Nematology, 2000, Vol. 2(5), 477-487

Molecular characterisation of *Heterorhabditis indica* isolates from India, Kenya, Indonesia and Cuba

Colin M. STACK [1], Subbana G. EASWARAMOORTHY [2], Usha K. METHA [2], Martin J. DOWNES [1], Christine T. GRIFFIN [1] and Ann M. BURNELL [1,*]

[1] *The Institute for Bioengineering and Agroecology, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland*

[2] *Sugarcane Breeding Institute, Indian Council for Agricultural Research, Coimbatore, Tamil Nadu 641 007, India*

Accepted for publication: 20 November 1999

Summary – Isolates of *Heterorhabditis* were identified as *H. indica* using the following molecular diagnostic features: hybridisation to a *H. indica* specific satellite DNA probe; *Alu*I and *Mbo*I restriction profiles of the rDNA ITS PCR product and the *Alu*I profile of the rDNA IGS PCR product. The Kenyan isolates represent a distinct subgroup of *H. indica.* These isolates lacked one of the two *Hinf*I restriction sites which are present in the rDNA ITS product of all the other isolates tested and they also differed from other *H. indica* isolates in their rDNA IGS *Hae*III restriction profile. The Indian isolates are interfertile. The Kenyan isolates are interfertile but only one Kenyan isolate, Ki3, produced viable progeny when crossed with *H. indica* LN2. The four Indonesian isolates are interfertile, but only one Indonesian isolate (INA H1) produced viable hybrids when crossed with *H. indica* LN2. INA H1 was also interfertile with the Kenyan isolate Ki3.

Résumé – ***Caractérisation moléculaire d'isolats d'*Heterorhabditis indica *provenant d'Inde, du Kenya, d'Indonésie et de Cuba*** **–** Des isolats d'*Heterorhabditis* ont été identifiés comme *H. indica* par l'utilisation des techniques de caractérisation moléculaire suivantes: hybridation avec une sonde spécifique du DNA satellite de *H. indica*, produits des profils de restriction par PCR de l'ITS du rDNA par *Alu*I et *Mbo*I et produit de PCR de l'IGS du rDNA par *Alu*I. Les isolats keniyans constituent un sous-groupe distinct d'*H. indica.* Un des deux sites de restriction de *Hinf*I, présent dans les produits de l'ITS du rDNA de tous les autres isolats étudiés, est absent dans ces isolats qui différaient également dans leurs profils de restriction de l'IGS du rDNA par *Hae*III. Les isolats d'Inde sont interfertiles. Les isolats kenyans sont inter-fertiles mais un seul de ces isolats, Ki3, a produit une descendance viable après croisement avec *H. indica* LN2. Les quatre isolats indonésiens sont interfertiles, mais un seul d'entre eux (INA H1) a produit des hybrides viables après croisement avec *H. indica* LN2. INA H1 a été également interfertile avec l'isolat kenyan Ki3.

Keywords – crossbreeding, DNA probe, entomopathogenic nematode, molecular diagnostics, rDNA ITS, rDNA IGS, satellite DNA.

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae, together with their symbiotic bacteria *Xenorhabdus* spp. and *Photorhabdus* sp., respectively, are currently being mass produced commercially and used to control a variety of soil-dwelling insect pests in Europe, USA, Australia and China (see reviews by Kaya and Gaugler, 1993; Ehlers, 1996). The biological control potential of EPN has stimulated numerous surveys in an effort to find new indigenous isolates and possibly also new species of *Heterorhabditis* and *Steinernema* (reviewed by Hominick *et al.*, 1996). Rapid and reliable diagnostic tests are required for species identification in such surveys and DNA fingerprinting techniques are now becoming more widely used as a first screen to determine the species composition of newly isolated EPN collections. These molecular approaches can then be supplemented by morphological, morphometric and cross-breeding techniques to confirm the identification of putative new species (reviewed by Hominick *et al.*, 1997).

Although *Steinernema* and *Heterorhabditis* share many similarities in their mode of life and morphology, these similarities result from convergent evolution and are not indicative of a close phylogenetic relationship between the two families (Poinar, 1993; Sudhaus, 1993; Blaxter *et al.*, 1998). *Steinernema* also appears to be more species rich than is *Heterorhabditis.* Twenty two *Steinernema*

* Corresponding author, e-mail: ann.burnell@may.ie

species are recognised by Hominick *et al.* (1997). Adams *et al.* (1998) carried out a phylogenetic analysis based on rDNA internal transcribed spacer 1 DNA sequences of nine described species and one putative species of *Heterorhabditis* and they suggested that three pairs of sister taxa may be conspecific, thereby delimiting six species of *Heterorhabditis*. The study of Adams *et al.* (1998) did not include *H. brevicaudis* (Liu, 1994).

Current biogeographic data suggests that two species of *Heterorhabditis*, *H. indica* and *H. bacteriophora*, have a global distribution. *H. indica* occurs widely in the tropics and subtropics, having been isolated in southern India (Poinar *et al.*, 1992); Sri Lanka (Amarasinghe *et al.*, 1994); peninsular Malaysia (Mason *et al.*, 1996); Indonesia (Griffin *et al.*, 1999b); the Caribbean region (Arteaga Hernandez & Mráček, 1984; Joyce *et al.*, 1994a; Grenier *et al.*, 1996a; Constant *et al.*, 1998); Egypt (Grenier *et al.*, 1996a) and in subtropical and warm temperate zones in Japan (Yoshida *et al.*, 1998). Curran and Driver (1994) presented data for *Hae*III restriction digests of the rDNA intergenic spacer region of a range of tropical isolates of *Heterorhabditis* from north Australia, Egypt, the Caribbean region, Florida, USA and Hawaii. They distinguished between two restriction profiles designated D1 and D1a. This distinction between the D1 and D1a profiles was based on a size polymorphism of the large *Hae*III restriction fragment from the rDNA IGS fragment.

