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Heterorhabditis baujardi sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations

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Summary – A survey of entomopathogenic nematodes in Vietnam yielded several *Heterorhabditis* isolates. The majority belonged to *H. indica*; their morphometrics are given in this paper. Three isolates collected in forests in Backan, Ninhbinh and Kontum provinces, Vietnam are here described as a new species *Heterorhabditis baujardi* sp. n. The new species is distinguished from the other ten valid *Heterorhabditis* species by a combination of morphological, morphometrical, and DNA characters. *Heterorhabditis baujardi* sp. n. is morphometrically similar to *H. indica*, but can be separated from this species by the shape of the gubernaculum and the number of normal pairs of genital papillae. The gubernaculum of *H. baujardi* sp. n. with the proximal end ventrally curved resembles that of *H. bacteriophora*. *Heterorhabditis baujardi* sp. n. can be separated from this latter species by a shorter body length of infective juveniles and longer spicules, longer gubernaculum, and a higher spicule length to anal body diameter ratio of males. The canonical discriminant analysis of morphometrical characters of both infective juveniles and males failed to discriminate *Heterorhabditis baujardi* sp. n. from *H. indica*. However, the new species was easily distinguished from *H. downesi*, *H. marelatus*, *H. megidis* and *H. bacteriophora*. *Heterorhabditis baujardi* sp. n. was slightly separated from *H. bacteriophora* by variables of the infective juveniles, but was clearly distinguished by variables of the males. Cross-breeding tests using isolates of the new species and *H. indica* did not yield fertile progeny. Analysis of the ITS1 sequence of rDNA of *H. baujardi* sp. n. revealed substantial differences with other known ITS1 *Heterorhabditis* sequences. Phylogenetic relationships between *Heterorhabditis* species and the usefulness of morphological and molecular characters for identification of species from this group are discussed.

Keywords – cross-breeding test, entomopathogenic nematodes, *Heterorhabditis*, ITS regions, phylogeny, rDNA, systematics, taxonomy.

Species of the families Steinernematidae and Heterorhabditidae are frequently used for biological control of insects. The better adapted these entomopathogenic nematodes (epn) are to the environmental conditions under which such a control is attempted, the more efficient the insect control that can be expected (Bedding, 1990). Therefore, it is important to search for indigenous populations and species.

Currently, the genus *Heterorhabditis* Poinar, 1976 contains ten valid species. Surveys for heterorhabditids conducted in different parts of the world have revealed their global distribution (Hominick *et al.*, 1997; Stock *et al.*, 2001). A survey of epn in Vietnam yielded several isolates of *Steinernema* of which three new species have previously been described (Phan *et al.*, 2001a, b). The survey also yielded several *Heterorhabditis* isolates. The majority belonged to *H. indica* but three isolates are here described as a new species, *Heterorhabditis baujardi* sp. n. The new species is separated from other *Heterorhabditis* species by differences in morphology, morphometrics, ITS1 sequences and results of cross-breeding tests.

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Table 1. *Populations of* Heterorhabditis *spp. used in Canonical Discriminant Analysis.*

Species	Location	Isolates	Source data
H. baujardi sp. n.	Sathay, Kontum, Vietnam	H.TN40	this paper
H. baujardi sp. n.	Babe, Backan, Vietnam	H.BB32	this paper
H. baujardi sp. n.	Cucphuong, Ninhbinh, Vietnam	H.CP13	this paper
H. bacteriophora	Brecon, Australia		Poinar, 1976
H. bacteriophora	Logan, Utah, USA		Poinar & Georgis, 1990
H. bacteriophora	Oliva, Córdoba, Argentina	OLI	Doucet *et al.*, 1996
H. bacteriophora	Río Cuarto, Córdoba, Argentina	RIV	Doucet *et al.*, 1996
H. downesi	Wexford, Ireland	K122	Stock *et al.*, 2001
H. downesi	Hornbaek, Denmark	EU94	Stock *et al.*, 2001
H. downesi	Kesckemet, Hungary	EU349	Stock *et al.*, 2001
H. indica	Babe, Backan, Vietnam	H.BB9	this paper
H. indica	Cucphuong, Ninhbinh, Vietnam	H.CP16	this paper
H. indica	Cucphuong, Ninhbinh, Vietnam	H.CP6	this paper
H. indica	Cucphuong, Ninhbinh, Vietnam	H.CP8	this paper
H. indica	Huongson, Hatinh, Vietnam	H.HS5	this paper
H. indica	Dienbien, Laichau, Vietnam	H.MP11	this paper
H. indica	Dienbien, Laichau, Vietnam	H.MP16	this paper
H. indica	Thaian, Ninhthuan, Vietnam	H.NT3	this paper
H. indica	Sathay, Kontum, Vietnam	H.TN48	this paper
H. indica	Tamil Nadu, India		Poinar *et al.*, 1992
H. indica	Pakistan		Anis *et al.*, 2000
H. marelatus	Oregon, USA		Liu & Berry, 1996
H. marelatus	California, USA		Stock *et al.*, 1996
H. marelatus	Oregon, USA	OH10	Stock, 1997
H. marelatus	California, USA		Stock, 1997
H. marelatus	California, USA		Stock, 1997
H. megidis	Ohio, USA		Poinar *et al.*, 1987
H. megidis	Maisieres, Henegouwen, Belgium	VBM30	Miduturi, 1997
H. megidis	Snellegem, West-Flanders, Belgium	Sn5	Miduturi, 1997

Materials and methods

Sampling

Soil samples (*ca* 500 ml) were collected in forests of the Vietnamese provinces of Backan, Ninhbinh and Kontum (Table 1) and were taken to a depth of 20 cm. Coordinates and altitudes of the sampling sites were registered using GARMIN GPS12CX. Entomopathogenic nematodes were extracted from soil by the *Galleria* baiting method (Bedding & Akhurst, 1975). Infective juveniles were collected from *Galleria* cadavers using the method of White (1927) and stored at 15°C in aerated water.

Morphological observations

For light microscopy, nematodes were reared on *G. mellonella.* We used infective juveniles (IJ) collected during the week after their first emergence from the insect cadavers; adults of the first generation were dissected from the cadavers (Nguyen & Smart, 1995). Nematodes were killed, fixed in hot 4% formalin (50-60°C), and kept in this solution for 48 h. Fixed nematodes were transferred to anhydrous glycerine according to Seinhorst's (1959) rapid method as modified by De Grisse (1969) and mounted on slides using coverglass supports to avoid flattening. All measurements were made using a drawing tube attached to an Olympus BX50 light microscope. The measurements of the tails of IJ included the cuticle part.

Statistical analyses

Morphometrics of infective juveniles and males from 29 populations belonging to six valid species of *Heterorhabditis* (Table 1) were used in a Canonical Discriminant Analysis (CDA) performed with STATISTICA (version

Table 2. *Morphometrics of infective juveniles (in μm) of* Heterorhabditis indica *Poinar* et al.*, 1992 from Vietnam. Measurements in form: mean ± SD (range).*

