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A survey of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae (Nematoda: Rhabditida) in the north-west of Iran

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Summary – During 2002-2004, a survey of entomopathogenic nematodes was conducted for the first time in Iran throughout the three provinces in the north-west of the country. Soil samples were tested for the presence of steinernematid and heterorhabditid nematodes by baiting with *Galleria mellonella* larvae. Of the 833 soil samples studied 27 were positive for entomopathogenic nematodes (3.2%), with 17 (2.0%) containing *Heterorhabditis* and ten (1.2%) *Steinernema* isolates. Morphological and molecular studies were carried out to characterise isolates. The *Heterorhabditis* isolates were identified as *Heterorhabditis bacteriophora* and *Steinernema* as *Steinernema carpocapsae*, *S. bicornutum* and *S. feltiae*. *Heterorhabditis bacteriophora* was the most common species, which was isolated from 17 sites across the three provinces. *Steinernema feltiae* was the most common species of *Steinernema*, which was isolated from eight sites but in only two provinces. *Steinernema carpocapsae* and *S. bicornutum* were each isolated from only one site. *Steinernema* spp. were isolated mainly from orchards and grasslands but *Heterorhabditis* was isolated mainly from grasslands and alfalfa fields.

Keywords – *Heterorhabditis bacteriophora*, *Steinernema bicornutum*, *Steinernema carpocapsae*, *Steinernema feltiae*.

Entomopathogenic nematodes (EPN) from Heterorhabditidae (Poinar, 1976) and Steinernematidae (Travassos, 1927) families are obligate insect parasites, which can infect and kill a broad range of insect hosts (Kaya & Gaugler, 1993). These nematodes are symbiotically associated with entomopathogenic bacteria *Photorhabdus* (Boemare *et al*., 1993) and *Xenorhabdus* (Thomas & Poinar, 1979). These nematodes have been used successfully as biological control agents of insect pests.

The only life cycle difference between *Heterorhabditis* and *Steinernema* is in the first generation. *Steinernema* species are amphimictic; this means that for successful reproduction, male and female infective juveniles (IJ) must enter the host, whereas *Heterorhabditis* species are hermaphroditic and only one IJ in the host is sufficient for successful reproduction. In the second generation of both nematode genera reproduction is amphimictic (Poinar, 1990). However, a hermaphroditic steinernematid species was isolated from Indonesia by Griffin *et al*. (2001).

Entomopathogenic nematodes have a global distribution (Hominick, 2002). The only continent where they have not been found is Antarctica. However, biotic and abiotic factors cause the distribution EPN to differ across different regions. Factors such as soil texture, temperature and host availability are thought to be important in determining their distribution (Hominick & Briscoe, 1990; Griffin *et al*., 1991; Stock *et al*., 1999).

The current survey is the first to be conducted in Iran, and focused on three of the 30 provinces, East Azarbaijan, West Azarbaijan and Ardabil. The goal of this study was to survey entomopathogenic nematodes from the families Heterorhabditidae and Steinernematidae in the north-west of Iran, to identify the species present and to distinguish their ecosystem, habitat and soil type preferences.

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Materials and methods

Collection of soil samples

In total, 833 soil samples were collected randomly from different cultivated and non-cultivated areas of north-west Iran during 2002-2004. Each soil sample was a composite of 5-20 random sub-samples taken in the same location, but at least 10 m away from each other and to a depth of 30 cm, using a small shovel. Between samples, the shovel was thoroughly rinsed with water and air dried to prevent contamination of the next sampling unit. The soil was thoroughly mixed on a plastic sheet and half of each sample was used for extraction of EPN.

Nematode isolation and propagation

EPN were recovered from soil samples using an insect baiting method, described by Bedding and Akhurst (1975). Ten last instar *Galleria mellonella* (L.) larvae were placed in a 300 ml jar containing moistened soil obtained from one of the samples and stored at room temperature (25 ± 2°C) for 2 weeks. The traps were checked every two days from the 5th day. Dead larvae from each container were placed in White (1929) traps to collect emerging IJ and were replaced by fresh larvae. To verify the pathogenicity of collected nematodes and to establish new cultures, emerging nematodes were pooled for each sample and used to infect fresh *G. mellonella* larvae.

Taxonomic studies – morphological characterisation

For morphological studies, nematodes were examined live or heat killed in Ringer's solution heated to 60°C. All nematodes used in this study were reared in *G. mellonella* larvae. Ten *G. mellonella* larvae were exposed to *ca* 1000 IJ in a Petri dish lined with two moistened filter papers at room temperature (25±3°C). For isolating mature females and males of the first and second generations, the infected larvae were dissected in Ringer's solution 4 and 7 days after infection, respectively.

Heat-killed nematodes were placed in triethanolamin-formalin (TAF) fixative (Kaya & Stock, 1997) and processed to anhydrous glycerine for mounting by a slow evaporation method (Poinar, 1976). Morphology and morphometric studies were conducted using an Olympus BX41 microscope equipped with differential interference elements and drawing tube.

The following characters were measured in males and/or IJ: total body length; maximum body diam.; anal body diam.; excretory pore position; distance from anterior end to base of pharynx; gubernaculum length; spicule length (measured along the curvature in a line along the centre of the spicule); gubernaculum length divided by spicule length (%); distance from anterior end to nerve ring position; ratio a (total body length divided by maximum body diam.); ratio b (total body length divided by distance from anterior end to base of pharynx); ratio c (body length divided by tail length); ratio D (excretory pore position divided by distance from anterior end to base of pharynx); ratio E (excretory pore position divided by tail length); spicule length divided by anal body diam. and tail length (measured with consideration of the extra cuticular sheath of the second-stage juvenile).