H. bacteriophora occurs in regions of continental and Mediterranean climate in both the northern and southern hemispheres (reviewed by Hominick *et al.*, 1996). To date, *H. megidis* has been recorded only from the northern hemisphere (Poinar *et al.*, 1987; Smits *et al.*, 1991; Miduturi *et al.*, 1996; Menti *et al.*, 1997; Yoshida *et al.*, 1998; Griffin *et al.*, 1999a), where it typically has a more northerly and more restricted distribution than *H. bacteriophora*, although *H. megidis* can be locally common in coastal regions of North West Europe (Griffin *et al.*, 1999a). The remaining described species of *Heterorhabditis* appear to have a more restricted distribution: *H. zealandica* has been isolated in New Zealand and Tasmania (Wouts, 1979; Poinar, 1990; Curran & Driver, 1994); *H. marelatus* has been isolated so far only in Oregon and California, USA (Liu & Berry, 1996; Stock, 1997); *H. brevicaudis* has been isolated in south east China (Liu, 1994) and *H. argentinensis* in Argentina (Stock, 1993), although it is possible that *H. argentinensis* and *H. bacteriophora* may be conspecific (Adams *et al.*, 1998). *Heterorhabditis* species distributions can also be influenced by altitude (Constant *et al.*, 1998), soil type (Kaya, 1990; Griffin *et al.*, 1994) and vegetation cover (Strong *et al.*, 1996).

We report here the isolation of new isolates of *H. indica* from India and Kenya. We have utilised a range of molecular diagnostic techniques in conjunction with crossbreeding in the identification of these new *H. indica* isolates and of isolates of *H. indica* previously isolated from India, Indonesia and Cuba. Our data indicate that the *Hae*III rDNA IGS restriction profile designated D1a by Curran and Driver (1994) also occurs in the *H. indica* type species LN2 and that the Kenyan isolates represent a distinct subgroup within the D1a group of *H. indica*.

Materials and methods

Nematode isolates

H. indica LN2, the *H. indica* type species (Poinar *et al.*, 1992), is maintained at the Sugarcane Breeding Institute (SBI), Coimbatore, India. It was originally isolated from soil samples collected at Ramanathapuram 20 km north of Coimbatore, Tamil Nadu. Coimbatore (at 11°N latitude and 77°E longitude), is 120 km from the Arabian sea, 270 km from the Indian ocean and 310 km from the Bay of Bengal. The soil samples (soil type, red loam) were collected from fallow land (the previous crop was monsoon sorghum) and were baited in the laboratory with top borer (*Scirpophaga excerptalis*, Pyralidae: Lepidoptera) larvae. LN2B — *in situ* baiting with *S. excerptalis* larvae was carried out in a field at Somayanur, Coimbatore, from which groundnuts had been harvested. The soil type was red loam. The *S. excerptalis* larvae were placed individually in small plastic lids covered with brass wire mesh. The insect traps were left in the soil for four days and were then returned to the laboratory at SBI and checked for nematode infection. LN4 — this isolate was obtained from an infected white grub (*Holotrichia serrata*, Scarabaeidae: Coleoptera) larva collected from a sugarcane field at Thirupattur, 25 km north of Coimbatore. The soil type was heavy clay.

In collaboration with personnel from the Kenya Agricultural Research Institute (KARI), 21 soil samples, each of about 500 g, were collected from five locations in coastal Kenya and two locations inland, in August 1994. One sample was taken near Tambia village, four from the WAU/KARI centre at Mtwapa, one from coral-based soil at an elevated (8 m) coastal site near Mombasa, 13 from vegetation fringing a beach at Kanamai, one from far inland in the south-west near Lolgorien, and one from near

the Sand river in the same area as the last. The samples were flown to NUI Maynooth, where each sample was divided and each half was baited with five late instar *Galleria mellonella* larvae and incubated at 28°C. *Heterorhabditis* isolates were recovered only at Kanamai, in a vegetation belt extending 15 m inland from the beach edge. Bioluminescent cadavers (indicating the presence of *Heterorhabditis*) were recovered from two of the 13 samples taken from there.

Four isolates were collected in a survey of five Indonesian islands by Griffin *et al.* (1999b).

Extracted DNA samples from four isolates displaying the D1a IGS restriction profile (FLGS10, JAM23, JAM79, ST09, ES10) were obtained from Dr Felice Driver (CSIRO, Canberra, Australia). The source and geographic origin of the other *Heterorhabditis* species and isolates included in this study are listed in Table 1.

MOLECULAR CHARACTERISATION

DNA was isolated according to Smits *et al.* (1991). The internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of the rDNA cistron were amplified by means of the polymerase chain reaction (PCR) as described by Joyce *et al.* (1994a, b). Amplification products were digested with restriction endonucleases following the manufacturer's instructions using 5-12 μl PCR product in a 15 μl reaction volume. The entire digest was loaded on a 2% agarose gel and electrophoresed in 1 × TBE at 5 V/cm for 3.5 h. Restriction fragments were visualised by ethidium bromide staining.

The dot blot procedure utilised the *H. indica* species-specific satellite DNA probe used by Grenier *et al.* (1996a). Genomic DNA (100 ng) samples were denatured by adding 1 M NaOH and 200 mM EDTA pH 8.2 to each sample to give a final concentration of 0.4 M NaOH, 10 mM

Table 1. *Source and geographic origin of the* Heterorhabditis *species and isolates included in this study.*

Species	Isolate	Geographic Origin	Source
H. indica	LN2	Coimbatore, India	Easwaramoorthy[1]
H. indica	LN2B	Coimbatore, India	This study
H. indica	LN4	Coimbatore, India	This study
H. indica	Ki3	Kanami, Kenya	This study
H. indica	K4A	Kanami, Kenya	This study
H. indica	INA H1	West Java, Indonesia	Griffin *et al.* (1999b)
H. indica	INA H9	Ambon, Indonesia	Griffin *et al.* (1999b)
H. indica	INA H17	Seram, Indonesia	Griffin *et al.* (1999b)
H. indica	INA H23	Moluccas, Indonesia	Griffin *et al.* (1999b)
H. indica	P2M	Artemisia, Cuba	Mracek[2]
H. indica	D1	Darwin, Australia	Bedding[3]
H. indica	FLGS10	Florida, USA	Curran & Driver[3]
H. indica	JAM23	Jamaica	Curran & Driver
H. indica	JAM79	Jamaica	Curran & Driver
H. indica	ST09	Virgin Islands	Curran & Driver
H. indica	ES10	Egypt	Curran & Driver
H. bacteriophora	HP88	Utah, USA	Akhurst[3]
H. zealandica	NZH3	New Zealand	Bedding[3]
H. marelatus	OH-10	Oregon, USA	Liu[4]
H. hepialus	Bodega Bay	California, USA	Stock[5]
H. megidis	HL81	Leeuwarden, The Netherlands	Westerman[6]
H. 'Irish type'	K122	Wexford, Ireland	Griffin *et al.* (1994).