Characters [1)]	*H. indica* H.BB9	*H. indica* H.CP6	*H. indica* H.CP8	*H. indica* H.CP16	*H. indica* H.HS5	*H. indica* H.MP11	*H. indica* H.MP16	*H. indica* H.NT3	*H. indica* H.TN48	*H. indica* (after Poinar *et al*., 1992)
n	25	25	25	25	25	25	25	25	25	25
L	583 ± 20 (547-643)	558 ± 28 (490-614)	575 ± 21 (538-619)	566 ± 17 (511-600)	595 ± 19 (557-626)	590 ± 19 (550-624)	602 ± 15 (578-636)	593 ± 21 (542-638)	563 ± 55 (348-622)	528 ± 26 (479-573)
Body diam.	21 ± 0.9 (20-23)	19 ± 0.9 (18-22)	20 ± 0.7 (18-21)	20 ± 1 (17-22)	20 ± 0.7 (19-22)	20 ± 1 (17-21)	20 ± 0.8 (19-22)	21 ± 0.5 (20-22)	20 ± 0.8 (18-21)	20 ± 6 (19-22)
Tail length	99 ± 4.9 (90-109)	96 ± 5.6 (86-105)	98 ± 5.9 (87-109)	96 ± 3.5 (90-102)	101 ± 3.7 (94-107)	98 ± 3.9 (91-106)	100 ± 4.7 (87-109)	100 ± 4.6 (90-108)	99 ± 6.9 (88-112)	101 ± 6 (93-109)
Anal body diam.	13 ± 0.4 (12.5-14)	13 ± 0.8 (11-14)	13 ± 0.7 (11-14)	13 ± 0.7 (11-14)	13 ± 0.7 (12-15)	13 ± 0.6 (12-14)	14 ± 0.9 (11-16)	14 ± 0.5 (13-14)	13 ± 0.6 (11-14)	n/a
Nerve ring	82 ± 2.4 (78-89)	82 ± 3.4 (75-91)	87 ± 6.1 (78-100)	81 ± 3.7 (74-95)	85 ± 4.1 (79-99)	85 ± 3.2 (80-91)	85 ± 3.7 (77-95)	83 ± 2.4 (79-87)	83 ± 4.5 (75-89)	82 ± 4 (72-85)
Excretory pore	97 ± 2.4 (92-104)	95 ± 3.5 (90-105)	100 ± 4.8 (92-110)	97 ± 4.8 (86-110)	99 ± 5.0 (92-118)	99 ± 3.4 (91-106)	102 ± 4.3 (92-111)	98 ± 2.8 (91-103)	96 ± 4.6 (88-103)	98 ± 7 (88-107)
Pharynx	117 ± 3.1 (111-125)	112 ± 2.4 (108-117)	115 ± 2.2 (111-119)	112 ± 2.4 (108-116)	117 ± 4.5 (99-123)	118 ± 2.8 (113-124)	119 ± 4.4 (122-128)	118 ± 3.2 (111-124)	117 ± 5.4 (108-124)	117 ± 3 (109-123)
a	28 ± 1.2 (25-30)	29 ± 1 (27-31)	30 ± 1 (27-31)	29 ± 1.1 (26-30)	30 ± 1 (28-32)	30 ± 1.3 (27-33)	30 ± 1 (28-32)	29 ± 0.9 (27-30)	29 ± 2.7 (27-33)	26 ± 4 (25-27)
b	5.0 ± 0.1 (4.7-5.2)	5.0 ± 0.2 (4.4-5.3)	5.0 ± 0.2 (4.6-5.4)	5.0 ± 0.1 (4.7-5.3)	5.1 ± 0.3 (4.8-6.1)	5.0 ± 0.1 (4.8-5.2)	5.1 ± 0.2 (4.6-5.4)	5.0 ± 0.2 (4.5-5.2)	4.8 ± 0.4 (2.8-6.3)	4.5 ± 0.34 (4.3-4.8)
c	5.9 ± 0.2 (5.5-6.2)	5.8 ± 0.2 (5.3-6.3)	5.9 ± 0.3 (5.6-6.7)	5.9 ± 0.2 (5.6-6.3)	6.0 ± 0.2 (5.3-6.2)	6.0 ± 0.2 (5.7-6.4)	6.0 ± 0.3 (5.6-7.3)	5.9 ± 0.2 (5.6-6.3)	5.7 ± 0.6 (3.1-6.6)	5.3 ± 0.5 (4.5-5.6)
d	0.83 ± 0.02 (0.8-0.87)	0.85 ± 0.03 (0.81-0.9)	0.86 ± 0.04 (0.8-0.93)	0.86 ± 0.04 (0.75-0.95)	0.85 ± 0.08 (0.77-1.19)	0.84 ± 0.02 (0.79-0.88)	0.86 ± 0.03 (0.8-0.9)	0.84 ± 0.02 (0.81-0.88)	0.82 ± 0.02 (0.79-0.85)	0.84 ± 0.05 (0.79-0.9)
e	0.97 ± 0.04 (0.9-1.06)	0.99 ± 0.06 (0.89-1.15)	1.02 ± 0.07 (0.91-1.25)	1.02 ± 0.07 (0.91-1.23)	0.98 ± 0.07 (0.89-1.12)	1.01 ± 0.04 (0.95-1.09)	1.02 ± 0.05 (0.92-1.18)	0.98 ± 0.05 (0.9-1.1)	0.97 ± 0.06 (0.88-1.09)	0.94 ± 0.07 (0.83-1.03)
f	0. 21 ± 0.01 (0.2-0.24)	0.20 ± 0.01 (0.18-0.22)	0.20 ± 0.01 (0.18-0.24)	0.21 ± 0.01 (0.19-0.24)	0.20 ± 0.01 (0.19-0.21)	0.20 ± 0.01 (0.18-0.23)	0.20 ± 0.01 (0.19-0.25)	0.21 ± 0.01 (0.19-0.23)	0.20 ± 0.01 (0.18-0.22)	0.20 ± 0.05 (0.18-0.22)

[1)] d = distance from anterior end to excretory pore/pharynx length; e = distance from anterior end to excretory pore/tail length; f = body diam./tail length.

5.5) computer package. *Heterorhabditis indica* Poinar *et al.*, 1992 was represented by two populations described in literature (Poinar *et al.*, 1992; Anis *et al.*, 2000) and nine Vietnamese populations of which the morphometrics are shown in Tables 2 and 3.

DNA EXTRACTION, PCR, DNA PURIFICATION AND SEQUENCING

DNA was extracted from a single hermaphroditic female using a modification of the method of Joyce *et al.* (1994c). For each isolate, a specimen was cut in 8 μl of worm lysis buffer (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatin). The nematode fragments were transferred in 4 μl of the buffer to an Eppendorf tube to which 5 μl of double distilled H_2O and 1 μl of proteinase K (600 μg/ml) were added. After freezing (−70°C 1 h) the tubes were incubated at 65°C for 1 h, and then at 95°C for 10 min.

After centrifugation (1 min; 13 000 *g*) of the tubes, 10 μl of the DNA suspension were added to a PCR reaction mixture containing 4 μl 10× PCR buffer, 1 μl $MgCl_2$ (25 mM), 1 μl dNTP mixture (10 mM each), 0.2 μl (500 nM) of each primer, 1.5 U *Taq* polymerase and 33.3 μl double distilled water to a final volume of 50 μl. The forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) were used in the PCR reaction

Table 3. *Morphometrics of males (in μm) of* Heterorhabditis indica *Poinar* et al.*, 1992 from Vietnam. Measurements in form: mean ± SD (range).*

Characters[1)]	*H. indica* H.BB9	*H. indica* H.CP6	*H. indica* H.CP8	*H. indica* H.CP16	*H. indica* H.HS5	*H. indica* H.MP11	*H. indica* H.MP16	*H. indica* H.NT3	*H. indica* H.TN48	*H. indica* (after Poinar *et al*., 1992)
n	15	15	15	15	15	15	15	15	15	12
L	623 ± 48 (550-715)	753 ± 38 (708-847)	786 ± 44 (703-838)	733 ± 48 (638-823)	856 ± 57 (761-936)	706 ± 45 (631-818)	974 ± 51 (929-1046)	815 ± 44 (727-895)	689 ± 81 (506-818)	721 ± 64 (573-788)
Body diam.	45 ± 3 (38-48)	46 ± 2 (43-50)	46 ± 3 (38-50)	38 ± 3 (34-41)	46 ± 3 (38-50)	53 ± 3 (46-58)	47 ± 6 (38-53)	58 ± 6 (53-79)	49 ± 4 (38-50)	42 ± 7 (35-46)
Anal body diam.	24 ± 2 (22-27)	20 ± 1 (18-22)	26 ± 2 (23-30)	20 ± 2 (18-22)	20 ± 1 (18-23)	18 ± 1 (16-21)	17 ± 2 (15-19)	20 ± 1 (18-22)	24 ± 2 (21-29)	23 ± 8 (19-24)
Excretory pore	89 ± 6 (80-103)	90 ± 4 (83-97)	92 ± 6 (80-100)	66 ± 5 (58-77)	79 ± 5 (70-88)	86 ± 5 (76-93)	69 ± 4 (64-72)	92 ± 4 (84-97)	71 ± 5 (63-78)	123 ± 7 (109-138)
Pharynx	101 ± 3 (97-108)	98 ± 3 (93-101)	109 ± 4 (101-114)	94 ± 3 (91-101)	95 ± 6 (86-105)	94 ± 3 (89-99)	99 ± 3 (94-101)	102 ± 3 (97-107)	95 ± 6 (85-103)	101 ± 4 (93-109)
Tail length	30 ± 3 (25-34)	31 ± 3 (26-34)	32 ± 4 (25-42)	31 ± 2 (27-35)	29 ± 3 (25-33)	31 ± 3 (27-40)	30 ± 1 (29-32)	32 ± 2 (30-35)	34 ± 5 (27-41)	28 ± 2 (24-32)
Testis reflexion	85 ± 7 (72-99)	70 ± 2 (60-80)	99 ± 11 (70-117)	72 ± 13 (45-89)	82 ± 10 (57-95)	82 ± 9 (70-99)	123 ± 14 (108-144)	93 ± 9 (79-111)	97 ± 13 (77-117)	91 ± 26 (35-144)
Spicule length	36 ± 3 (30-40)	36 ± 3 (27-39)	38 ± 3 (33-43)	35 ± 2 (32-38)	38 ± 2 (35-42)	37 ± 3 (32-44)	40 ± 3 (36-42)	43 ± 2 (38-45)	39 ± 3 (34-44)	43 ± 3 (35-48)
Gubernaculum length	19 ± 2 (16-22)	19 ± 1 (17-21)	19 ± 2 (17-23)	18 ± 2 (16-22)	18 ± 2 (16-21)	16 ± 2 (11-21)	17 ± 3 (13-19)	21 ± 1 (19-23)	20 ± 2 (17-24)	21 ± 3 (18-22)
GS	0.53 ± 0.04 (0.45-0.57)	0.52 ± 0.04 (0.46-0.63)	0.50 ± 0.05 (0.44-0.57)	0.52 ± 0.05 (0.44-0.62)	0.48 ± 0.04 (0.39-0.55)	0.42 ± 0.05 (0.32-0.5)	0.43 ± 0.04 (0.37-0.46)	0.49 ± 0.04 (0.42-0.57)	0.52 ± 0.04 (0.45-0.57)	0.50 ± 0.1 (0.4-0.6)
SW	1.48 ± 0.2 (1.1-1.78)	1.81 ± 0.2 (1.33-2.09)	1.50 ± 0.1 (1.23-1.69)	1.72 ± 0.1 (1.56-1.94)	1.89 ± 0.2 (1.63-2.18)	2.09 ± 0.2 (1.8-2.41)	2.37 ± 0.3 (2.12-2.74)	2.13 ± 0.2 (1.74-2.39)	1.62 ± 0.2 (1.32-1.97)	n/a

