RESEARCH/INVESTIGACIÓN

OCCURRENCE AND DISTRIBUTION OF HETERORHABDITID POPULATIONS IN THE HAWAIIAN ISLANDS

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ABSTRACT

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A survey of entomopathogenic nematodes (EPN) in the Hawaiian Islands recovered *Heterorhabditis indica* and previously undescribed *Heterorhabditis* species. Using *Galleria* bait, morbid larvae were recovered from 187 of the 275 samples collected within 100 m of the ocean. Entomopathogenic nematodes were recovered from 21% of morbid larvae. *Heterorhabditis indica* and *Heterorhabditis* spp. occurred in 7% and 8% of the total samples, respectively. Half of the heterorhabditids recovered were from the island of Kauai. *Heterorhabditis indica* was detected on all islands and *Heterorhabditis* spp. on all islands but Molokai. In filter paper bioassays, the Oahu strains of *H. indica* were the most infective EPN tested, causing 100% mortality of *G. mellonella* larvae within 48 hr after inoculation with 10 or more infective juveniles. *Heterorhabditis* was easily isolated from coastal areas of Hawaii.

Key words: biological control, entomopathogenic nematode, Hawaii, *Heterorhabditis*.

RESUMEN

Myers, R. Y., B. S. Sipes, T. K. Matsumoto, C. L. Mello, and J. S. Mello. 2015. Ocurrencia y distribución de las poblaciones heterorhabdítidos en las islas de Hawai. Nematropica 45:198-207.

En una prospección de nematodos entomopatógenos (EPN) en el archipiélago de Hawái se recuperaron *Heterorhabditis indica* y una especie no descrita de *Heterorhabditis*. Mediante el uso de trampas de *Galleria*, se recuperaron larvas mórbidas en 187 de las 275 muestras recolectadas en la zona dentro de los 100 m de distancia al océano. Nematodos entomopatógenos fueron recuperados en un 21% of las larvas mórbidas. *Heterorhabditis indica* y *Heterorhabditis* spp. aparecieron en un 7% y 8% del total de las muestras, respectivamente. La mitad de los heterorrabditidos recuperados, provenían de la isla de Kauai. *Heterorhabditis indica* se detectó en todas las islas y *Heterorhabditis* spp. en todas las islas excepto Molokai. En bioensayos en papel de filtro, las cepas de *H. indica* de Oahu fueron las más infectivas de todas las ensayadas, causando 100% de mortalidad en las larvas de *G. mellonella* dentro de las 48 hr después de la inoculación con 10 o más juveniles. *Heterorhabditis* se asiló fácilmente de áreas costeras de Hawái.

Palabras clave: control biológico, nematodo entomopatógeno, Hawái, *Heterorhabditis*.

INTRODUCTION

The establishment of invasive species in the U.S. comes at an economic cost of $120 billion/year (Pimentel *et al.*, 2005). For this reason, regulatory agencies have strict importation laws on plants, animals, and microorganisms that may be detrimental to indigenous species or cause economic harm to agriculture. In some cases, the most effective and sustainable method to control invasive pests is to allow the introduction of natural enemies to combat the problem. In a recent example, the Hawaii Department of Agriculture granted an expedited amendment in 2011 for the entomopathogenic fungus, *Beauveria bassiana*, to be removed from the List of Restricted Microorganisms (Part A) and added to the List of Nonrestricted Microorganisms for control of coffee berry borer, *Hypothenemus*

hampei. This action occurred with strong grower support after researchers demonstrated the effectiveness of *B. bassiana* and documented the existence of native strains. Interest was raised by local growers to conduct a similar study to determine if the entomopathogenic nematodes *Heterorhabditis* spp., currently on the List of Restricted Animals (Part A), were prevalent in the Hawaiian Islands and, if so, would be an effective management tool for invasive insect pest control.

The use of entomopathogenic nematodes (EPN) for insect management is not a new concept in Hawaii. A commercial strain of *Steinernema carpocapsae* was evaluated for its biological control potential against tropical pest species (Hara *et al.*, 1989). While effective against *Liriomyza trifolii* (agromyzid leafminer), *Cylas formicarius elegantulus* (sweet potato weevil), and *Chrysodeixis chalcites* (green garden looper), *S. carpocapsae* caused low mortality in *Cosmopolites sordidus* (banana root borer) and *Adoretus sinicus* (Chinese rose beetle) (Hara *et al.*, 1989). With increased mobility and a broader host range, *Heterorhabditis* or other species of *Steinernema* may offer better control of some insect pests.

