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Natural Occurrence of Entomopathogenic Nematodes (Rhabditida: *Steinernema, Heterorhabditis*) in the Azores

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Abstract: A soil survey for entomopathogenic nematodes was conducted throughout the nine islands of the Azorean archipelago. Forty-six out of 1,180 samples (3.9%) were positive, with *Heterorhabditis* spp. isolated from 30 sites on six islands and *Steinernema* spp. isolated from 16 sites on three islands. São Miguel and Terceira Islands were positive for both genera, and Pico Island was positive only for *Steinernema*. Entomopathogenic nematodes were found from sea level up to 750 m. Seventy percent of the samples positive for *Heterorhabditis* were collected below 150 m, whereas 62.5% of the samples positive for *Steinernema* were collected above 300 m. *Heterorhabditis* was not isolated above 450 m. *Steinernema* was collected mostly in loamy-sand and sandy-loam soils with a pH below 6, whereas *Heterorhabditis* was mostly collected in sandy and loamy-sand soils with pH higher than 6. *Steinernema* and *Heterorhabditis* were found in cropland, orchards, and pastures, while *Heterorhabditis* was found also in woodland and native vegetation.

Key words: Azores, biological control, entomopathogenic nematodes, *Heterorhabditis* spp., Insecta, island, natural occurrence, *Steinernema* spp., survey.

Entomopathogenic nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae are important biological control agents of several insect pests (Begley, 1990; Klein, 1990; Laumond et al., 1979; Poinar, 1979). In the Azores, these nematodes have been assayed against the Japanese beetle, *Popillia japonica* (Lacey et al., 1993; Lacey et al., 1994; Simões et al., 1994), and against the armyworm, *Mythimna* (*Pseudaletia*) *unipuncta*. These two insects are major pests of pastures that support the cattle-breeding and diary industries. These industries constitute the main sources of income for the archipelago.

The first laboratory and field trials with steinernematids and heterorhabditids against Japanese beetle grubs in very limited areas of Terceira Island were conducted with strains of *Steinernema carpocapsae, S. glaseri,* and *Heterorhabditis bacteriophora* from the United States and France (Simões et al., 1994). The results from those small-scale tests showed that these nematodes controlled the Japanese beetle. However, their use would require extensive introductions of exotic nematodes, which could upset the natural fragility of the island environment. This constraint lead to the development and implementation of an extensive survey program for the isolation of EPNs in the Azorean archipelago as a part of a large program for the survey of local endemic biological control agents that could be useful for insect pest control in these islands.

Soil-dwelling nematodes in the Azores are poorly characterized. Other than identification of some plant-parasitic nematodes (Sturhan, 1973) and some animal parasites of the orders Strongylida, Ascaridida, Oxyurida, and Spirurida (Afonso-Roque, 1989), there are no known references to other representatives of the phylum Nemata in the Azores. The objective of this study was to survey the nine islands of the archipelago to determine the occurrence of *Steinernema* and *Heterorhabditis* in relation to altitude and environmental factors, including soil type and vegetation, and to isolate strains of these nematodes for use against appropriate insect pests.

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Materials and Methods

A soil sampling was conducted in the nine islands of the Azorean archipelago for entomopathogenic nematodes survey. Samples were taken at different altitudes from sea level to 1,100 m and within the diverse plant communities of the islands (croplands, orchards, pastures, woodlands, and native vegetation, including endemic plants such as green heather, *Erica azorica,* and Azorean juniper, *Juniperus brevifolia*). The survey was conducted on Pico in June 1991, on São Jorge in June 1992, on Faial in June 1993, on Terceira in June 1994, on Santa Maria in November 1994, on Flores and Corvo in February 1995, on Graciosa in April 1995, and on São Miguel from April 1992 to February 1995. In all, 1,180 sites were sampled for EPN throughout the archipelago (Table 1). At each sampling site, in an area of approximately 500 m^2, 10 subsamples (10 to 15 cm deep) of about 150 cm^3 of soil were taken with a shovel. The subsamples were pooled and ca. 1 kg of soil was placed in a plastic bag and transported to the laboratory. Site location, date, elevation, and associated vegetation were recorded. Before the next sample was taken, the shovel was washed with 70% alcohol.