[1] Sugar Cane Breeding Institute, Coimbatore, India;
[2] Institute of Entomology, Czech Academy of Sciences, Ceske Budejovice, Czech Republic;
[3] CSIRO, Canberra, Australia;
[4] Oregon State University, OR, USA;
[5] University of California, Davis, CA, USA;
[6] Van Hall Instituut, Leeuwarden, The Netherlands.

EDTA and the samples were then boiled for 10 min in a water bath. The samples were then transferred by vacuum suction onto a positively charged nylon membrane (Amersham Life Sciences Ltd., Amersham, Buckinghamshire, UK) in a slot blot apparatus (Schleicher & Schuell, D37582 Dassel, Germany) as per manufacturer's instructions. The DNA was then fixed onto the nylon membrane by UV cross-linking using Stratagene's Stratalinker (Stratagene, La Jolla, CA 92037, USA). The recombinant pUC plasmids Hi12 and HP88s9 which contained respectively the *H. indica* and *H. bacteriophora* satellite DNA monomers were obtained from Dr Pierre Abad, INRA, Antibes, France and amplified using PCR. The PCR primers used to amplify the *H. indica* monomer from the Hi12 plasmid were 5′-CTGAAGCACTTGGGACAGAGC-3′ and 5′-CTCCTCGTTGAGGACGGGAGT-3′ (Abadon *et al.*, 1998; Grenier, pers. comm.). The *H. bacteriophora* monomer was amplified from the HP88s9 plasmid using the following PCR primers 5′-AGCTATGCCAGAATGATCGCC-3′ and 5′-AGATTCTCTGTACGATGAGTA-3′ (Grenier *et al.*, 1996b; Grenier, pers. comm.). DNA was amplified using the following conditions: one cycle of 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 0.5 min annealing at 52°C for 1 min and extension at 72°C for 2 min, with a final cycle of extension at 72°C for 5 min.

The probes were labelled using the ECL direct nucleic acid detection system (Amersham) following the manufacturer's instructions. Hybridisations were conducted at 42°C overnight. After hybridisation, the filters were washed first with 0.5 × SSC for 40 min at 42°C and then with 0.1 × SSC for 5 min at room temperature. After post-hybridisation washes, filters were exposed to Hyperflim-ECL (Amersham) following the manufacturer's instructions. The membrane was then stripped and reprobed using an 18S rDNA probe as a loading control. The 18S probe was obtained by PCR from *H. indica* LN2 genomic DNA using the primers: 18SR2B 5′-TACAAAGGGCAGGGACGTATT-3′ and 18S1.2 5′-GGCGATCAGATACCGCCCTAGTT-3′ (T.O. Powers, pers. comm.).

Cross-breeding tests

Cross-breeding studies were carried out as described by Dix *et al.* (1992) with the following controls being set up for each cross: virginity test — 20 virgin females were placed on a lipid agar plate that had been inoculated and pre-incubated with the primary form of the LN2 bacteria; self-cross — ten virgin females and ten males of the same isolate were placed on lipid agar plates containing the bacterial symbiont. The result of any cross between different isolates was taken as valid only if there were no progeny in the virginity test and there were progeny in the self-cross. At least ten second generation virgin females were used for each cross.

Results

Restriction profiles of the rDNA internal transcriber spacer (ITS) region

All of the tropical isolates of *Heterorhabditis* yielded a *ca* 1 kb fragment upon PCR amplification with the ITS primers. These amplification products were digested with the diagnostic restriction endonucleases used by Joyce *et al.* (1994a) for species diagnosis in *Heterorhabditis* (*viz. Alu*I, *Hinf*I and *Mbo*I). When digested with *Alu*I (Fig. 1) the Indian isolates LN2B and LN4 shared the same restriction profile as the *H. indica* LN2 type species, as did the Indonesian isolates, the P2M isolate from Cuba, and the Kenyan isolates. When digested with *Mbo*I, the Kenyan isolates shared the same restriction profile

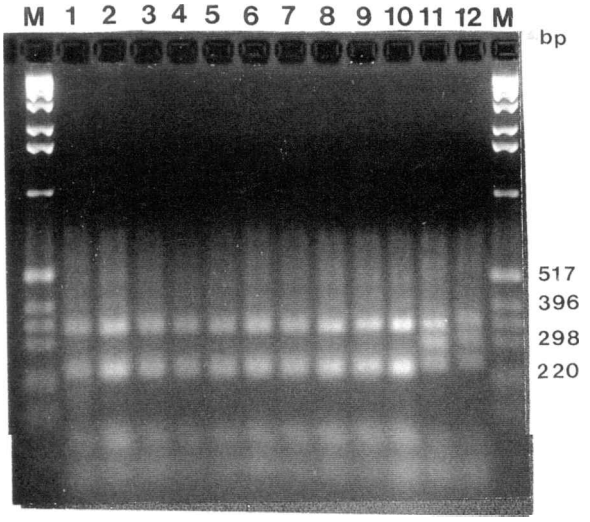

Fig. 1. Alu*I restriction digests of the PCR amplification products of the rDNA internal transcribed spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel and stained with ethidium bromide. M: 1 kb marker; 1:* H. indica *LN2; 2: P2M; 3: LN2B; 4: LN4; 5: INA H23; 6: INA H9; 7: INA H17; 8: INA H1; 9: Ki3; 10: K4A; 11:* Heterorhabditis *'Irish type' K122; 12:* H. megidis *HL81.*

as *H. indica* LN2 and P2M (Fig. 2). The Indonesian isolates also displayed the same *Mbo*I restriction profile as *H. indica* LN2 (data not shown). When digested with *Hinf*I (Fig. 3) the Indian, Indonesian and Cuban isolates but not the Kenyan isolates, possessed the same restriction profile as *H. indica* LN2. The Kenyan isolates have only a single *Hinf*I restriction site in the ITS rDNA fragment, yielding two restriction fragments of *ca* 620 and 450 bp, unlike all the other *H. indica* isolates tested, which possess two *Hinf*I sites in this region, yielding three restriction fragments of *ca* 450, 360 and 240 bp.