A number of invasive insects in Hawaii have the potential to be managed by EPN. *Heterorhabditis* may be an effective biological control agent of *Aethina tumida* (small hive beetle) (Shapiro-Ilan *et al.*, 2010), *Frankliniella occidentalis* (western flower thrips) (Ebssa *et al.*, 2001), *Opogona sacchari* (banana moth) (Peña *et al.*, 1990), and *Euscepes postfasciatus* (West Indian sweet potato weevil) (Figueroa *et al.*, 1993). Integrated pest management (IPM) strategies utilizing EPN to complement *B. bassiana* are being explored for control of *H. hampei*. EPN sprayed on fallen cherries could be used as an alternative to sanitation by causing mortality of *H. hampei* larvae and adults and subsequently reducing the population of future generations in the field. A commercial isolate of *S. carpocapsae* contributed to 23.7% mortality of *H. hampei* larvae and 26.6% adult mortality when applied to infested coffee berries (Manton *et al.*, 2012). Castillo and Marbán-Mendoza (1996) showed that strains of *Heterorhabditis* spp. from Costa Rica were as effective against *H. hampei* as the commercial strain of *S. carpocapsae*. If endemic EPN are better adapted to Hawaii's tropical environment, persistence in the field may be improved.

In 1991, Hara *et al.* surveyed the Hawaiian Islands to document existing EPN. They demonstrated not only the existence of *Steinernema* on Maui but also the presence of *Heterorhabditis* on five of the main Hawaiian Islands. The study found *Heterorhabditis* to be restricted to coastal regions with positive sites located 100 m from beaches. Similar results were seen in surveys from the Azores where 70% of samples positive for *Heterorhabditis* were collected below 150 m (Rosa *et al.*, 2000) and in Indonesia where 100% of positive samples were recovered from coastal sites (Griffin *et al.*, 2000). In Guadaloupe and neighboring islands, Constant *et al.* (1998) found *H. indica* almost exclusively in beach and limestone cliff regions, comprising 73% and 13% of positive sites, respectively.

The objective of our study was to document natural populations of *Heterorhabditis* in the Hawaiian Islands to strengthen the case for importation of commercial strains. A secondary objective was to discover pathogenic, better adapted isolates to control insect pests in the tropics.

MATERIALS AND METHODS

Survey of natural habitats

Soil samples were collected from coastal regions on the Hawaiian Islands of Kauai, Oahu, Molokai, Maui, and Hawaii. Collection sites were located under vegetation and within 100 m of the shoreline. Coordinates were recorded with a Garmin Oregon 550t GPS. A 500 cm^3 soil sample was collected with a garden spade from a depth of 5 to 30 cm. The soil was placed into plastic bags (15 cm x 23 cm, 2 mil), labeled, and stored in a cooler. Soil temperature was measured at select sites on Kauai where *Heterorhabditis* was previously recovered using a handheld traceable thermometer (Fisher Scientific, Waltham, MA) inserted 4-cm deep before sample collection. After nematode extraction, soil pH and salinity were measured in the laboratory with an Orion 320 Basic PerpHect (Thermo Scientific, Waltham, MA) and an Oakton ECTestr (Eutech Instruments, Waltham, MA), respectively.

The *Galleria* baiting method (Bedding and Akhurst, 1975) was used to recover EPNs from soil. Soil samples were placed in a 473-cm^3 plastic container, baited with six *Galleria mellonella*, and stored in darkness at 28°C. After 1 wk, infected *G. mellonella* were rinsed and placed on modified White traps (Kaya and Stock, 1997) for nematode collection. Infective juveniles (IJ) were stored in water at 25°C. Nematode cultures were maintained through re-inoculations of *G. mellonella* larvae (Dutky *et al.*, 1964).