In the laboratory, soil samples were bioassayed with the ''*Galleria* trap'' method (Bedding and Akhurst, 1975). Each soil sample was placed in a 1-liter container along with 10 final instar *Galleria mellonella* and incubated at 23 °C for 1 week. Each day, the containers were inverted to encourage maximal contact of *G. mellonella* with the soil. After incubation the insects were removed and examined for mortality. Dead insects from each sample were rinsed and placed on a moist filter paper in a petri dish for 3 to 4 days. *Galleria mellonella* larvae showing signs of parasitism by steinernematids or heterorhabditids (Poinar, 1979) were transferred to a modified White trap (White, 1929) to collect the infective juveniles (IJ). Living insects from each site were also rinsed and placed on a filter paper in a petri dish for 3 to 4 days at 23 °C and then observed for parasitism. When more than 200 IJs were recovered, the pathogenicity of the harvested IJs was tested by exposing them to five final instar *G. mellonella.* If fewer than 200 IJ were available, 5 IJ were surface-sterilized in sterile Ringer solution with 10% sodium hypochloride for 10 minutes. Then they were rinsed 3 times with sterile Ringer solution, finally, suspended in 10 µl of sterile Ringer solution, and injected into a *G. mellonella* larva. Controls were performed with Ringer solution. Injected *G. mellonella* were incubated as 23 °C to observe mortality and the presence of IJ. Parasitized *G. mellonella* were placed in a White trap to collect the IJ, which were stored at 10 °C in distilled water. *Steinernema* and *Heterorhabditis* were identified by life cycle and morphology of the IJ and adults (Poinar, 1990). The collected isolates are part of the Azorean Entomopathogen Culture Collection (AzECC) and are currently stored in water at 10 °C and passed through *G. mellonella* every 6 months.

Table 1. Positive samples for *Steinernema* and *Heterorhabditis* spp. in the Azorean archipelago.

			Positive samples			
		No. of	*Steinernema*		*Heterorhabditis*	
Island	Area (km^2)	tested samples	No.	Percent	No.	Percent
Santa Maria	97	85	0		1	(1.2)
São Miguel	757	259	10	(3.9)	12	(4.6)
Terceira	402	230	4	(1.7)	5	(2.2)
São Jorge	246	173	0		7	(4.1)
Graciosa	62	72	0		3	(4.2)
Pico	433	94	2	(2.1)	0	
Faial	172	142	0		2	(1.4)
Flores	142	113	0		0	
Corvo	17	12	0		0	
	2,328	1,180	16	(1.4)	30	(2.5)

Soils positive for EPN were analysed for pH, organic matter, and soil texture. The analyses were conducted at the Soil Analysis Laboratory of the Department of Agricultural Sciences, University of Azores, Terceira.

The distribution of positive samples was analyzed for altitude, plant communities, and type soil of each site. Data are reported as means and standard errors, and were analyzed with contingency tables and Chi squared tests, $P = 0.05$.

Results

Of the 1,180 soil samples taken on the nine islands from 1991 to 1995, 3.9% were positive for EPN (Table 1). *Heterorhabditis* spp. were collected from 30 samples on six islands: Santa Maria (1), São Miguel (12), Terceira (5), São Jorge (7), Graciosa (3), and Faial (2). *Steinernema* spp. were isolated from 16 samples on three islands: São Miguel (10), Terceira (4), and Pico (2) (Fig. 1). Nine *S. carpocapsae* and one *S. glaseri* were identified in the genus *Steinernema* and 19 *H. bacteriophora* in the genus *Heterorhabditis* (Table 2). The identification of the other *Steinernema,* if not concluded, seems to be *S. carpocapsae*. Also, the isolates of *Heterorhabditis* seem to be *H. bacteriophora*. Both genera were present on São Miguel and Terceira islands. Pico Island was positive only for *Steinernema,* whereas Santa Maria, São Jorge, Graciosa, and Faial islands were positive only for *Heterorhabditis*. On São Miguel, the largest island in the archipelago, the greatest number of positive samples was found. *Heterorhabditis* spp. were more abundant in the western part of the island, whereas *Steinernema* spp. were more abundant in the eastern part (Fig. 1). Throughout the archipelago, positive samples for EPNs were taken from sea level up to 750 m (Table 3). Twenty-one positive samples for *Heterorhabditis* were taken below 150 m, representing 70% of the isolations of this genus, whereas 10 isolates of *Steinernema* (62.5% of the isolations of this genus) were collected above 300 m. This distribution in altitude was significantly different (χ^2 = 16.518, df = 4, P = 0.0003). *Steinernema* was found above 450 m, but *Heterorhabditis* was not (Table 3). Most of the positive samples (65.2%) were taken in loamy-sand and sandy-loam, with an equal prevalence of *Steinernema* and *Heterorhabditis*. *Steinernema* were extracted from soils with a pH below 6, whereas *Heterorhabditis* were mostly collected from soils with pH higher than 6 (χ^2 = 19.871, df = 3, P = 0.0005). Distribution of these two genera was not affected by soil organic matter (χ^2 = 8.932, df = 3, P = 0.0628), although we found *Heterorhabditis* in soils with a higher percentage of organic matter than those in which we found *Steinernema* (Table 4).

