*Nematology*, 1999, Vol. 1(3), 321-332

# Isolation and characterisation of *Heterorhabditis* spp. (Nematoda: Heterorhabditidae) from Hungary, Estonia and Denmark

Christine T. GRIFFIN, Ilona DIX, Susan A. JOYCE, Ann M. BURNELL and Martin J. DOWNES

*Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland*

Accepted for publication: 31 May 1998

**Summary –** Targeted surveys were conducted for the entomopathogenic nematode *Heterorhabditis* in areas of Denmark, Estonia and Hungary. Isolates were identified by IEF, PCR and cross-fertility tests as belonging to three distinct taxonomic groups: *H. bacteriophora*, the north-west European (NWE) type of *H. megidis* and the Irish type of *Heterorhabditis*. The Irish and NWE types of *Heterorhabditis* were both present in Denmark (at six and four sites, respectively), while only the NWE type was recovered in Estonia. *H. bacteriophora* was the dominant heterorhabditid identified in Hungary (ten sites), but the Irish type was also detected at two sites. This is the first report of the Irish type of *Heterorhabditis* on continental Europe. Co-occurrence of two *Heterorhabditis* types at a single site was noted in Denmark (Irish and NWE) and in Hungary (Irish and *H. bacteriophora*). *Heterorhabditis* was recovered at 38.5% of sites (n = 26) in Denmark (north coast of Sjælland), 27.3% of the coastal sites (n = 22) in Estonia, and 32.6% of sites (n = 46) in Hungary.

**Résumé –** ***Isolation et caractérisation d'espèces d'*Heterorhabditis** ***(Nematoda: Heterorhabditidae) originaires de Hongrie, d'Estonie et du Danemark –*** Des prospections ciblées ont été effectuées dans certaines régions du Danemark, d'Estonie et de Hongrie pour rechercher les nématodes du genre *Heterorhabditis*. Les souches, identifiées par les méthodes de concentration isoélectrique, de PCR et d'hybridation, appartiennent aux trois groupes taxinomiques d'*Heterorhabditis*: *H. bacteriophora*, le groupe de l'Europe du nord-ouest (NWE) de *H. megidis* et le groupe irlandais d'*Heterorhabditis*. Le groupe irlandais et le groupe NWE sont tous les deux présents au Danemark (dans six et quatre sites, respectivement), mais seul le dernier groupe a été rencontré en Estonie. *H. bacteriophora*, présent dans dix sites, est l'espèce dominante d'*Heterorhabditis* en Hongrie, mais le groupe irlandais a été également détecté dans deux sites. C'est la première fois que le groupe irlandais est rencontré en Europe continentale. La présence simultanée de deux types d'*Heterorhabditis* est signalée au Danemark (groupe irlandais et groupe NWE) et en Hongrie (groupe irlandais et *H. bacteriophora*). Des *Heterorhabditis* ont été collectés sur 38.5% des 26 sites du Danemark (côte nord de Sjælland), 27.3% des 22 sites du littoral estonien et 32.6% des 46 sites de Hongrie.

**Keywords:** biological control, entomopathogenic nematode, geographic distribution, identification, soil survey.

Entomopathogenic nematodes of the families Heterorhabditidae and Steinernematidae have considerable potential for the control of insect pests. The non-feeding infective juvenile (IJ) can survive in the soil for several months until susceptible insects are encountered. The IJs enter insects and, together with their symbiotic bacteria, kill them within days.

Surveys for entomopathogenic nematodes have been conducted in many parts of the world, including Australia (Akhurst & Bedding, 1986), the United States (Akhurst & Brooks, 1984; Hara *et al.*, 1991) and Europe (Mrácek, 1980; Deseö & Miller, 1985; Burman *et al.*, 1986; Blackshaw, 1988; Vänninen *et al.*, 1989; Hominick & Briscoe, 1990; Griffin *et al.*, 1991; 1994b), for the purposes both of recovering potentially useful isolates and of gaining an insight into the ecology of the nematodes.

Smits *et al.* (1991) identified *Heterorhabditis* isolates from Europe, on the basis of restriction length polymorphisms, as being of three types: *Heterorhabditis bacteriophora*, the north-west European (NWE) group and the Irish group. Current evidence suggests that the *H. bacteriophora* group is the dominant *Heterorhabditis* in southern and central Europe. Thus, Smits *et al.* (1991) identified isolates from Italy, Spain, central Germany (Darmstadt), and Moldova as *H. bacteriophora*, and the species has also been isolated in the south of France (Grenier *et al.*, 1996). Mrácek and Jenser (1988) identified a heterorhabditid isolated near Budapest as *H. heliothidis*, which is now considered to be conspecific with *H. bacteriophora* (Poinar, 1990). Isolates from the Netherlands, Poland and the north of Germany were assigned by Smits *et al.* (1991) to the NWE type of *Heterorhabditis*. This type has also been isolated in the south of England (Hominick *et al.*, 1995) and in Belgium (Miduturi *et al.*, 1996). While most of the records of it have been from northern Europe, the type was also recovered in north-

ern Greece (Menti *et al.*, 1997). The Irish type of *Heterorhabditis* is, to date, the only heterorhabditid to have been isolated in Ireland, where it is relatively common in sandy coastal grasslands (Griffin *et al.*, 1994a). It was also found associated with coastal sites in Britain (Griffin *et al.*, 1994b; Hominick *et al.*, 1995), but has not previously been recorded outside of these islands.

The NWE type of *Heterorhabditis* and *H. megidis* share the same rDNA restriction profiles for all the restriction enzymes tested by Joyce *et al.* (1994a). There is strong evidence, however, that the Irish type represents a separate species. Irish type isolates possess a distinctive IEF protein electrophoregram (Joyce *et al.*, 1994b) and a distinctive repetitive DNA restriction profile (Smits *et al.*, 1991). Also, they can be distinguished from NWE type *Heterorhabditis* by mtDNA and rDNA ITS restriction profiles for selected enzymes (Joyce *et al.*, 1994a), and they are reproductively isolated from *H. megidis* and NWE isolates (Dix *et al.*, 1992; Griffin *et al.*, 1994b; Joyce *et al.*, 1994b). Adams *et al.* (1998) sequenced 716 bases of the rDNA ITS1 region of nine putative species of *Heterorhabditis* and observed 35 nucleotide substitutions between the Irish type isolate K122 and *H. megidis*, two of which are autapomorphies for K122. There is thus cumulative evidence that *H. megidis* and NWE type are conspecific but the Irish type is a distinct species.

