Potential of endemic entomopathogenic nematodes against emerging insect pests in Hawai‘i

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Abstract

Entomopathogenic nematodes (EPNs) parasitize insects utilizing mutualistic bacteria to infect and kill the host, allowing the nematode to feed and reproduce within the insect cadaver. Consequently EPNs are highly sought after for their biological control potential. A survey for EPNs was conducted on O’ahu and Hawai’i Island using a modified baiting method. One hundred seven soil samples were collected and baited with *Tenebrio molitor* (mealworm) larvae. Forty-seven of the 107 sites contained at least one nematode infected mealworm containing nematodes. Mealworm mortality was attributed to EPNs, fungal contamination, parasitoids or an unknown variable in 16%, 10%, 1% and 73% of samples respectively. Eighty-two EPN isolates were passed through two subsequent inoculations in order to confirm their entomopathogenic nature. A total of 41 EPN isolates were recovered through three rounds of reinoculation and recovery. PCR analysis and sequencing were conducted on third generation nematodes, targeting the ITS region. Sequencing analysis suggested three groups of *Oscheius*. *Oscheius* was recovered from 96% of locations sampled on Hawai’i Island and O’ahu. Four *Oscheius* isolates (BI 1a, BI 12a, OJ 4a, OJ 5b) recovered from the soil survey were tested alongside endemic *Steinernema feltiae* and *Heterorhabditis indica* to evaluate their efficacy against *Tenebrio molitor* larvae at three inoculum levels (3, 9 and 15 nematodes/mealworm). The most effective nematodes as indicated by LT_{50} were: *S. feltiae* at 3 nematodes/mealworm (46.4 hours), *Oscheius* BI 12a at 9 EPN/mealworm (39.7 hours), and *S. feltiae* at 15 nematodes/mealworm (35.2 hours). Three *Oscheius* isolates, *S. feltiae* and *H. indica*, were further evaluated against all stages (larvae, pre-pupae, pupae and adults) of *Hypothenemus hampei*, the coffee berry borer (CBB). CBB
mortality attributed to nematodes was highest with *H. indica* and *S. feltiae* at 17.6% and 16.7% respectively. Coffee berry borer mortality not attributed to EPNs or *Beauveria bassiana* may have resulted from the EPNs symbiotic bacteria physiological or physical causes. This series of experiments suggests that *Oscheius* is a common EPN found in Hawai‘i that displays behavior and levels of biological control similar to established EPNs. The presence of endemic *Oscheius* isolates in Hawaii is a welcomed finding that will add to the sparse list of biological control organisms in Hawaii. The utilization of the prospective endemic EPN *Oscheius* is an excellent option as *Heterorhabditis* and *Steinernema* spp. (aside from *Steinernema carpocapsae*) are currently on Hawaii’s List of Restricted Animals and cannot be imported into the state of Hawaii.
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Entomopathogenic Nematodes

Introduction

The evolution of nematode-insect association consists of three fundamental stages: free-living, phoresy, and necromeny, which branches into parasitism and entomopathogeny (Dillman, 2012). Free-living nematodes are microbiotrophs that transiently interact with insects (Dillman, 2012). Phoretic nematodes utilize insects as vectors for dispersal and/or shelter, but do not have any nutritional association with the insects (Houck and Oconnor, 1991; Kruitbos et al. 2008; Dillman, 2012). Necromenic nematodes are those that have adapted to utilize insect cadavers as a food source, adopting a saprophytic life strategy. Necromenic nematodes do not contribute to the insect’s death (Dillman, 2012). According to Dillman et al. (2012), necromeny subdivides into parasitism and entomopathogeny branches, which encompassed the parasitic mermithid nematodes and the entomopathogenic nematodes Heterorhabditis and Steinernema.

Parasitic nematodes actively infect and feed on a living insect host, causing varying degrees of harm and/or death to the insect (Dillman, 2012). This group of nematodes includes mermithids which are obligate parasites of arthropods, predominantly insects (Poinar, 1975; Poinar, 1990; Kaiser, 1991). Mermithids can be found in either aquatic or terrestrial ecosystems, depending on their insect host (Cuthbert, 1968; Molloy and Jammback, 1975). Mermithids enter, parasitize, and mature within the infected arthropod until the host resources are exhausted. The mermithid then forcefully emerges from the host, generally killing the arthropod host in the process (Nickle, 1972; Poinar
Adult mermithids vary in size from 0.5 mm to 50 cm in length, significantly larger than EPNs (Nickle, 1972).

Entomopathogenic nematodes (EPNs) have adapted and developed an intimate relationship with unique mechanisms to transmit mutualistic insect pathogenic bacteria, which rapidly kill an insect host (Dillman, 2012). Nematodes in Steinernematidae and Heterorhabditidae are EPNs, obligate parasites of insects, and have been extensively studied (Poinar, 1990). Entomopathogeny differs from parasitism in that EPN utilize pathogenic bacteria and bacteria carried by the EPN maybe virulent or avirulent against the insect host. Virulent bacteria allows the nematode to be immediately virulent to the insect host (Dillman, 2012).

The term “entomopathogenic” is frequently used in pathology and parasitology, but has an unreliable definition. Entomopathogenic typically referring to “microorganisms and viruses capable of causing disease in an insect host” (Onstad, 2006; Dillman, 2012). Dillman et al. (2012) revisited and redefined entomopathogenic in regards to nematodes by establishing the following critical characteristics: (i) nematodes have a mutualistic-symbiotic relationship with bacteria, which is involved in the pathogenesis, (ii) the relationship between the nematode and the bacteria might be facultative, although it is maintained over subsequent generations (at least 2–3), and (iii) insect death is rapid, in less than 5 days (Dillman, 2012).

EPNs are found in two families; the Heterorhabditidae (Poinar, 1990) and the Steinernematodae (Travassos, 1927). Poinar (1990) reported that EPNs host range spans nearly all-insect orders and provides a significantly broader range than any other microbial control agent. In vitro test have shown EPNs to be generalists, and lethal to
nearly every insect tested, making them ideal candidates for biocontrol (Woodring and Kaya, 1988; Kaya et al., 1993; Kaya, 2000). More than 70 species of *Steinernema* and 20 species of *Heterorhabditis* are currently recognized (Stock, 2015). EPNs are cosmopolitan in their distribution and found in a variety of climates, soils and temperatures globally (Hara et al., 1991; Kaya et al., 1993). Survival is primarily limited by the availability of susceptible hosts and abiotic factors, including soil texture, overabundant or insufficient moisture, extreme temperatures, and exposure to ultraviolet radiation (Kaya et al., 2000).

The life cycles of Heterorhabditae and Steinernematidae have been studied extensively and are similar. Both families emerge in the soil as third stage infective juveniles (IJs) (Poinar, 1990). IJs are morphologically adapted to remain in the environment for prolonged periods of time, possessing features like a double cuticle, an increased number of mitochondria, and mouthparts that reflect their non-eating behavior, while waiting for a host insect (Poinar, 1990). Heterorhabditae and Steinernematidae also share similarities in infectivity time, length of life cycles and the presence of an entomopathogenic bacteria (Hazir, 2003; Grewal 1999).

EPNs wait in the soil for an insect host, responding to physical and chemical stimuli when they detect insects (Poinar, 1990; Kaya et al., 1993). IJs typically employ one of two foraging strategies when seeking out an insect host: ambush or cruise (Grewal et al. 1994; Lacey and Kaya, 2000; Gaugler, 2002). Ambusher EPNs utilize an energy-conserving approach, either standing on tail or lying in wait for an insect to pass, i.e. *Steinernema carpocapsae* and *S. scapterisci* (Lacey and Kaya, 2000; Gaugler, 2002). Cruiser EPNs actively seek out a host, i.e. *S. glaseri* (Lacey and Kaya, 2000; Gaugler,
Once a suitable host is found, *Steinernema* IJ exploit the insects natural openings (mouth, anus or spiracles) to gain entry, while *Heterorhabditae* IJ can also utilize a dorsal tooth/hook to break the outer cuticle of an insect and enter (Poinar, 1990). Cruiser EPNs have been found to be better in controlling insect pests than ambusher EPNs as they actively seek out a host (Gaugler, *et al.*, 1997).

Under ideal conditions, a single EPN is capable of killing an individual insect pest. However, ideal circumstances depend on the interaction between the nematode species, bacteria, and the insect host (Belair *et al.*, 2003; Campos-Herra and Gutierrez, 2009), host habitat (Gaugler and Kaya, 1990), and the time of EPN infection (Haukeland and Lola-Luz, 2010).