[1)] SW = spicule length/anal body diam.; GS = gubernaculum length/spicule length.

for amplification of the complete ITS region (Joyce *et al.*, 1994c). The amplification profile was carried out using a PTC-100 thermocycler, which was preheated at 92°C for 2 min followed by 35 cycles of 92°C for 30 s, 54°C for 30 s and 72°C for 2 min, and then 72°C for 10 min. After DNA amplification, 5 μl of product was loaded on a 1% agarose gel for DNA checking. Amplified products were purified using a Qiagen Gel Purification Kit (Qiagen Ltd, Leusden, The Netherlands). DNA fragments were sequenced with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Lennik, Belgium). The resulting products were purified using a Centriflex Gel Filtration Cartridge (Edge Biosystems Inc., Gaithersbugs, MD, USA) and run on ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, USA). The DNA sequences were edited with Chromas 1.45. The original ITS sequence of *H. baujardi* sp. n. is deposited at Genbank under accession number AF548768.

Sequence alignment and phylogenetic analysis

The DNA sequence of *H. baujardi* sp. n. was aligned using Clustal X 1.64 (default options) with other ITS1-rDNA sequences obtained from GenBank: *H. zealandica* Poinar, 1990 (AF029705); *H. argentinensis* Stock, 1993 (AF029706); *H. hawaiiensis* Gardner *et al.*, 1994 (AF029707); *H. bacteriophora* Poinar, 1976 (AF029708); *H. indica* Poinar *et al.*, 1992 (AF029710); *H. megidis* Poinar *et al.*, 1987 (AF029711); *H. downesi* Stock *et al.*, 2001 (=*Heterorhabditis* sp. 'Irish K122', AF029712); *H. marelatus* Liu & Berry, 1996 (AF029713) and two outgroup taxa *Caenorhabditis elegans* (Maupas, 1899) (X03680) and *Pellioditis typica* Stefanski, 1922 (AF036946). Two alignments were generated. The first one was limited to nine *Heterorhabditis* species (Fig. 2); the second contained the nine *Heterorhabditis* species along with two outgroup taxa (available by request from

the first author). Both alignments were slightly manually edited.

Equally weighted maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP* (4.0 beta version) (Swofford, 1998). A heuristic search procedure was used with the following settings: ten replicates of random taxon addition, tree-bisection-reconnection branch swapping, multiple trees retained, no steepest descent, and accelerated transformation. Gaps were treated as missing data. Bootstrap analysis with 1000 replicates and Decay analysis were calculated as measures of support for individual clades for MP trees. For ML analysis, the appropriate substitution model of DNA evolution that best fitted the data set was determined by the Akaike Information Criterion with MODELTest 3.06 (Posada & Crandall, 1998). Bootstrap analysis with 100 replicates was conducted to assess the degree of support for ML tree clades. Kishino-Hasegava (KHTest) test was performed as implemented in PAUP. Pairwise divergences between taxa were computed by PAUP* as the absolute distance values and the percent mean distance values adjusted for missing data.

CROSSBREEDING TESTS

Cross-breeding tests with one isolate of *H. baujardi* sp. n. (TN40) and one isolate of *H. indica* (LN2) were carried out on lipid agar plates prepared according to Dunphy and Webster (1989). The plates were inoculated with the primary form of the bacterial symbiont from the appropriate nematode strain and incubated at 30°C for 48 h. For each cross test, 20 virgin females and 20 males of the appropriate strains were placed on the plate and incubated at 25°C; the self-test for controls comprised 20 virgin females and 20 males of the same isolate. Results were checked after 2-3 days (Griffin *et al.*, 1994; Stock *et al.*, 1996).

Heterorhabditis baujardi* **sp. n.**
(Fig. 1)

MEASUREMENTS

See Tables 4, 5.

DESCRIPTION

Male

Body slender, curved ventrally or C-shaped when heat-killed. Cuticle looking smooth under a light microscope. Head truncate or slightly rounded. Amphids inconspicuous. Mouth opening funnel-shaped or cup-shaped. Stoma shallow. Pharynx muscular; procorpus and metacorpus cylindrical; isthmus indistinct; basal bulb pyriform, valve distinct. Nerve ring surrounding isthmus or just above basal bulb. Cardia prominent and protruding into intestine lumen. Monorchic gonad reflexed. Spicule paired, slightly ventrally curved. Gubernaculum about 50% of spicule length, proximal end without knob, ventrally curved. Bursa peloderan, with nine normal pairs of papillae: three pairs located anterior to the cloacal aperture (two pairs very close together and the anteriormost pair well in advance) and six caudal pairs with anterior most approaching level of cloacal aperture. Tail conoid without mucron. Phasmids inconspicuous.

Female

Hermaphroditic female with robust, spiral-shaped or C-shaped body when heat-killed. Cuticle smooth. Head truncate or slightly rounded. Labial region with six distinct lips surrounding mouth, each with a labial papilla. Amphidial apertures inconspicuous. Mouth opening funnel-shaped or cup-shaped. Stoma shallow. Pharynx with cylindrical procorpus and metacorpus; isthmus indistinct; basal bulb pyriform, valve distinct. Cardia prominent, protruding into intestine lumen. Didelphic, amphidelphic gonad, reflexed. Vulva a transverse, slightly protruding slit near middle of body. Vagina short, straight with muscular walls. Tail longer than anal body diameter. Phasmids inconspicuous. Amphimictic female similar to hermaphroditic female but much smaller and tail blunt.

Infective juvenile

Body long, slender, often enclosed in cuticle of second-stage. This cuticle with distinct longitudinal ridges. Anterior end rounded. Labial region with a large dorsal tooth. Mouth and anus closed. Pharynx long and narrow, isthmus distinct and surrounded by nerve ring, basal bulb elongated with valve. Cardia prominent. Hemizonid distinct and located at isthmus level. Excretory pore just below hemizonid. Anterior intestinal region forming a pouch filled with rod-like symbiotic bacteria. Bacteria also occurring in lumen of intestine. Phasmids inconspicuous.

* Named in memory of the late Dr Pierre Baujard.