Here we report the results of a sampling programme in Hungary, Denmark and Estonia. There are no previous reports of entomopathogenic nematodes from Denmark or Estonia. In Hungary, limited sampling in the vicinity of Budapest recovered a single *H. bacteriophora* isolate (Mrácek & Jenser, 1988). The present sampling programme was part of a broader search in Europe aimed at the recovery of novel wild type strains of *Heterorhabditis* with useful traits. Therefore, sampling was directed at sites at which it was considered likely that the genus was present, *viz* sandy soils, and especially coastal sites, which we (Griffin *et al.*, 1994b) and others (*e.g.* Hara *et al.*, 1991; Amarasinghe *et al.*, 1994) have found to be a suitable habitat for heterorhabditids.

## Materials and methods

### Survey and sampling

Potential sampling sites in Denmark, Estonia and Hungary were initially identified from maps and geological data and subsequently by local inspection. Sites in Denmark and Estonia were all coastal (Fig. 1A, B). In Denmark, sampling was restricted to the north coast of Sjælland (Fig. 1A). Sampling in Hungary was conducted in two regions where soils are relatively sandy, near Kecskemét and Debrecen, respectively (Fig. 1C, D). Between the rivers Danube and Tisza, in and around the Kiskungsági National Park (south of Kecskemét; Bács-Kiskun county), there are extensive sand hills consisting of river deposited sand reworked by the wind. The second area in Hungary was to the north and east of Debrecen (Hajdú-Bihar and Szabolcs-Szatmár counties).

Soil sampling and baiting with *Galleria mellonella* were as described by Griffin *et al.* (1994b) with the addition that samples found to be positive for *Heterorhabditis* were baited a second time. The main sampling programme was conducted in October-November, 1991. Targeted sampling to confirm some of the original findings was carried out in Sjælland in December, 1992 and in Hungary in June, 1994.

### Molecular characterisation

The isolates from the main sampling programme were identified from protein isoelectric focusing electrophoregrams (pH range 3-10) of soluble proteins from IJ as described by Joyce *et al.* (1994b). This diagnosis was subsequently confirmed by restriction enzyme digest of the internal transcribed spacer (ITS) region of rDNA as described by Joyce *et al.* (1994a), using the *Mbo*I restriction enzyme. Isolates from later targeted sampling programmes were characterised using rDNA ITS restriction profiles only.

In the case of isolates expressing the HP88 restriction profile, the rDNA non transcribed spacer (NTS) region was amplified as described by Joyce *et al.* (1994c) and the *ca* 1.4 kb amplification product DNA was digested using the enzymes *Hae*III and *Hpa*II.

### Cross-breeding studies

Interstrain crosses were performed on lipid agar plates as described by Dix *et al.* (1992). At least 60 second generation virgin females were used for each interstrain cross. Reference strains used for the identification of newly isolated nematodes are shown in Table 1.

## Results

*Heterorhabditis* was isolated in Sjælland (Denmark), Estonia and Hungary. There was no difference between

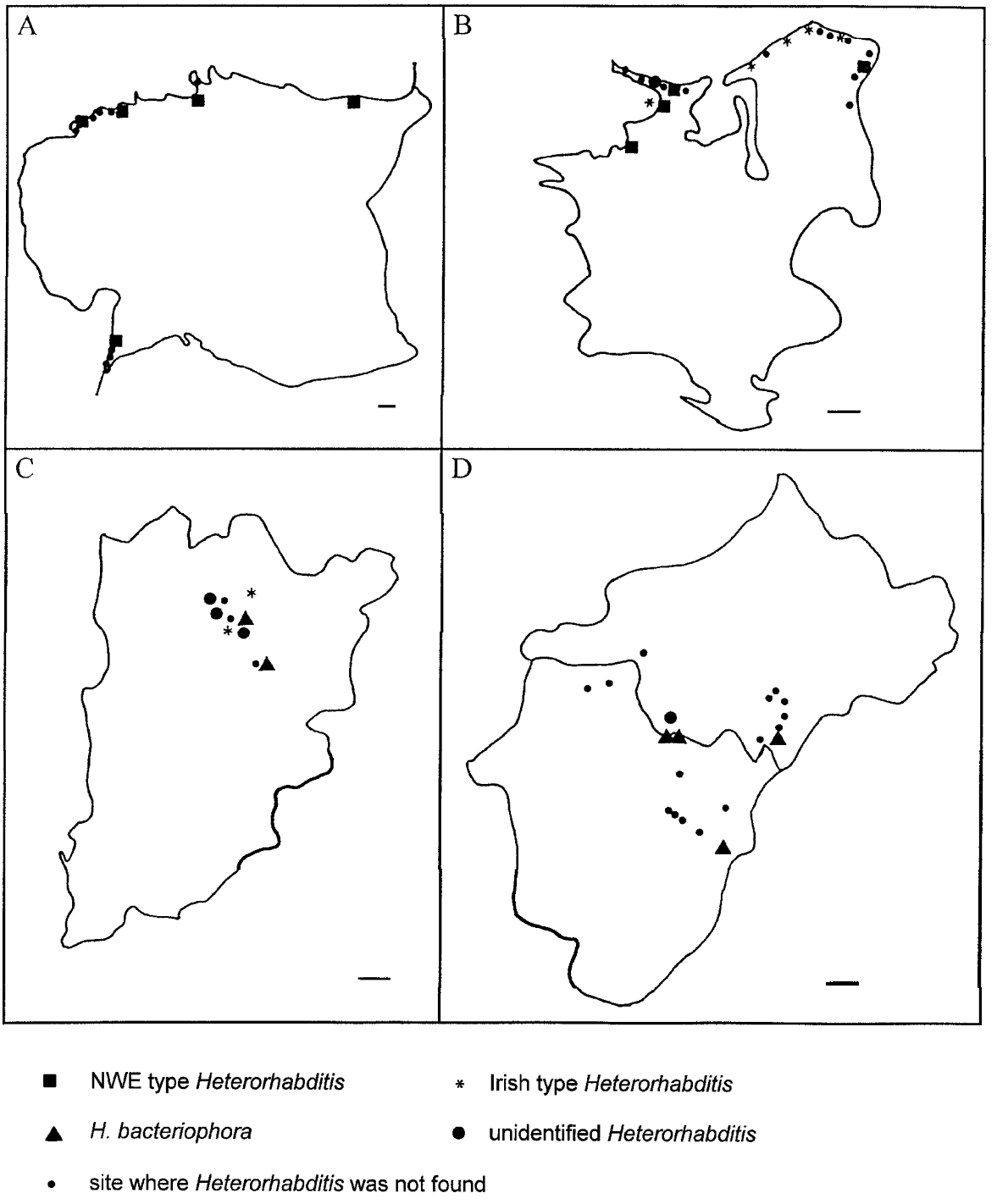


**Fig. 1.** *Sites sampled for* Heterorhabditis *A: Estonia; B: Sjælland, Denmark; C: Kecskemét region (Bács-Kiskun county), Hungary and D: Debrecen region (Hajdú-Bihar and Szabolcs-Szatmár counties), Hungary. (Each symbol may represent more than one site; scale bar = 10 km).*