Morphological identification was made using taxonomic criteria suggested by Stock and Kaya (1996) and Hominick *et al*. (1997). Additionally, morphological features of males and IJ of representative isolates of each species group were examined using scanning electron microscopy. For this purpose specimens were processed following protocols described by Nguyen and Smart (1995).

Taxonomic studies – molecular characterisation

Extraction of DNA

DNA was extracted from a single IJ with the following method: the nematode was crushed in 15 μl 1× PCR Buffer and transferred to a precooled sterilised 0.2 ml tube containing 10 μl of the same buffer. The tube was incubated at −70°C for 15 min and thawed at 60°C then inoculated with 2 μl of 60 μg ml^{-1} proteinase K. The tube was incubated at 65°C for 2 h, and then heated at 95°C for 15 min. After centrifugation at 16 000 *g* for 15 min, the supernatant containing nematode DNA was collected and stored at −70°C until use.

PCR amplification and sequencing

The ITS and 28s regions were amplified by PCR in a 50 μl reaction containing: 15 μl of worm lysis mix, 5 μl of 10× PCR Buffer, 2 μl of dNTP mix, 1.2 unit of *Taq* DNA polymerase, 1 μl of each primer and double distilled water to final volume. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used in the PCR reaction for amplification of the complete ITS region (Joyce *et al*., 1994). The primers for the amplification of a partial 28S rDNA were 5′-CG ATAGCGAACAAGTACCGAGAG-3′ (forward) and 5′-

CCTGCTCAGGCATAGTTCACCATC-3′ (reverse) (Qiu *et al*., 2004).

Amplified products were purified using a Qiagen Purification kit (Qiagen, Leusden, The Netherlands). Purified DNA was sequenced with Barcode Sequencing Service of AGOWA Inc. The DNA sequences were edited with Chromas 2.01. The sequences are deposited in Genbank (Table 1).

Sequence alignment and phylogenetic analysis

The DNA sequences were aligned using Clustal X 1.64 (Thompson *et al*., 1997) with the (ITS1-5.8S-ITS2 for *Heterorhabditis* spp. and 28S for *Steinernema* spp.) sequences of other *Heterorhabditis* and *Steinernema* species obtained from GenBank. For *Heterorhabditis* analysis, sequences of the following taxa were used: *Heterorhabditis bacteriophora* Poinar, 1976 (EF043438); *H. indica* Poinar *et al*., 1992 (EF043445); *H. megidis* Poinar *et al*., 1987 (EU163272); *H. downesi* Stock *et al*., 2002 (EF043442); *H. marelatus* Liu & Berry, 1996 (EF043441); *H. zealandica* Poinar, 1990 (EF530041) and *Cervidellus alutus* (Siddiqi, 1993) as the outgroup taxon (AF331911).

For *Steinernema* analysis we used some of the sequences which have been deposited in GenBank by Stock *et al.* (2001) including *S. affine* (Bovien, 1937) (AF331899), *S. ceratophorum* Jian, Reid & Hunt, 1997 (AF331888), *S. cubanum* Mráček, Hernandez & Boemare, 1994 (AF331889), *S. intermedium* (Poinar, 1986) (AF331909), *S. karii* Waturu, Hunt & Reid, 1997 (AF331902), *S. monticolum* Stock, Choo & Kaya, 1997 (AF331895), *S. oregonense* Liu & Berry, 1996 (AF331891), *S. riobrave* Cabanillas, Poinar & Raulston, 1994 (AF331893), *S. scapterisci* Nguyen & Smart, 1990 (AF331898), *S. feltiae* (Filipjev, 1934) (AF331906), *S. carpocapsae* (Weiser, 1955) (AF331900), *S. bicornutum* Tallosi, Peters & Ehlers, 1995 (AF331904) and *Caenorhabditis elegans* (Maupas, 1899) as the outgroup taxon (X03680).

Table 1. *Location, vegetation and soil type of sites with entomopathogenic nematodes of the genera* Heterorhabditis *and* Steinernema.

Species (GenBank Accession No.)	Isolate	Province	Locality	Vegetation	Soil type
H. bacteriophora (EU598222)	IRA1	East Azarbaijan	Jolfa	Alfalfa	sandy loam
H. bacteriophora (EU598223)	IRA2	East Azarbaijan	Marand	Alfalfa	sandy clay loam
H. bacteriophora (EU516355)	IRA3	East Azarbaijan	Marageh	Alfalfa	sandy loam
H. bacteriophora (EU598224)	IRA5	East Azarbaijan	Marageh	Orchard	clay loam
H. bacteriophora (EU598225)	IRA6	East Azarbaijan	Marand	Orchard	sandy clay loam
H. bacteriophora (EU598226)	IRA8	East Azarbaijan	Malekan	Grassland	clay loam
H. bacteriophora (EU598227)	IRA10	East Azarbaijan	Oskoo	Orchard	sandy clay loam
H. bacteriophora (EU598228)	IRA12	West Azarbaijan	Ormiyeh	Grassland	sandy loam
H. bacteriophora (EU598229)	IRA13	West Azarbaijan	Ormiyeh	Grassland	sandy clay loam
H. bacteriophora (EU598230)	IRA16	East Azarbaijan	Bonab	Grassland	sandy clay loam
H. bacteriophora (EU598231)	IRA19	Ardabil	Meshgin shahr	Grassland	sandy clay loam
H. bacteriophora (EU598232)	IRA24	East Azarbaijan	Ahar	Grassland	sandy loam
H. bacteriophora (EU598233)	IRA26	East Azarbaijan	Ahar	Alfalfa	sandy clay loam
H. bacteriophora (EU598234)	IRA29	East Azarbaijan	Kaleibar	Alfalfa	sandy clay loam
H. bacteriophora (EU598235)	IRA38	East Azarbaijan	Ajab shir	Alfalfa	sandy loam
H. bacteriophora (EU598236)	IRA40	East Azarbaijan	Mianeh	Grassland	sandy loam
H. bacteriophora (EU598237)	IRA41	East Azarbaijan	Bostan abad	Vegetable	sandy loam
S. bicornutum (EU598242)	IRA7	East Azarbaijan	Marand	Alfalfa	sandy clay loam
S. carpocapsae (EU598241)	IRA18	Ardabil	Meshgin shahr	Orchard	sandy clay loam
S. feltiae (EU598243)	IRA17	East Azarbaijan	Varzgan	Grassland	sandy clay loam
S. feltiae (EU598244)	IRA21	East Azarbaijan	Ahar	Grassland	sandy clay loam
S. feltiae (EU598245)	IRA22	East Azarbaijan	Ahar	Grassland	sandy clay loam
S. feltiae (EU598246)	IRA23	East Azarbaijan	Sarab	Cropland	sandy loam
S. feltiae (EU598247)	IRA25	East Azarbaijan	Ahar	Alfalfa	sandy loam
S. feltiae (EU598248)	IRA28	East Azarbaijan	Shabestar	Orchard	sandy clay loam
S. feltiae (EU598249)	IRA30	East Azarbaijan	Heris	Orchard	sandy loam
S. feltiae (EU598250)	IRA34	Ardabil	Meshgin shahr	Orchard	sandy clay loam