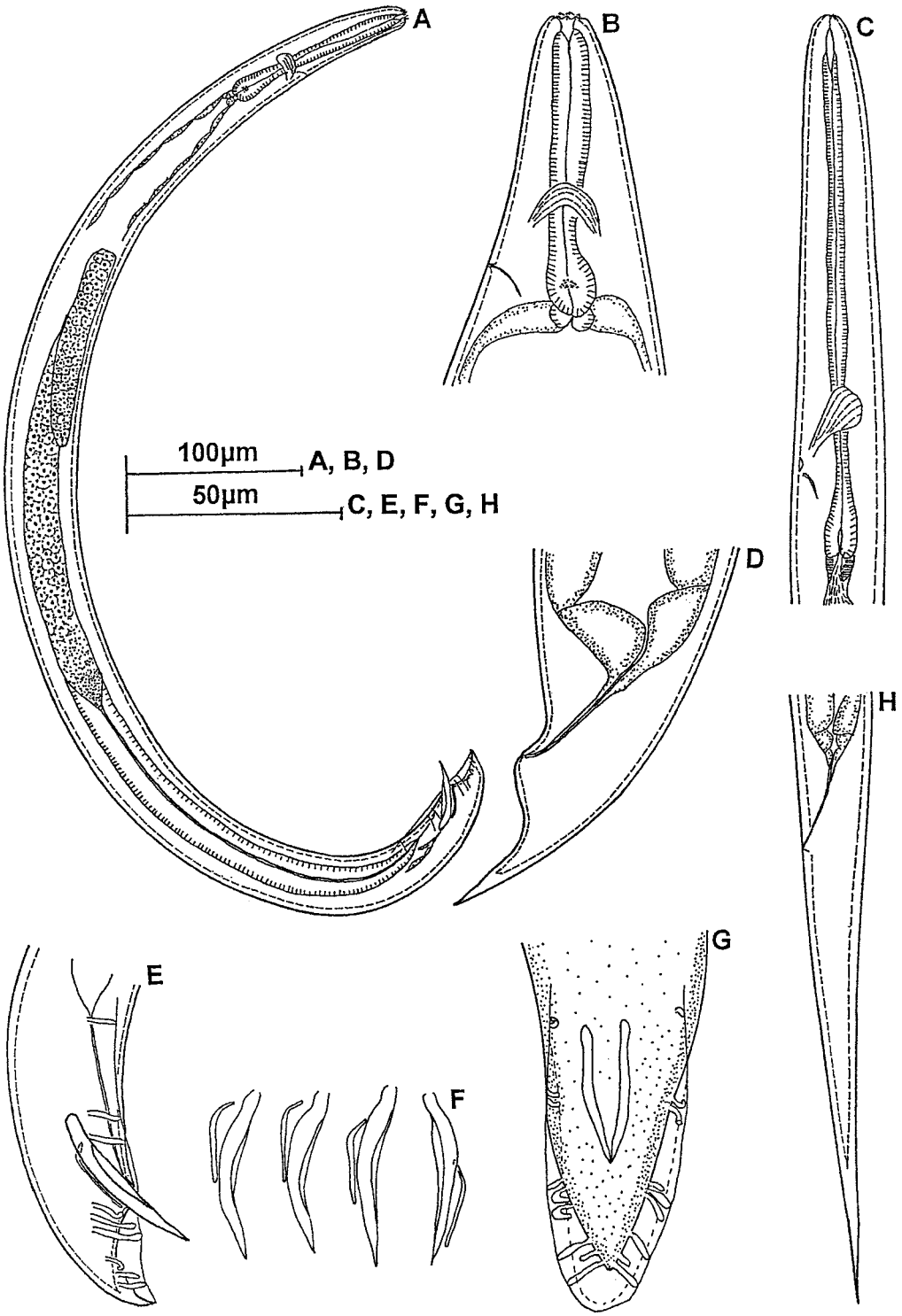


Fig. 1. Heterorhabditis baujardi *sp. n. A, E, F, G: male. A: Entire view; E: Tail in lateral view; F: Spicule and gubernaculum; G: Tail in ventral view. B, D: Hermaphroditic female. B: Pharyngeal region; D: Tail in lateral view. C, H: Infective juvenile. C: Pharyngeal region; H: Tail in lateral view.*

Table 4. *Morphometric characters (in μm) of adults of* Heterorhabditis baujardi *sp. n. Measurements in form: mean ± SD (range).*

Characters[1)]	Holotype	Sanhon, Sathay, Kontum Paratypes			Babe, Backan			Bong, Cucphuong, Ninhbinh		
	Male	Male	Hermaphroditic female	Amphimictic female	Male	Hermaphroditic female	Amphimictic female	Male	Hermaphroditic female	Amphimictic female
n		14	15	15	15	15	15	15	15	15
L	840	889 ± 45 (818-970)	3571 ± 290 (3135-4170)	1647 ± 205 (1335-2130)	898 ± 57 (782-996)	3647 ± 446 (3105-4680)	1988 ± 199 (1710-2385)	849 ± 127 (641-1072)	4089 ± 499 (3135-5400)	1855 ± 181 (1350-2160)
Body diam.	46	49 ± 2 (43-53)	208 ± 20 (180-240)	116 ± 17 (90-150)	46 ± 2 (41-50)	225 ± 37 (195-315)	141 ± 16 (120-165)	43 ± 4 (38-53)	274 ± 34 (195-330)	117 ± 14 (90-150)
Excretory pore	85	81 ± 7 (71-93)	173 ± 12 (156-192)	121 ± 14 (104-149)	86 ± 6 (76-95)	163 ± 19 (119-197)	121 ± 11 (105-136)	87 ± 9 (71-107)	163 ± 17 (131-194)	124 ± 12 (98-156)
Nerve ring	64	65 ± 7 (54-77)	136 ± 8 (119-147)	97 ± 13 (75-122)	66 ± 6 (57-75)	114 ± 10 (92-131)	90 ± 6 (75-101)	70 ± 7 (59-83)	127 ± 12 (96-146)	98 ± 10 (80-123)
Pharynx	107	116 ± 10 (105-132)	196 ± 7 (186-206)	149 ± 16 (131-185)	99 ± 4 (92-104)	183 ± 13 (153-210)	139 ± 7 (126-152)	101 ± 7 (91-115)	190 ± 17 (147-222)	145 ± 10 (123-173)
Testis reflexion	94	91 ± 13 (63-106)	–	–	93 ± 14 (71-113)	–	–	92 ± 13 (60-114)	–	–
Tail length	31	33 ± 3 (28-38)	86 ± 11 (66-114)	78 ± 7 (68-89)	30 ± 3 (26-35)	99 ± 7 (90-114)	87 ± 9 (77-110)	29 ± 2 (25-33)	101 ± 13 (74-126)	77 ± 6 (65-87)
Anal body diam.	21	22 ± 1 (20-24)	55 ± 5 (47-63)	32 ± 4 (27-41)	21 ± 2 (17-24)	49 ± 7 (42-66)	32 ± 3 (27-36)	20 ± 2 (16-27)	56 ± 7 (44-72)	30 ± 5 (24-47)
Spicule length	42	40 ± 3 (33-45)	–	–	40 ± 3 (31-43)	–	–	40 ± 3 (34-47)	–	–
Gubernaculum length	18	20 ± 1.5 (18-22)	–	–	20 ± 2 (14-23)	–	–	21 ± 2 (14-26)	–	–
V	–	–	45 ± 1 (43-48)	48 ± 1 (46-51)	–	44 ± 2 (40-48)	47 ± 2 (45-50)	–	44 ± 5 (37-64)	48 ± 3 (43-55)
a	18	18 ± 1 (16-22)	17 ± 1 (15-19)	14 ± 1 (12-16)	20 ± 2 (17-23)	16 ± 1 (14-19)	14 ± 2 (11-18)	20 ± 2 (16-24)	15 ± 2 (11-19)	16 ± 1 (13-19)
b	7.8	7.7 ± 0.7 (6.4-8.8)	18 ± 1 (16-21)	11 ± 1 (10-12)	9 ± 1 (8-10)	20 ± 3 (17-31)	14 ± 1 (13-17)	8 ± 1 (7-11)	22 ± 3 (14-28)	24 ± 2 (19-28)
c	27	27 ± 2 (24-33)	42 ± 4 (36-50)	21 ± 3 (19-32)	30 ± 3 (25-34)	37 ± 4 (30-46)	23 ± 2 (20-26)	30 ± 5 (22-39)	41 ± 5 (29-55)	24 ± 2 (19-28)
SW	2.03	1.82 ± 0.18 (1.38-2.08)	–	–	1.91 ± 0.27 (1.42-2.41)	–	–	2.01 ± 0.27 (1.50-2.60)	–	–
GS	0.44	0.50 ± 0.05 (0.44-0.61)	–	–	0.49 ± 0.05 (0.38-0.57)	–	–	0.52 ± 0.05 (0.38-0.59)	–	–

[1)] SW = spicule length/anal body diam.; GS = gubernaculum length/spicule length.

Bacterial associate

Unknown.

Type host and locality

Type host unknown. The type population was collected by baiting soil from soil collected in Sanhon, Sathay, Kontum forest (longitude 107°43′E, latitude 14°28′N, altitude 850 m a.s.l.).

Other localities

Other populations were found in Bong, Cucphuong, Ninhbinh (105°36′E, 20°20′N, 500 m a.s.l.), and Babe, Backan (105°34′E, 22°26′N, 700 m a.s.l.).