**Table 1.** *Origin and source of reference strains of* Heterorhabditis

| Name | Origin | ITS restriction profile | Source |
|---|---|---|---|
| HB1 | Brecon, Australia | *H. bacteriophora* | R.J. Akhurst* |
| HP88 | Utah, USA | *H. bacteriophora* HP88 | P. Westerman** |
| Darmstadt | Darmstadt, Germany | *H. bacteriophora* HP88 | T. Jackson*** |
| K122 | Wexford, Ireland | Irish type | Own collections |
| HF85 | Flevopolder, The Netherlands | *H. megidis* NWE type | P. Westerman** |

* CSIRO, Canberra, Australia.
** Van Hall Institute, Leeuwarden, The Netherlands.
*** New Zealand Pastoral Agriculture Research Institute Ltd., Lincoln, New Zealand.

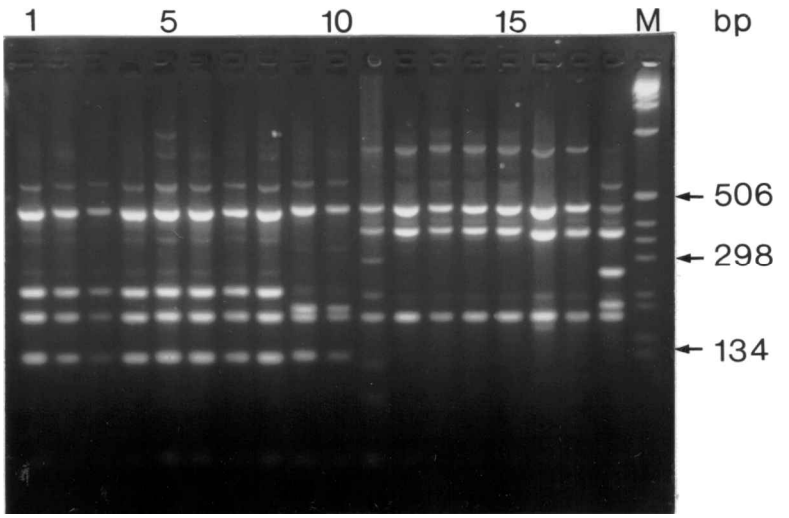


**Fig. 2.** Mbo*I restriction digests of the PCR amplification products of the rDNA internal transcribed spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel. 1: EU333; 2: EU335; 3: EU347; 4: EU348; 5: EU349; 6: EU94; 7: EU106; 8: K122; 9: EU85; 10: HF85; 11: EU339; 12: EU362; 13: EU367; 14: EU368; 15: EU369; 16: HP88; 17: Darmstadt; 18: HB1; M: 1 kb marker. Strains EU85 (lane 9) and EU106 (lane 7) are from Denmark, all other strains with the EU designation are from Hungary.*

the three countries in the proportion either of sites or of samples positive for *Heterorhabditis* (Table 2). Overall, the genus was recovered at 33% of sites, and in 16% of samples. Three species were detected (Table 2): *H. megidis* NWE type in Denmark and Estonia; Irish type *Heterorhabditis* in Denmark and Hungary, and *H. bacteriophora* (HP88 type restriction profile) in Hungary. The *Mbo*I restriction profiles of the rDNA ITS region from a representative sample of these isolates are shown in Fig. 2. The faint bands visible on this gel represent incomplete digestion products.

### Sjælland (Denmark) and Estonia

All of the sites sampled in Sjælland and Estonia (and hence all of the sites positive for *Heterorhabditis)* were within 1.5 km of the sea; the majority were within 200 m of it. In Estonia, *Heterorhabditis* was detected at six of the 22 sites sampled, and all isolates were identified as the NWE type (Table 2; Fig. 1A). Positive sites included meadow, recreational grassland areas, open coniferous forest with extensive grass cover and the margins of a cereal field (Table 3).

Both the Irish type and NWE type of *Heterorhabditis* were isolated in Sjælland (Table 2; Fig. 1B). Most of the sites from which *Heterorhabditis* was recovered were dune or turf recreational areas, often with shrubs and/or conifers present (Table 3). Both Irish and NWE types occurred together at one site, Gudmindrup Lyng. Six samples were taken at this site; the NWE and Irish types were each recovered from one sample. The Irish type

**Table 2.** *Occurrence of three types of* Heterorhabditis *(*H. bacteriophora *[H.b] Group, the North West European type of* H. megidis *[H.m] and Irish type* Heterorhabditis*) at sites in Hungary, Denmark and Estonia, October-November 1991.*

| Country | Number of sites and **samples** | Number (& %) of sites and **samples** with *Heterorhabditis* | Number of sites with *Heterorhabditis* identified to species | | | |
|---|---|---|---|---|---|---|
| | | | Total | H.b | H.m | Irish |
| Estonia | 22 | 6 (27.3%) *a* | 6 | 0 | 6 | 0 |
| | **81** | **9 (11.1%) *a*** | | | | |
| Sjælland (Denmark) | 26 | 10 (38.5%) *a* | 9 | 0 | 4 | 6 |
| | **85** | **15 (17.6%) *a*** | | | | |
| Hungary | | | | | | |
| Kecskemét | 22 | 10 (45.5%) *A* | 8 | 6 | 0 | 2 |
| | **39** | **12 (30.8%) *A*** | | | | |
| Debrecen | 24 | 5 (20.8%) *A* | 4 | 4 | 0 | 0 |
| | **57** | **6 (10.5%) *B*** | | | | |
| Total | 46 | 15 (32.6%) *a* | 12 | 10 | 0 | 2 |
| | **96** | **18 (18.8%) *a*** | | | | |