Molecular identification

For species identification, PCR was performed on single IJ specimens using general nematode primers and sequenced. Nematodes were tentatively identified

using morphological methods and prepared for PCR in 35-µL reactions (Cabos *et al.*, 2013). Primers ITS-F (5'-TGTAGGTGAACCTGCTGCTGGATC-3') and ITS-R (5'-CCTATTTAGTTTCTTTTCCTCCGC-3') (Saeki *et al.*, 2003) were used to amplify the ITS region. The PCR conditions were 95°C for 2 min, 40 cycles of 95°C for 45 sec, 56°C for 30 sec, 72°C for 20 sec, followed by 5 min 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 DNAsis Max 3.0 (Hitachi Solutions American, San Bruno, CA) and trimmed to include only sequences from both DNA strands. Sequences from different nematode samples were aligned using the Multiple Sequence Alignment in DNAsis Max using the Clustal W (1.6) algorithm. Nucleotide sequences from *H. hawaiiensis* sequence (AF029707), *H. indica* strain N-MID10 (HQ225902), *H. indica* (AY321483), and *Heterorhabditis* sp. SGmg3 (FJ751864) were obtained from the National Center for Biotechnology Information (NCBI) and used as reference sequences in the alignments. BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was used to shade multiple sequence alignments to highlight unique polymorphisms of each sequence.

Infectivity bioassay

Infectivity studies were conducted with selected strains recovered from the above survey; *H. indica* strains KM88, KM89, OM158, OM160, and HM173; and *Heterorhabditis* sp. KM105 and HM108. A single *G. mellonella* third instar was placed in each well of a 24-well tissue culture plate lined with a 1.5 cm circular piece of Whatman no. 1 filter paper. *Galleria* were raised in the laboratory on a cereal diet (Poinar, 1975). Each well was inoculated with 1, 3, 5, 10, 25, or 50 IJ in 50 µL of water. A 24-well plate holding *G. mellonella* larvae was inoculated with 50 µL water/well to serve as a negative control. The plates were sealed and stored in darkness in a 28°C incubator. Larval mortality was observed at 24-hr intervals for 72 hr. Six replications were conducted at each nematode concentration and strain. Data were analyzed by one-way ANOVA and means separated by the least significant means analysis (SAS, 2007).

RESULTS

Survey of natural habitats

Soil samples were collected at 275 sites from five Hawaiian Islands (Fig. 1). Morbid *G. mellonella* larvae were retrieved from 68% of the samples. Entomopathogenic nematodes were found in 21% of morbid larvae. Entomopathogenic fungi, including *Beauveria* and *Metarhizium*, were observed in some of the remaining cadavers. *Heterorhabditis indica* and a previously undescribed species of *Heterorhabditis* were the most prevalent EPN collected. *Heterorhabditis* spp. and *H. indica* were recovered from 8% and 7% of the total samples, respectively. Half of the positive heterorhabditid samples were collected from the island of Kauai. *Steinernema carpocapsae* was found only once during the survey and occurred at Hanapepe Park on Kauai. *Oscheius* spp. (Heterorhabditoides), *Rhabditis* spp. and *Rhabdias* spp., identified by sequence analysis of the ITS region, were also recovered from baited *G. mellonella* cadavers.

The predominant vegetation at sites positive for heterorhabditids were Ironwood (*Casuarina equisetifolia*), False kamani (*Terminalia catappa*), and Koa haole (*Leucaena leucocephala*) (Table 1). *Steinernema carpocapsae* was found under Kiawe (*Prosopis pallida*). The predominant vegetation at sites negative for the presence of EPN were Tree heliotrope (*Tournefortia argentea*), Naupaka (*Scaevola sericea*), and Pickleweed (*Batis maritima*). Soil pH ranged from 6.93 to 7.73 and salinity was 0.5 to 3.5 ms in samples positive for EPN. Negative soil samples had pH ranging as low as 5.7 and as high as 8.48 whereas salinity in negative samples was 1.2 to 13.7 ms. The prevalent soil type was sandy loam. Soil temperatures where *H. indica* were collected ranged from 23°C to 26°C whereas soils with *Heterorhabditis* spp. were 21°C to 23°C.