Table 5. *Morphometric characters (in μm) of third stage juveniles of* Heterorhabditis baujardi *sp. n. Measurements in form: mean ± SD (range).*

Characters[1)]	Sanhon, Sathay, Kontum	Babe, Backan	Bong, Cucphuong, Ninhbinh
n	25	25	25
L	551 ± 27 (497-595)	565 ± 12 (538-590)	568 ± 24 (487-622)
Body diam.	20 ± 2 (18-22)	20 ± 1 (18-22)	20 ± 1 (18-22)
Excretory pore	97 ± 3 (91-103)	97 ± 3 (93-102)	98 ± 4 (90-105)
Nerve ring	81 ± 3 (75-86)	81 ± 2 (76-87)	82 ± 3 (74-87)
Pharynx	115 ± 3 (107-120)	118 ± 2 (115-123)	117 ± 3 (107-124)
Tail length	90 ± 4 (83-97)	97 ± 3 (92-105)	90 ± 5 (79-100)
Anal body diam.	13 ± 0.7 (11-14)	13 ± 0.6 (11-14)	13 ± 0.7 (11-14)
a	28 ± 1 (26-30)	29 ± 1 (26-31)	28 ± 1 (26-32)
b	4.8 ± 0.2 (4.5-5.1)	4.8 ± 0.1 (4.6-5.0)	4.9 ± 0.2 (4.4-5.5)
c	6 ± 0.3 (6-6.7)	4.8 ± 0.1 (4.6-5)	6 ± 0.3 (5.8-7.3)
d	0.84 ± 0.03 (0.78-0.88)	0.82 ± 0.02 (0.8-0.86)	0.84 ± 0.03 (0.77-0.9)
e	1.08 ± 0.04 (0.98-1.14)	1 ± 0.04 (0.91-1.08)	1.09 ± 0.06 (0.96-1.28)
f	0.22 ± 0.01 (0.2-0.25)	0.2 ± 0.01 (0.19-0.24)	0.22 ± 0.01 (0.2-0.27)

[1)] d = distance from anterior end to excretory pore/pharynx length; e = distance from anterior end to excretory pore/tail length; f = body diam./tail length.

Type material

Holotype male and six paratype males are deposited at Ghent University, Institute for Zoology, K.L. Ledeganckstraat 35, 9000, Belgium. Slides with paratype males, hermaphroditic females, amphimictic females and infective juveniles deposited at the Department of Nematology, Institute of Ecology and Biological Resources, National Centre for Science and Technology, Hoang Quoc Viet 18, Nghiado, Caugiay, Hanoi, Vietnam and University of California, Riverside, USA.

Table 6. *Standardised coefficients for canonical variables of infective juveniles.*

Characters[1)]	Root 1	Root 2	Root 3
L	3.40767	0.87812	−0.62499
Tail length	−3.74362	−0.97244	−1.12955
a	−0.75283	1.18435	3.01354
d	−0.90062	−0.97507	−0.07157
b	−1.89238	−2.96626	−1.30098
e	−7.14977	−3.61236	2.73464
Anal body diam.	−0.99465	0.50880	0.06402
c	2.65349	−0.24782	−0.14841
Nerve ring	1.19136	1.88065	−2.27997
f	3.60816	3.35298	−3.61188
Pharynx	−1.20163	−2.45411	0.75157
Body diam.	−1.14304	−1.59606	3.58318
Eigenval	70.23605	15.4316	7.88344
Cum. Prop	0.738698	0.900998	0.983911

[1)] d = distance from anterior end to excretory pore/pharynx length; e = distance from anterior end to excretory pore/tail length; f = body diam./tail length.

Canonical discriminant analysis (CDA)

The CDA using 12 variables for infective juveniles and ten variables for males failed to discriminate the three *H. baujardi* sp. n. populations from *H. indica* (Fig. 2). However, the new species was easily distinguished from *H. downesi*, *H. marelatus*, *H. megidis* and *H. bacteriophora*. *Heterorhabditis baujardi* sp. n. was slightly separated from *H. bacteriophora* by variables of the infective juveniles, but was clearly distinguished by variables of the males. The standardised coefficients for canonical variables of infective juveniles and males are presented in Tables 6 and 7.

Morphological diagnosis and relationships with other species

Morphometrically and morphologically, *H. baujardi* sp. n. resembles *H. indica*. It can be distinguished from this species by the shape of the gubernaculum (proximal end without knob, ventrally curved *vs* flat, narrow, not reflexed at tip; Fig. 3D) and nine normal pairs of genital papillae *vs* seven pairs of normal papillae, the last two being generally atrophied, highly modified, or absent (Poinar *et al.*, 1992; Anis *et al.*, 2000).

The gubernaculum of *H. baujardi* sp. n. resembles that of *H. bacteriophora*. However, *H. baujardi* sp. n. can be separated from this latter species by a slightly shorter

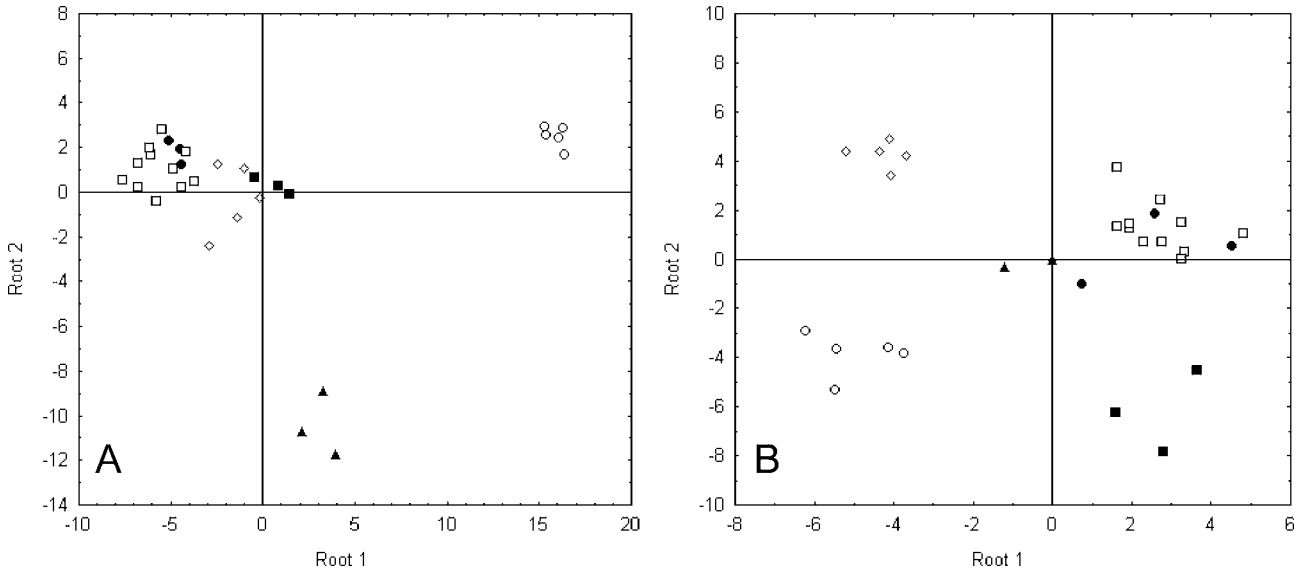


Fig. 2. *Results from Canonical Discriminant Analysis of* Heterorhabditis *spp. A: Performed with 12 morphometric variables of infective juveniles; B: Performed with ten morphometric variables of males. (◊)* H. bacteriophora*; (●)* H. baujardi *sp. n.; (■)* H. downesi*; (□)* H. indica*; (○)* H. marelatus*; (▲)* H. megidis.

Table 7. *Standardised coefficients for canonical variables of males.*

Characters[1)]	Root 1	Root 2	Root 3
Body diam.	−0.65493	0.48097	−0.02347
GS	−4.88931	2.09480	2.58116
Excretory pore	−2.77045	1.89810	1.54185
Anal body diam.	−1.62604	2.67516	3.45302
Spicule length	−4.61111	−2.39721	1.50087
Pharynx	0.76452	−0.83550	−1.95445
Testis reflexion	0.52298	0.14264	0.50003
L	−1.39635	0.85931	0.81471
Guberna-culum length	6.642034	−1.04697	−3.8823
SW	−1.3923	4.156534	2.618612
Eigenval	13.7838	11.9819	5.180156
Cum. Prop	0.435984	0.814973	0.978823

[1)] SW = spicule length/anal body diam.; GS = gubernaculum length/spicule length.