Molecular phylogenetic relationships were obtained by equally weighted maximum parsimony (MP) and maximum likelihood (ML) using PAUP* 4.0b8 (Swofford, 1998). MP was performed with a heuristic search with the following setting: one hundred replicates of random taxon addition (RTA), tree-bisection-reconnection (TBR) branch swapping, multiple trees retained, no steepest descent and accelerated transformation. All data were assumed to be unordered, all characters were treated as equally weighted, and gaps were treated as missing data. For ML analysis, the appropriate substitution model of DNA evolution that best fitted the data set was determined by the Akaike Information Criterion with Model Test 3.06 (Posada & Crandall, 1998). Bootstrap analysis with 1000 replicates was conducted as a measure of support for individual clades for MP and ML trees (Phan *et al*., 2003).

Cross-breeding tests

To further confirm the identity of the *Steinernema* isolates, cross-breeding tests were performed between at least one representative isolate of each species group, with known isolates of the species group to which they were provisionally assigned, using the haemolymph hanging drop technique (Poinar, 1967). The following species were used to confirm their identity: *S. feltiae* (4CFMO strain), *S. carpocapsae* (All strain) and *S. bicornutum* (strain Azar4).

For each treatment an individual IJ from each population was transferred into a drop of haemolymph, from *G. mellonella* larvae, on the under surface of the lid of a small Petri dish. The transfer was carried out under a dissecting microscope. Two ml of sterile water was added to each Petri dish and the Petri dishes were sealed with parafilm. After 3 days the male from one isolate was transferred to a drop containing a female of a known species and *vice versa*. Self-cross controls were also conducted in the same way. Fifteen replicates were conducted for each treatment and control. The Petri dishes were then incubated at 20°C for 2 weeks. The reproduction of the nematodes was observed and recorded during the experimental period. The experiment was repeated twice.

Results

Geographical sources of nematodes

Entomopathogenic nematodes were recovered from 27 sites (3.2%); 17 sites (2.0%) were positive for the occurrence of heterorhabditids and ten sites (1.2%) for steinernematids (Fig. 1; Table 1). Based on morphological and molecular characterisation one species of *Heterorhabditis* and three distinct species groups of *Steinernema* were found. All *Heterorhabditis* isolates were identified as *H. bacteriophora* and *Steinernema* species groups as *S. carpocapsae*, *S. bicornutum* and *S. feltiae*. Among steinernematids, *S. feltiae* was recovered from eight sites (80% of the steinernematids found) but *S. carpocapsae* and *S. bicornutum* were each isolated from only one site. No sample yielded more than one species of entomopathogenic nematode.

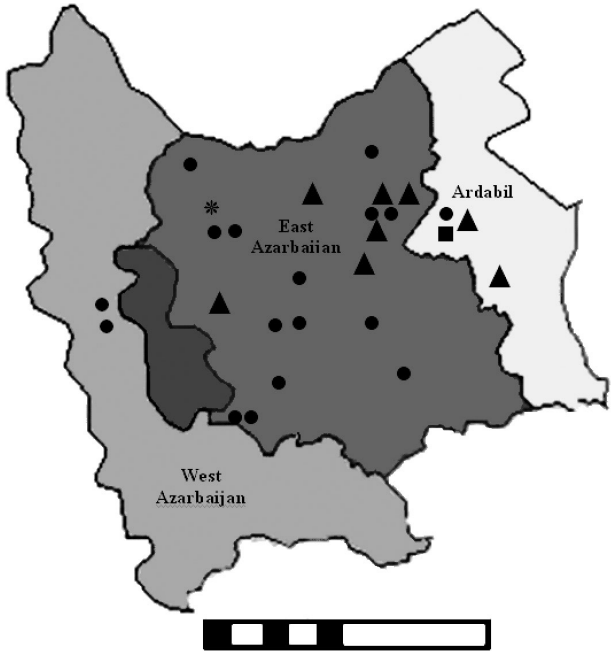

Fig. 1. *Distribution of entomopathogenic nematodes in the north-west of Iran. The names represent the three provinces (map scale 250 km).* ● Heterorhabditis bacteriophora*;* ■ Steinernema carpocapsae*;* * S. bicornutum*;* ▲ S. feltiae.