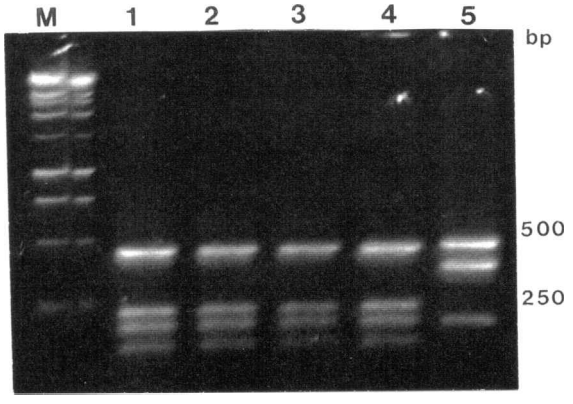

Fig. 2. Mbo*I restriction digests of the PCR amplification products of the rDNA internal transcribed spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel and stained with ethidium bromide. M: 1 kb marker; 1:* H. indica *LN2; 2: P2M; 3: Ki3; 4: K4A; 5:* H. bacteriophora *HP88.*

Restriction profiles of the rDNA intergenic spacer (IGS) region

The PCR amplification products for the IGS rDNA region of the tropical isolates varied in size from 1.5 kb to 1.7 kb. The amplified IGS rDNA fragments were restricted with the endonucleases *Alu*I and *Hae*III. A diagnostic *Alu*I restriction pattern was obtained for *H. indica* LN2 and this was shared by all the Indian, Indonesian, Cuban and Kenyan isolates (Fig. 4) and the D1 and D1a type isolates (Fig. 6). When *Hae*III was used to digest the IGS rDNA region, *H. indica* LN2 displayed a distinctive five fragment profile of 740, 280, 190, 160 and 120 bp (Fig. 5) and, with the exception of the Kenyan isolates, four of these fragments were shared by the other tropical isolates. The Kenyan isolates Ki3 (lane 9) and K4A (lane 10) had a distinct restriction profile yielding *Hae*III fragments of 700, 280 (a doublet), 185 and 120 bp. The largest *H. indica Hae*III fragment was highly polymorphic in size between the tropical isolates, ranging in size from 700 to 850 bp, with some isolates having fragments of intermediate size ca 740 bp. Size polymorphism in this fragment was the basis on which the types D1 and D1a were recognised by Curran and Driver (1994). The *Hae*III profiles of the other three species of *Heterorhabditis* included in Fig. 5 were all distinctly different from that of *H. indica*. As can be seen from Fig. 6, the *Hae*III rDNA IGS restriction profile designated D1a by Curran and Driver (1994) (lanes 4-7) is similar to that of *H. indica* LN2, but the *Hae*III pattern of the Kenyan isolates (Fig. 6, lane 3; Fig. 5, lanes 9,10) is unique.

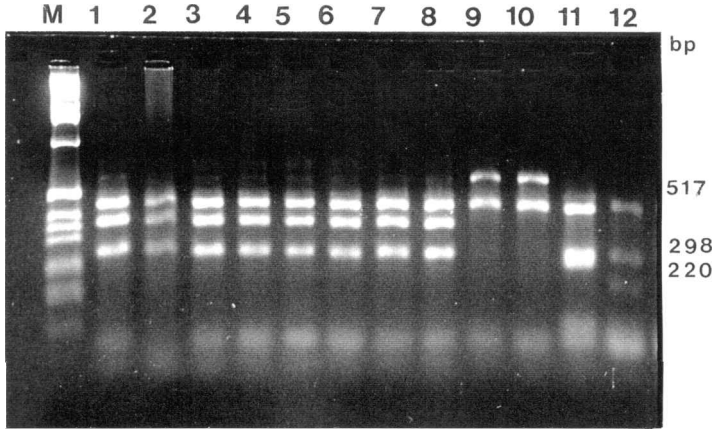

Fig. 3. Hinf*I restriction digests of the PCR amplification products of the rDNA internal transcribed spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel and stained with ethidium bromide. M: 1 kb marker; 1: LN2; 2: P2M; 3: LN2B; 4: LN4; 5: INA H23; 6: INA H9; 7: INA H17; 8: INA H1; 9: Ki3; 10: K4A; 11:* Heterorhabditis *'Irish type' K122; 12:* H. megidis *HL81.*

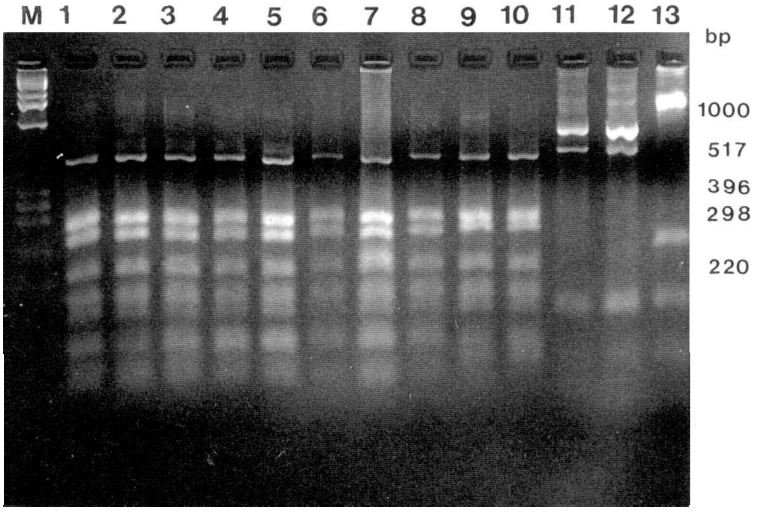

Fig. 4. Alu*I restriction digests of the PCR amplification products of the rDNA intergenic spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel and stained with ethidium bromide. M: 1kb size marker; 1: LN2; 2: P2M; 3: LN2B; 4: LN4; 5: INA H23; 6: INA H9; 7: INA H17; 8: INA H1; 9: Ki3; 10: K4A; 11:* Heterorhabditis *'Irish type' K122; 12:* H. megidis *HL81; 13:* H. bacteriophora *HP88.*