Entries followed by the same letter do not differ significantly ($P \leqslant 0.05$) from each other. Lower case letters: between countries (chi-square test); upper case letters: between regions of Hungary (Fisher's exact test). Normal letters: sites; bold letters: samples.

was recovered from the landward side of a dune ridge, with mainly shrubs (*Rosa rugosa*, *Empetrum nigrum* and heathers) and some grass, while the NWE type came from a grass/clover roadside verge bordering that area. An examination of the site descriptions for other *Heterorhabditis*-positive sites in Sjælland suggested a possible association between the presence of Irish *Heterorhabditis* and *Rosa rugosa*. Excluding Gudmindrup Lyng, where both nematode types occurred, roses were present at four of the five Irish-positive sites, but at none of the three NWE-positive sites. A test of association between nematode type and presence of roses at nematode-positive sites was significant at $P = 0.07$ (Fisher's exact test).

## Hungary

*H. bacteriophora* was the dominant type present in Hungary, but the Irish type was identified at two sites in the Kecskemét region (Table 3; Fig. 1C). *Heterorhabditis*-positive sites in Kecskemét included meadow, pasture, a grassy hillside with juniper, a rye field with associated verge, a wooded roadside verge, and the bank of a dried-up alkaline lake (Table 3). In the Debrecen area, only *H. bacteriophora* was detected (Fig. 1D). Sites in this region had the heaviest soils of all those sampled in any of the three countries, being sandy loams or sandy clay loams rather than sands. Positive sites here were pasture, waste grassland and the margins of a maize field (Table 2).

Overall, one third of the sites sampled in Hungary were positive for *Heterorhabditis* (Table 2). *Heterorhabditis* was detected at a higher proportion of sites in the Kecskemét region (46%) than in the Debrecen region (21%), and the difference was significant at $P = 0.054$ (Fisher's exact test). The difference between these two regions of Hungary reached significance at $P \leqslant 0.05$ when the proportion of positive samples was compared (31 *vs* 11%) (Table 2). When only the data for *H. bacteriophora* (which occurred in both regions) were considered, the prevalence remained higher in Kecskemét (6/22 sites, 6/39 samples) than Debrecen (4/24 sites, 4/59 samples) but the difference was not significant for either sites or samples.

In a restricted sampling programme in June 1994, several soil samples were taken at each of the two Kecskemét sites at which the Irish type *Heterorhabditis* isolates (EU167 and EU176) had been recovered in 1991. These were respectively, a wide roadside verge with hedgerow broadening to deciduous woodland, and a low-lying pasture with reeds (*Phragmites australis*) adjoining the sampled area. Irish type *Heterorhabditis* was again isolated in

**Table 3.** *Locations of sites where* Heterorhabditis *was detected in October-November 1991, the habitat from which positive samples were taken, and the identity of the isolated* Heterorhabditis *(H.m.:* H. megidis *NWE type; H.b.:* H. bacteriophora*; Irish: Irish type of* Heterorhabditis*; Het.: identified to genus only).*

| Site location | Sample/isolate code | Identity of isolate | Habitat at sample |
|---|---|---|---|
| Estonia | | | |
| Tallinn | EU6 | *H.m.* | Park: turf with *Elymus* |
| Keile Joa | EU17 | *H.m.* | Open conif. forest with grass cover |
| Lohusalu | EU21 | *H.m.* | Meadow |
| Taagupi | EU36 | *H.m.* | Grass |
| | EU38 | *H.m.* | Weedy margin of cereal field |
| Aa | EU70 | *H.m.* | Turf grass |
| | EU71 | *H.m.* | Grassy slope |
| Tsitre | EU80 | | Meadow |
| | EU81 | *Het.* | Meadow |
| Denmark: Sjælland | | | |
| Mørdrup | EU85 | *H.m.* | Grass (waste ground) |
| Hornbæk | EU94 | Irish | Open coniferous forest with ground oak, roses and grass |
| Gilleleje | EU103 | *Het.* | Grassy slope |
| | EU106 | **Irish | Shrubs and long grass |
| Rågeleje | EU114 | Irish | Meadow |
| W. of Rågeleje | EU116 | Irish | Grass |
| | EU121 | Irish | Grassy slope |
| Liseleje | EU132 | Irish | Dunes, some roses |
| | EU133 | *Het.* | Dunes, some roses |
| Nyrup | EU140 | *H.m.* | Long grass, scattered conifers |
| | EU141 | *Het.* | Long grass, scattered conifers |
| Lumsas | EU143 | *Het.* | Grass (parking place) |
| Gudmindrup Lyng | EU158 | Irish | Grass/rose/heather/*Vaccinium* |
| | EU162 | *H.m.* | Grass verge |
| Vindekilde | EU166 | *H.m.* | Old dunes, long grass |
| Hungary: Kecskemét | | | |
| Near Kecskemét | EU167 | ** ***Irish | Roadside verge with trees |
| *KNP IV | EU173 | *** *Het.* | Short grass, bank of dry alkaline lake |
| near KNP V | EU175 | *Het.* | Pasture |
| KNP V(1) | EU176 | ***Irish | Pasture |
| KNP V(2) | EU178 | *H.b.* | Rye field including verge |
| KNP V(3) | EU185 | *H.b.* | Sand hills; grass and juniper |
| KNP V(4) | EU188 | *H.b.* | Meadow on hillside |
| | EU190 | *Het.* | Pasture |
| KNP V(5) | EU193 | *H.b.* | Meadow |
| KNP V(6) | EU195 | *H.b.* | Meadow |
| | EU196 | *Het.* | Meadow |
| KNP VI (Bugac) | EU201 | *H.b.* | Cut pasture |

**Table 3.** *(Continued).*

| Site location | Sample/isolate code | Identity of isolate | Habitat at sample |
|---|---|---|---|
| Hungary: Debrecen | | | |
| Létavértes | EU222 | *H.b.* | Sheep pasture |
| Nyírbéltek | EU228 | *H.b.* | Pasture on hill |
| | EU229 | *Het.* | Pasture |
| Téglás | EU246 | *H.b.* | Grass with shrubs (waste ground) |
| Téglás-Újfehértó | EU249 | *H.b.* | Margins of maize field |
| Újfehértó | EU254 | *Het.* | Grass (waste ground) |

* KNP IV-VI: Kiskunsagi National Park, Regions IV-VI.
** Irish type re-isolated in Nov. 1992 (Sjælland) or June 1994 (Kecskemét: EU333, EU335, EU347, EU348 and EU349).
*** *Hb.* Re-isolated in June 1994 (EU339 near Kecskemét; EU369 at KNP IV; EU362 & EU367 at KNP V(1)).

the verge (site of EU167), where it was detected in 5/20 samples, but it was not isolated in any of seventeen samples from the pasture (site of EU176). This time, *H. bacteriophora* was present at both sites (verge: 1/20 samples; pasture: 2/17 samples), though it had not been detected at either site in the 1991 sampling programme.