Molecular identification

Specimens tentatively identified as *H. indica* were 100% aligned (Fig. 2) with the corresponding sequence reported by Adams *et al.* (1998) for *H. hawaiiensis*. The *H. indica* strains from three Hawaiian islands had 100% identity as did the *H. indica* strain N-MID10 from Midway Island. One nucleotide difference existed between *H. indica* strains from Hawaii and the *H. indica* strain from India in the evaluated portion of the ITS region. Sequencing of the D2/D3 domains with the 501 (Thomas *et al.*, 1997) and 391 primers (Nadler and

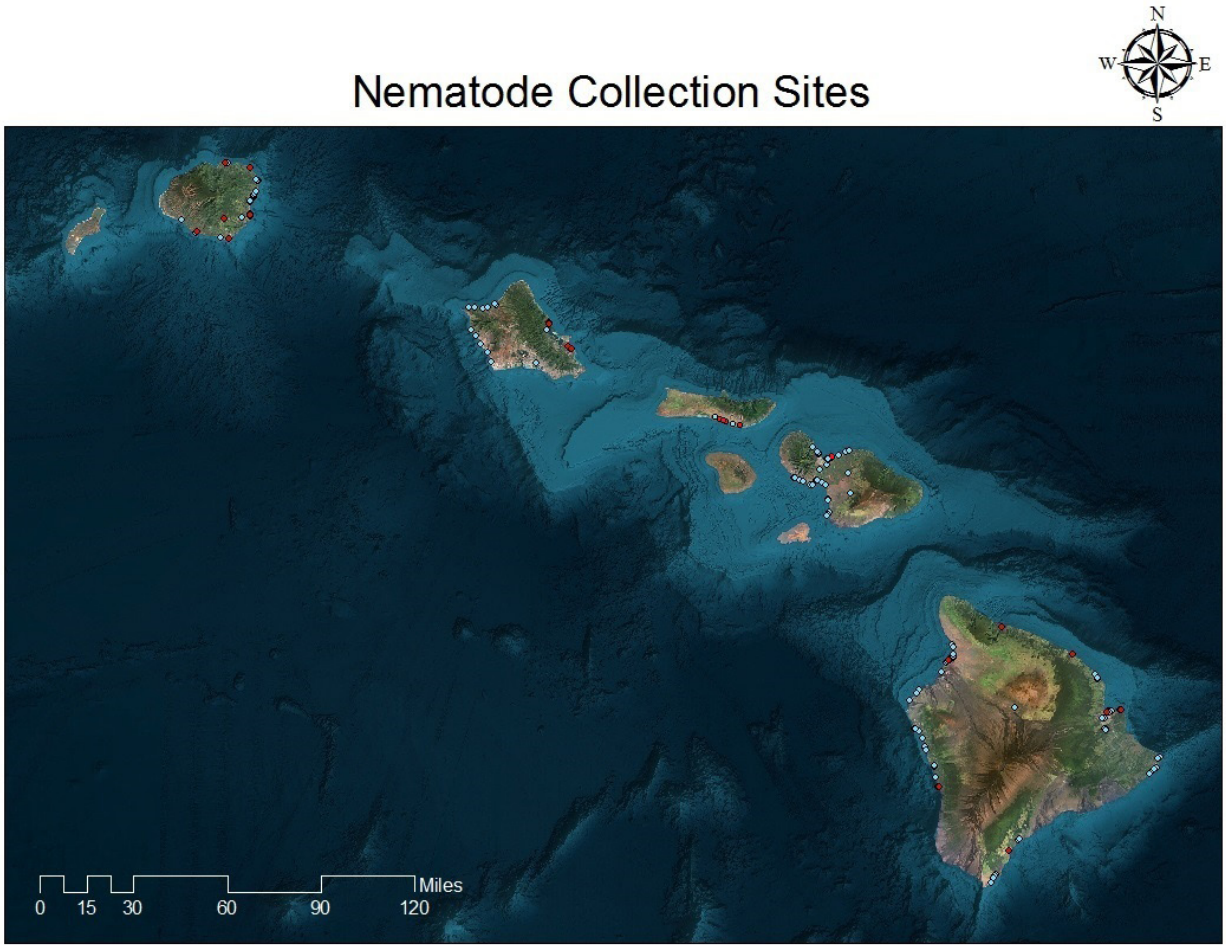

Fig. 1. Distribution of *Heterorhabditis* in Hawaii. Collection sites where *Heterorhabditis* were recovered are marked by a red circle and sites that were negative for the presence of *Heterorhabditis* are marked with a blue circle.