The majority of *H. bacteriophora* isolates were found in grasslands and alfalfa fields (grasslands 41.2%, alfalfa fields 35.3%). Orchards (mainly apple) and vegetable plots yielded the remaining positive samples. *Steinernema feltiae* was isolated mainly from orchards and grasslands in similar proportion (37.5%) followed by alfalfa fields and cereals (12.5%). *Steinernema carpocapsae* and *S. bicornutum* were isolated from an orchard and alfalfa field, respectively (Table 1).

In terms of species diversity, orchards and alfalfa fields displayed the greatest diversity of species: *H. bacteriophora*, *S. feltiae* and *S. carpocapsae* in orchards, and the

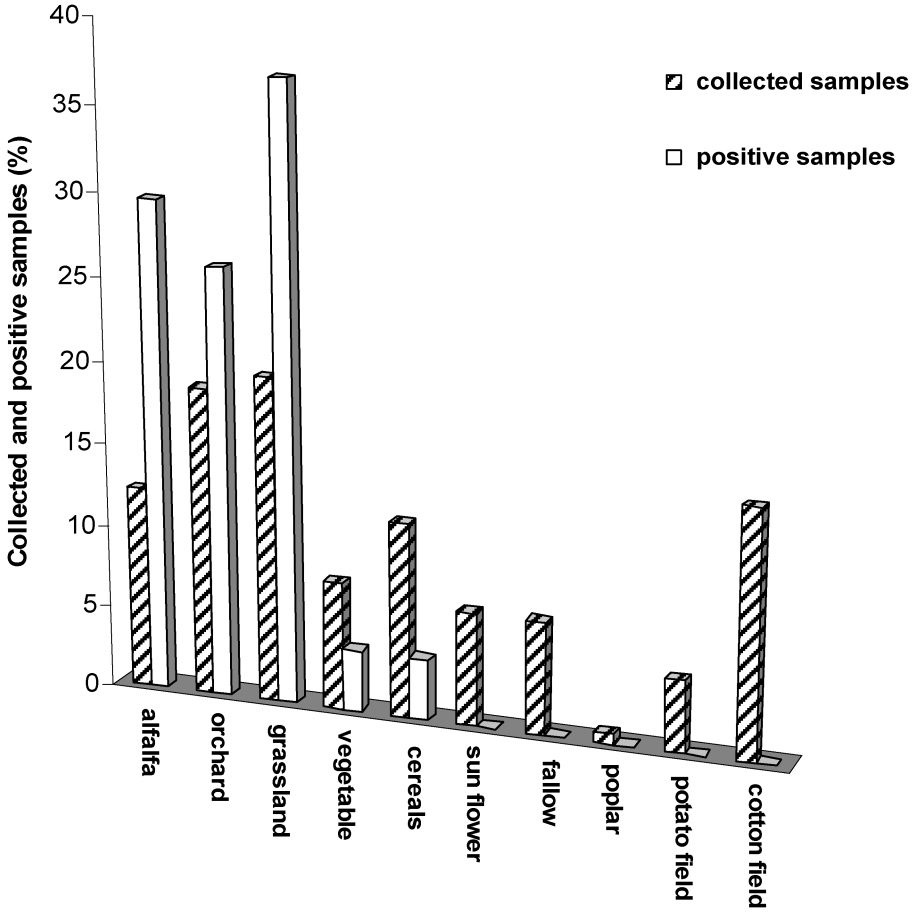

Fig. 2. *Percentages of soil samples from different habitats collected and positive for entomopathogenic nematodes. Total number of samples taken: 833. Total number of positive samples: 27.*

alfalfa fields were represented by *H. bacteriophora*, *S. feltiae* and *S. bicornutum*.

In terms of EPN recovery there was a significant difference at $P < 0.05$ between habitats when tested with chi-square ($\chi^2 = 20.58$, df $= 9$). Grassland showed the highest recovery rate (1.2%) followed by alfalfa field (0.96%), orchards (0.84%), cropland (0.12%) and vegetable (0.12%) (Fig. 2). The habitats sunflower, fallow, poplar, potato field and cotton field were completely negative for nematodes.

The survey was only conducted in 8 months of the year because in other months sampling was not possible due to weather conditions. In every month of sampling we found sites with EPN. A chi-square test for association between sampling period and frequency of nematode recovery showed significance at $P < 0.01$ ($\chi^2 = 24.49$, df $= 7$). EPN recovery was highest in May (40.7%) followed by April (25.9%), October (11.1%), September (7.4%) and June, July, August and November (3.7%) (Fig. 3).

A chi-square test for association between soil texture and frequency of nematode recovery showed significance at $P < 0.01$ ($\chi^2 = 16.18$, df $= 3$). EPN recovery in sandy-clay-loam soil (55.5%) was higher than in other soil textures. There were no positive samples from clay soil (Fig. 4).

Discussion

The present study records for the first time the occurrence of EPN in Iran. Iran, which covers an area 1 648 195 km^2, has a variety of different climatic regions. It stretches from Khazar Lake (the Caspian Sea) in the north, bordered by forest with moderate temperatures and