Entomopathogenic nematodes were present in diverse habitats in the islands. *Heterorhabditis* occurred in all habitat types, but *Steinernema* was not found in woodland or native vegetation habitats (Table 5). Three of 32 (9.4%) soil samples from native vegetation contained *Heterorhabditis*. Pasture and croplands, which occupy the largest area on the islands, produced the smallest percentage of positive samples—3.8% and 3.2%, respectively. Orchards, representing a small part of the cultivated surface in the Azores, had 8.5% of the positive samples.

Discussion

The 3.9% prevalence in the Azores is similar to that reported by Hara et al. (1991) in the Hawaiian Islands (6.8%), Blackshaw (1988) in Northern Ireland (3.8%), Ehlers et al. (1991) in Italy (5%), Shamseldean and Abd-Elgawad (1994) in Egypt (9.5%), and Choo et al. (1995) in Korea (4.6%). However, this rate is lower than those reported by Mràcek and Webster (1993) in western Canada (20%), Liu and Berry (1995) in Oregon (11.8%), Akhurst and Brooks (1984) in North Carolina (20.2%), Stock (1995) in the Pampean region of Argentina (13.2%), Burman et al. (1986) in Sweden (25%), Griffin et al. (1991) in Ireland (10.5%), Gwynn and Richardson (1996) in Great Britain (12.8%), Garcia del Pino (1996) in the Catalonia region of Spain (23.3%), Armarasinghe et al. (1994) in the southwestern coastal zone of Sri Lanka (30%), and Yoshida et al. (1998) in Japan (10%). High prevalences were observed by Mràcek (1980) in Czechoslovakia (36.8%), Sturhan and Liscová (1999) in Slovak Republic (35%), and Hominick and Briscoe (1990) in England (48.6%).

Steinernema and *Heterorhabditis* were found only on the eastern and central groups of the Azores, which are closest to Europe and Africa. Most of the plant and animal species found on the archipelago were introduced from these continents (Sousa, 1985). *Heterorhabditis* was present on six of the nine islands, whereas *Steinernema* was found only