*Heterorhabditis* and *Steinernematids* differ from non-EPNs in that they have evolved a symbiotic association with bacteria in the genera *Photorhabdus* and *Xenorhabdus*, respectively (Poinar, 1990; Akhurst and Boemare, 2000). The EPN protects its bacteria from environmental elements (sunlight, soil and water) and host defense mechanisms (Poinar, 1990). Once the IJ enters the host’s hemocoel, the symbiotic bacterium is released (Poinar, 1990; Kaya *et al.*, 1993). The bacteria feed on the hemocel fluids, multiplying, and secreting toxins which kill the insect host within 48 hours of infection (Poinar, 1990; Kaya *et al.*, 1993). IJs consume the bacteria and insect's deteriorating tissue (Selvan *et al.*, 1993; Fenton *et al.*, 2000). The bacteria and developing nematodes feed and reproduce within the insect cadaver for several generations (Poinar, 1990; Akhurst *et al.*, 2000). Once the food source has been exhausted, new IJs are produced and exit the insect cadaver to seek out a new insect host (Selvan *et al.*, 1993; Grewal and Georgis, 1999).
In Rhabditidae, nematodes within the genus *Oscheius* can also be characterized as insect-parasitic. *Oscheius* has not been described as an EPN but as a nematode that parasitizes insects and has great potential as a biological control agent (Godfrey *et al*., 2005; Dillman, 2012). Within *Oscheius*, nematodes are categorized into the groups; *Insectivora* or *Dolichura*, comprised of 14 species and 13 species respectively (Sudhaus, 1976). Nematodes in the genus *Oscheius* can act as entomopathogens (Zhang *et al*., 2008; Ye *et al*., 2010; Torres-Barragan *et al*., 2011), scavengers (Sudhaus, 1993), and kleptoparasites of cadavers already occupied by EPNs (Campos-Herra *et al*., 2015b). *Oscheius* has been observed co-infecting insect cadavers and coexisting with EPNs (Campos-Herra, 2015a; Campos-Herra *et al*., 2015b). This interaction can be expected in nature as *Oscheius* can be facultative scavengers and/or kleptoparasites (Campos-Herra *et al*., 2015b).

*Oscheius chongmingensis*, *O. carolinensis* and *Caenorhabditis briggsae* have been identified as potential new insect pathogens (Zhang, 2008; Abebe *et al*., 2010; Ye *et al*., 2010; Torres-Barragan, 2011). *Oscheius chongmingensis* and *C. briggsae* are associated with the insect pathogenic bacteria genus *Serratia*, whereas *O. carolinensis* likely has additional insect pathogenic bacterial associations (Zhang *et al*., 2008; Abebe *et al*., 2010; Ye *et al*., 2010). Under the updated entomopathogenic criteria (Dillman *et al*., 2012), *O. chongmingensis* and *O. carolinensis* have been reclassified as true entomopathogens (Zhang *et al*., 2008; Ye *et al*., 2010; Torres-Barragan *et al*., 2011; Dillman *et al*., 2012).

*Heterorhabditidoides* was described as a new genus of EPN by Zheng *et al*. (2008), however clear morphological similarity between *Heterorhabditidoides* and
Oscheius was reported and the two genera were unified, resulting in the reclassification of *H. chongmingensis* into *Oscheius* (Ye et al. 2010; Liu et al. 2012). Given the recent findings and advances with nematodes in the genus *Oscheius*, particularly the reclassification of *O. chongmingensis* and *O. carolinensis* as EPNs, it is critical that *Oscheius* spp. is explored in depth to determine its host range, infectivity and efficacy potential on hosts along with its potential as a biocontrol agent.

**Objectives**

The objectives of the proposed research are:

1) To identify and confirm the presence of endemic EPNs on Hawai’i and O’ahu Islands, and

2) To evaluate the biological control capabilities of endemic EPNs.

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Entomopathogenic Nematodes of Hawai‘i

Abstract

Entomopathogenic nematodes (EPNs) parasitize insects utilizing mutualistic bacteria to infect and kill the host, allowing the nematode to feed and reproduce within the insect cadaver. Consequently EPNs are highly sought after for their biological control potential. A survey for EPNs was conducted on O‘ahu and Hawai‘i Island using a modified baiting method. One hundred seven soil samples were collected and baited with Tenebrio molitor (mealworm) larvae. Soil samples were observed daily for 5 days and morbid T. molitor larvae were placed on white traps. Forty-seven of the 107 sites contained at least one mealworm containing nematodes. Mealworm mortality was attributed to EPNs, fungal contamination, parasitoids or an unknown variable in 16%, 10%, 1% and 73% of samples respectively. Eighty-two EPN isolates were passed through two subsequent inoculations in order to confirm their entomopathogenic nature. A total of 41 EPN isolates were recovered through three rounds of reinoculation and recovery. PCR analysis and sequencing was conducted on third generation nematodes, targeting the ITS region. Sequencing analysis suggested three groups of Oscheius. Oscheius was recovered from 96% of locations sampled on Hawai‘i Island and O‘ahu. The Oscheius isolates and an isolate preliminarily identified as Heterocephalobellus sp. occurred in 76%, 12%, 8% and 4% of positive locations respectively. This survey suggests that Oscheius is a common EPN in Hawai‘i.

Introduction

Hara et al. (1991) surveyed six of the seven main Hawaiian Islands (Kaua‘i, O‘ahu, Maui, Molokai, Lanai and Hawai‘i Island) in 1991 to document the presence of
entomopathogenic nematodes. *Heterorhabditis* was found on five of the six islands surveyed (exception of Lanai) and *Steinernema* spp. was only recovered on Maui. Of the 361 soil samples collected by Hara *et al.* (1991), 24 (6.8%) were positive for EPNs, 22 (6.3%) were *Heterorhabditis* sp. and two (0.5%) were positive for *Steinernema* sp. (Hara *et al.*, 1991). *Heterorhabditis* sp. was recovered exclusively from coastal regions, located 150 m or closer to the shoreline. *Steinernema* spp. isolates were collected more inland, between 300 and 900 m from shore (Hara *et al.*, 1991). These findings are consistent with EPN surveys conducted in California, Indonesia and the Azores, where *Heterorhabditis* sp. were collected within 150 m of the ocean and *Steinernema* spp. were collected from inland locations greater than 300 m from the ocean (Stock *et al.*, 1999; Griffin *et al.*, 2000; Rosa *et al.*, 2000).

In 2014, *H. indica* was recovered from Kaua’i, O’ahu, Molokai, Maui and Hawai’i Island, and an undiscovered *Heterorhabditis* species was recovered from all islands except Molokai (Myers *et al.*, 2015). *Heterorhabditis* was recovered within 100 m of the ocean shore, with 187 of 275 samples collected yielding morbid larva, and 21% of the morbid larva containing EPNs (Myers *et al.*, 2015).

*Heterorhabditis* and *Steinernema* spp. (aside from *S. carpocapsae*) are currently on Hawaii’s List of Restricted Animals (Part A) (HDOA, 2006) and cannot be imported without a permit. Documenting the existence of native EPN populations would facilitate permitting and ultimate application of EPN for insect control. Hara *et al.* (1991), and Myers *et al.* (2015), found *Heterorhabditis* on the five major Hawaiian Islands, however *Steinernema* has not been characterized outside of two isolated locations on Maui in 1991 (Hara *et al.*, 1991; Myers *et al.*, 2015). If native populations are found, *Steinernema,*
Heterorhabdits and Oscheius may be removed from the List of Restricted Microorganisms as Beauveria bassiana was in 2012, after native Hawaiian strains were confirmed (Matsumoto, personal communication). The confirmation of native EPN populations in Hawai’i will provide more options for nematode-based biological control, implementation with Integrated Pest Management (IPM) and management of invasive insect pests, such as Hypothenemus hampei (coffee berry borer) and Oryctes rhinoceros (coconut rhinoceros beetle).

