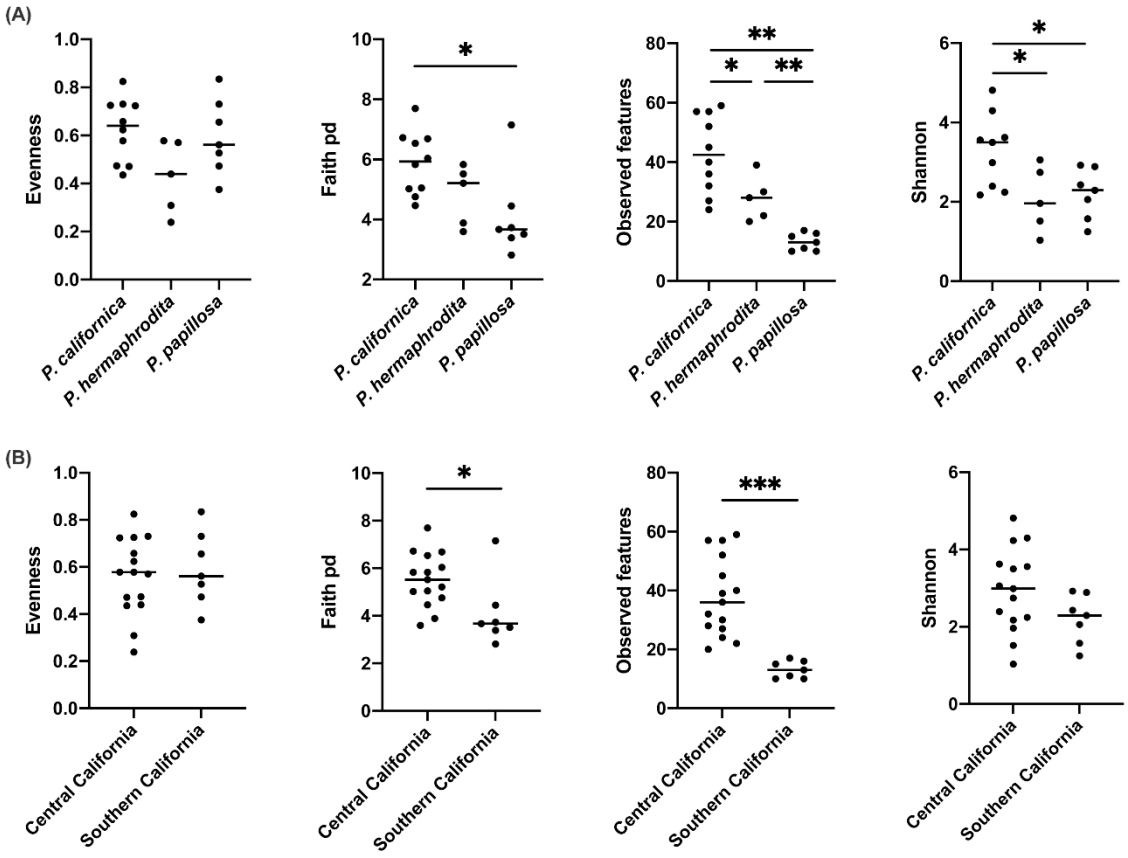

Figure 4.1 The comparison of microbiome alpha diversity of nematode-associated microbial communities. (A) *Phasmarhabditis* species and (B) location affect the richness of the microbial composition in nematode. Kruskal-Wallis test, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