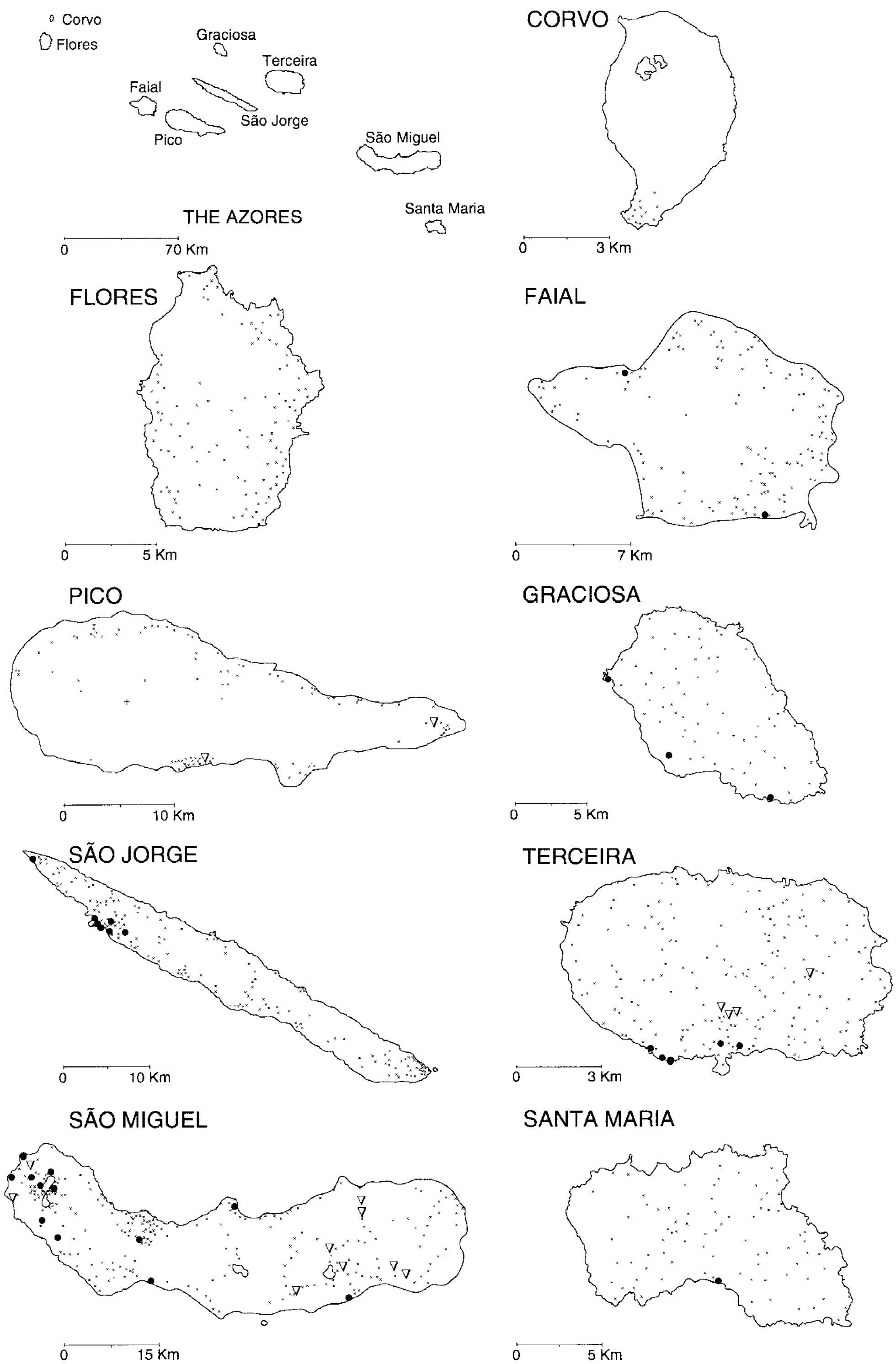

FIG. 1. Geographical distribution of the sampling sites (x) in the nine islands of the Azores with the identification of the positive samples for entomopathogenic nematodes ([∇] *Steinernema;* [●] *Heterorhabditis*).

TABLE 2. Locality, altitude, and habitat in the Azorean archipelago where *Steinernema* and *Heterorhabditis* spp. were isolated.