**Materials and Methods**

Survey. Soil samples were collected on the islands of O’ahu and Hawai’i Island from inland regions. Collection sites were located under vegetation greater than 150 m from the shoreline (Table 1.1). Each sample site was catalogued by recording the location, vegetation, and general soil type. A 500 cm³ soil sample was collected with a garden spade to a depth of 5–30 cm. The soil was placed into plastic bags (15 cm x 23 cm, 2 mil), labeled, and stored in an insulated cooler. Soil order was determined by overlaying the collection locations with the Hawai’i Soil Atlas (CTHAR, 2015). Soil was screened (1-cm mesh) to remove rocks, debris and establish a uniform texture. A 50 cm³ subsample of soil was placed into a petri dish (90 mm x 20 mm), lined with Whatman no. 1 (90 mm) filter paper on the top and bottom and saturated with 3-5 ml water to activate the nematodes (Mohammed *et al.* 2012). Soil samples were baited with late stage Tenebrio molitor larvae and held at 25°C in the dark. After 2 days and continuing daily for 1 week, dead *T. molitor* were removed and sterilized in 10% NaOCl for 30 seconds, rinsed three times in distilled water for 30 seconds. An individual morbid *T. molitor* was placed onto a modified White trap (Kaya and Stock, 1997) for nematode collection.
White traps were monitored using a Leica WILD MZ8 observing microscope for presence of nematodes and a Leica DMIR B inverted microscope for morphological identification. Any white trap with nematodes emerging was re-inoculated onto *T. molitor* to confirm death was a result of EPNs, not bacteria, fungus or other means. Confirmation of EPN status was conducted by pipetting 1 ml of the putative EPN (about 1000 IJ) onto the lid and base of a filter paper-lined petri dish (60 mm x 15 mm) containing two *T. molitor* larvae. After 2 days, dead *T. molitor* were removed and sterilized as described and transferred to white traps. IJ emerging from the white traps were counted, adjusted to 1000 IJ/ml and stored in water at 15°C in darkness. Nematode cultures were maintained through re-inoculation of *T. molitor* larvae.

**Identification.** Identification of the nematodes collected from the mealworms was conducted through sequencing and visual observation. PCR was performed on single IJ specimens using general nematode primers and sequenced to determine nematode species. Nematodes were tentatively identified using morphological characteristics under an observing microscope. An individual nematode was placed in a 10 µl drop of water on a clean microscope slide. The nematode was cut using a Roboz surgical micro 45° angle tool (model number RS-9421-06). The water drop containing the nematode was pipetted into a 50 µl tube and prepared for PCR in 35 µl reactions (Cabos *et al.*, 2013). Primers ITS-F (5’-TGTAGGGAACCTGCTGCTGGATC-3’) and ITS-R (5’-CCTATTTAGTTTCTTTTCCCTCCGC-3’) (Saeki *et al.*, 2003) were used to amplify the ITS region. The PCR conditions were 95°C for 2 minutes, 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, followed by 5 minutes at 72°C. A phiX174 DNA/HaeIII marker and 10 µl of each PCR product were separated on a 1.2%
agarose gel, stained with GelRed in 1xTAE, and visualized under UV light. For samples with visible bands, the remaining 25 µL of the PCR product was purified and sequenced by Eurofins MWG Operon (Huntsville, AL).

Forward and reverse sequences were assembled using Genious (Biomatters Auckland, New Zealand) and trimmed to only include sequences from both DNA fragments. A contingent sequence was created using the DNA sequence assembly program Cap3 (NCBI). The contingent sequences were compared with sequences from the National Center for Biotechnology Information (NCBI) and the closest match from the Genbank catalogue was used for identification purposes alongside morphological characteristics. Nematode DNA from each population was amplified, sequenced and assessed a minimum of two times.

**Results**

*Survey.* Soil samples were collected from 107 sites on O’ahu and Hawai’i Island (Figs. 1.1 and 1.2). Morbid *T. molitor* larvae containing EPNs were retrieved from 77% of the 107 sites (82 positive samples). The 82 samples containing EPNs were reinoculated onto *T. molitor*. From the second inoculation, morbid larvae containing EPNs were recovered from 63% of samples, resulting in 52 positive samples. The third round of inoculation yielded morbid *T. molitor* larvae containing EPNs in 79% of samples. These 41 samples represented 38% of the original 107 sites sampled positive for EPNs.

Additionally, 8.4% of all cultures experienced fungal contamination, and < 1% of dead larvae were killed by parasitoids. Koch’s postulate was satisfied by extracting EPNs from infected *T. molitor* cadavers obtained from collected soil samples,
establishing pure cultures through multiple generations of re-infecting healthy *T. molitor* larvae and the ability to reisolate the same EPNs from infected insect cadavers.

Color change of *T. molitor* larvae occurred as a dark brown/black, however color change tended to be patchy (Fig. 1.3) not always spreading consistently throughout the cadaver. *T. molitor* larvae typically turn black and dry out upon naturally induced death, however, when infected cadavers are cut open, they are typically moist, retained their original shape and are full of lipids which are induced by the EPNs mutualistic bacteria.

Positive sites on O’ahu were primarily on the north and western portion of the island, following major highways and roadways (Fig. 1.2). Positive sites on Hawai’i Island were concentrated on the east side, migrating out from Hilo, also following major roadways (Fig. 1.1). The prevalent soil order was Oxisol on O’ahu and Andisol on Hawai’i Island.

The predominant habitat where positive *Oscheius* was observed was agricultural land on both O’ahu and Hawai’i Island (Table 1.1). *Oscheius* isolates were collected under a wide range of trees, the most common being Ohia (*Ohia lehua*) on Hawai’i Island and Kiawe (*Prosopis pallida*) on O’ahu. It is possible that *Oscheius* is located at a lower soil depth than other samples were taken at or it could have hitchhiked on machinery used for excavation since it was not observed in other samples. Another possibility is that these dead hosts could be found at lower soil depths. In both O’ahu and Hawai’i Island, distribution of *Oscheius* isolates indicates a branching pattern from the major cities, following primary roadways and highways.
Figure 1.1 Locations from which soil was collected on Hawai‘i Island. Each site that is positive for EPN is represented by a dark square.
Figure 1.2 Locations from which soil was collected on O’ahu Island. Each site that is positive for EPN is represented by a dark square.
Figure 1.3 Color changes of *Tenebrio molitor* larvae induced by *Oscheius*. Death by natural causes, no color change (A), slight color change induced by *Oscheius* infection (B), partial color change induced by *Oscheius* (C, D), complete color change induced by *Oscheius* (E)

*Identification.* Sequencing of samples revealed three different species of *Oscheius*, and one isolate whose closest match is *Heterocephalobellus* sp. (Table 1.1). The *Oscheius* isolates and an isolate preliminarily identified as *Heterocephalobellus* sp. occurred 76%, 12%, 8% and 4% of positive locations respectively. All *Oscheius* species had a sequence identity of 99% when compared to the GenBank catalogue. *Oscheius* isolates were recovered from 20% and 36% of samples on Hawai‘i Island and O‘ahu Island respectively. *Oscheius* sp. was recovered once. One *Oscheius* isolate was recovered twice, in both cases in recently excavated areas. Big Island isolate BI 29a did not yield a high sequence identity or query coverage match to any EPNs. One of three sequences yielded a match with 82% sequence identity and 51% query coverage to *Heterocephalobellus* sp. The next closest matches were to three variations of “uncultured organism clone *ciidir*”, with an 88% sequence identity and 33% query cover. For this reason, BI 29a is preliminarily being identified as *Oscheius* sp. Future research will be required to confirm the identity of this nematode, this will require molecular cloning and resequencing of the nematodes DNA.
<table>
<thead>
<tr>
<th>Sample Location</th>
<th>Reference number</th>
<th>Order</th>
<th>Habitat</th>
<th>Nominal Oxicast</th>
<th>Sample for isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA ARS Park, Hilo</td>
<td>KF938879</td>
<td>Roadside</td>
<td>Oxicast</td>
<td>Oxicast</td>
<td>Camp Timberline, Maui</td>
</tr>
<tr>
<td>Lava Tree State Park, Kona</td>
<td>KF938879</td>
<td>Residential</td>
<td>Oxicast</td>
<td>Oxicast</td>
<td>Mauna Kea, Hilo</td>
</tr>
<tr>
<td>Pauoa, Hilo</td>
<td>KF938879</td>
<td>Residential</td>
<td>Oxicast</td>
<td>Oxicast</td>
<td>Mountain View, Maui</td>
</tr>
<tr>
<td>Devedo Park, Hilo</td>
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<td>Residential</td>
<td>Oxicast</td>
<td>Oxicast</td>
<td>Hilo</td>
</tr>
<tr>
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<td>Roadside</td>
<td>Oxicast</td>
<td>Oxicast</td>
<td>Ke'e, Pupukea</td>
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</tbody>
</table>