The average number of bait insects parasitised (luminescent) in the *Heterorhabditis*-positive samples from Kecskemét was similar to that for Denmark and Estonia, while significantly more insects were parasitised in the Debrecen samples (Fig. 3). To test whether the different parasitisation levels in the Debrecen and Kecskemét samples was due to differences in species composition between the two areas, a comparison was made testing only those samples in which *H. bacteriophora* was identified. *H. bacteriophora*-positive samples from Kecskemét yielded less than half as many parasitised cadavers per sample as the *H. bacteriophora*-positive Debrecen samples (Kecskemét: $x = 2.3 \pm 0.76$, $n = 6$; Debrecen: $x = 6.0 \pm 2.04$, $n = 4$) and the difference was significant at $P = 0.086$ (Student's t test).

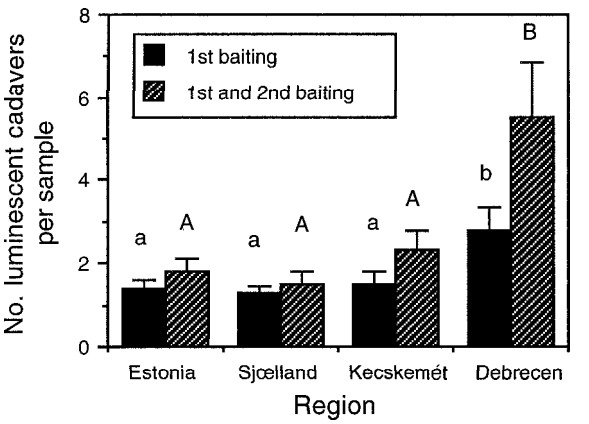


**Fig. 3.** *Mean number of luminescent* Galleria mellonella *cadavers per* Heterorhabditis*-positive soil sample from Estonia, Denmark (Sjælland), and Hungary (Kecskemét and Debrecen). Data are for first baiting and total (first and second) baiting. Bars accompanied by the same letter are not significantly different ($P \leqslant 0.05$, Duncan's multiple range test); lower case letters: first baiting; upper case letters: total baiting.*

## Cross-breeding studies

Hungarian and Danish isolates which display an Irish type profile are interfertile with each other and with the Irish type isolate K122, from Ireland (Table 4). When the females of the Irish type isolate EU114 (Denmark) were crossed with males of the NWE type isolate EU85 (also from Denmark), embryonic lethal progeny resulted (not in table). Crosses between Hungarian isolates with the Irish type profile and Hungarian isolates with the HP88-type profile gave rise to embryonic lethal progeny (data not shown).

Hungarian isolates having the same ITS region *Mbo*I restriction profile as the *H. bacteriophora* HP88 strain were not interfertile when crossed with it. The cross of HP88 females and Hungarian HP88 type males (EU362 and EU369) gave sterile F1 progeny while the reciprocal cross resulted in embryonic lethals. Crosses set up with Darmstadt (a European isolate with the HP88 type profile and interfertile with HP88) gave the same results with these isolates as did the cross with HP88 itself (Table 5). None of the restriction enzymes used to digest either the ITS region or the NTS region of the rDNA gene complex revealed a difference between the Hungarian HP88 type

**Table 4.** *Crosses between* Heterorhabditis *isolates sharing the "Irish type" rDNA ITS restriction profile and resulting in fertile progeny (+ ).*

| Female | Male | | | | |
|---|---|---|---|---|---|
| | K122 (Ireland) | EU94 (Denmark) | EU106 (Denmark) | EU333 (Hungary) | EU349 (Hungary) |
| K122 | + | + | + | + | + |
| EU94 | + | + | + | + | + |
| EU349 | + | + | + | + | + |

**Table 5.** *Crosses between isolates sharing the* H. bacteriophora *HP88 type rDNA ITS restriction profile.*

| Female | Male | | | |
|---|---|---|---|---|
| | HP88 (USA) | Darmstadt (Germany) | EU362 (Hungary) | EU369 (Hungary) |
| HP88 | + | + | F1 | F1 |
| Darmstadt | + | + | n.d. | F1 |
| EU369 | e.l. | e.l. | + | + |

+ : cross resulted in fertile progeny.
e.l.: embryonic lethal.
F1: sterile F1 adults.
n.d.: not determined.

isolates and HP88 itself. Fig. 4 shows a *Hae*III digestion profile of the rDNA NTS region of five Hungarian *H. bacteriophora* HP88 type isolates. All of these isolates share the same NTS restriction profile as the HP88 isolate, having four restriction fragments of *ca* 620, 332, 197 and 148 bp. The faint bands visible on this gel most probably represent incomplete digestion products.