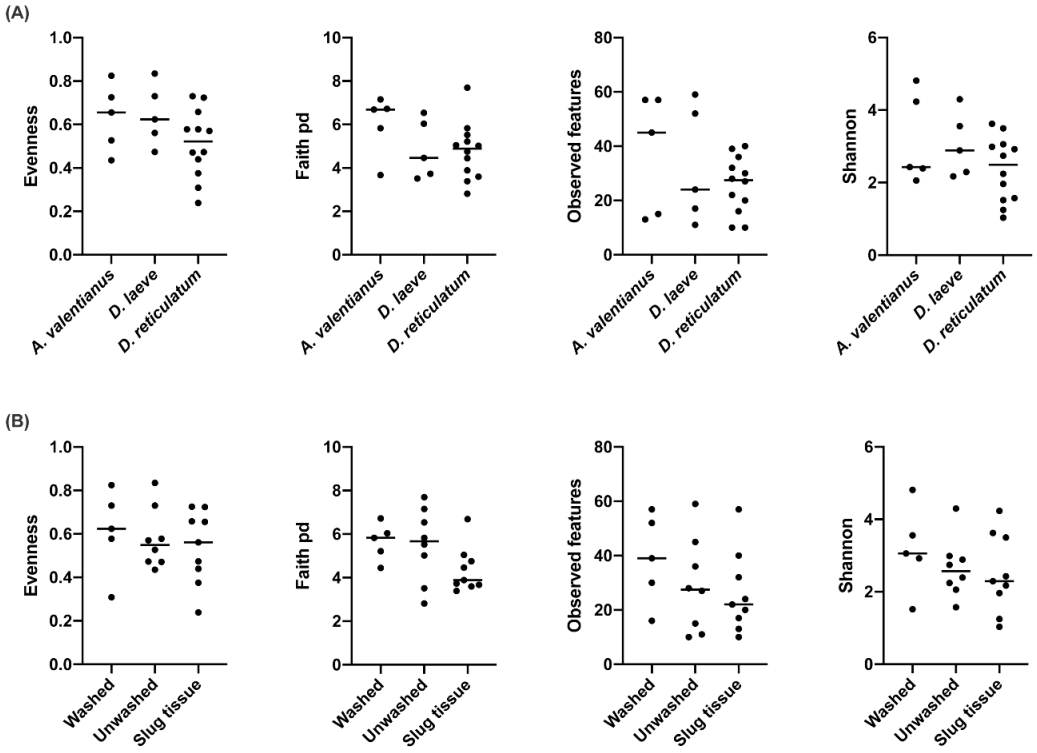

Figure 4.2 Comparison of microbiome alpha diversity across different host species or sample collection strategy. (A) Gastropod host and (B) collection strategy are not associated with differences in the diversity of the *Phasmarhabditis* microbiome.

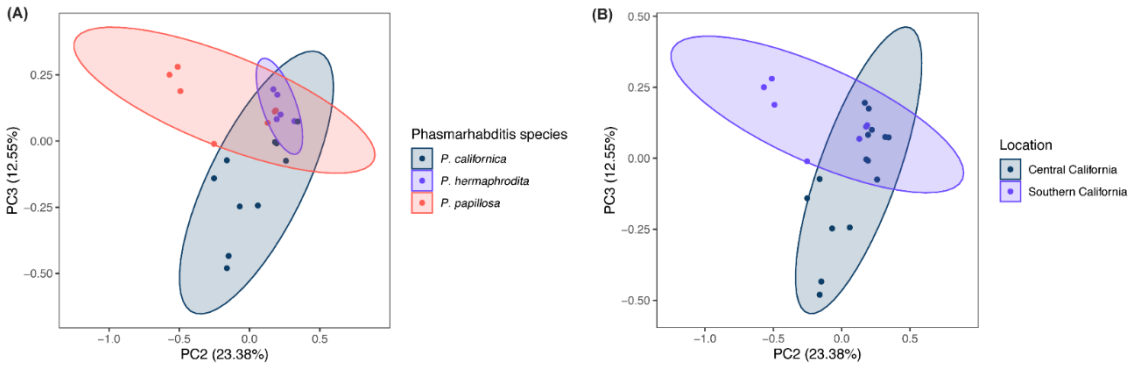

Figure 4.3 Principal coordinate analysis (PCoA) plots of nematode microbiomes based on Bray Curtis distance. PCoA plots showing (A) *Phasmarhabditis* species and (B) location. Percent variance explained is shown in parentheses for each axis. Ellipses show 95% confidence intervals.

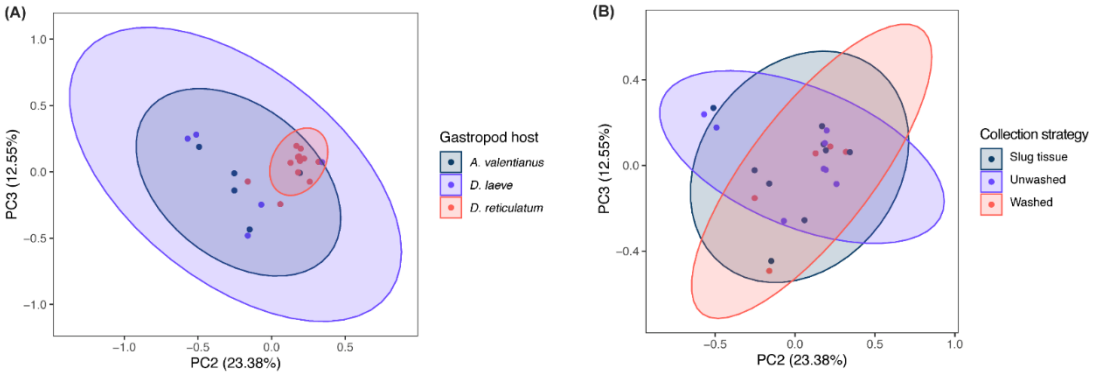

Figure 4.4 PCoA plots of nematode microbiome of different gastropod hosts, based on Bray Curtis distance. PCoA plots with samples clustered by (A) gastropod host and (B) collection strategy; % variance explained shown in parentheses for each axis. Ellipses show 95% confidence intervals.