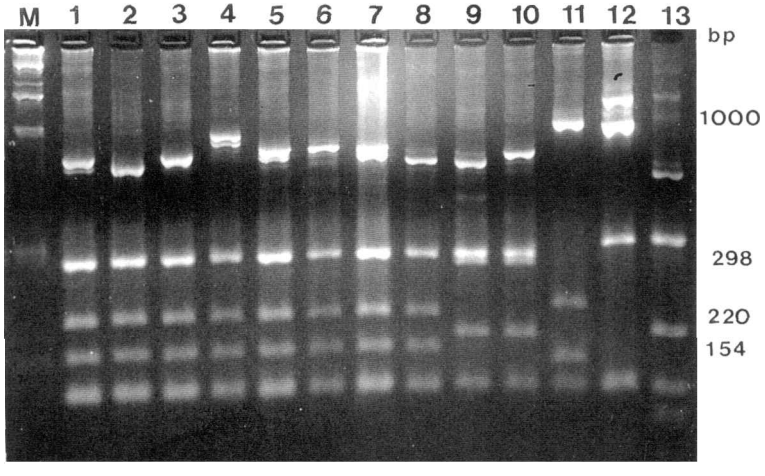

Fig. 5. Hae*III restriction digests of the PCR amplification products of the rDNA intergenic spacer region of* Heterorhabditis *isolates, separated on a 2% agarose gel and stained with ethidium bromide. M: 1kb size marker; 1: LN2; 2: P2M; 3: LN2B; 4: LN4; 5: INA H23; 6: INA H9; 7: INA H9; 8: INA H1; 9: Ki3; 10: K4A; 11:* Heterorhabditis *'Irish type' K122; 12:* H. megidis *HL81; 13:* H. bacteriophora *HP88.*

USE OF *H. INDICA* SPECIES SPECIFIC SATELLITE DNA PROBE

When Southern blots of total genomic DNA of the tropical isolates were probed with the *H. indica* species-specific satellite DNA probe described by Abadon *et al.* (1998), the probe hybridised only with the isolates classified from PCR analysis as being *H. indica*, including the Kenyan isolates Ki3 and K4A (Fig. 7A). No hybridisation was detected by this probe to any of the other species included as controls on the Southern blot. This blot was then reprobed with the *H. bacteriophora* specific satellite DNA probe described by Grenier *et al.* (1996), and this probe hybridised only with the HP88 isolate of *H. bacteriophora* included on the Southern blot (data not shown).

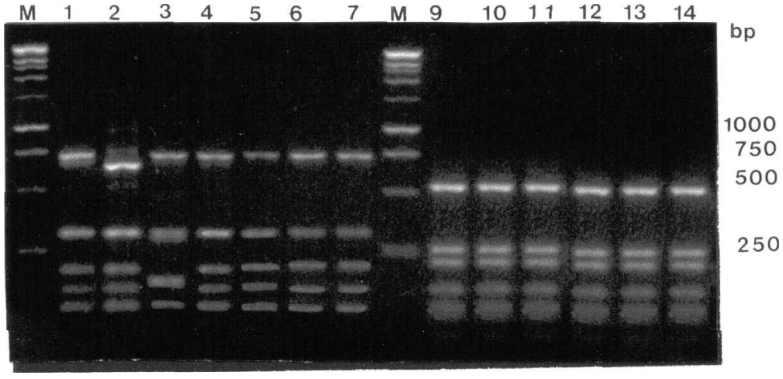

Fig. 6. Hae*III and* Alu*I restriction digests of the PCR amplification products of the rDNA intergenic spacer region of* Heterorhabditis *isolates, including isolates designated type D1a by Curran and Driver (1994). M: 1kb size marker; 1:* H. indica *LN2; 2: D1; 3: K4A; 4: ES10; 5: JAM79; 6: ST09; 7: FLGS10; M: 1 kb marker; 9:* H. indica *LN2; 10: D1; 11: ES10; 12: JAM79; 13: ST09; 14: FLGS10. Lanes 1 to 7 contain* Hae*III digests and lanes 9 to 14 contain* Alu*I digests. The digests were separated on a 2% agarose gel and stained with ethidium bromide.*

Table 2. *The results of cross-breeding experiments of six* Heterorhabditis *isolates from India, Indonesia and Kenya.*

Female	Male					
	H. indica LN2 India	LN 4 India	INA H1 Indonesia	INA H23 Indonesia	Ki3 Kenya	K4A Kenya
H. indica LN2	+	+	+	0	+	0
LN 4	+	+	0	0	+	+
INA H1	+	+	+	+	+	0
INA H23	0	0	+	+	0	0
Ki3	+	+	+	0	+	+
K4A	0	0	0	0	+	+

+ cross resulted in fertile progeny;
0 no progeny detected.

Cross breeding analysis

Selected cross-breeding results are presented in Table 2. All the Indian isolates are interfertile. The Kenyan isolates, which differed from *H. indica* in their *Hinf*I ITS restriction profile and their *Hae*III IGS restriction profile, are interfertile amongst each other but only one Kenyan isolate, Ki3, produced viable progeny when crossed with the *H. indica* LN2 type species. The Indonesian isolates are interfertile, but surprisingly, only one of the Indonesian isolates tested (INA H1) produced viable hybrids when crossed with *H. indica* LN2. The INA H1 isolate was also interfertile with the Kenyan isolate Ki3.

Discussion

The results presented here confirm and extend previous studies which show that a combination of molecular diagnostic tools can be reliably used for species identification in *Heterorhabditis.* Isolates of *Heterorhabditis* from India, Kenya, Indonesia and Cuba hybridised to the *H. indica* specific satellite DNA probe (Grenier *et al.*, 1996; Abadon *et al.*, 1998). *H. indica* specific DNA restriction profiles were also obtained for all the tropical isolates of *Heterorhabditis* investigated here when the ITS rDNA region was restricted using the diagnostic restriction enzymes *Alu*I and *Mbo*I (Joyce *et al.*, 1994a). The utility of restriction digests of the rDNA ITS region in species diagnosis of EPN has been confirmed in several studies (Joyce *et al.*, 1994a, b; Miduturi *et al.*, 1996; Hominick *et al.*, 1997; Yoshida *et al.*, 1998; Griffin *et al.*, 1999a; Pamjav *et al.*, 1999). Restriction of the rDNA IGS region with *Alu*I also yielded a *H. indica* specific restriction profile for all the tropical isolates tested in this study.