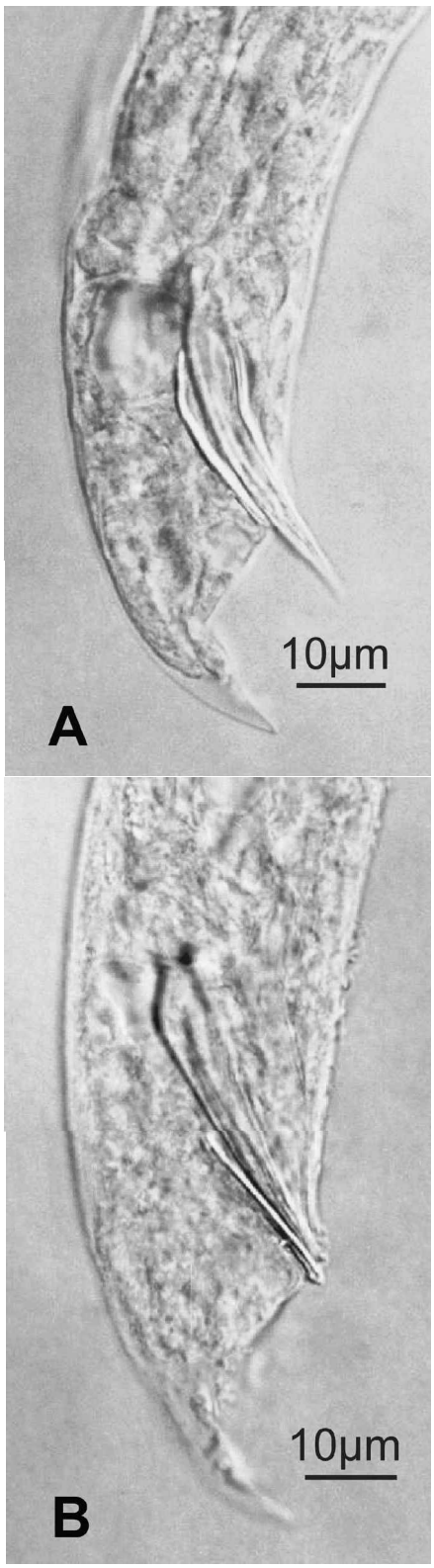
body length (563 (487-622) *vs* 588 (512-670) μm) of infective juveniles, slightly longer spicules (40 (31-47) *vs* 36 (31-41) μm), slightly longer gubernaculum (20 (14-26) *vs* 18 (14-21) μm), and a higher ratio SW (1.94 (1.38-2.61) *vs* 1.28 (1.20-1.34)) of males.

The new species differs from the tropical *H. brevicaudis* by a wider anal body diameter (13 *vs* 8.8), a lower ratio e (1.06 *vs* 1.6) of infective juveniles and shorter spicules (40 *vs* 47), a higher ratio GS (0.51 *vs* 0.4), a lower ratio SW (1.94 *vs* 2.3) of males.

Molecular characterisation and relationships with other species

The polymerase chain reaction amplified a single DNA product of about 850 bp for each sample from the three populations. As the three PCR products had an identical sequence, only one isolate was used for further analysis. The ITS1 region of the *Heterorhabditis* species ranged from 378 (*H. downesi*) to 406 bp (*H. baujardi* sp. n.). The ITS1 region exhibited the following base composition: A – 24 (20-26)%, C – 22 (20-23)%, G – 25 (24-27)%, T – 30 (28-32)%.

The length of the alignment with nine *Heterorhabditis* sequences generated by Clustal X (Fig. 4) was 427 bp. The ITS1 sequence of *H. baujardi* sp. n. differs from 51 (*H. hawaiiensis*) to 99 substitutions (*H. zealandica*) from that of other species. The pairwise divergence between taxa ranged from 0.5 to 25% (Table 8). MP analysis of this alignment revealed 427 characters, of



which 93 were parsimony informative. In an unrooted MP tree *H. baujardi* sp. n. clustered with *H. indica* + *H. hawaiiensis* with high bootstrap value (Fig. 4A) and was supported by 29 autapomorphies (unique substitutions) (Fig. 5).

The length of the *Heterorhabditis* sequence alignment with outgroup taxa was 491 bp. MP analysis of this alignment indicated that among 492 characters, 141 were parsimony informative. The phylogenetic trees obtained from MP (tree length = 557) and ML (DNA model = GTR + G, - ln likelihood = 2687.38) had a similar topology (Fig. 4B). *Heterorhabditis baujardi* sp. n. clustered with *H. indica* + *H. hawaiiensis* (clade of sub-tropical and tropical species) with high to moderate bootstrap support. The position of *H. marelatus* was not well resolved. The KHT-est did not reveal significant difference ($P = 0.4148$) between the topology when *H. zealandica* clustered with the *H. megidis* + *H. downesi* clade, which required a two steps longer tree than the maximum parsimonious one.

Crossbreeding tests

Crossbreeding tests were restricted to one isolate of the new species (H.TN40) and the morphologically and morphometrically closely related *H. indica* (LN2). No fertile progeny was produced when both species were crossed with each other; when both species were self-crossed, progeny was produced.

Discussion

The morphological traits of males and IJ provide most of the useful taxonomic characters for *Heterorhabditis* species (Stock & Kaya, 1996; Hominick *et al*., 1997). Following multivariate analysis of morphometric characters of *Heterorhabditis* spp., Stock and Kaya (1996) suggested that both body length and tail length of IJ and the body length and testis reflexion of males contribute most in the discrimination of species. Unlike Stock and Kaya (1996) who used characters from individuals obtained from single populations from each species, we used the means of the morphometrical characters and the ratios for several populations and concluded that the body length and testis reflexion of males do not contribute much to the discrimination of *Heterorhabditis* spp. Our data further indi-

Fig. 3. *LM photographs of mail tail ends showing spicules and characteristic gubernaculum of* Heterorhabditis baujardi *sp. n. (A) and* H. indica *(B).*

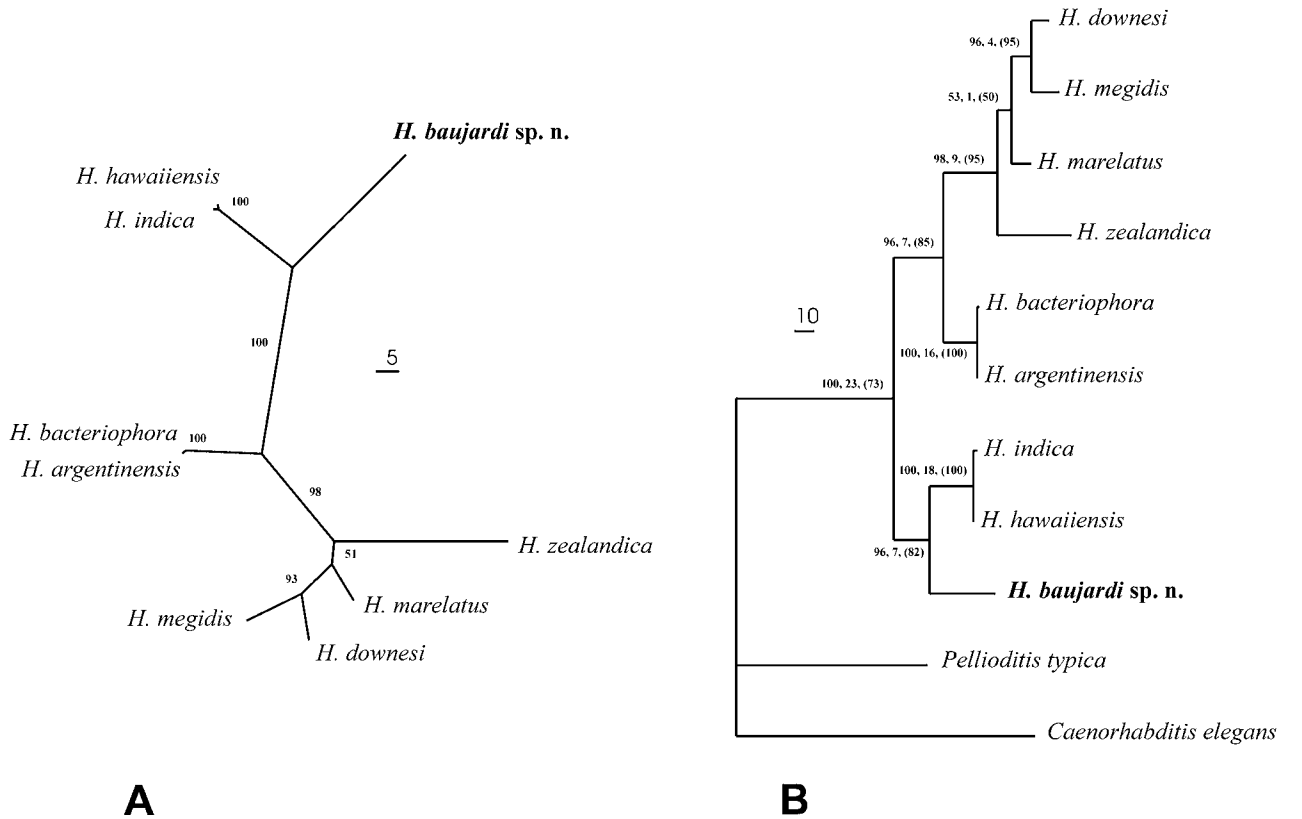


Fig. 4. *Single maximum parsimony tree obtained from analysis of the alignment of sequences from A: Nine* Heterorhabditis *species only (Tree length = 220, CI = 0.9000, RI = 0.8721, RC = 0.7849, g1 = − 1.0688) and the alignment of sequences from B: Nine* Heterorhabditis *and two outgroup species (Tree length = 557, CI = 0.8654, RI = 0.7396, RC = 0.6400, g1 = − 0.9535). Bootstrap and decay values are given in appropriate clades. Bootstraps for ML tree are in brackets. Scales indicate the number of nucleotide changes.*