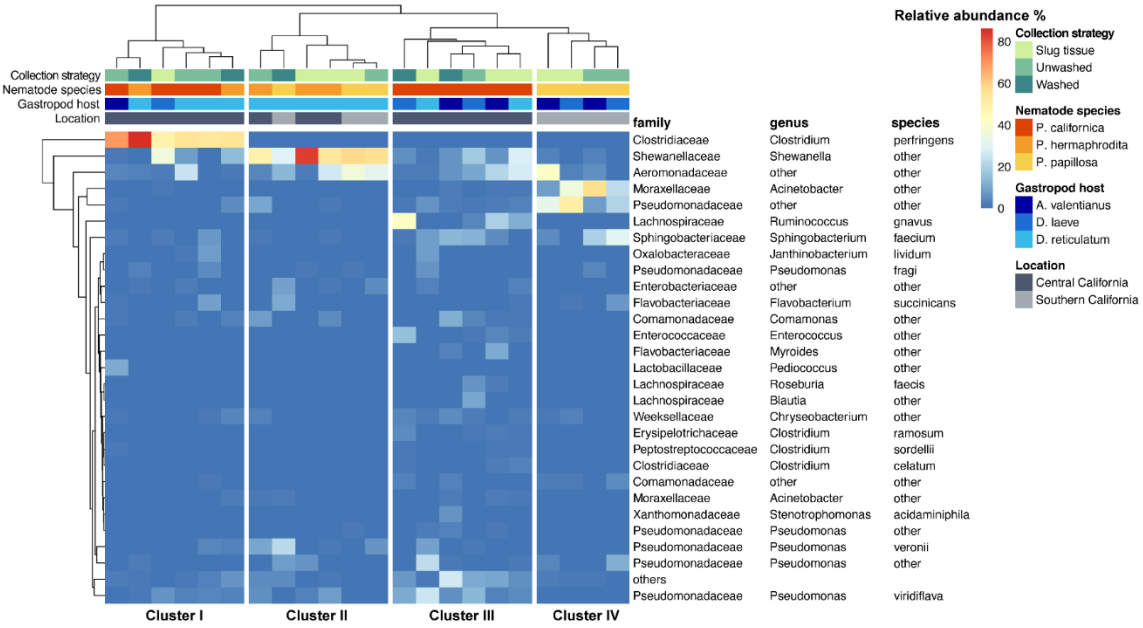

Figure 4.5 Heatmap of the nematode microbiome at species level. Species with relative abundance >5% across all samples are displayed.

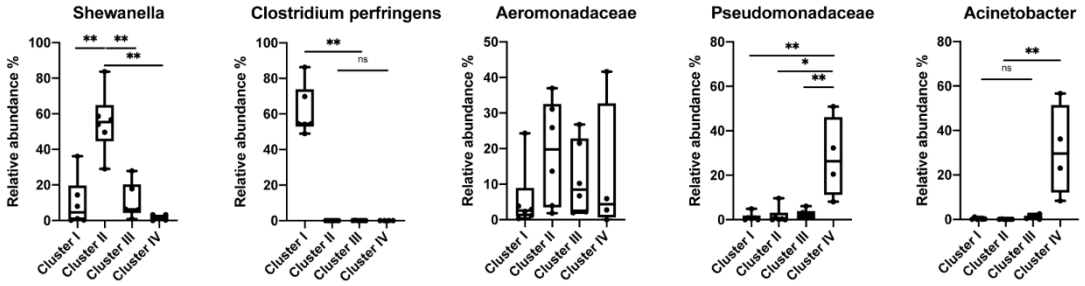

Figure 4.6 Relative abundance of the most abundant species in the *Phasmarhabditis* species. Mann-Whitney U-test, *, $p<0.05$; **, $p<0.01$. Boxplots show inter-quartile range, whiskers minimum to maximum.

Tables

Table 4.1 permANOVA analysis reveals the microbial differences between gastropod hosts, locations, *Phasmarhabditis* species, or washed/unwashed/slug tissue. Bolded values indicate statistical significance.