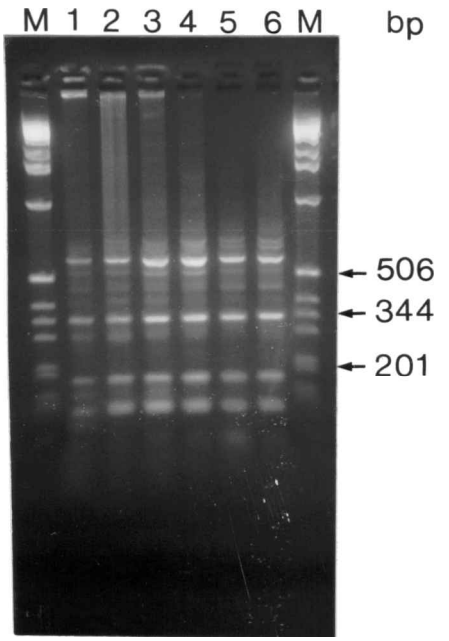


**Fig. 4.** Hae*III restriction digests of the PCR amplification products of the rDNA non-transcibed spacer region of Hungarian isolates of the HP88 (*H. bacteriophora*) type separated on a 2% agarose gel. 1: HP88; 2: EU339; 3: EU362; 4: EU367; 5: EU368; 6: EU369; M: 1 kb DNA ladder (Gibco BRL).*

## Discussion

Isoelectric focusing electrophoregrams of soluble proteins from IJ and the pattern of the restriction enzyme digest of the ITS region of rDNA revealed that both NWE type *H. megidis* and Irish type *Heterorhabditis* occur in Denmark. Isolates showing the Irish type PCR profile were also recovered from soil samples collected in the Kecskemét region of Hungary. The cross-breeding studies showed that these Hungarian and Danish Irish type isolates are interfertile with each other and with the Irish type isolate K122 (from Ireland). This indicates that the Irish type of *Heterorhabditis* is not restricted to the coastal region of Ireland and Britain (Griffin *et al*., 1994b; Hominick *et al*., 1995), but occurs in central and northern Europe as well.

NWE type *H. megidis* predominates on the northern continental fringe, being the only *Heterorhabditis* isolated to date from the Netherlands (Hominick *et al.*, 1995), Belgium (Miduturi *et al.*, 1996), northern Germany and Poland (Smits *et al.*, 1991) and Estonia (present study). The NWE type has also been identified from the south east of England (Hominick *et al.*, 1995) where the climate of Britain most resembles that of the continental fringe. In contrast, the Irish type *Heterorhabditis* is the only one recovered in Ireland and in all but the south east of Great Britain. This might suggest that it is a species of mild, wet climates. However, its discovery at central European sites in Hungary and on a portion of the continental fringe in Denmark, where it occurs with the NWE type, suggests that the picture is more complicated: further sampling effort will clearly be necessary to delineate its distribution range. The apparent absence of the Irish type in Estonia, with its relatively long cold winters, would support the thesis that the Irish type is a species of equitable climates.

Moreover, bearing in mind that soils sampled were of a similar, sandy texture, it is striking that Irish type *Heterorhabditis* occurred at a lower prevalence toward the climatically more extreme centre of the continent (Hungary, 4% of sites) than in Denmark (23% of sites) or in Ireland and Britain, where it was detected at an average of 12% of sites (Griffin *et al.*, 1994b). This suggests that it is less successful in the conditions prevailing in Hungary, which would accord with the poorer heat tolerance of Irish type isolates than of Hungarian *H. bacteriophora* reported by Finnegan *et al.* (1999). The site at which the Irish type was detected in both of the Hungarian samplings (November, 1991 and June, 1994) was a roadside verge shaded by trees — virtually a small woodland; few other wooded sites were included in this study. In the Negev region of Israel, *Heterorhabditis* densities were higher in plots with a high degree of shade (Glazer *et al.*, 1996). Perhaps in Hungary, Irish type *Heterorhabditis* is favoured by woodland, where lower temperatures and higher soil moisture would be expected than in the neighbouring grassland. While the other Hungarian site at which the Irish type was recovered was open grassland, it was bordered by reeds, indicating a high water table. The Irish type was not recovered from this site during the repeat sampling in June 1994; however, at that time of the year the nematodes may have been below the 10 cm soil depth to which it was sampled. In warm regions, *Heterorhabditis* tends to be found in the deeper soil layers during the summer, especially in soil which is more exposed to solar radiation (Glazer *et al.*, 1996). The Irish type isolates from Denmark and Hungary have not yet been fully characterised for biological traits; given the diversity amongst isolates of the type from Britain and Ireland (Hass, 1996) it would not be surprising if locally adapted ecotypes were detected amongst them.

A number of *Heterorhabditis* isolates collected in Hungary show rDNA ITS and rDNA NTS DNA restriction profiles identical to that of the American isolate of *H. bacteriophora*, HP88, using key diagnostic restriction enzymes. However, the Hungarian isolates are not interfertile with HP88. HP88-compatible nematodes do occur in Europe, however, as evidenced by the cross-breeding success with the Darmstadt strain. The incompatibility between HP88 and the Hungarian HP88-like isolates is at the post-mating level, and results in either hybrid inviability or hybrid sterility depending on the direction of the cross. Embryos were formed in all of the crosses that were carried out. These embryos grew into apparently normal but sterile adults if the maternal partner for the cross was from either the HP88 or Darmstadt strain. Whether genetic or cytoplasmic maternal factors play an important role in the reproductive isolation between these isolates has not been determined.

The results of this survey provide further evidence of a geographic separation in Europe between the *H. megidis*-like nematodes (the Irish and NWE types of *Heterorhabditis*), found predominantly in northern Europe, and the *H. bacteriophora* group found in southern and central Europe. However, the occurrence of the Irish group in Hungary (present study) and of *H. megidis* in Greece (Menti *et al.*, 1997) clearly indicates that a division between a northern '*megidis*' group and a southern *H. bacteriophora* group is too simplistic.

The recovery rate of *Heterorhabditis* was much higher in this study, in which sites with sandy soils were specifically targeted for sampling, than in non-targeted surveys conducted in neighbouring areas of Europe. Thus, *Heterorhabditis* was recovered at one third of sites in Hungary, but was not recovered in the survey of Czechoslovakia (Mrácek, 1980). Similarly, the recovery rate of *Heterorhabditis* at 38.5% of sites in Sjælland is considerably higher than in West-Flanders (Belgium) (5% of sites) (Miduturi *et al.*, 1996) or in the Netherlands (13% of sites) (Hominick *et al.*, 1995). The efficacy of targeted sampling in recovering strains of *Heterorhabditis* was previously demonstrated in Ireland and Britain: Griffin *et al.* (1994b) recovered *Heterorhabditis* from 4-45% of sandy sites, while in general surveys, it either was not detected (Blackshaw, 1988; Boag *et al.*, 1992; Gwynn & Richard-

son, 1996) or was present at only one site per survey (Hominick & Briscoe, 1990; Griffin *et al*., 1991). The relatively high prevalence of *Heterorhabditis* in Sjælland and Estonia in the present study also contrasts with the apparent rarity of the genus across the Baltic sea in Sweden, Finland and Norway (Burman *et al*., 1986; Vänninen *et al*., 1989; Haukeland, 1993); but this may again reflect the relative amounts of sampling effort in the favoured habitat. Targeted sampling in Norway yielded a *Heterorhabditis* isolate from a sandy soil within 50 m of the sea (Haukeland Salinas, 1996).