Table 8. *Pairwise distances between taxa (below diagonal: total character differences, above diagonal: mean character differences adjusted for missing data).*

Species	1	2	3	4	5	6	7	8	9
H. baujardi sp. n	–	0.13947	0.13421	0.23467	0.24274	0.26121	0.20789	0.20153	0.20102
H. indica	53	–	0.00526	0.22039	0.23497	0.24796	0.19726	0.16000	0.16489
H. hawaiiensis	51	2	–	0.22039	0.23497	0.24796	0.19726	0.16000	0.16489
H. downesi	88	80	80	–	0.06133	0.15241	0.06989	0.14785	0.14516
H. megidis	92	86	86	23	–	0.14516	0.07796	0.15733	0.15467
H. zealandica	99	91	91	57	54	–	0.13067	0.19519	0.19519
H. marelatus	79	72	72	26	29	49	–	0.12202	0.11936
H. bacteriophora	79	60	60	55	59	73	46	–	0.00254
H. argentinensis	79	62	62	54	58	73	45	1	–

cate that morphometrical characters such as ratio e, ratio f and body diameter of IJ and spicule length, gubernaculum length and ratio SW of males play a significant role in discrimination. Consequently, these morphometrical characters and ratios should be considered when identifying and describing *Heterorhabditis* spp. The comparison of the species positions in the score plots obtained in both analyses reveals common conclusions: the clear differen-

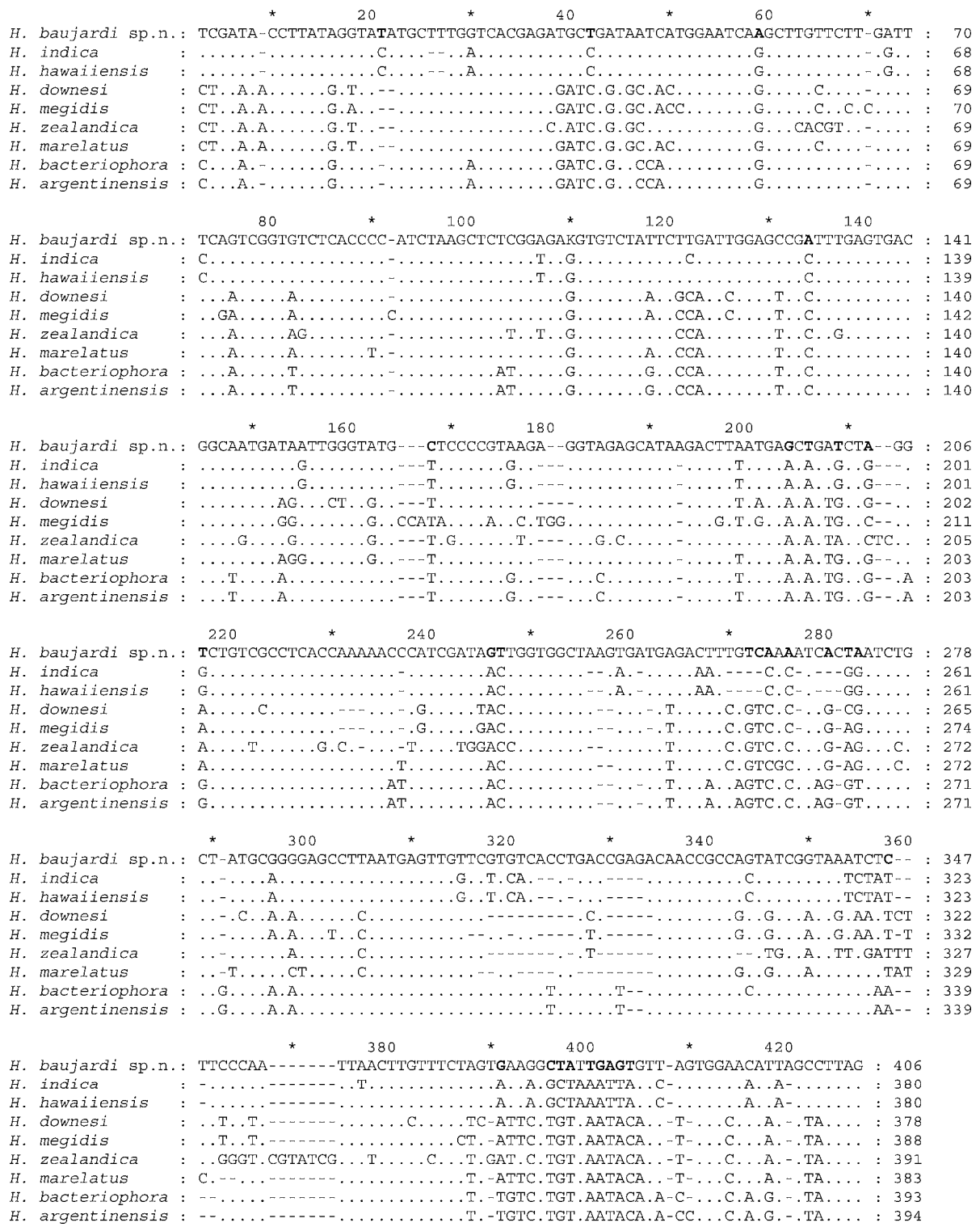


Fig. 5. *Multiple sequence alignment of the ITS1-rDNA of* Heterorhabditis *species. Autapomorphies for* H. baujardi *sp. n. are indicated in bold type.*

tiation of *H. marelatus* and *H. megidis* from other *Heterorhabditis* spp. and the high similarity of *H. indica* and *H. bacteriophora* based on morphometrics of IJ, but with good discrimination on the base of male morphometrics.

Molecular techniques such as allozyme electrophoresis (Akhurst, 1987), isoelectric focusing (Griffin *et al.*, 1994; Joyce *et al.*, 1994b), DNA restriction fragment length polymorphisms (Smits *et al.*, 1991; Joyce *et al.*, 1994a; Stack *et al.*, 2000), RAPD analysis (Gardner *et al.*, 1994; Liu & Berry, 1995; Stock *et al.*, 1996; Hashmi & Gaugler, 1998) and DNA sequencing (Liu *et al.*, 1997, 1999; Adams *et al.*, 1998) provide useful tools for taxonomic studies of *Heterorhabditis* at both population and species level. The results of IEF of soluble proteins obtained by Joyce *et al.* (1994b) allowed the separation of four groups of *Heterorhabditis* isolates presently recognised as four species: *H. bacteriophora*, *H. megidis, H. downesi* and *H. indica.* Analysing the genetic variability using RAPD markers, Hashmi and Gaugler (1998) concluded that the banding patterns positively correlated with the morphological classification of *Heterorhabditis.* However, *H. hawaiiensis* could not be separated from *H. indica* nor *H. marelatus* from *H. hepialius* (Stock *et al.*, 1996). After studying ITS1 sequences (Adams *et al.*, 1998) and ND4 sequences (Liu *et al.*, 1999), both research groups concluded that the sequence divergence between species was congruent with the differentiation of these species on the basis of morphological and biological criteria. Both studies did not reveal differences in appropriate DNA fragments between *H. marelatus* and *H. hepialius* that justified synonymisation of these species by Stock (1997). Based on the high degree of ITS1 sequence identity and lack of autapomorphic characters, Adams *et al.* (1998) suggested that *H. indica* may be conspecific with *H. hawaiiensis* and *H. bacteriophora* conspecific with *H. argentinensis.*

The molecular phylogeny of the genus *Heterorhabditis* has been reconstructed based on the analyses of sequences from the partial 26S gene of rDNA (Curran & Driver, 1994), partial 18S gene of rDNA (Liu *et al.*, 1997), ITS1 of rDNA (Adams *et al.*, 1998) and partial ND4 gene of mitochondrial DNA (Liu *et al.*, 1999). If the analysis of the partial 18S gene showed that this fragment is too conservative and not useful for phylogenetic study of this genus, the analyses of other regions of DNA revealed some resolved relationships between species. The phylogenetic estimation based on the ND4 gene (Liu *et al.*, 1999), however, differed from that inferred from the ITS1 region; *e.g.*, *H. bacteriophora* was genetically related to *H. megidis*, but not with *H. marelatus* as in the ITS-based phylogeny. This difference might be due to some bias in one of the data sets. Detailed phylogenetic analyses of the genus were conducted by Adams *et al.* (1998).