Table 1: Sample Location, Habitat, Soil order, nominal oxicast, and reference numbers for isolates for positive locations containing endomycorrhizal.
<table>
<thead>
<tr>
<th>Reference number</th>
<th>Name</th>
<th>Order</th>
<th>Habit</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1938579</td>
<td>Ochius Oxisol</td>
<td>Agricultural Park</td>
<td>O1 26a</td>
<td>Waimanalo Experimental Station, Wainialu Ridgeview Estates, Center</td>
</tr>
<tr>
<td>K1938579</td>
<td>Ochius Oxisol</td>
<td>Agricultural Roadside</td>
<td>O1 22b</td>
<td>Hekia Koa Pier, Ko Olinao, Hekia, North Shore</td>
</tr>
<tr>
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<td>Ochius Oxisol</td>
<td>Agricultural Roadside</td>
<td>O1 19a</td>
<td>Punalu′u Central, Whitemore, Waialua</td>
</tr>
<tr>
<td>K1938579</td>
<td>Ochius Oxisol</td>
<td>Fossil</td>
<td>O1 11b</td>
<td>Laniakea, Waialua</td>
</tr>
<tr>
<td>K1938579</td>
<td>Ochius Oxisol</td>
<td>Lithofossil</td>
<td>O1 10a</td>
<td>Waialua, Waialua</td>
</tr>
<tr>
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<td>Ochius Oxisol</td>
<td>Agricultural Mollic</td>
<td>O9 9b</td>
<td>Makaha Towers, Waianae</td>
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<tr>
<td>K1938579</td>
<td>Ochius Oxisol</td>
<td>Agricultural Mollic</td>
<td>O9 7b</td>
<td>Waiheʻe Rd, Waianae</td>
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<td>Agricultural Mollic</td>
<td>O9 6b</td>
<td>Hauula Rd, Waianae</td>
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<tr>
<td>L19611142</td>
<td>Ochius Oxisol</td>
<td>Park</td>
<td>O9 5b</td>
<td>Laniakea Ranch, Waianae</td>
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<tr>
<td>L19613267</td>
<td>Ochius Oxisol</td>
<td>Park</td>
<td>O9 4b</td>
<td>Makaha Community Park, Ewa</td>
</tr>
</tbody>
</table>

Table 1. Sample location, habitat, soil order, nematode and reference numbers for isolates for positive locations containing nematophagetic nematodes Ochius.
Discussion

*Oscheius* was isolated from inland areas on O’ahu and Hawai’i Island (Fig. 1.1 and 1.2). No *Steinernema* or *Heterorhabditis* were recovered.

These EPN discovery rates are higher than those found in previous EPN surveys (Hara *et al*. 1991; Myers *et al*. 2015). Myers *et al*. (2015) also recovered *Oscheius* spp. during the survey of Hawai’i, however *H. indica* was the most prevalent species collected. Differences in discovery rates could be attributed to substituting *T. molitor* larvae in place of *Galleria mellonella* larvae and use of a modified soil extraction technique that incorporated significantly more water and less soil. The combination of these three factors could potentially favor *Oscheius* over *Steinernema* and/or *Heterorhabditis* in the EPN baiting process.

Surveys for *Oscheius* have been conducted elsewhere around the world, including China, Columbia, India, and Italy. Several *Oscheius* species have been recovered including *O. chongmingensis*, *O. columbiana*, *O. nadarajani* and *O. onirici* (Stock *et al*. 2005; Zhang *et al*. 2008; Ali *et al*. 2011; Torrini *et al*, 2015). Zhang *et al*. (2008) discovered *O. chongmingensis* and found the 18S and ITS rDNA regions to be closely related to nematodes in the Rhabditidae family. Molecular data combined with morphological data confirmed and established *O. chongmingensis* to be the first discovered EPN in the Rhabditidae family. This is the first time an EPN did not belong to the two core families of Heterorhabditidae and Steinernematidae.

*Oscheius tipulae* is one of the most common nematode species found in non-desert regions of the world (Félix *et al*. 2006). *Oscheius* was first discovered and recorded in Hawai’i by Félix *et al*. (1999), as *Oscheius* strains JU134, JU136 and JU142.
As *Oscheius* is known to be cosmopolitan in its distribution, it is highly likely that it was introduced into Hawai‘i, unbeknownst in soil, plants and ship ballast. This hypothesis is in accordance with the theory proposed by Hara *et al.* (1991) that *Heterorhabditis* was likely introduced into Hawai‘i in the ballasts of trade ships. In addition, Poinar (1990) advocated that the importation of potted plants, introduction of unintentional nematodes by growers and the dumping of ballasts from early sailing ships as primary reasons for the presence of *S. carpocapsae* and *S. feltiae* in Australia and New Zealand. Pimentel *et al.* (2005) speculated that 95% of invasive species introduced into the United States are attributed to accidental human introductions, many imported with plants or in soil, or ballast carried on ships.

The distribution of *Oscheius* can be attributed to human movement of plants, soils, agricultural products (Poinar 1990; Hara 1991; Pimentel *et al.* 2005) and potentially phoretic distribution (Eng *et al.* 2005). Nematodes have been known to utilize insects, arthropods and other organisms as vectors for dispersal and/or shelter (Houck and Oconner, 1991; Kruitbos *et al.* 2008). By utilizing host or non-host organisms, EPNs are able to either infect the insect host, or survive long enough to allow the infective juvenile nematodes to emerge somewhere other than the site of initial infection and spread (Downes and Griffin, 1996; Eng *et al.* 2005). *Steinernema glaseri* (Lacey *et al.* 1995), *S. feltiae* (Epsky *et al.* 1988; Gouge and Hague, 1995), and *H. marelatus* are a few species of EPN that utilize phoracy for distribution (Eng *et al.* 2005).

Little is known about the host range and virulence of endemic *Oscheius* isolates. Cockroaches, pill bugs, centipedes, millipedes and landhoppers were occasionally observed in soil collected at or near the sampling areas. The primary hosts of these
endemic *Oscheius* strains are unknown, however *Oscheius* is known to exhibit scavenging (Sudhaus, 1993) and potentially kleptoparasitic (Campos-Herra *et al.*, 2015) behavior in addition to their entomopathogenic nature (Zhang *et al.*, 2008; Ye *et al.*, 2010; Torres-Barragan *et al.*, 2011).

Color change of an insect cadaver is commonly attributed to the EPNs insect pathogenic bacteria taking effect, killing the host. For example, the bacterium associated with *Herorhabditis, Photorhabdus*, induces a brick red color change whereas the bacterium associated with *Steinernema, Xenorhabdus*, produces a pale orange to brown color change in the insect cadaver (Morris 1985; Samish and Glazer, 1992; Vasconcelos *et al*. 2004). *Oscheius chongmingensis* induces a red color change in infected insect cadavers (Zhang *et al*. 2008).

In some *Oscheius* isolates, nematodes emerging from the insect cadavers were mixed stages (juvenile, dauer infective juveniles and adults). Dillman has reported similar findings, and has molecularly identified these mixed stages as being all the same *Oscheius* species (Dillman, personal communication). Dillman theorizes that this is likely due to *Oscheius* not completely utilizing all resources within the cadaver prior to emerging. Similar behavior has been observed by necromantic nematodes such as *Pristionchus* (Waller *et al*. 2010). *Oscheius* is known to exhibit necromantic behavior. Necomeny is widely considered to be the evolutionary transition stage between parasitism and entomopathogeny (Dillman *et al*. 2012). This could explain why some members of the *Oscheius* genus are considered to be true entomopathogens while the status of others are still being evaluated.
The transition from necromeny into entomopathogenicity may evolve at different rates, even within populations of the same species (Campos-Herera et al. 2015). Habitat, climate, resource availability, and other key environmental factors induce changes in natural selection and genetic variation between individuals, or within a population, likely influencing this evolutionary transformation from necromeny into entomopathogenicity. Isolates from secluded areas with divergent natural histories could behave as necromenics, while neighboring populations are exhibiting the premature stages of entomopathogenicity (Campos-Herera et al. 2015).

The presence of endemic Oscheius isolates in Hawaii is a welcomed finding that will add to the sparse list of biological control organisms in Hawaii. The utilization of the prospective endemic EPN Oscheius is an excellent option as the EPNs Heterorhabditis and Steinernema spp. (aside from Steinernema carpocapsae) are currently on Hawaii's List of Restricted Animals (Part A) and cannot be imported into the state (HDOA, 2006). The identification and classification of new endemic EPNs, such as Oscheius, would expedite the process of approving EPN based biocontrol agents by demonstrating their existing prevalence.

References


major heterochronic changes relative to *Caenorhabditis elegans*. Proceedings of the Royal Society of London B: Biological Sciences. 266(1429):1617-1621.