Hudspeth, 1998) showed high similarity between the Hawaiian strains of *H. indica* and *H. indica* strains Khatatba and Kerr (data not shown).

Two different sequences were revealed in specimens of *Heterorhabditis* spp. One nucleotide difference and two deletions were observed in samples KM105 (Kauai) and HM108 (Hawaii Island) (Fig. 3). The remaining *Heterorhabditis* sp. sequenced representing strains from all islands were identical. The closest match in GenBank was to *Heterorhabditis* sp. SGmg3, a new species from Meghalaya, India. Sequence alignment suggests SGmg3 and the endemic Hawaiian strains are two separate species. In maintaining the inventory of collected strains, it was observed that KM105 and HM108 had better infectivity and were easier to maintain on *G. mellonella* larvae than strains of the other undescribed *Heterorhabditis* species.

Infectivity bioassay

Heterorhabditis indica caused significantly higher mortality in *G. mellonella* larvae than *Heterorhabditis* sp. and the control ($P < 0.0001$). At combined inoculum levels, the Oahu strain OM158 caused 94% mortality 72 hr after inoculation. Within 48 hr, 100% mortality occurred in larvae inoculated with 5 or more IJ. Other *H. indica* strains performed similarly with 80 to 90% mortality observed in inoculated larvae. Death of the host occurred more rapidly at higher inoculum levels. Mortality of 2 and 4% was observed in larvae inoculated with *Heterorhabditis* sp. KM105 and HM108, respectively. No mortality occurred when larvae were introduced to only 1 to 3 IJ of *Heterorhabditis* sp. strains.

Table 1. Location and associated vegetation of sites containing the entomopathogenic nematode *Heterorhabditis*.

Strain and Species	Location	Vegetation
KM88, KM89 *H. indica*	Shipwreck Beach, Po `ipū, Kauai	Ironwood (*Casuarina equisetifolia*), Koa haole (*Leucaena leucocephala*), Seashore paspalum (*Paspalum vaginatum*), Christmas berry (*Schinus terebinthifolia*)
KM102 *Heterorhabditis* spp.	Ahukini Pier, Lihue, Kauai	Koa haole (*Leucaena leucocephala*), Guinea grass (*Panicum maximum*), Spider lily (*Crinum* sp.)
KM105 *Heterorhabditis* spp.	Ahukini Rd, Lihue, Kauai	Ilie'e (*Plumbago zeylanica*), Kalinga (*Cyperus* sp.)
KM179 *Heterorhabditis* spp.	Ahukini Rd, Lihue, Kauai	Koa haole (*Leucaena leucocephala*), Ilie'e (*Plumbago zeylanica*)
KM193 *Heterorhabditis* spp.	Anini Beach Road, Kauai	False kamani (*Terminalia catappa*), Mangrove (*Rhizophora* sp.)
KM234 *H. indica*	Hanalei Beach Park, Kauai	Ironwood (*Casuarina equisetifolia*), Bermuda grass (*Cynodon dactylon*)
OM158 *H. indica*	Kalama Beach Park, Oahu	Ironwood (*Casuarina equisetifolia*), Sea grape (*Coccoloba uvifera*), Naupaka (*Scaevola sericea*)
OM160 *H. indica*	Kailua Beach Park, Oahu	Ironwood (*Casuarina equisetifolia*)
OM163 *Heterorhabditis* spp.	Kualoa Beach Park, Oahu	Kou (*Cordia subcordata*), False kamani (*Terminalia catappa*), Naupaka (*Scaevola sericea*), Maunaloa (*Canavalia cathartica*), Bermuda grass (*Cynodon dactylon*)
OM228 *Heterorhabditis* spp.	Ko'olina Lagoon, Oahu	Coconut palm (*Cocos nucifera*)
MKM245, MKM246 *H. indica*	Kakahai'a Beach Park, Molokai	Kiawe (*Prosopis pallida*), Hau (*Hibiscus tiliaceus*)
MKM248, MKM249 *H. indica*	One Ali'i Park, Molokai	Ironwood (*Casuarina equisetifolia*), Naupaka (*Scaevola sericea*), and Bermuda grass (*Cynodon dactylon*)
MM206 *H. indica*	Kanaha Pond, Wailuku, Maui	Ironwood (*Casuarina equisetifolia*), Ākulikuli kai (*Lycium sandwicense*), Bermuda grass (*Cynodon dactylon*)
HM9 and HM10 *Heterorhabditis* spp.	Kings Landing, Hilo, Hawaii	Ironwood (*Casuarina equisetifolia*), False kamani (*Terminalia catappa*)
HM27 *Heterorhabditis* spp.	Laupahoehoe Point, Hawaii	Maunaloa (*Canavalia cathartica*), Ilie'e (*Plumbago zeylanica*)
HM38 *Heterorhabditis* spp.	Bayfront, Hilo, Hawaii	Horsetail (*Conyza* sp.), Fireweed (*Erechtites valerianifolia*)
HM108 *Heterorhabditis* spp.	Waipio Valley, Hawaii	Ironwood (*Casuarina equisetifolia*)
HM173 *H. indica*	Ho'okena Beach Park, Hawaii	False kamani (*Terminalia catappa*)