	Overall p-value	**Group 1**	**Group 2**	**pseudo-F**	**p-value**	**q-value**
Gastropod host	**p=0.017**	*A. valentianus*	*D. laeve*	0.834	0.578	0.578
		A. valentianus	*D. reticulatum*	2.569	**0.02**	0.06
		D. laeve	*D. reticulatum*	2.109	0.056	0.084
Location	**p=0.007**	Central California	Southern California	3.028	**0.007**	**0.007**
***Phasmarhabditis* species**	**p=0.003**	*P. californica*	*P. hermaphrodita*	1.989	0.093	0.093
		P. californica	*P. papillosa*	3.148	**0.005**	**0.015**
		P. hermaphrodita	*P. papillosa*	2.303	0.056	0.084
Collection strategy	p=0.61	Slug tissue	Unwashed	0.861	0.49	0.735
		Slug tissue	Washed	1.249	0.255	0.735
		Unwashed	Washed	0.473	0.877	0.877

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CHAPTER 5

Conclusions and Final Remarks

Phasmarhabditis plays an important role as a biological control agent across multiple European countries (Wilson et al., 1993; Wilson & Rae, 2015). Its use has not been observed in the United States due to agricultural policies of introducing a foreign organism into the country. As seen in Chapter 2, it has now been shown that *Phasmarhabditis* species are spread throughout the state of California in the United States. Further survey work has also shown that the nematodes exist north of the state as well in Oregon (McDonnell, 2018). Three species of *Phasmarhabditis* have been found to exist in California as well as one unidentified species. *P. californica* and *P. hermaphrodita* seem to occupy the northern and central regions of California while *P. papillosa* seems to occupy the southern section of the state. The argument that *Phasmarhabditis* nematodes cannot be used within the United States due to their non-existence within the area is now a moot argument. However, an argument can be made that *Phasmarhabditis* should not be used nationally since they have not been surveyed for or discovered in other areas of the United States. If further surveys are performed across multiple locations spanning throughout the entire United States, *Phasmarhabditis* may have a role to play as a nationally recognized biological control agent against gastropods.

The commercialized European strain of *P. hermaphrodita* (Nemaslug®) is extremely effective at controlling multiple pestiferous gastropods throughout its regions of use (Rae et al., 2007). As seen in chapter 3, locally discovered strains of *Phasmarhabditis* (including *P. hermaphrodita*, *P. californica*, and *P. papillosa*) are also

effective at controlling at least one invasive gastropod pest, *T. pisana*, at both adult and juvenile stages (Schurkman et al., 2022; Tandingan De Ley et al., 2020). The efficacy of the local strains should be tested against various other pestiferous and beneficial gastropods in order to understand the effects the use of *Phasmarhabditis* would have on various gastropod populations and their ecosystems. However, as seen in chapter 3, the local strains seem to be capable of controlling some pestiferous gastropods, and therefore their use as a biological control agent within the United States should be heavily considered.

The previous understanding of *Phasmarhabditis* and its mode of action to kill its gastropod hosts has been a topic of confusion. Some research has hinted towards *Phasmarhabditis* using an EPN like method of killing where it uses mutualistic bacteria to assist in the killing of the host (Tan & Grewal, 2001; Wilson et al., 1995). However, other research suggested that *Phasmarhabditis* does not form mutualistic relationships with very specific species of bacteria like EPNs (Rae et al., 2010). All research performed analyzed the microbiome of gastropods and nematodes which were stored in the laboratory for a period of time. The maintenance of the gastropods and nematodes inside of a laboratory allow time for the microbiome of the organisms to shift depending upon local conditions. Therefore, it is preferable to assess the microbiomes of organisms in their natural or native habitat when investigating these bacterial relationships (Dirksen et al., 2016). In chapter 4, I analyzed the microbiomes of multiple *Phasmarhabditis* species in their native or natural habitat, directly from their gastropod host, across multiple geographic regions. This was done in order to search for any microbial patterns

within the nematodes which may be indicative of important bacterial relationships with *Phasmarhabditis* species. The most commonly occurring bacteria within the *Phasmarhabditis* species identified (*P. hermaphrodita*, *P. californica*, and *P. papillosa*) were *Shewanella*, *Clostridium perfringens*, Aeromonadaceae, Pseudomanodaceae, and *Acinetobacter*. No clear-cut evidence was found to indicate that *Phasmarhabditis* kills its gastropod hosts in an EPN like fashion. However, the research within chapter 4 is limited to only *Phasmarhabditis* within Central and Northern California. More research is needed to fully explore the relationship between *Phasmarhabditis* species and their microbiomes.

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