Infectivity Potential of Entomopathogenic Nematodes of Hawai’i

Abstract

The efficacies of six endemic nematodes, consisting of four *Oscheius* isolates, *Steinernema feltiae* and *Heterorhabditis indica*, were evaluated against *Tenebrio molitor* larvae at three inoculum levels (3, 9 and 15 nematodes/mealworm). The most effective nematodes as indicated by LT$_{50}$ were: *S. feltiae* at 3 nematodes/mealworm (46.4 hours), *Oscheius* BI 12a at 9 EPN/mealworm (39.7 hours), and *S. feltiae* at 15 nematodes/mealworm (35.2 hours). At 3 EPN/mealworm *S. feltiae* (46.4 hours) was followed by *Oscheius* isolates OJ 4a, BI 1a, OJ 5b and BI 12a and *H. indica* (63.5 hours). At 9 EPN/mealworm *Oscheius* BI 12a (39.7 hours) was followed by *S. feltiae*, *H. indica* and *Oscheius* isolates OJ 4a, BI 1a and OJ 5b (95.0 hours). At 15 EPN/mealworm *S. feltiae* (35.2 hours) was followed by *Oscheius* isolates OJ 4a and BI 1a, *H. indica* and *Oscheius* isolates BI 12a and OJ 5b (71.7 hours). Three *Oscheius* isolates, *S. feltiae* and *H. indica*, were further evaluated against all stages (larvae, pre-pupae, pupae and adults) of *Hypothenemus hampei*, the coffee berry borer (CBB). CBB mortality attributed to nematodes was highest with *H. indica* and *S. feltiae* at 17.6% and 16.7% respectively. CBB mortality associated with *Beauveria bassiana* ranged from 4.5% to 26.7%. Coffee berry borer mortality not attributed to EPNs or *B. bassiana* may have resulted from the EPNs symbiotic bacteria physiological or physical causes.

Introduction

Entomopathogenic nematodes (EPNs) have a wide host range which spans nearly all-insect orders, and provides a significantly broader range than many other microbial control agents (Poinar, 1990). *In vitro* test have shown EPNs to be generalists, lethal to
nearly every insect tested (Kaya et al., 2000). Under ideal conditions, a single EPN is capable of killing an individual insect host. However, ideal circumstances depend upon the interaction between the nematode species and the insect host (Belair et al., 2003; Campos-Herra and Gutierrez, 2009), host immunity (Gaugler et al., 2002), host habitat (Gaugler and Kaya, 1990), and the time of EPN infection (Haukeland and Lola-Luz, 2010). The infectivity of different species of EPN varies with different insect pests. With *Steinernema* spp., insect susceptibility varied greatly depending on the species tested (Belair et al., 2003). *Steinernema feltiae* has a highly variable LC₅₀, ranging from 0.27 to 99.61 IJ/cm², when tested against *Leptinotarsa decemlineata*, *Spodoptera littoralis* and *Trichoplusia ni Hübner* hosts (Campos-Herrera and Gutierrez, 2009). Generally EPNs are able to infect and kill their insect host within 48 hours of infection (Poinar, 1990; Kaya and Gaugler, 1993).

In Hawai‘i, commercial strains (introduced from the continental United States) of *S. carpocapsae* were evaluated for their biocontrol applicability under *in vitro* conditions against tropical insect pests of Hawai‘i. *Steinernema carpocapsae* gave promising results against *Liriomyza trifolii* (agromyzid leafminer), *Cylas formicarius elegantulus* (sweet potato weevil), and *Chrysodeixis chalcites* (green garden looper) (Hara et al., 1989). In a separate *in vitro* test (Ebssa et al., 2001), *S. feltiae*, *S. carpocapsae* and *H. bacteriophora* strains were tested against *Frankliniella occidentalis* (western flower thrips). The most virulent EPN strains yielded high levels of mortality against late second instar larvae and pre-pupae in high moisture environments (Ebssa et al., 2001). *In vitro* experiments carried out by Mohammed et al. (2012) found *S. feltiae* and *S. carpocapsae* to be highly effective against *Pieris rapae* (imported cabbageworm) and provide high levels of control.
against 4th instar P. rapae larvae. Furthermore, the combination of S. feltiae and S. carpocapsae further increased the level of insect mortality over that of a single EPN species (Mohammed et al., 2012).

The newly categorized EPN Oscheius spp. is currently being explored for its potential as a biocontrol agent. Oscheius colombiana, O. pheropsophi and O. amsactae have been found necrotizing Cyrtomenus bergi (burrower bug), Pheropsophus aequinoctialis (bombardier beetle) and Amsacta moori (Lepidoptera: Arctidae) (red hairy caterpillar), respectively, naturally in the field (Nguyen and Smart, 1994; Stock, 2005). Under laboratory conditions, Oscheius sp. has been effective against Galleria mellonella (greater wax moth) and Corcyra cephalonica (rice moth) (Katti et al., 2006). Among Oscheius sp., Steinernema sp. and Heterorhabdits sp. against Euproctis sp. (hairy caterpillar) larvae and Conogethes punctiferalis (shoot borer) larvae and pupae, Oscheius sp. was the most virulent against shoot borer pupae. IJ penetration into shoot borer larvae was greatest by Steinernema sp. (16 IJs/larvae), followed by Heterorhabdits sp. (11 IJs/larvae) and Oscheius sp. (6 IJs/larvae) (Pervez et al., 2012). Since successful in vitro testing has been observed with other EPNs, more studies will be conducted for an invasive pest to coffee emerging in Hawai‘i, Hypothenemus hampei Coffee Berry Borer (CBB).

The CBB is the most destructive insect pest of coffee worldwide, causing economic losses exceeding $500 million annually (Jaramillo et al., 2011; Messing et al., 2012). CBB was first identified in Hawai‘i in August 2010 on a Kona coffee farm on the Big Island (Burbano et al., 2011). In 2014, CBB was found and quarantined in the Waialua region of O‘ahu (HDOA, 2014). EPNs have been documented parasitizing CBB
naturally in India and Mexico (Varaprasad et al., 1994; Castillo et al., 2002). *Steinernema* and *Heterorhabditis* were evaluated under field condition and were successfully able to penetrate fallen coffee cherries and parasitize the immature CBB (Lara et al., 2004).

An ideal EPN species is effective against the target insect if the EPN is quick to infect and kill at low dosage. An effective EPN can be utilized at low population densities and still provide effective levels of insect control. To determine the effectiveness of low population densities of endemic *Oscheius* sp., *S. feltiae* and *Heterorhabditis* sp., the mortality of *T. molitor* and CBB was evaluated.

The objective of these series of experiments was to evaluate the biological control potential of endemic *Oscheius* isolates alongside endemic *S. feltiae* MG14 and *H. indica* OM160 against *T. molitor* and CBB.

**Materials and Methods**

*Tenebrio* 1. To determine differences among endemic *Oscheius* isolates, the time need to kill *T. molitor* was recorded. Four isolates of *Oscheius* (BI 1a, BI 12a, OJ 4a and OJ 5a-1) were compared to *S. feltiae* MG 14, *Heterorhabditis* OM 160 and a water control. Individual larvae of *T. molitor* were placed into a petri plate (60 mm x 15 mm) lined with Whatman no. 1 (55 mm) filter paper. A 500µl aliquot of water was applied to the top and bottom of the petri plate. Each petri plate was infested with 3 IJ/100cm² and stored in an incubator at 25°C in complete darkness. Each day, the top and bottom filter papers were rehydrated with 250 µl of water. Any insect larvae clinging to the top of the petri plate were returned to the bottom of the plate. Petri dishes were observed for 3 days. Each treatment was replicated four times and the experiment was repeated four times. Lethal
time (LT\textsubscript{50}) was calculated using a nonlinear regression (logistic 3p) model in JMP 12.2. The model was

\[ y = \frac{c}{(1 + e^{-a \times (x - b)})} \]

where \(y\) is % mortality, \(x\) is time in hours, \(a\) is growth rate \((b)\) is the inflection point and \(c\) is the asymptote.

\textit{Tenebrio 2.} Isolates of \textit{Oscheius} BI 1a, BI 12a, OJ 4a and OJ 5a were compared to \textit{S. feltiae} MG 14, \textit{Heterorhabditis} OM 160 and a water control to determine pathogenicity against larvae of \textit{T. molitor} larvae. Individual larvae of \textit{T. molitor} were placed into a petri plate (60 mm x 15 mm) lined with Whatman no. 1 (55 mm) filter paper. A 500\textmu l aliquot of water was applied to the top and bottom of the petri plate to moisten the filter paper. Each petri plate was then infested with 3, 9 or 15 nematodes in 20 \textmu l of water and placed in an incubator at 25°C in complete darkness. The larvae were observed every 24 hours for mortality over 5 days. Each day, the top and bottom filter papers were rehydrated with 250 \textmu l of water. Any larvae clinging to the top of the petri plate were returned to the bottom of the plate. Larval morality was evaluated by no response to physical prodding, rigor mortis, and/or a color change. Each treatment was replicated 4 times and the experiment was repeated four times. Lethal time (LT\textsubscript{50}) was calculated as described previously.