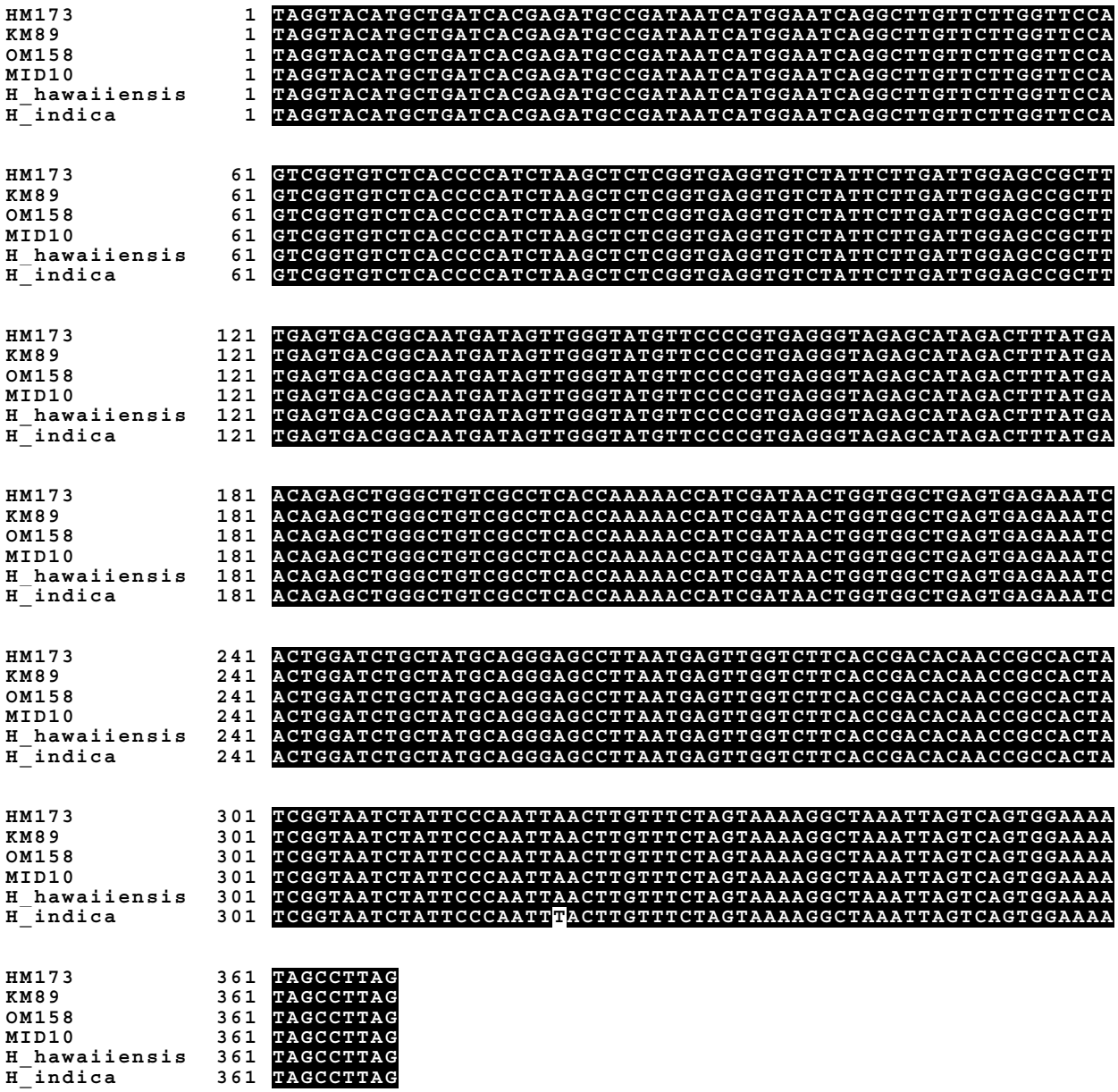

Fig. 2. Multiple sequence alignment of *Heterorhabditis indica* strains.