The PCR amplification product which we obtained for the *H. indica* rDNA IGS varied in size from 1.2 to 1.6 kb. Hominick *et al.* (1997) also observed length heterogeneity in the rDNA IGS region among geographic isolates of single *Steinernema* spp. and they suggested that this heterogeneity may make restriction profiles of this DNA fragment unreliable for species identification. The length heterogeneity which we detected in the *H. indica* IGS PCR amplification product did not affect the *Alu*I restriction profile of the product and a clear-cut seven restriction fragment pattern was common to all the *H. indica* isolates

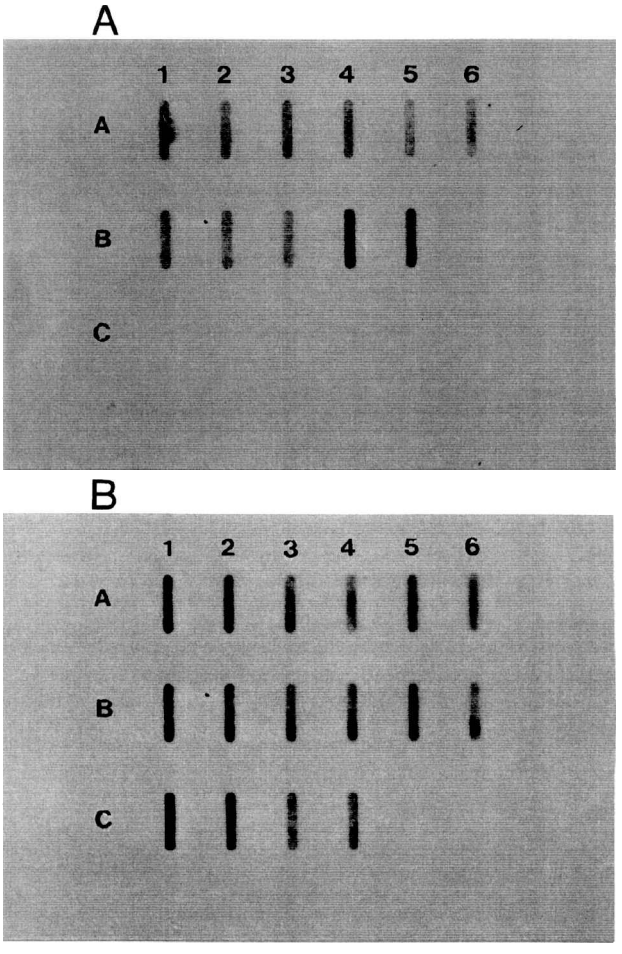

Fig. 7. *A: Slot blot analysis of genomic DNA (100 ng) of the* Heterorhabditis *isolates using a* H. indica *specific satellite DNA probe; B: The membrane used for Fig. 7A was stripped and reprobed using an rDNA 18S probe as a loading control. A1: H. indica LN2; A2: D1; A3: P2M; A4: LN2B; A5: LN4; A6: INA H23; B1: INA H9; B2: INA H17; B3: INA H1; B4: Ki3; B5: K4A; B6:* H. bacteriophora *HP88; C1:* H. zealandica *NZH3; C2:* H. marelatus *OH-10; C3:* H. hepialus *Bodega Bay; C4:* Heterorhabditis *'Irish type' K122.*

tested. When the IGS region was digested with *Hae*III, *H. indica* LN2 displayed a distinctive five fragment profile. All of the *H. indica* isolates, with the exception of the Kenyan isolates, also displayed a five fragment profile. Four of these fragments were conserved among the isolates, but the largest fragment (of *ca* 740 in *H. indica* LN2) was polymorphic among the *H. indica* isolates. This fragment, which seems to be associated with the length polymorphism in the *H. indica* IGS region, is the basis of the D1/D1a polymorphism observed by Curran and Driver (1994). In most species studied to date, the rDNA IGS contains tandem arrays of subrepeats (Gerbi, 1985; Williams *et al.*, 1990; Vahidi & Honda, 1991; Novak *et al.*, 1993; Linares *et al.*, 1994; Crease, 1995). This results in the length of the IGS region being variable both between and within species. The length heterogeneity of the *H. indica* IGS PCR product did not affect the *Alu*I restriction profile of this fragment, which suggests that *Alu*I has a restriction site within a tandem repetitive unit in the rDNA IGS of *H. indica.* In contrast, *Hae*III appears not to cut within this tandem repeat region with the result that one of the *Hae*III restriction fragments is polymorphic, its length presumably depending on the number of *Alu*I repeat units which it contains.

The *Hae*III rDNA IGS restriction profile of the D1a isolate is similar to that of *H. indica* LN2, but the *Hae*III pattern of the Kenyan isolates is unique. The large *Hae*III fragment of the Kenyan isolate K4A is the same size as that of D1a, thus the Kenyan isolates appear to represent a distinct subgroup within D1a. Tropical isolates from Darwin (North Australia), Puerto Rico, Hawaii, the Virgin Islands and Egypt were found by Curran and Driver (1994) to possess the D1 profile, while the D1a profile was described by these authors from isolates collected in Puerto Rico, Jamaica, Florida, the Virgin Islands and Egypt. Comparison of the D1 and D1a *Hae*III and *Alu*I rDNA IGS restriction profiles with those of *H. indica* LN2, indicates that the isolates designated D1 and D1a by Curran and Driver (1994) belong to *H. indica.*