\textit{Coffee Berry Borer.} Ten CBB-infested coffee cherries sourced from Kona Rainforest Organic Coffee Company, Captain Cook, Kona Hawai’i were placed in a 7 ml cup (Solo model P325-3). All coffee cherries were positioned with the CBB entry holes facing up. Coffee cherries were inoculated with 1500 IJ delivered in 1 ml of water directly into the
CBB-entry holes using a pipette. *Oscheius* isolates BI 1a, BI 12a and OJ 4a, *S. feltiae* MG 14, *Heterorhabditis* OM 160, and a water control was evaluated. The cup was placed within a HiPlas 309-07-B container with a 2.5-cm-diameter screened hole removed and covered with Parafilm to increase humidity. The double container was placed into a 4-l plastic container and covered a 23 cm x 13 cm screened lid. The plastic container was placed into a Darwin Chamber (model no. IN034-LT-DA) set to 25°C, 70% relative humidity and 12 hours light/12 hours dark for 1 week. A HOBO Pro v2 probe was placed within a control container within the plastic container to record temperature and relative humidity. At the end of the week, coffee cherries were dissected and the number of living and dead CBB larvae, pre-pupae, pupae and adults were tallied. All life stages of CBB, dead or alive, were placed into a drop of water, dissected, and observed for the presence of nematodes. All morbid larvae were evaluated for EPN infection. Darkening or color change of the cadaver was deemed indicative of EPN infection (Gaugler, 2002). A turgid cadaver body was also considered a sign of EPN infection. Larvae infected by non-toxic bacteria were indicated by color change of the body and softening of the cadaver. Cadavers with visible mycelia were considered killed by fungi. Each treatment was replicated 10 times and the entire experiment was repeated twice. Lethal time (LT_{50}) was calculated as described previously.

**Results**

*Tenebrio* 1. All six endemic nematode isolates were pathogenic to *T. molitor* larvae. Larval mortality ranged from 56.3% to 87.5% in the 72-hour timeframe. The isolates differed in their lethality to *T. molitor* larvae (Fig. 2.1). The model showed *S. feltiae*
having the lowest LT$_{50}$ time at 37.5 hours, followed by OJ 4a (*Oscheius* sp.), BI 1a (*O. tipulae*), BI 12a (*O. myriophilus*), OJ 5b (*O. tipulae*) and *H. indica* (Table 2.1).

*Tenebrio 2.* All 6 endemic nematode isolates were pathogenic towards *T. molitor* larvae, causing larval mortality from 75.0% to 100.0% in the 120-hour timeframe. At 3 IJ/100cm$^2$, *S. feltiae* had the lowest LT$_{50}$ of 46.4 hours, followed by OJ 4a, BI 1a, BI 12a, OJ 5b and *H. indica* (Table 2.2). At 9 IJ/100cm$^2$, BI 12a had the lowest LT$_{50}$ time of 39.7 hours, followed by *S. feltiae, H. indica, OJ 4a, BI 1a and OJ 5b*. At 15 IJ/100cm$^2$, *S. feltiae* had the lowest LT$_{50}$ time of 35.2 hours, followed by OJ 4a, *H. indica*, BI 1a, BI 12a and OJ 5b. With *S. feltiae* and *H. indica*, LT$_{50}$ decreased as IJ inoculum increased as expected (Fig. 2.2).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Mortality (A)</th>
<th>Inflection Point (B)</th>
<th>Asymptote (C)</th>
<th>$R^2$</th>
<th>LT$_{50}$</th>
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<tbody>
<tr>
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<td>47.84</td>
<td>81.25</td>
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<td>BI 12a</td>
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<td>46.49</td>
<td>91.94</td>
<td>1.00</td>
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</tr>
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<td>OJ 5b</td>
<td>0.959</td>
<td>48.23</td>
<td>56.25</td>
<td>1.00</td>
<td>50.4</td>
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<tr>
<td><em>S. feltiae</em></td>
<td>0.094</td>
<td>34.85</td>
<td>89.73</td>
<td>1.00</td>
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</tr>
<tr>
<td><em>H. indica</em></td>
<td>0.094</td>
<td>52.33</td>
<td>93.97</td>
<td>1.00</td>
<td>53.6</td>
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</table>
Figure 2.1 Curves of *Tenebrio molitor* mortality caused by 6 endemic EPN isolates. BI 1a (*Oscheius*), BI 12a (*Oscheius*), OJ 4a (*Oscheius*), OJ 5b (*Oscheius*), *Steinernema feltiae* and *Heterorhabditis indica*. 
Figure 2.2 Mortality curves of BI 1a (A), BI 12a (B), OJ 4a (C), OJ 5b (D) isolates of Oscheius, Steinernema feltiae (E), and Heterorhabditis indica (F). Light grey, dark grey and black lines represent 3, 9 and 15 IJ/100cm$^2$ respectively.
<table>
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<th>Isolate</th>
<th>R²</th>
<th>Asymptote</th>
<th>Injection Point</th>
<th>Infection Mortality</th>
<th>% Mortality</th>
<th>% Dose</th>
<th>II Dose</th>
<th>II</th>
<th>LT 50</th>
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</thead>
<tbody>
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<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
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<tr>
<td>S. Pelican</td>
<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
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<tr>
<td>L.</td>
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<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
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<tr>
<td>OI 56</td>
<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>OI 48</td>
<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BI 12a</td>
<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BI 1a</td>
<td>46.8</td>
<td>98.0</td>
<td>98.0</td>
<td>96.0</td>
<td>96.0</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: LT 50 values of BI 1a, BI 12a, OI 48, OI 56 isolates of *O. cholerae, Shewanella Pelicanus, and Helicobacter Indica at 3.9 and 15 II/100cm².
In general, average infection times at 3 IJ/100cm² and 15 IJ/100cm² were comparable, however, infectivity at 9 IJ/100cm² was variable (Fig. 2.2). The rate at which mealworms died was variable, particularly with Oscheius isolates at 9 IJ/100cm². The intermediate level of 9 IJ/100cm² was the slowest and least effective in Oscheius isolates yet the most effective in BI 12a. All Oscheius isolates utilized were highly variable in regard to their infection averages. In the second experiment, 75% to 100% mealworm mortality was observed compared to a variability of 56.5% to 87.5% in the first experiment.

Coffee Berry Borer. Morbid CBB larvae, pre-pupae, pupae and adults were recovered from all 5 isolates of endemic EPN tested (Fig. 2.3). An average of 9 CBB (varying life stages) were observed per cherry. Total CBB mortality was highest with S. feltiae at 13.7%, followed by OJ 4a, BI 12a, H. indica, BI 1a and the control (Table 2.3). Of the total dead CBB, between 8.7% and 17.6% was attributed to EPNs. An entomopathogenic fungi or unknown factors accounted for most CBB mortality. CBB death attributed to nematodes was highest with H. indica and S. feltiae at 17.6% and 16.7% respectively. Of the three Oscheius isolates evaluated, BI 12a was the only isolate able to kill CBB. IJs were only found in morbid adult CBB from S. feltiae, BI 12a and H. indica in three, two and one instance respectively. Single, live IJs of S. feltiae and H. indica were recovered from morbid CBB, whereas multiple live IJs were recovered from BI 12a. Twenty-one nematodes were recovered from BI 12a, suggesting reproduction occurred within the CBB cadaver.
Table 2.3 *Hypothenemus hampeii* mortality induced by isolates of *Oscheius*, *Steinernema* and *Heterorhabditis* or by an entomopathogenic fungus.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Average CBB/cherry</th>
<th>Dead (%)</th>
<th>Nematode</th>
<th>Fungus</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI 1a</td>
<td>9</td>
<td>9.2</td>
<td>0.0</td>
<td>6.3</td>
<td>93.7</td>
</tr>
<tr>
<td>BI 12a</td>
<td>9</td>
<td>12.2</td>
<td>8.7</td>
<td>13.0</td>
<td>78.3</td>
</tr>
<tr>
<td>OJ 4a</td>
<td>10</td>
<td>11.5</td>
<td>0.0</td>
<td>4.5</td>
<td>95.5</td>
</tr>
<tr>
<td>S. feltiae</td>
<td>9</td>
<td>13.7</td>
<td>16.7</td>
<td>8.3</td>
<td>75.0</td>
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<tr>
<td>H. indica</td>
<td>9</td>
<td>9.9</td>
<td>17.6</td>
<td>11.8</td>
<td>70.6</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>8.2</td>
<td>0.0</td>
<td>26.7</td>
<td>73.3</td>
</tr>
</tbody>
</table>

The presence of an entomopathogenic fungus was a contributing factor distinguishing Trial #1 from Trial #2. Trial #1 had very little fungus present, if at all, whereas in Trial #2 significant fungal infection was observed. In Trial #2, the entomopathogenic fungus frequently encased the entire coffee cherry and/or adult CBB. Trial #2 comprised 93% of all CBB death attributed to an entomopathogenic fungus. All other morbid CBB death not linked to EPNs or the entomopathogenic fungus were categorized as unknown.