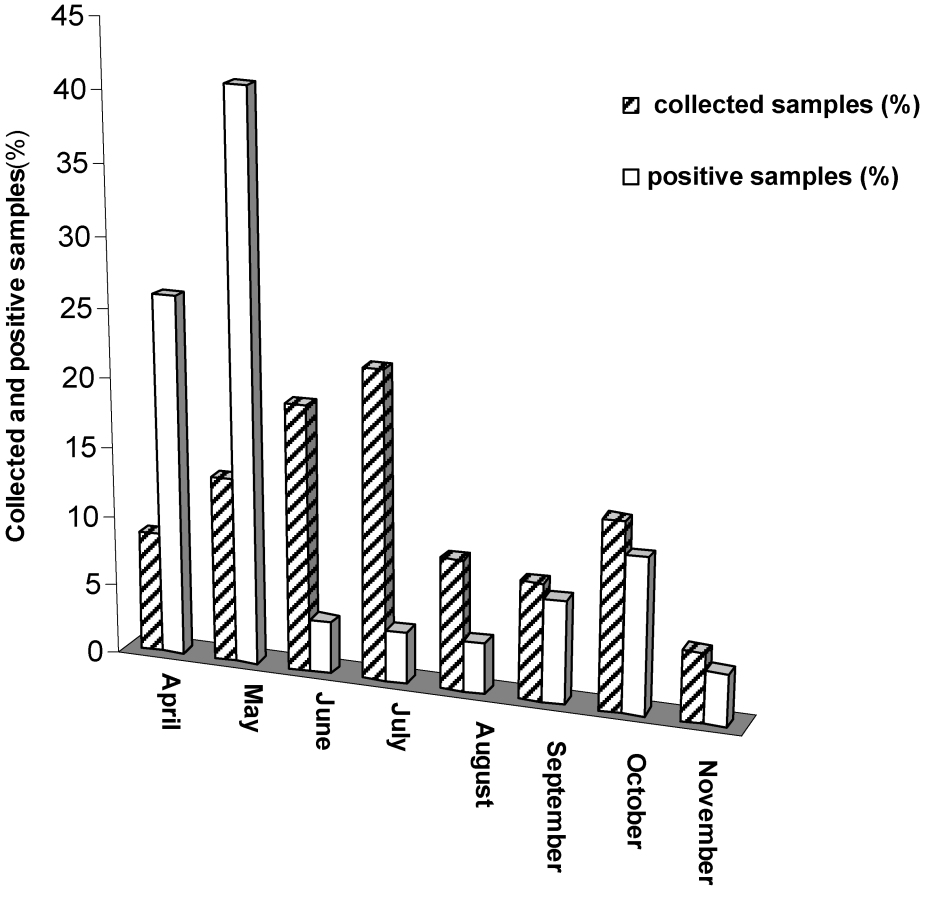

Fig. 3. *Percentages of soil samples and positive for entomopathogenic nematodes in different months. Total number of samples taken: 833. Total number of positive samples: 27.*

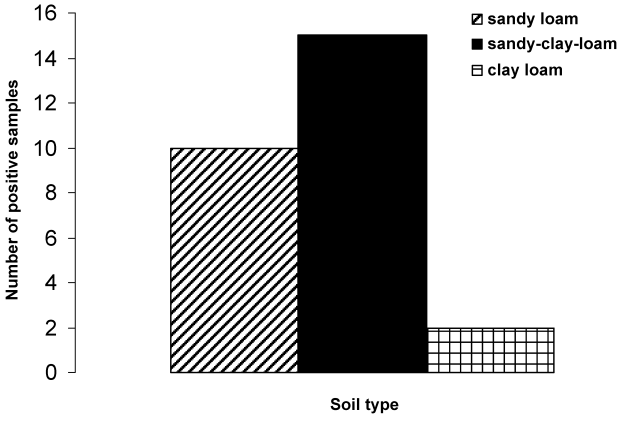

Fig. 4. *Recovery of entomopathogenic nematodes from different soil types.*

high humidity, to the Persian Gulf in the south, which has high temperature and humidity. The multiplicity of climatic zones makes it suitable for a wide variety of organisms. More than 2000 plant species grow in Iran. With such high plant diversity, which also results in a high diversity of insects, it is reasonable to suppose that Iran could harbour a number of different species and strains of EPN.

The north-west of Iran is a mountainous region with a cool continental climate. The region shares a border with Turkey, where three surveys have been conducted with the isolation of several known EPN species and at least one new species (Hazir *et al*., 2003a). Özer *et al*. (1995) recovered *S. feltiae* from the coast of the Black Sea, and Susurluk *et al*. (2002) isolated *H. bacteriophora*, a *Heterorhabditis* sp. and *S. feltiae* from Ankara. Hazir *et al*. (2003a) isolated *H. bacteriophora*, *S. feltiae*, *S. affine* and an undescribed *Steinernema* species from Turkey. Hazir *et al*. (2003b) subsequently described the new species as *S. anatoliense*. In their survey the most common species

was *S. feltiae*, which was isolated from ten sites in six regions, followed by *H. bacteriophora* from seven sites in seven regions, *S. affine* from four sites in two regions and *S. anatoliense* from one site.

The first goal of the present work was to identify native species of EPN. In biological control programmes, using native biocontrol agents is often preferable to using exotic ones, since they are adapted to local conditions. Novel species and strains may have superior traits, making them suitable for direct commercial exploitation or as a source of genetic diversity for breeding improved strains (Choo *et al*., 1995).

Although EPN were recovered at a low rate (3% of sites) in our study, we isolated four species from a somewhat small region with variable climate and vegetation. One reason for this low recovery rate could be that only *Galleria mellonella* was used as a trap insect, and it may not be an appropriate host for all EPN species/strains (Spiridonov & Moens, 1999). Using only room temperature for baiting the soil samples could be another important reason. However, such a low recovery rate is not unusual and has been recorded in some other surveys conducted in other regions of the world (Choo *et al*., 1995; Rosa *et al*., 2000; Hazir *et al*., 2003a).

The habitats with high EPN recovery, such as grassland, orchard and alfalfa, were ones in which chemical control usage was low. We conclude that agricultural regions with high chemical pest control input have a lower recovery rate of EPN compared with areas with less chemical use. Chemical pesticides can impact on EPN both directly and indirectly, by reducing host abundance.