An important step towards achieving an effective nematode bacterium complex for pest control is to seek naturally occurring endemic EPN isolates, as such isolates are likely to possess physiological traits that are adapted to local climatic and ecological conditions. The Kenyan isolates described in this study clearly belong to a distinct sub-group of *H. indica* and it is probable that these isolates also share a distinctive phenotype. The two Kenyan isolates are interfertile but only one of these isolates (Ki3) produced viable progeny when crossed with the *H. indica* LN2 type species. All of the Indonesian *Heterorhabditis* isolates were indistinguishable from *H. indica* in the diagnostic molecular tests and they were interfertile with each other, but only one of the Indonesian isolates (INA H1) was interfertile with *H. indica* LN2 and INA H1 was also interfertile with the Ki3 isolate from Kenya. These results suggest that, although *H. indica* has a global distribution in tropical and subtropical regions of the world, geneflow within the species may be quite restricted. We have previously observed reproductive incompatibility be-

tween isolates of *H. bacteriophora* from Europe (Griffin *et al.*, 1999a) and from North America (Dix *et al.*, unpubl.). We have not determined whether this reproductive isolation is caused by genetic means or is the result of cytoplasmic factors. Cytoplasmic factors such as endosymbiont bacteria (see reviews by Werren, 1997; Johanowicz & Hoy, 1998) and chromosomal factors such as transposons (reviewed by Kidwell, 1990; Petrov *et al.*, 1995) have been shown to be frequent causes of reproductive incompatibility in arthropods. Endosymbiont bacteria have recently been shown to cause reproductive incompatibility in filarial nematodes (Hoerauf *et al.*, 1999). Since the reproductive incompatibility observed in this study does not yield clear groups of compatible and incompatible strains and in view of the consistency of the DNA based diagnostic tests, any assignment of biological species within the *H. indica* group would be injudicious at present. Adams (1988) evaluates phylogenetic and biological species concepts in the delimitation of species in the *H. bacteriophora* group.

The global distribution of *H. indica* throughout the tropics and subtropics suggests that *H. indica* possesses a range of phenotypic characters which give it an advantage in this climatic zone. Such phenotypes might include adaptation to high temperatures, desiccation tolerance and good dispersal ability. It is unlikely that the widespread distribution of *H. indica* in the tropics arises because this species was extant before the continents began breaking up and drifting. The best supported hypothesis of phylogenetic relationships among *Heterorhabditis* species, in a phylogeny derived from the rDNA ITS 1 DNA sequence data, puts *H. indica* as the most ancient *Heterorhabditis* lineage (Adams *et al.*, 1998). However, the phylogenetic tree for *Heterorhabditis* and *Steinernema* of Reid (1994) shows that the genetic distance between *Heterorhabditis* species is considerably less than that between *Steinernema* species and given the small genetic distance detected by Reid (1994) between the most divergent of the *Heterorhabditis* species in his study (a D value of *ca* 0.04 as calculated by the method of Nei and Li, 1979), it seems unlikely that *H. indica* speciated before the Jurassic/Cretaceous break up of Pangaea (an estimated 200 million years ago).

Acknowledgement

We acknowledge the support of the European Community STD-3 Programme (TS3 CT 940273).

References

ABADON, M., GRENIER, E., LAUMOND, C. & ABAD, P. (1998). A species-specific satellite DNA from the entomopathogenic nematode *Heterorhabditis indicus*. *Genome* 41, 148-153.

ADAMS, B.J. (1998). Species concepts and the evolutionary paradigm in modern nematology. *Journal of Nematology* 30, 1-21.

ADAMS, B.J., BURNELL, A.M. & POWERS, T.O. (1998). A phylogenetic analysis of the genus *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. *Journal of Nematology* 30, 22-39.

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.

ARTEAGA HERNANDEZ, E.M. & MRÁČEK, Z. (1984). *Heterorhabditis heliothidis*, a parasite of insect pests in Cuba. *Folia Parasitologia* (Praha) 31, 11-17.

BLAXTER, M.L., DE LEY, P,. GAREY, J.R., LIU, L.X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J.R., MACKEY, L.Y., DORRIS, M., FRISSE, L.M., VIDA, J.T. & THOMAS, W.K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71-75.

CONSTANT, P., MARCHAY, L., FISHER-LE SAUX, M., BRIAND-PANOMA, S., & MAULÉON, H. (1998). Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Guadeloupe islands. *Fundamental and Applied Nematology* 21, 667-672.

CREASE, T.J. (1995). Ribosomal DNA evolution at the population-level — nucleotide variation in intergenic spacer arrays of *Daphnia pulex*. *Genetics* 141, 1327-1337.

CURRAN, J. & DRIVER, F. (1994). Molecular taxonomy of *Heterorhabditis*. In: Burnell, A.M., Ehlers, R-U. & Masson, J.P. (Eds). *Genetics of Entomopathogenic Nematode-Bacterium Complexes*. Luxembourg, European Commission Publication EUR 15681 EN, pp. 178-187.

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

EHLERS, R.-U. (1996). Current and future use of nematodes in biocontrol: practice and commercial aspects with regard to regulatory policy issues. *Biocontrol Science and Technology* 6, 303-317.

GERBI, S.A. (1985). Evolution of ribosomal DNA. In: MacIntyre, R.J. (Ed.) *Molecular evolutionary genetics*. New York, NY, USA, Plenum Press, pp. 419-517.

GRENIER, E., BONIFASSI, E., ABAD, P. & LAUMOND, C. (1996a). Use of species specific satellite DNAs as diagnostic probes in the identification of Steinernematidae and

Heterorhabditidae entomopathogenic nematodes. *Parasitology* 113, 483-489.

GRENIER, E., LAUMOND, C., & ABAD, P. (1996b). Molecular characterisation of two species-specific tandemly repeated DNAs from entomopathogenic nematodes *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida). *Molecular and Biochemical Parasitology* 83, 47-56.

GRIFFIN, C.T., JOYCE, S.A., DIX, I. BURNELL, A.M. & DOWNES, M.J. (1994). Characterisation of the entomopathogenic nematode *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., DIX, I., JOYCE, S.A., BURNELL, A.M. & DOWNES, M.J. (1999a). Isolation and characterisation of *Heterorhabditis* spp. from Hungary, Estonia and Denmark. *Nematology* 1, 321-332.

GRIFFIN, C.T., CHAERANI, R., FALLON, D., REID, A.P. & DOWNES, M.J. (1999b). Occurrence and distribution of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis indica* in Indonesia. *Journal of Helminthology* 74, 143-150.

HOERAUF, A., NISSENPAHLE, K., SCHMETZ, C., HENKLEDUHRSEN, K., BLAXTER, M.L., BUTTNER, D.W., GALLIN, M.Y., ALQAOUD, K.M., LUCIUS, R. & FLEISCHER, B. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomososoides sigmodontis* and results in filarial infertility. *Journal of Clinical Investigation* 103, 11-17.