**Discussion**

In general, mortality of *T. molitor* was similar at the lowest (3 IJ/100cm²) and highest (15 IJ/100cm²) inoculum levels tested. It is possible that at 3 IJ/100cm², the EPNs do not encounter or interact with other EPNs and proceed to infect the host uninterrupted. At 9 IJ/100cm², interactions and competition for entry into the insect between nematodes may have taken place, which potentially delayed infection. At 15
IJ/100cm², the interaction and competition among EPNs likely still occurs, however, the density of EPNs is high enough that some will infect the host in a timely manner. This behavior was not exhibited in *S. feltiae* and *H. indica*, as larval mortality was positively correlated to increased EPN inoculum levels.

Comparison of the LT₅₀ values of *T. molitor* at 3 IJ/100cm² from the first and second experiments showed a lower LT₅₀ across all isolates. The difference in LT₅₀ values between the two experiments can be attributed to the two additional days in the second experiment. A more comprehensive representation of the infection times is clearly represented by the increase in mealworm mortality.

The ranking of LT₅₀ values among nematode isolates remained the same in both experiments. In both experiments, *S. feltiae* had the lowest LT₅₀ value followed by OJ 4a, BI 1a, BI 12a, OJ 5b and *H. indica*. The consistency observed might be attributable to the nematode foraging strategy. EPNs are evaluated based on behavioral characteristics and categorized into 3 behavioral classes; ambushers, cruisers or intermediate foragers which utilize both ambusher and cruiser behaviors (Lewis, 2002). In general, cruisers are more effective at infecting stationary hosts, whereas ambushers are more effective at infecting fast moving hosts (Grewal *et al.* 1993). *Heterorhabditis bacteriophora, H. indica, H. megidis, S. anomaly* and *S. glasseri* utilize cruiser behavior, whereas *S. carpocapsae* and *S. scapterisci* are ambushers. *Steinernema feltiae* is an intermediary, utilizing a combination of both ambushing and cruiser characteristics (Grewal *et al.* 1993; Lewis, 2002). The mealworms utilized in this experiment were very active, thus nematodes that exhibit ambushing behavior are favored. *Steinernema feltiae*, an intermediary, had the lowest LT₅₀, whereas *H. indica*, a cruiser, had the highest LT₅₀.
The LT$_{50}$ of the Oscheius isolates are much closer to *H. indica* than to *S. feltia*, suggesting the Oscheius isolates likely exhibit cruiser behavior. This hypothesis is plausible as *O. myriophilus* is a necromenic nematode (Poinar 1986; Torres-Barragan 2011). As a parasite of dead insects, *O. myriophilus* has to actively seek out a non-moving host, likely exhibiting a cruiser behavior.

*Oscheius carolinensis*, a recently classified EPN, is thought to have evolved an entomopathogenic lifestyle more recently than *Heterorhabditis* and *Steinernema* (Goodrich-Blair and Clarke, 2007; Chaston and Goodrich-Blair, 2010; Torres-Barragan et al. 2011). Thus their foraging strategy for insect parasitism is less evolved than other EPNs (Dillman et al. 2012) and is in the process of developing. This theory, in addition to the two additional days provided in the second experiment, could explain the variation in time between *Oscheius* isolates compared to *S. feltiae* and *H. indica*.

Under ideal conditions, a single EPN is capable of killing an individual insect. EPNs were observed swimming out of the CBB cadaver upon dissection and were easily identified by their morphological characteristics (double cuticle, movement pattern, etc.). While CBB death attributed to EPNs was recorded, the evaluation did not account for death potentially attributed to the EPN mutualistic endosymbiotic bacteria without the presence of an EPN.

Upon entering a host, IJs encounter various defense mechanisms, primarily encapsulation of the nematode and nodulation of its endosymbiotic bacteria within the hemolymph (Gaugler, 2002). If encapsulated, the nematode will likely die, however the nodulated bacteria that the nematode released upon infection can continue to multiply and can forcibly discharge from the nodules, proceeding to kill the host (Gaugler, 2002). It is
highly likely that the majority of CBB death observed, not attributed to an entomopathogenic fungi, could be credited to EPNs and/or their mutualistic bacteria. This phenomenon of host death by bacteria without nematode reproduction has been observed in ticks (Hassanain et al. 1997). This possibility could not be confirmed due to the natural black color of the adult CBB, so phenotypic observation of bacterial induced death via color change was not possible. However, within the coffee cherry, three morbid CBB larvae were observed, all exhibiting phenotypic color change. *Heterorhabditis indica*, in both cases yielded a red color change, characteristic of its *Photorhabdus* bacteria.

Reproduction was observed once in CBB, with isolate BI 12a. *S. feltiae* and *H. indica*, utilize amphimictic modes of reproduction in which a male and female nematode are necessary for reproduction (Dix et al. 1992). Nematodes in the genus *Oscheius* are hermaphroditic, allowing hermaphroditic females to reproduce (Wood, 1988; Baille et al. 2008). As hermaphrodites, *Oscheius* is better suited to reproduce within the insect host and potentially infect neighboring insects.

*Oscheius* has been evaluated and compared to the Steinernematids and Heterorhabditids. Pervez et al. (2012) found *Steinernema* sp. (IISR 02) and *Oscheius* sp. (IISR 07) to be most promising in controlling insect pests of ginger. Five of the six EPN isolates tests, including three of four *Oscheius* sp. isolates, were 100% virulent against hairy caterpillar larvae. *Oscheius* sp. (IISR 07) was found to be the most virulent against shoot borer pupae, inducing 100% mortality. In a separate study, Ali et al. (2008) concluded that there was no significant variation in mortality of the *Helicoverpa armigera*, *Galleria mellonella*, *Corcyra cephalonica* induced by Steinernematids,
Heterorhabditids and *Oscheius*, indicating that no one group is superior to the other (Gaugler and Kaya, 1990; Ali *et al*., 2008).

The presence of entomopathogenic fungi was a contributing factor distinguishing Trial #1 and Trial #2 in the CBB infectivity assay. Trial #1 had very little entomopathogenic fungi present, if at all, whereas Trial #2 had significant entomopathogenic fungi. Cherries collected at the same time were used in both trials; however, Trial #2 was started 3 days after Trial #1. De La Rosa (1997) found that the mean lethal time for the entomopathogenic fungi *Beauveria bassiana* infection is between 4.3 and 7.5 days. This is within the time period of Trial #1, which showed the initial onset of the entomopathogenic fungi, and well within Trial #2 where the entomopathogenic fungi was extensive. Additionally, the HOBO probe indicated that the relative humidity within the container holding the cherries was 95%, 20% higher than the thermostat setting of 75% relative humidity. High humidity favors *B. bassiana* development and principally attacks CBB during these periods of high humidity (Moore, 1989; Murphy and Moore, 1990). A combination of the increased humidity and time lapse between Trial #1 and Trial #2 allowed the entomopathogenic fungi ideal conditions and additional time to sporulate and spread. This was evident in the drastic increase in entomopathogenic fungi associated CBB death observed in Trial #2.

The presence of entomopathogenic fungi was not expected in this experiment, however there is potential to utilize entomopathogenic fungi alongside EPNs. EPNs primarily target soil dwelling pests, while entomopathogenic fungi would predominantly target the pest above ground (Acevedo *et al*., 2007). Barbercheck and Kaya (1991) evaluated *B. bassiana* alongside *H. bacteriophora* and *S. carpocapsae*, finding that the
combination provided a greater level of control on *Spodoptera exigua* than either individually. Shapiro *et al.* (2004) observed increased mortality of *Curculio caryae* (Coleoptera: Curculionidae) larvae when using *S. carpocapsae* alongside *B. bassiana* at high concentrations or *H. indica* alongside *M. anisopliae* at low concentrations. Acevedo *et al.* (2007) observed a combination of entomopathogenic fungi and EPNs provided faster mortality at the cost of IJ reproduction. Co-infection of entomopathogenic fungi and EPNs has been observed, however only one can reproduce successfully per host, and that will be determined by which infects first (Barbercheck and Kaya, 1991; Acevedo *et al.* 2007).