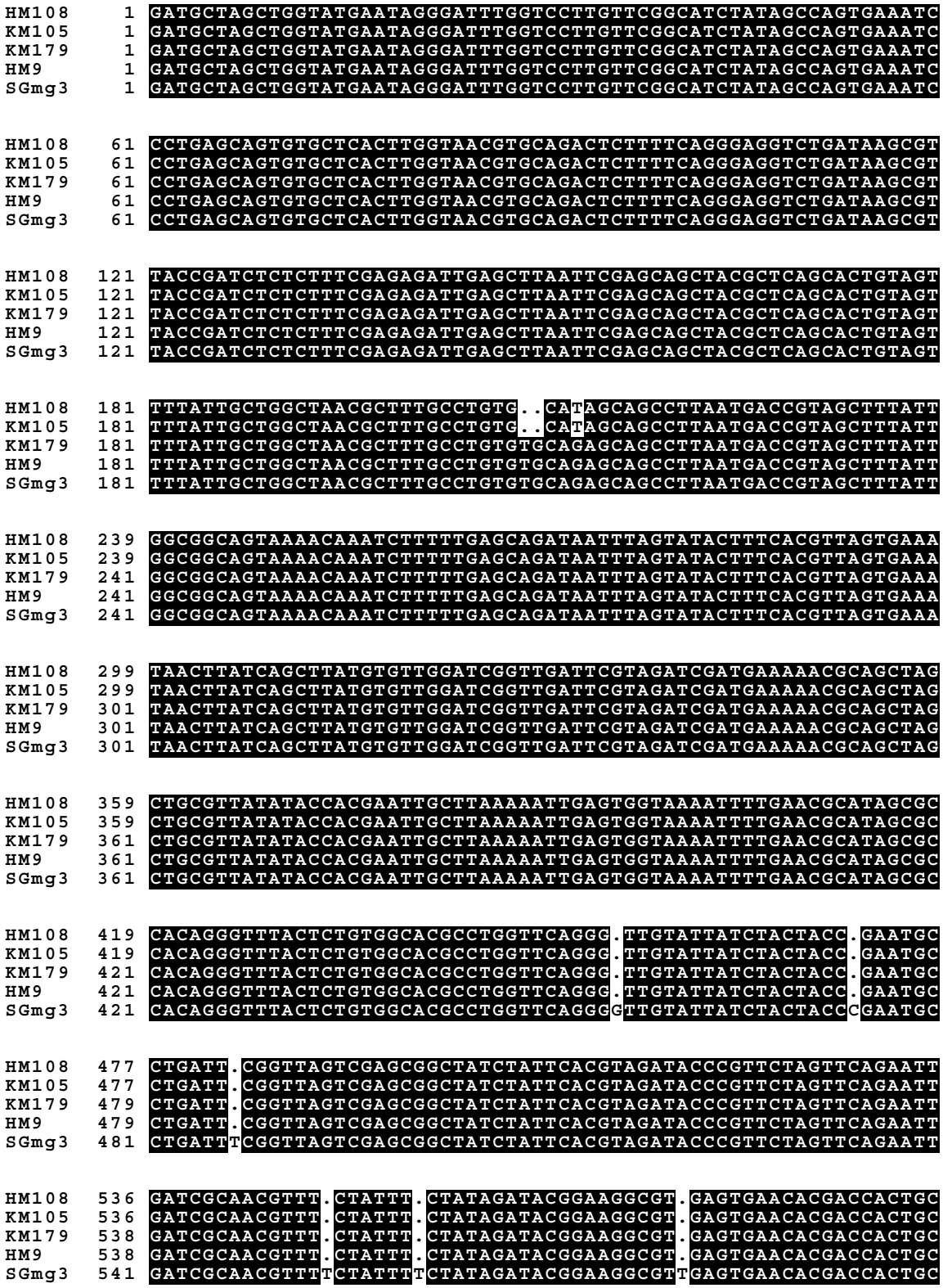

Fig. 3. Multiple sequence alignment of undescribed *Heterorhabditis* spp. endemic to Hawaii.