Heterorhabditis bacteriophora was the most common and widely distributed entomopathogenic nematode in the north-west of Iran, followed by *S. feltiae*. Both of these species are widely distributed in the world (Hominick *et al*., 1996; Adams *et al*., 2006). *Steinernema feltiae* is widely distributed in temperate regions, whilst *H. bacteriophora* typically occurs in regions with continental and mediterranean climates (Hominick, 2002). *Heterorhabditis* appears to be the dominant EPN genus in mediterranean countries of the Middle East, such as West Bank Palestinian Territory and Egypt (Glazer *et al*., 1991; Iraki *et al*., 2000; Salama & Abd-Elgawad, 2001).

The north-west of Iran is a mountainous region with a temperate-cold climate in which the annual temperature can range from −20 to 39°C (Table 2). We found *H. bacteriophora* at 17 sites with four different habitats (alfalfa, orchard, vegetable and grassland) and with some differences in climate and, therefore, it is likely that the respective isolates may have different ecological traits. For example, there is a large difference between Mianeh and Kaleibar in the number of days with maximum temperature equal to 30°C and above, 128 and 14, respectively (Fig. 5); therefore, the two *H. bacteriophora* strains from these localities may have different heat tolerances and survival rates. Different nematode species/strains have different temperature optima and ranges for activity and survival (*e.g*., Grewal *et al*., 1994; Griffin & Downes, 1994).

Table 2. *Characteristics of some of the sampling localities positive for entomopathogenic nematodes.*

Locality	No. of EPN isolates	No. of EPN species	Sampling time	Elevation (m)	Average of minimum temperature (°C)	Average of maximum temperature (°C)	No. of days with maximum temperature equal to 30°C and above	No. of days with minimum temperature equal to 0°C and below	Temperature records lowest (°C)	Temperature records highest (°C)	Annual rainfall (mm)
Ahar	5	2	Apr 2003	1390	5.5	15.8	20	104	−14	31.2	274.3
Kaleibar	1	1	June 2003	1796	2.8	14.2	14	*	*	*	*
Marageh	2	1	Sep 2003	1477.7	8.3	19.2	84	84	−8.6	39.8	249.2
Meshgin shahr	3	3	May 2003	1568.5	5.2	14.4	6	114	−10.6	37.4	362.3
Mianeh	1	1	June 2003	1110	7.6	20.9	128	82	−13.8	38.6	276.6
Ormiyeh	2	1	June 2003	1313	5.5	17.8	58	110	−9.2		296.1
Oskoo	1	1	July 2002	1361	7.7	18.8	94	79	−16	33.8	218.8
Sarab	1	1	Aug 2004	1682	1.8	15.7	27	149	−20.6	33.6	287.5

* Data not available.

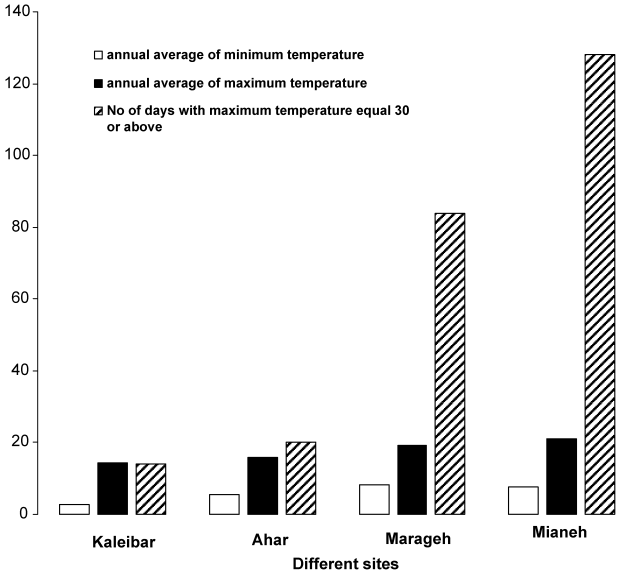

Fig. 5. *Some meteorological factors at four sites from which* Heterorhabditis bacteriophora *was recovered.*

Steinernema carpocapsae and *S. bicornutum* were each found at only one site, in orchard and alfalfa field, respectively. The reason for the low recovery of these two species is not known. *Steinernema carpocapsae* appears to have a global distribution but sometimes at low frequency as in central and northern Europe (Hominick, 2002). This species, originally described as indigenous to the Czech Republic, was not recovered by Mráček and Becvar (2000) even though they sampled intensively in suitable habitats, including the type locality. In an extensive survey of Germany, *S. carpocapsae* was found at only one of 1193 sites (Sturhan, 1999), a recovery rate similar to that of the present survey.

The natural hosts of EPN in north-west Iran are unknown, but alfalfa weevil is the major insect pest in alfalfa fields and codling moth in orchards. Because of the frequent occurrence of *H. bacteriophora* and other EPN in these two habitats it is possible that these insects are amongst their natural hosts. Accordingly, the isolated steinernematids and heterorhabditids will be evaluated in the future for their control potential against these and other target pests.