Endemic *Oscheius* isolates are a welcomed addition to the sparse list of endemic biocontrol options available in Hawaii. Especially since a reduction in chemical based controls have been observed due to probable negative impacts on the environment, as well as potential adverse effects on non-target animal and human health (Brun *et al.*, 1989; Lacey *et al.*, 2000). Utilization of the potential EPN *Oscheius* is an excellent option as *Heterorhabditis* and *Steinernema* spp. (aside from *Steinernema carpocapsae*) are currently on Hawaii's List of Restricted Animals (Part A) and cannot be imported into the state (HDOA, 2006). The identification and classification of new endemic EPNs would expedite the process of approving EPN based biocontrol agents by demonstrating their existing prevalence.

**References**

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Conclusion

The objective of this research was to identify endemic entomopathogenic nematodes (EPNs) in Hawaii and evaluate their biological control potential against Hypothenemus hampei (coffee berry borer), an insect pest crippling the local coffee industry. A survey to identify endemic EPNs was conducted on O’ahu and Hawai’i Island. Three species of the insect-associated nematode Oscheius were recovered.

The evolution of nematode-insect association consists of three fundamental stages: free-living, phoresy, and necromeny, which branches into parasitism and entomopathogeny (Dillman, 2012). Entomopathogeny differs from parasitism in that entomopathogenic nematodes have adapted and developed an intimate relationship with unique mechanisms to transmit mutualistic insect pathogenic bacteria, which rapidly kill an insect host (Dillman, 2012). Nematodes are characterized as EPNs based on the following critical characteristics: (i) nematodes use a mutualistic-symbiotic relationship with bacteria, which is involved in the pathogenesis, (ii) the relationship between the nematode and the bacteria might be facultative, although it is maintained over subsequent generations (at least 2–3), and (iii) insect death is rapid, in less than 5 days (Dillman, 2012).

Nematodes in the genus Oscheius are predominantly recognized as insect-associated nematodes, however novel research has found several members of Oscheius to be entomopathogenic in nature. Recent exploration of mutualistic-symbiotic bacteria associated with nematodes in the genus Oscheius has found O. chongmingensis and C. briggsae to be associated with the insect pathogenic bacteria genus Serratia, while O. carolinensis likely has additional insect pathogenic bacterial associations (Zhang et al.,
Oscheius chongmingensis and O. carolinensis have been classified as EPNs (Zhang et al., 2008; Ye et al., 2010; Torres-Barragan et al., 2011; Dillman et al., 2012) upon the confirmation of mutualistic symbiotic bacteria and fulfilling the EPN criteria established by Dillman et al. (2012).

The color change of an insect cadaver is a characteristic response commonly associated with an EPNs symbiotic bacteria. For example, the bacterium associated with Heterorhabditis, Photorhabdus, induces a brick red color change while the bacterium associated with Steinernema, Xenorhabdus, produces a brown to black color change in the insect cadaver (Morris 1985; Samish and Glazer, 1992; Vasconcelos et al. 2004). Endemic Oscheius infection and mortality of T. molitor larvae was consistently observed within 120 hours (5 days) of inoculation and color change of the morbid larvae was frequently observed. Color change of T. molitor was typically a deep brown to black and appeared patchy at times, not encompassing the entire cadaver. Most Oscheius species not currently recognized as EPNs as their mutualistic-symbiotic bacteria has yet to be identified, however the aforementioned observations suggest such a relationship exists.

Oscheius has been evaluated and compared to Steinernema and Heterorhabditis EPN. Pervez et al. (2012) found Steinernema sp. (IISR 02) and Oscheius sp. (IISR 07) to be most promising in controlling insect pests of ginger. In a separate study, Ali et al. (2008) concluded that there was no significant variation in mortality of the gram pod weevil, greater wax moth or rice moth induced by Steinernematids, Heterorhabditids or Oscheius. No one group was superior to the other (Gaugler and Kaya, 1990; Ali et al., 2008). These results are comparable to the results of this thesis, suggesting the endemic Oscheius isolates are equally effective as EPNs. Endemic Oscheius are comparable to S.
feltiae and H. indica species against mealworms (Tenebrio molitor) and Oscheius isolate BI 12a was equally effective as S. feltiae against the coffee berry borer (Hypothenemus hampei).

Given the recent findings and advances with nematodes in the genus Oscheius, particularly the acceptance of O. chongmingensis and O. carolinensis as EPNs, findings from this thesis suggest Oscheius from Hawaii are also likely to be EPN. Additional testing to confirm the presence of mutualistic-symbiotic bacteria in endemic Oscheius isolates is required. However numerous observations throughout the extraction, rearing and infection process of this thesis strongly suggest mutualistic-symbiotic bacteria are present in the three endemic Oscheius isolates isolated.

The entomopathogenic fungi observed during the CBB experiment that was conducted in this thesis greatly contributed to CBB mortality. Barbercheck and Kaya (1991) evaluated the entomopathogenic fungi Beauveria bassiana alongside the EPNs H. bacteriophora and S. carpocapsae, and results showed that the combination provided a greater level of control on Spodoptera exigua (Lepidoptera: Noctidae) than individually. Shapiro et al. (2004) observed increased mortality of Curculio caryae larvae when using S. carpocapsae alongside B. bassiana at high concentrations or H. indica alongside Metarhizium anisopliae at low concentrations. As EPNs primarily target soil dwelling pests, and entomopathogenic fungi predominantly target the pest above ground (Acevedo et al. 2007), there is potential to utilize entomopathogenic fungi alongside EPNs. The confirmation of native EPN populations in Hawai‘i will provide more options for nematode-based biological control. Implementation with Integrated Pest Management (IPM) of emerging invasive insect pests, such as Hypothenemus hampei (coffee berry
borer) and *Oryctes rhinoceros* (coconut rhinoceros beetle) will provide possible control methods. Along with other biological control methods such as *B. bassiana* may provide control for future insect pests to come.

**References**


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Appendix
There were a variety challenges that were encountered throughout the duration of these experiments. From these challenges, a series of modifications to optimize the techniques and protocols of the experiments were thought of, including:

A problem with the mealworm assays was that infection time was highly variable among the three concentrations of IJs used and reproduction of EPNs within the cadaver was not confirmed. If morbid mealworms were placed on white traps it would determine if nematode reproduction occurred. Reproduction would be important to record as it would represent the inoculum level required to overwhelm the host’s natural immunity and/or defense mechanisms (encapsulation) allowing reproduction to occur. In the event no EPNs emerged, but a color change was present, the EPN was likely encapsulated by the host’s natural defense however the bacteria proceeded to kill the host. In the event IJ(s) were able to infect the host, its fecundity could be reduced by the host’s defenses. Additionally, a single IJ, or multiple amphimictic IJs of the same sex would be unable to reproduce.

Mature, red cherries were picked directly from coffee trees and the number of adult CBB within the cherry was impossible to determine, aside from the single adult present in the entry hole. The number of CBB found within the coffee cherries tested was highly variable. It would have been more effective to bore holes into a ripe uninfected coffee cherry, and allow a pre-determined number of adult CBB to infest the cherry (De La Rosa et al. 1997). This would allow statistical analysis of morbid adult CBB to be calculated.
Each of the ten cherries, which were inoculated, were treated as an individual repetition, unfortunately they were placed into a single container and inoculated individually. Separating each cherry into their own container would eliminate the possibility of nematodes from neighboring cherry inoculations infecting neighboring adult CBBs, which emerged from the cherry.

The large percentage of CBB death attributed to an unknown factor was potentially induced by the EPNs mutualistic endosymbiotic bacteria. Based on the black color of the adult CBBs exoskeleton, phenotypic observation of the characteristic color change initiated by EPN bacteria was not possible. If a DNA sample of morbid adult CBB, currently categorized as unknown, was taken and run through a PCR based detection assay using primers based on the nematodes specific endosymbiotic bacteria, CBB death could be associated with the bacteria. In the event the CBB death presently categorized as unknown is found linked to the bacteria, roughly 90% of all CBB death would be attributed to EPNs.

The presence of entomopathogenic fungi was a significant problem in the CBB infectivity assay. Now knowing the mean lethal time of the entomopathogenic fungi *B. bassiana* is between 4.3 and 7.5 days, a better option for future experiments would be to use fresh cherries in each experiment with no delay after collection. If cherries are setup within their own container, as mentioned above, this would also potentially limit the spread of entomopathogenic fungi between cherries. Additionally, the increased humidity encountered as a result of the Parafilm over the container holding the cherries increased the relative humidity approximately 20%. The removal of Parafilm would allow relative humidity to be regulated in a much more controlled manner. A
combination of the increased humidity and the time lapse between starting Trial #1 and Trial #2 likely provided entomopathogenic fungi ideal conditions and additional time to sporulate and spread as seen in the CBB assay.