DISCUSSION

This study confirmed that *Heterorhabditis* is easily isolated from coastal areas of Hawaii. *Heterorhabditis indica* was recovered from all islands surveyed and *Heterorhabditis* spp. was verified on all islands except Molokai. *Heterorhabditis indica* was first discovered in Hanalei, Kauai in a survey conducted by Hara *et al.* (1991). The species was later described by Gardner *et al.* (1994) as *H. hawaiiensis* and sequenced by Adams *et al.* (1998). Nguyen and Hunt (2007) classified *H. hawaiiensis* as a junior synonym of *H. indica*. Our sequence comparison showed one nucleotide difference between the two. Little is known about the host range and pathogenicity of *H. indica*. The absence of obvious insect hosts was noted during visual observation of the sampling site. On occasion, pillbugs, cockroaches, and collembola would be detected when collecting the soil samples. Yellow sticky traps left at sites positive for *Heterorhabditis* were inconclusive. The primary hosts of these endemic strains are unknown as is how they persist in a somewhat desolate environment. This study confirms EPN do persist in these locations as we were able to recover *Heterorhabditis* from five of the same sites Hara *et al.* (1991) sampled 23 years ago.

It is interesting to note that the *H. indica* sequence alignments were not only identical among specimens across the Hawaiian Islands but also had 100% identity to specimens recovered from Midway Island. This lends strong support to the theory presented by Hara *et al.* (1991) that *Heterorhabditis* may have been brought to Hawaii in the ballasts of ships. Poinar (1990) considered the importation of potted plants, introduction of the nematodes by growers, and the dumping of ballasts from early sailing ships as reasons for the existence of *Steinernema feltiae* and *S. carpocapsae* in Australia and New Zealand. Pimentel *et al.* (2005) speculated that 95% of invasive species to the U.S. were accidental human introductions, many by plants or soil and water ballasts. Mauléon *et al.* (2006) supported Poinar's (1993) theory that *H. indica* could have descended from a marine nematode and demonstrated that crustaceans found near sampling sites could be alternative hosts.

Once established in a new habitat, further distribution of EPN can occur by adult insects (Timper *et al.*, 1988) and potentially from humans moving sand inadvertently or deliberately, especially from beach parks where *Heterorhabditis* was recovered (Hara *et al.*, 1991). It would be worthwhile to conduct further surveys to determine how far ranging the distribution of these endemic Hawaiian strains has become. In preliminary tests, thirteen samples taken from coffee farms on the mountain slopes above positive coastal sites on Kauai and Hawaii were negative for EPN.

Infectivity studies demonstrated *H. indica* to be more effective than *Heterorhabditis* sp. at causing mortality in *G. mellonella*. *Heterorhabditis indica* is highly pathogenic with mortality of infected hosts occurring within 24 to 48 hr with as little as 1 IJ/larva. *Heterorhabditis* sp. has shown promise in other studies by infecting alternate life stages or different host insects than *H. indica* (Myers, unpublished data). Although the results may not correlate directly to infectivity of other species, it warrants further investigation into the potential of these endemic strains for use in insect pest control.

Georgis *et al.* (2006) promoted the evaluation of multiple EPN species and strains when developing a biological control program. Host susceptibility and adaptation of nematodes to environmental conditions especially temperature can vary. Soil temperatures ranged from 21°C to 23°C in locations where *Heterorhabditis* spp. was recovered whereas *H. indica* was found at temperatures of 23°C – 26°C. Since native habitats determine an EPN's temperature tolerance (Kaya, 1990), it would be prudent to utilize endemic strains when possible. The endemic strains recovered in this survey hold promise for controlling invasive insect pests and persisting after application when utilized in Hawaiian agricultural systems.

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