The aim of our research was primarily the recovery of strains of *Heterorhabditis*. The targeting of sandy soil is considered a key strategic feature in this regard. Another factor which we consider may contribute to the relatively high prevalence of *Heterorhabditis* recorded in this and the study of Griffin *et al*. (1994b) is that each sample consisted of many (*ca* 40) subsamples, a strategy designed to maximise the recovery of organisms such as *Heterorhabditis* which have a clumped distribution (Stuart & Gaugler, 1994). Bednarek (1986) recommended that at least 50 (sub)samples should be collected to allow one to be convinced that entomopathogenic nematodes will be detected if present. Nevertheless, even where several samples (each of about 40 subsamples) were collected at a given site, we are not convinced that failure to detect *Heterorhabditis* reflects its absence from that site; in Ireland, *Heterorhabditis* may sometimes be present at very low frequencies in the sandy coastal habitats to which it is restricted (Downes, 1995).

While *Heterorhabditis* was recovered from a higher proportion of sites in Kecskemét than Debrecen, higher proportions of bait insects were parasitised in the positive Debrecen samples, suggesting a higher nematode population density in these samples. Nematode population densities are affected by several factors. However, the Debrecen soils were notably less sandy than those in any of the other regions sampled in this study, and a number of studies (Ishibashi & Kondo, 1987; Kung *et al*., 1990) suggest that IJ of entomopathogenic nematodes survive longer in heavier soils. They would also be expected to disperse less. Heavier soils may then have fewer, more concentrated populations while sands may have more numerous, dispersed populations.

Two species of *Heterorhabditis* were found to occur together at the same site twice in this study. Irish type *Heterorhabditis* occurred together with *H. bacteriophora* at a site in Hungary, and it occurred together with NWE type *H. megidis* at a Danish site. Reports of the co-occurrence of heterorhabditid species are rare; but Amarasinghe *et al*. (1994) recovered *H. indica* and an unidentified heterorhabditid from a single sample in Sri Lanka. Coexistence may be facilitated by species having different foraging strategies (Koppenhöfer & Kaya, 1996) or other differences of behaviour or life cycle. Little is known of the host preferences of European heterorhabditids; however, *H. bacteriophora* and the Irish type are not closely related (Smits *et al*., 1991; Adams *et al*., 1998) and show different biological characteristiscs (Griffin *et al*., 1994a; Hass, 1996). On the other hand, Irish type *Heterorhabditis* and NWE type *H. megidis*, which were found together at one site in Denmark, are closely related (Smits *et al*., 1991; Adams *et al*., 1998) and members of the two types are similar in several biological characteristics (Griffin *et al*., 1994a; Hass, 1996). It is unknown whether the two *Heterorhabditis* types parasitise the same host species where they occur together. Although not expected on theoretical grounds, *Steinernema feltiae* and *S. affinis* were found parasitising the same species (and sometimes even the same individual larva) of bibionid fly in Denmark (Bovien, 1937; Poinar & Lindhardt, 1971). However, there is some indication of association of Irish type *Heterorhabditis* with roses in Sjælland. Irish type was more likely than the NWE type to be found at sites with Rosaceae, and at the site where the two types occurred together, the Irish type was in a sample taken from shrub (including Rosaceae) dominated area. This perhaps indicates that in Denmark the Irish type is specialising on hosts associated with these plants or with their associated community. Such an association occurs, for example, between *H. marelata* and bush lupins in Bodega Bay, California (Strong *et al*., 1996).

The occurrence together of Irish type *Heterorhabditis* and of the NWE type of *H. megidis* presents an exciting opportunity to study how these closely related but distinct species avoid competitive displacement in Danish sand dunes.

## Acknowledgements

We are grateful to the directorate of Kiskunsági National Park for permission to collect within the Park, and to Dr J. Kelemen and Mr F. Pál-Szabó for help in the planning and execution of the sampling in the Kecskemét region. This work was carried out with the financial assistance of the EU (ECLAIR contract AGRE 0002).

## References


ADAMS, B.J., BURNELL, A.M. & POWERS, T.O. (1998). A phylogenetic analysis of the genus *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer I DNA sequence data. *Journal of Nematology* 30, 22-39.

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

AKHURST, R.J. & BROOKS, W.M. (1984). The distribution of entomophilic nematodes (Heterorhabditidae and Steinernematidae) in North Carolina. *Journal of Invertebrate Pathology* 44, 140-145.

AMARASINGHE, L.D., HOMINICK, W.M., BRISCOE, B.R. & REID, A.P. (1994). Occurrence and distribution of entomopathogenic nematodes in Sri Lanka. *Journal of Helminthology* 68, 277-286.

BEDNAREK, A. (1986). Estimation of the occurrence in soil of infective juveniles of entomogenous nematodes (Rhabditida: Steinernematidae, Heterorhabditidae). *Revue de Nématologie* 9, 414-416.

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

BOAG, B., NEILSON, R. & GORDON, S.C. (1992). Distribution and prevalence of the entomopathogenic nematode *Steinernema feltiae* in Scotland. *Annals of Applied Biology* 121, 355-360.

BOVIEN, P. (1937). Some types of association between nematodes and insects. *Videnskabelige Meddelelser fra Dansk naturhistorisk Forening* 101, 1-144.

BURMAN, M., ABRAHAMSON, K., ASCARD, J., SJÖBERG, A. & ERIKSSON, B. (1986). Distribution of insect parasitic nematodes in Sweden. *Proceedings, 4th International Colloquium of Invertebrate Pathology, Veldhoven, The Netherlands*: 312 [Abstr.].

DESEÖ, K.V. & MILLER, L.A. (1985). Efficacy of entomogenous nematodes, *Steinernema* spp., against clearwing moths, *Synanthedon* spp., in north Italian apple ochards. *Nematologica* 31, 100-108.

DIX, I., BURNELL, A.M., GRIFFIN, C.T., JOYCE, S.A., NUGENT, M.J. & DOWNES, M.J. (1992). The identification of biological species in the genus *Heterorhabditis* (Nematoda: Heterorhabditidae) by cross-breeding second generation amphimictic adults. *Parasitology* 104, 509-518.