HOMINICK, W.M., REID, A.P. BOHAN, D.A. & BRISCOE, B.R. (1996). Entomopathogenic nematodes: biodiversity, geographical distribution and convention on biological diversity. *Biocontrol Science and Technology* 6, 317-332.

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

JOHANOWICZ, D.L. & HOY, M.A. (1998). The manipulation of arthropod reproduction by *Wolbachia* endosymbionts. *Florida Entomologist* 81, 310-317.

JOYCE, S.A., BURNELL, A.M. & POWERS, T.O. (1994a). The characterisation of *Heterorhabditis* isolates by restriction enzyme analysis of PCR amplified fragments of mtDNA and rDNA genes. *Journal of Nematology* 26, 260-270.

JOYCE, S.A., REID, A. & CURRAN, J. (1994b). Application of the 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*. Luxembourg, European Commission Publication EUR 15681 EN, pp. 178-187.

KAYA, H.K. (1990). Soil ecology. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, USA, CRC Press, pp. 93-115.

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

KIDWELL, M.G. (1990). Evolutionary aspects of hybrid dysgenesis in *Drosophila*. *Canadian Journal of Zoology* 68, 1716-1726.

LINARES, A.R., BOWEN, T. & DOVER, G.A. (1994). Aspects of non-random turnover involved in the concerted evolution of intergenic spacers within the ribosomal DNA of *Drosophila melanogaster*. *Journal of Molecular Evolution* 39, 151-159.

LIU, J. (1994). [A new species of the genus *Heterorhabditis* from China (Rhabditida: Heterorhabditidae).] *Acta Zootaxonomica Sinica* 19, 268-272.

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

MASON, J.M., RAZAK, A.R. & WRIGHT, D.J. (1996). The recovery of entomopathogenic nematodes from selected areas within Peninsular Malaysia. *Journal of Helminthology* 70, 303-307.

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.

NEI, M. & LI, W.-H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States* 76, 5269-5273.

NOVAK, E.M., DE MELLO, M.P., GOMES, H.B.M., GALINDO, I., GUEVARA, P., RAMIREZ J.L. & DA SILVERIA J.F. (1993). Repetitive sequences in the ribosomal intergenic spacer of *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* 60, 273-280.

PAMJAV, H., TRIGA, D. BUZAS, Z., VELLAI, T., LUCSKAI, A., ADAMS, B., REID, A.P., BURNELL, A.M., GRIFFIN, C., GLAZER, I., KLEIN, M.G. & FODOR, A. (1999). Novel application of PhastSystem polyacrylamide gel electrophoresis using restriction fragment length polymorphism-internal transcribed spacer patterns of individuals for molecular identification of entomopathogenic nematodes. *Electrophoresis*, 20,1266-1273.

PETROV, D.A., SCHUTZMAN, J.L., HARTL, D.L., & LOZOVSKAYA, E.R. (1995). Diverse transposable elements are mobilised in hybrid dysgenesis in *Drosophila virilis*. *Proceedings of the National Academy of Sciences of the United States* 92, 8050-8054.

POINAR, G.O. JR. (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. 23-64.

POINAR, G.O. JR. (1993). Origins and phylogenetic relationships of the entomophilic rhabditids *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* 16, 333-338.

POINAR, G.O. JR., JACKSON, T. & KLEIN, M. (1987). *Heterorhabditis megidis* sp. n. (Heterorhabditidae: Rhabditida) parasitic in the Japanese beetle *Popillia japonica* (Scarabidae: Coleoptera), in Ohio. *Proceedings of the Helminthological Society of Washington* 53, 53-59.

POINAR, G.O. JR., KARUNAKAR, G.K. & DAVID, H. (1992). *Heterorhabditis indicus* n. sp. (Rhabditida: Nematoda) from India: Separation of *Heterorhabditis* spp. by infective juveniles. *Fundamental and Applied Nematology* 15, 467-472.

REID, A.P. (1994). Molecular taxonomy of *Steinernema*. In: Burnell, A.M., Ehlers, R.-U. & Masson, J.P. (Eds). *Genetics of entomopathogenic nematode-bacterium complexes*. Luxembourg, European Commission Publication EUR 15681 EN, pp. 178-187.

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

STOCK, S.P. (1993). A new species of thegenus *Heterorhabditis* Poinar, 1976 (Nematoda: Heterorhabditidae) parasitizing *Graphognathus* sp. larvae (Coteoptera: Curculionidae) from Argentina. *Research and Reviews in Parasitology* 53, 103-107.

STOCK, S.P. (1997). *Heterorhabditis hepialus* Stock, Strong & Gardner, 1996a junior synonym of *H. marelatus* Liu & Berry, 1996 (Rhabditida: Rhabditidae) with a redescription of the species. *Nematologica* 43, 455-463.

STOCK, S.P., STRONG, D. & GARDNER, S. (1996). Identification of *Heterorhabditis* (Nematoda: Heterorhabditidae) from California with a new species isolated from the larvae of the ghost moth *Hepialius* [*sic*] *californicus* (Lepidoptera: Hepialidae) from Bodega Bay Natural Reserve. *Fundamental and Applied Nematology* 19, 585-592.

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 lupine. *Oecologica* 108, 39-45.

SUDHAUS, W. (1993). Die mittels symbiontischer Bakterien entomopathogenen Nematoden Gattungen *Heterorhabditis* and *Steinernema* sind keine Schwestertaxa. *Verhandlungen der Deutschen Zoologischen Gesellschaft* 86, 146.

VAHIDI, H. & HONDA, B.M. (1991). Repeats and subrepeats in the intergenic spacer of rDNA from the nematode *Meloidogyne arenaria*. *Molecular and General Genetics* 227, 334-336.

WERREN, J.H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology* 42, 587-609.

WILLIAMS, S.M., ROBBINS, L.G., CLUSTER, P.D., ALLARD, R.W. & STROBECK, C. (1990). Superstructure of the *Drosophila* ribosomal gene family. *Proceedings of the National Academy of Sciences of the United States* 87, 3156-3160.

WOUTS, W.M. (1979). The biology and life cycle of a New Zealand population of *Heterorhabditis heliothidis* (Heterorhabditidae). Nematologica 25, 191-202.

YOSHIDA, M., REID, A.P., BRISCOE, B.R., & HOMINICK, W.M. (1998). Survey of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Japan. *Fundamental and Applied Nematology* 21, 185-198.