In our study, the month in which samples were collected had a statistically significant effect on the proportion that was positive for EPN. The highest proportion of positive samples were taken in spring (April and May) followed by autumn (September, October) and then summer. As we conducted sampling only once per site, and not during successive seasons, our results may be due partly to the characteristics of the particular regions and sites that were chosen for sampling in each month. However, the pattern of EPN recovery is, as expected, based on seasonal weather patterns. In north-west Iran, winters are cold with heavy snowfall and subfreezing temperatures during December and January. Spring and autumn are relatively mild, while summers are dry and hot. Most of the relatively low annual rainfall occurs from October to April. Spring, therefore, is the season when temperature and moisture are optimum for EPN activity and survival, and populations of potential hosts are also high. With the onset of the hot, dry season, the EPN populations declined until autumn, when precipitation and soil moisture increased again. Brown and Gaugler (1997) demonstrated that drought can markedly delay EPN emergence from host cadavers. There is some evidence of seasonality of occurrence in different EPN surveys (Mráček, 1980; Akhurst & Bedding, 1986; Blackshaw, 1988; Hominick & Briscoe, 1990). By contrast, Campbell *et al*. (1995) and Glazer (1996) reported an absence of seasonal fluctuation in EPN densities. Moisture, temperature and host frequency are the most important abiotic and biotic factors affecting EPN density and distribution, but factors that affect EPN population dynamics are still poorly understood (Půža & Mráček, 2005).

Soil type is one of the most important factors affecting EPN distribution in soil. Generally, soils with high sand and low clay content yield more positive results. In our survey the majority of positive samples were sandy-clay-loam and sandy loam. Similar results were reported by a number of other researchers (*e.g*., Hominick & Briscoe, 1990; Griffin *et al*., 1991; Liu & Berry, 1995; Stock *et al.*, 1999; Hazir *et al*., 2003a). Clay soil yielded negative results in our survey. Nematodes have a poor survival rate in clay soil due to smaller soil pores.

Based on these findings in the northwest of the country and the multiplicity of climatic zones in Iran, we predict that more species and possibly new species may be discovered in future surveys of Iran.

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References

ADAMS, B.J., FODOR, A., KOPPENHÖFER, H.S., STACKEBRANDT, E., STOCK, S.P. & KLEIN, M.G. (2006). Biodiversity and systematics of nematode-bacterium entomopathogens. *Biological Control* 37, 32-49.

AKHURST, R.J. & BEDDING, R.A. (1986). Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. *Journal of the Australian Entomology Society* 25, 241-244.

BEDDING, R.A. & AKHURST, R.J. (1975). A simple technique for detection of insect parasitic rhabditid nematodes in soil. *Nematologica* 21, 109-110.

BLACKSHAW, R.P. (1988). A survey of insect parasitic nematodes in Northern Ireland. *Annals of Applied Biology* 113, 561-565.

BOEMARE, N.E., AKHURST, R.J. & MOURANT, R.G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology* 43, 249-255.

BROWN, I.M. & GAUGLER, R. (1997). Temperature and humidity influence emergence and survival of entomopathogenic nematodes. *Nematologica* 43, 363-375.

CAMPBELL, J.F., LEWIS, E.E., YODER, F. & GAUGLER, R. (1995). Entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) seasonal population dynamics and impact on insect populations in turfgrass. *Biological Control* 5, 598-606.

CHOO, H.Y., KAYA, H.K. & STOCK, S.P. (1995). Isolation of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in Korea. *Japanese Journal of Nematology* 25, 44-51.

GLAZER, I. (1996). Survival mechanisms of entomopathogenic nematodes. *Biocontrol Science and Technology* 6, 373-378.

GLAZER, I., LIRAN, N. & STEINBERGER, Y. (1991). A survey of entomopathogenic nematodes (Rhabditida) in the Negev desert. *Phytoparasitica* 19, 291-300.

GREWAL, P.S., SELVAN, S. & GAUGLER, R. (1994). Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. *Journal of Thermal Biology* 19, 245-253.

GRIFFIN, C.T. & DOWNES, M.J. (1994). Recognition of low temperature active isolates of entomopathogenic nematode *Heterorhabditis* spp. (Rhabditida: Heterorhabditidae). *Nematologica* 40, 106-115.

GRIFFIN, C.T., MOORE, J.F. & DOWNES, M.J. (1991). Occurrence of insect-parasitic nematodes (Steinernematidae, Heterorhabditidae) in the Republic of Ireland. *Nematologica* 37, 92-100.

GRIFFIN, C.T., O'CALLAGHAN, K.M. & DIX, I. (2001). A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes? *Parasitology* 122, 181-186.

HAZIR, S., STOCK, S.P., KAYA, H.K., KOPPENHÖFER, A.M. & KESKIN, N. (2001). Developmental temperature effects on five geographic isolates of the entomopathogenic nematode *Steinernema feltiae* (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology* 77, 243-250.

HAZIR, S., KESKIN, N., STOCK, S.P., KAYA, H.K. & OZCAN, S. (2003a). Diversity and distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Turkey. *Biodiversity and Conservation* 12, 375-386.

HAZIR, S., STOCK, S.P. & KESKIN, N. (2003b). A new entomopathogenic nematode, *Steinernema anatoliense* n. sp. (Rhabditida: Steinernematidae), from Turkey. *Systematic Parasitology* 55, 211-220.

HOMINICK, W.M. (2002). Biogeography. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 115-143.

HOMINICK, W.M. & BRISCOE, B.R. (1990). Occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in British soils. *Parasitology* 100, 295-302.

HOMINICK, W.M., REID, A.P., BOHAN, D.A. & BRISCOE, B.R. (1996). Entomopathogenic nematodes – biodiversity, geographical distribution and the convention on biological diversity. *Biocontrol Science and Technology* 6, 317-331.

HOMINICK, W.M., BRISCOE, B.R., DEL PINO, F.G., HENG, J.A., HUNT, D.J., KOZODOY, E., MRAČEK, Z., NGUYEN, K.B., REID, A.P., SPIRIDONOV, S., STOCK, S.P., STURHAN, D., WATURU, C. & YOSHIDA, M. (1997). Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology* 71, 271-298.