Sample*	Locality	Altitude (m)	Habitat	Nematode	Reference number for isolate
SMa81	Praia Formosa	5	pasture	*Heterorhabditis* sp.	Az24
SM02	Pico de Lima	225	woodland	*H. bacteriophora*	Az35
SM50	Península	275	cropland	*H. bacteriophora*	Az38
SM66	Cerrado da Ladeira	275	pasture	*H. bacteriophora*	Az32
SM68	Túnel (Sete Cidades)	275	pasture	*H. bacteriophora*	Az36
SM72	Lomba do Vasco	425	pasture	*H. bacteriophora*	Az37
SM78	Rabo de Asno	215	orchard	*H. bacteriophora*	Az141
SM80	Candelária	125	cropland	*H. bacteriophora*	Az142
SM81	Ginetes	125	cropland	*S. carpocapsae*	Az143
SM84	Ponta do Escalvado	50	pasture	*H. bacteriophora*	Az144
SM86	Ponta dos Mosteiros	10	pasture	*H. bacteriophora*	Az145
SM88	Lombinha (Mosteiros)	250	cropland	*S. carpocapsae*	Az146
SM118	Furnas	320	cropland	*S. carpocapsae*	Az149
SM145	Ribeira Quente	20	orchard	*Heterorhabditis* sp.	Az147
SM154	Praia do Pópulo	5	woodland	*H. bacteriophora*	Az148
SM170	Pico do Ferro	580	pasture	*Steinernema* sp.	Az154
SM176	Salto do Cavalo	645	pasture	*Steinernema* sp.	Az155
SM187	Faial da Terra	300	pasture	*S. carpocapsae*	Az150
SM188	Lomba do Alcaide	260	pasture	*S. carpocapsae*	Az151
SM200	Cassepe da Costa	450	pasture	*S. carpocapsae*	Az152
SM201	Cassepe da Costa	395	pasture	*S. carpocapsae*	Az153
SM229	Furnas	380	pasture	*Steinernema* sp.	Az157
SM247	Ribeirinha	5	cropland	*H. bacteriophora*	Az156
T3	Igreja Velha	5	pasture	*Heterorhabditis* sp.	Az161
T4	Negrito	5	pasture	*Heterorhabditis* sp.	Az162
T5	São Bartolomeu	20	orchard	*Heterorhabditis* sp.	Az163
T149	Agualva	200	pasture	*S. carpocapsae*	Az20
T181	Figueiras Pretas	100	cropland	*Heterorhabditis* sp.	Az164
T201	Junco	300	pasture	*Steinernema* sp.	Az165
T202	Achada	300	pasture	*Steinernema* sp.	Az166
T204	Achada	300	pasture	*Steinernema* sp.	Az167
T205	Carreirinha	50	woodland	*Heterorhabditis* sp.	Az168
SJ4	Ponta dos Rosais	250	native vegetation	*H. bacteriophora*	Az28
SJ29	Morro Grande	25	cropland	*H. bacteriophora*	Az29
SJ30	Morro Grande	50	pasture	*H. bacteriophora*	Az34
SJ31	Fonte das Eiras	25	pasture	*H. bacteriophora*	Az30
SJ76	Cais da Queimada	25	native vegetation	*H. bacteriophora*	Az33
SJ140	Santo Amaro	275	cropland	*H. bacteriophora*	Az31
SJ163	Velas	50	cropland	*Heterorhabditis* sp.	Az169
G14	Alto do Sul	40	orchard	*Heterorhabditis* sp.	Az158
G42	Serra Branca	330	pasture	*Heterorhabditis* sp.	Az159
G46	Porto Afonso	20	pasture	*Heterorhabditis* sp.	Az160
P1	São João	10	orchard	*S. carpocapsae*	Az27
P53	Piedade	250	cropland	*S. glaseri*	Az26
F87	Alto da Laginha	20	pasture	*H. bacteriophora*	Az39
F107	Fajã	10	native vegetation	*H. bacteriophora*	Az140

* Samples notation indicates the island of survey, as follows: SMa = Santa Maria Island, SM = São Miguel Island, T = Terceira Island, SJ = São Jorge Island, G = Graciosa Island, P = Pico Island, and F = Faial Island.

on three. The distribution of EPN within the archipelago does not seem to be an artifact resulting from the number of samples taken from each island because on São Miguel, where the sample density was lowest, both genera were found. Preponderance of *Heterorhabditis* over *Steinernema* has been also reported for the Hawaiian Islands, where 92% of samples were positive for *Heterorhabditis* (Hara et al., 1991), Puerto Rico (Roman and Figueroa, 1995), the French West Indies (E. Mauleon, pers. comm.) to Egypt (Sham-

TABLE 3. Distribution in altitude of positive soil samples for entomopathogenic nematodes (EPN) (*Steinernema, Heterorhabditis* spp.).

Altitude (m)	Number of samples	Positive samples		
		EPN	*Steinernema*	*Heterorhabditis*
0–149	523	23 (4.4%)	2 (0.4%)	21 (4.0%)
150–299	336	11 (3.3%)	4 (1.2%)	7 (2.1%)
300–449	191	9 (4.7%)	7 (3.7%)	2 (1.0%)
450–599	92	2 (2.2%)	2 (2.2%)	0
600–749	30	1 (3.3%)	1 (3.3%)	0
750–1,100	8	0	0	0

seldean and Abd-Elgawad, 1994), and Sri Lanka (Amarasinghe et al., 1994). In contrast, steinernematids dominate in a few surveys in Europe (Burman et al., 1986; Garcia del Pino, 1996; Griffin et al., 1991; Hominick et al., 1995; Steiner, 1996; Sturhan and Liscová, 1999; Yoshida et al., 1998).