DOWNES, M.J. (1995). Conceptual approaches to effects of interacting host size and density on genetic diversity in entomopathogenic nematode populations. In: Griffin, C.T., Gwynn, R.L. & Masson, J.P. (Eds). *Ecology and transmission strategies of entomopathogenic nematodes*. Brussels, Belgium, European Commission, pp. 69-76.

FINNEGAN, M.M., DOWNES, M.J., O'REGAN, M. & GRIFFIN, C.T. (1999). Effect of salt and temperature stresses on survival and infectivity of *Heterorhabditis* spp. infective juveniles. *Nematology* 1, 69-78.

GLAZER, I., KOZODOI, E., SALAME, L. & NESTEL, D. (1996). Spatial and temporal occurrence of natural populations of *Heterorhabditis* spp. (Nematoda: Rhabditida) in a semiarid region. *Biological Control* 6, 130-136.

GRENIER, E., BONIFASSI, E., ABAD, P. & LAUMOND, C. (1996). Use of species-specific satellite DNAs as diagnostic probes in the identification of Steinernematidae and Heterorhabditidae entomopathogenic nematodes. *Parasitology* 113, 483-489.

GRIFFIN, C. T., FINNEGAN, M.M. & DOWNES, M.J. (1994a). Environmental tolerances and the dispersal of *Heterorhabditis*: survival and infectivity of European *Heterorhabditis* following prolonged immersion in seawater. *Fundamental and Applied Nematology* 17, 415-421.

GRIFFIN, C.T., JOYCE, S.A., DIX, I., BURNELL, A.M. & DOWNES, M.J. (1994b). Characterisation of the entomopathogenic nematodes *Heterorhabditis* (Nematoda: Heterorhabditidae) from Ireland and Britain by molecular and cross-breeding techniques, and the occurrence of the genus in these islands. *Fundamental and Applied Nematology* 17, 245-253.

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.

GWYNN, R.L & RICHARDSON, P.N. (1996). Incidence of entomopathogenic nematodes in soil samples collected from Scotland, England and Wales. *Fundamental and Applied Nematology* 19, 427-431.

HARA, A.H., GAUGLER, R., KAYA, H.K. & LEBECK, L.M. (1991). Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiian Islands. *Environmental Entomology* 20, 211-216.

HASS, B. (1996). *Persistence of entomopathogenic nematodes of the genus* Heterorhabditis *Poinar, 1975 with emphasis on effects of natural ageing under starvation*. Ph.D. Thesis, National University of Ireland, 179 p.

HAUKELAND, S. (1993). Entomopathogenic nematodes found in Norway. *Norwegian Journal of Agricultural Science* 7, 17-27.

HAUKELAND SALINAS, S. (1996). [Beneficial nematodes — natural occurrence in Norway.] *Gartneryrket* 7, 13-17.

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., & BRISCOE, B.R. (1995). Prevalence and habitat specificity of steinernematid and heterorhabditid nematodes isolated during soil surveys of the UK and the Netherlands. *Journal of Helminthology* 69, 1-6.

ISHIBASHI, N. & KONDO, E. (1987). Dynamics of the entomogenous nematode *Steinernema feltiae* applied to soil with

and without nematicide treatment. *Journal of Nematology* 19, 404-412.

JOYCE, S.A., BURNELL, A.M. & POWERS, T.O. (1994a). Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. *Journal of Nematology* 26, 260-270.

JOYCE, S.A., GRIFFIN, C.T. & BURNELL, A.M. (1994b). The use of isoelectric focusing and polyacrylamide gel electrophoresis of soluble proteins in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). *Nematologica* 40, 601-612.

JOYCE, S.A., REID, A., DRIVER, F. & CURRAN, J. (1994c). Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.P. (Eds). *Genetics of entomopathogenic nematode-bacterium complexes.* Brussels, Belgium, European Commission, pp. 41-48.

KOPPENHÖFER, A.M. & KAYA, H.K. (1996). Coexistence of two steinernematid nematode species (Rhabditida: Steinernematidae) in the presence of two host species. *Applied Soil Ecology* 4, 221-230.

KUNG, S.-P., GAUGLER, R. & KAYA, H.K. (1990). Soil type and entomopathogenic nematode persistence. *Journal of Invertebrate Pathology* 55, 401-406.

MENTI, H., WRIGHT, D.J. & PERRY, R.N. (1997). Desiccation survival of populations of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. *Journal of Helminthology* 71, 41-46.

MIDUTURI, J.S., MOENS, M., HOMINICK, W.M., BRISCOE, B.R. & REID, A.P. (1996). Naturally occurring entomopathogenic nematodes in the province of West-Flanders, Belgium. *Journal of Helminthology* 70, 319-327.

MRÁČEK, Z. (1980). The use of "*Galleria* traps" for obtaining nematode parasites of insects in Czechoslovakia (Lepidoptera: Nematoda, Steinernematidae). *Acta Entomologica Bohemoslovaca* 77, 378-382.

MRÁČEK, Z. & JENSER, G. (1988). First report of entomogenous nematodes of the families Steinernematidae and Heterorhabditidae from Hungary. *Acta Phytopathologica et Entomologica Hungarica* 23, 153-156.

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

POINAR, G.O., JR & LINDHARDT, K. (1971). The re-isolation of *Neoaplectana bibionis* Bovien (Nematoda) from Danish bibionids (Diptera) and their possible use as biological control agents. *Acta Entomologica Scandinavica* 2, 301-303.

SMITS, P.H., GROENEN, J.T.M. & DE RAAY, G. (1991). Characterization of *Heterorhabditis* isolates using DNA restriction length polymorphism. *Revue de Nématologie* 14, 445-453.

STRONG, D. R., KAYA, H.K., WHIPPLE, A.V., CHILD, A.L., KRAIG, S., BONDONNE, M., DYER, K. & MARON, J.L. (1996). Entomopathogenic nematodes: natural enemies of root-feeding caterpillars on bush lupin. *Oecologia* 108, 39-45.

STUART, R.J. & GAUGLER, R. (1994). Patchiness in populations of entomopathogenic nematodes. *Journal of Invertebrate Pathology* 64, 39-45.

V¯NNINEN, I., HUSBERG, G.B. & HOKKANEN, H. (1989). Occurrence of entomopathogenic fungi and entomoparasitic nematodes in cultivated soil in Finland. *Acta Entomologica Fennica* 53, 65-71.