IRAKI, N., SALAH, N., SANSOUR, M.A., SEGAL, D., GLAZER, I., JOHNIGK, S.A., HUSSEIN, M.A. & EHLERS, R.-U. (2000). Isolation and characterization of two entomopathogenic nematode strains, *Heterorhabditis indica* (Nematoda, Rhabditida), from the West Bank, Palestinian Territories. *Journal of Applied Entomology* 124, 271-380.

JOYCE, S.A., REID, A., DRIVER, F. & CURRAN, J. (1994). Application of polymerase chain reaction (PCR) methods to identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.P. (Eds). *Proceeding of Symposium & Workshop, St Patrick's College, Maynooth, Co. Kildare, Ireland*. European Commission, DGXII, Luxembourg, pp. 178-187.

KAYA, H.K. & GAUGLER, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology* 38, 181-206.

KAYA, H.K. & STOCK, S.P. (1997). Techniques in insect nematology. In: Lacey, L.A. (Ed.). *Manual of techniques in insect pathology*. Biological Techniques Series. San Diego, CA, USA, Academic Press, pp. 281-324.

LIU, J. & BERRY, R.E. (1995). Natural distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Oregon soils. *Environmental Entomology* 24, 159-163.

LIU, J. & BERRY, R.E. (1996). *Heterorhabditis marelatus* n. sp. (Rhabditida: Heterorhabditidae) from Oregon. *Journal of Invertebrate Pathology* 67, 48-54.

MRÁČEK, Z. (1980). The use of 'Galleria traps' for obtaining nematode parasites of insect in Czechoslovakia (Lepidoptera; Nematoda, Steinernematidae). *Acta Entomologica, Bohemoslov* 77, 378-382.

MRÁČEK, Z. & BECVAR, S. (2000). Insect aggregations and entomopathogenic nematode occurrence. *Nematology* 2, 297-301.

NGUYEN, K.B. & SMART JR, G.C. (1995). Scanning electron microscope studies of *Steinernema glaseri* (Nematoda: Steinernematidae). *Nematologica* 41, 183-190.

ÖZER, N., KESKIN, N. & KIRBAS, Z. (1995). Occurrence of entomopathogenic nematodes (Steinernematidae: Heterorhabditidae) in Turkey. *Nematologica* 41, 639-640.

PHAN, K.L., SUBBOTIN, S.A., NGUYEN, N.C. & MOENS, M. (2003). *Heterorhabditis baujardi* sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations. *Nematology* 5, 367-382.

POINAR JR, G.O. (1967). Description and taxonomic position of the DD-136 nematode (Steinernematidae; Rhabditoidea) and its relationship to *Neoaplectana carpocapsae* Weiser. *Proceedings of Helminthological Society of Washington* 34, 199-209.

POINAR JR, G.O. (1976). Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida: Heterorhabditidae n. fam.). *Nematologica* 21 (1975), 463-470.

POINAR JR, G.O. (1990). Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, USA, CRC Press, pp. 23-61.

POSADA, D. & CRANDALL, K.A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.

PŮŽA, V. & MRÁČEK, Z. (2005). Seasonal dynamics of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* as a response to abiotic factors and abundance of insect hosts. *Journal of Invertebrate Pathology* 89, 116-122.

QIU, L., FANG, Y., ZHOU, Y., PANG, Y. & NGUYEN, K.B. (2004). *Steinernema guangdongense* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from southern China with a note on *S. serratum* (*nomen nudum*). *Zootaxa* 704, 1-20.

ROSA, J.S., BONIFASSI, E., AMARAL, J., LACEY, L.A., SIMOES, N. & LAUMOND, C. (2000). Natural occurrence of entomopathogenic nematodes (Rhabditida: *Steinernema*, *Heterorhabditis*) in the Azores. *Journal of Nematology* 32, 215-222.

SALAMA, H.S. & ABD-ELGAWAD, M.M. (2001). Isolation of heterorhabditid nematodes from palm tree planted areas and their implications in the Red Palm Weevil control. *Journal of Pest Science* 74, 43-45.

SPIRIDONOV, S.E. & MOENS, M. (1999). Two previously unreported species of steinernematids from woodlands in Belgium. *Russian Journal of Nematology* 7, 39-42.

STOCK, S.P. & KAYA, H.K. (1996). A multivariate analysis of morphometric characters of *Heterorhabditis* species (Nemata, Heterorhabditidae) and the role of morphometrics in the taxonomy of the species of the genus. *Journal of Parasitology* 82, 806-813.

STOCK, S.P., PRYOR, B.M. & KAYA, H.K. (1999). Distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in natural habitats in California, USA. *Biodiversity and Conservation* 8, 535-549.

STOCK, S.P., CAMPBELL, J.F. & NADLER, S.A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* 87, 877-889.

STURHAN, D. (1999). Prevalence and habitat specificity of entomopathogenic nematodes in Germany. In: Gwynn, R.L., Smits, P.H., Griffin, C., Ehlers, R.-U., Boemare, N. & Masson, J.P. (Eds). *COST 819 Entomopathogenic nematodes – Application and persistence of entomopathogenic nematodes.* European Commission, Luxembourg, DG XII, pp. 123-132.

SUSURLUK, A., DIX, I. & STACKEBRANDT, E. (2002). Identification and ecological characterisation of three entomopathogenic nematode-bacterium complexes from Turkey. *Nematology* 3 (2001), 833-841.

SWOFFORD, D.L. (1998). *PAUP*. Phylogenetic analysis using parsimony. Version 4*. Sunderland, MA, USA, Sinauer Associates, 128 pp.

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876-4882.

WHITE, G.F. (1929). A method for obtaining infective nematode larvae from cultures. *Science* 66, 302-303.

WRIGHT, P.J. (1992). Cool temperature reproduction of steinernematid and heterorhabditid nematodes. *Journal of Invertebrate Pathology* 60, 143-152.