Six of the Azorean isolates were used in a comparative characterization assay by means of species-specific satellite DNA. These isolates were confirmed as *S. carpocapsae, S. glaseri,* and *H. bacteriophora* (Grenier et al., 1996a; Grenier et al., 1996b). *Steinernema carpocapsae* appears to have a global distribution. In Europe this species is dominant in Italy (Ehlers et al., 1991), France (Grenier et al., 1996a; Poinar, 1990), and Spain (Garcia del Pino, 1996). *Heterorhabditis bacteriophora* is also present on all continents except Antarctica (Grenier et al., 1996b; Hominick et al., 1996). In Europe this species is located mostly in the southern countries, which could explain its presence in the Azores. In contrast, in Ireland and Great Britain, where temperatures are lower, only *H. megidis* is found. The presence of *S. glaseri* in the Azores accords with this species' wide distribution, which includes North America (Poinar, 1990), South America (Doucet, 1990; Pizano et al., 1985), Europe (Agüera de Doucet and Gabarra, 1994), and Asia (Stock et al., 1995).

Altitude had a clear influence on the distribution of both genera in the islands. *Heterorhabditis* was most abundant from soil samples at lower altitudes, and its relative abundance decreased with altitude. Above 300 m *Steinernema* became the more abundant. In the Hawaiian Islands there was also a distinctly higher prevalence of *Heterorhabditis* at sea level and *Steinernema* above 300 m (Hara et al., 1991). In the Azorean archipelago, soil temperature and humidity depend on altitude and are probably the determinants for the distribution of these nematodes. In the western part of São Miguel Island, which has a lower elevation than the eastern part and, thus, higher soil temperatures, *Heterorhabditis* is the predominant genus. On the eastern side, *Steinernema* is more common. Afonso-Roque (1989) demonstrated a difference in the distribu-

TABLE 4. Distribution by soil type of positive samples for *Steinernema* and *Heterorhabditis* spp.

Nematode	Soil type	Percent positive samples	Percent organic matter (Mean ± SEM)	pH (Mean ± SEM)
Steinernema	Sandy	6.3 (1)	6.1	6.0
	Loamy-sand	43.8 (7)	5.6 ± 1.6	5.4 ± 0.2
	Sandy-Loam	37.5 (6)	5.8 ± 1.1	5.2 ± 0.2
	Loamy	12.5 (2)	11.3 ± 0	5.2 ± 0
Heterorhabditis	Sandy	36.7 (11)	5.5 ± 0.2	6.3 ± 0.2
	Loamy-sand	33.3 (10)	4.4 ± 0.5	6.1 ± 0.3
	Sandy-Loam	23.3 (7)	5.2 ± 1.5	5.6 ± 0.3
	Loamy	6.7 (2)	2.7 ± 0.4	5.9 ± 0.5

TABLE 5. Distribution among habitats of positive samples for entomopathogenic nematodes (EPN) (*Steinernema, Heterorhabditis* spp.).

Habitat	Number of samples tested	Positive samples		
		EPN	*Steinernema*	*Heterorhabditis*
Pasture	633	24 (3.8%)	11 (1.7%)	13 (2.1%)
Cropland	339	11 (3.2%)	4 (1.2%)	7 (2.0%)
Orchard	59	5 (8.5%)	1 (1.7%)	4 (6.8%)
Woodland	75	3 (4.0%)	0	3 (4.0%)
Native vegetation	32	3 (9.4%)	0	3 (9.4%)
Others	42	0	0	0
	1,180	46 (3.9)	16 (1.4)	30 (2.5)

tion of ruminant-parasitic nematodes below and above 200 m, suggesting that the distribution of those nematodes was related to temperature and rainfall in each part of the island. Temperature and rainfall also affect the distribution of insects that potentially could be hosts for these EPN.

Although most *Heterorhabditis* were extracted from soil sampled near sea level, these soils have a low percentage of sand. These soils are very similar to those where *Steinernema* was found. This finding was quite different from that observed in Hawaii (Hara et al., 1991), Ireland (Blackshaw, 1988; Griffin et al., 1991), and Sweden (Burman et al., 1986), where *Heterorhabditis* was found in sandy soils and *Steinernema* in silty soils. However, we observed that *Heterorhabditis* occurred more often in soils with lower organic matter content and more alkaline pH than did *Steinernema*. Steiner (1996) also reported a distribution related to soil pH among *Steinernema* species.

We collected 46 isolates of EPNs representing at least three species. These isolates provide a pool of potential biological agents for the control of important insect pests in the islands without introducing new organisms. The most pressing need is to determine their efficacy against the Japanese beetle and the armyworm.

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