The alignment of nucleic acid or protein sequences is a critical step in comparative molecular and evolutionary studies. Alignment procedures can influence the outcome of phylogenetic studies (Morrison & Ellis, 1997; Hickson *et al.*, 2000; Subbotin *et al.*, 2001). In our study, the Clustal X 1.64 program generated an alignment with fewer numbers of gaps and visually fewer numbers of ambiguously aligned regions (positions 315-334 in alignment) than that created by the Malign program and presented in Adams *et al.* (1998). As a result, the sequence analysis of the Clustal alignment showed fewer substitution divergences between taxa than that from the Malign alignment. The algorithm of the Malign program using parsimony of the resulting tree as the global optimal criterion for alignment can be considered problematic for the estimation of the optimal alignment. In addition, the Clustal algorithm using sequence similarity as a criterion cannot guarantee optimum sequence homology (Morrison & Ellis, 1997). We can expect that the putative secondary structure model of this region will help to produce a multiple sequence alignment that is closer to the true one than to the output from any computerised algorithm. Although the computer programs generated different ITS1 alignments, the topologies of the best-supported hypothesis for phylogenetic relationships obtained in both analyses were, with the exception for the position of *H. zealandica* and *H. marelatus*, very similar. In the trees generated by Adams *et al.* (1998), *H. zealandica* had sister relationships with *H. megidis* + *H. downesi*, but with weak support. The KHTest using our data set, however, did not reveal significant differences between these topologies. Thus, the above-mentioned fact may indicate significant phylogenetic signals in both ITS1 sequence data sets.

In our phylogenetic tree, the tropical and subtropical species *H. indica*, *H. hawaiiensis* and *H. baujardi* sp. n. form one clade separated from those species known mainly from temperate regions. *H. brevicaudis*, isolated from south-east China and not included in our study, may also belong to the tropical and subtropical species clade.

Obviously, the results obtained from classical taxonomy in combination with molecular analyses show that both approaches are complementary and valuable for identifying and characterising species of *Heterorhabditis* and confirm conclusions made by Stock and Kaya (1996).

Our study reveals that *H. baujardi* sp. n. is morphometrically close to *H. indica* but differs from this species in the disposition of the genital papillae and gubernaculum shape. In addition the cross-breeding test, useful for distinguishing *Heterorhabditis* species (Dix *et al*., 1992; Griffin *et al*., 1994; Stock *et al*., 1996; Stack *et al*., 2000), revealed reproductive incompatibility between *H. baujardi* sp. n. and *H. indica*. The relatively high level of sequence divergence from other *Heterorhabditis* species and the presence of nucleotide autapomorphies in the ITS sequences of *H. baujardi* sp. n. can be considered as relevant evidence for its separate specific status.

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References

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

AKHURST, R.J. (1987). Use of starch gel electrophoresis in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). *Nematologica* 33, 1-9.

ANIS, M., SHAHINA, F., REID, A.P. & MAQBOOL, M.A. (2000). Redescription of *Heterorhabditis indica* Poinar *et al*., 1992 (Rhabditida: Heterorhabditidae) from Pakistan. *Pakistan Journal of Nematology* 18, 11-27.

BEDDING, R.A. (1990). Logistics and strategies for introducing entomopathogenic nematodes technology into developing countries. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, USA, CRC Press, pp. 233-245.

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

CURRAN, J. & DRIVER, F. (1994). Molecular taxonomy of *Heterorhabditis*. In: Burnell, A.M., Ehlers, R.U. & Masson, J.P. (Eds). *COST 812. Biotechnology: Genetics of entomopathogenic nematode-bacterium complexes. Proceedings of Symposium and Workshop, St. Patrick's College, Maynooth, Co. Kildare, Ireland*. European Commission, DG XII, Luxembourg, pp. 41-48.

DE GRISSE, A.T. (1969). Redescription ou modifications de quelques techniques utilisées dans l'étude de nématodes phytoparasitaires. *Mededelingen Rijksfaculteit Landbouwwetenschappen Gent* 34, 315-359.

DIX, I., BURNELL, A.M., GRIFFIN, C.T., JOY, S.A., NUGENT, M.J. & DOWN, 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.

DOUCET, M.M.A. DE., BERTOLOTTI, M.A. & CAGNOLO, S.R. (1996). On a new isolate of *Heterorhabditis bacteriophora* Poinar, 1975 (Nematoda: Heterorhabditidae) from Argentina: life cycle and description of infective juveniles, female, males and hermaphrodites of 2nd and 3rd generations. *Fundamental and Applied Nematology* 19, 415-420.

DUNPHY, G.B. & WEBSTER, J.M. (1989). The monoxenic culture of *Neoaplectana carpocapsae* DD-136 and *Heterorhabditis heliothidis*. *Revue de Nématologie* 12, 113-123.

GARDNER, S.L., STOCK, S.P. & KAYA, H.K. (1994). A new species of *Heterorhabditis* from Hawaiian Islands. *Journal of Parasitology* 80, 100-106.

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.

HASHMI, G. & GAUGLER, R. (1998). Genetic diversity in insect-parasitic nematodes (Rhabditida: Heterorhabditidae). *Journal of Invertebrate Pathology* 72, 185-189.

HICKSON, R., SIMON, C. & PERREY, S.W. (2000). The performance of several multiple-sequence alignment programs in relation to secondary-structure features for an rRNA sequence. *Molecular Biology and Evolution* 17, 530-539.

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.

JOYCE, S.A., BURNELL, A.M. & POWERS, T.O. (1994a). Characterisation of *Heterorhabditis* isolates by PCR amplification of segment 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

identification of entomogenic 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.

LIU, J. & BERRY, R.E. (1995). Determination of PCR conditions for RAPD analysis in entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae). *Journal of Invertebrate Pathology* 65, 79-81.

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

LIU, J., BERRY, R.E. & MOLDENKE, A.F. (1997). Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rDNA gene sequences. *Journal of Invertebrate Pathology* 69, 246-252.

LIU, J., BERRY, R.E. & BLOUIN, M.S. (1999). Molecular differentiation and phylogeny of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) based on ND4 gene sequences of mitochondrial DNA. *Journal of Parasitology* 85, 709-715.

MAUPAS, E. (1899). La mue et l'enkystement chez les nématodes. *Archives de Zoologie Expérimentale et Générale* 7, 563-628.

MIDUTURI, J.S. (1997). *Bionomics of naturally occurring entomopathogenic nematodes in Belgium.* PhD thesis, Ghent University, 163 pp.

MORRISON, D.A. & ELLIS, J.T. (1997). Effects of nucleotide sequence alignment on phylogeny estimation: a case of 18S rDNA of Apicomplexa. *Molecular Biology and Evolution* 14, 428-441.

NGUYEN, K.B. & SMART, JR, G.C. (1995). Morphometrics of infective juveniles of *Steinernema* spp. and *Heterorhabditis bacteriophora* (Nemata: Rhabditida). *Journal of Nematology* 27, 206-212.

PHAN, K.L., NGUYEN, N.C. & MOENS, M. (2001a). *Steinernema sangi* sp. n. (Rhabditida: Steinernematidae) from Vietnam. *Russian Journal* of *Nematology* 9, 1-7.

PHAN, K.L., NGUYEN, N.C. & MOENS, M. (2001b). *Steinernema loci* sp. n. and *Steinernema thanhi* sp. n. (Rhabditida: Steinernematidae) from Vietnam. *Nematology* 3, 503-514.

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

POINAR, 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.

POINAR, G.O. & GEORGIS, R. (1990). Characterization and field application of *Heterorhabditis bacteriophora* strain HP88 (Heterorhabditidae: Rhabditida). *Revue de Nématologie* 13, 387-393.

POINAR, G.O., JACKSON, T. & KLEIN, M. (1987). *Heterorhabditis megidis* sp. n. (Heterorhabditidae: Rhabditida) parasitic in the Japanese beetle, *Popillia japonica* (Scarabaeidae: Coleoptera) in Ohio. *Proceedings of Helminthological Society of Washington* 54, 53-59.

POINAR, G.O., 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.

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

SEINHORST, J.W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67-69.

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

STACK, C.M., EASWARAMOORTHY, S.G., METHA, U.K., DOWNES, M.J., GRIFFIN, C.T. & BURNELL, A.M. (2000). Molecular characterisation of *Heterorhabditis indica* isolates from India, Kenya, Indonesia and Cuba. *Nematology* 2, 477-487.

STEFANSKI, W. (1922). Excrétion chez les nématodes libres. *Archiwum Nauk Biologicznych Towarzystwa Naukowego Warszawskiego* 1, 1-33.

STOCK, S.P. (1993). A new species of the genus *Heterorhabditis* Poinar, 1975 (Nematoda: Heterorhabditidae) parasitizing *Graphognathus* sp. larvae (Coleoptera: Curculionidae) from Argentina. *Research and Reviews in Parasitology* 53, 103-107.

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

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 species of the genus. *Journal of Parasitology* 82, 806-813.

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

STOCK, S.P., GRIFFIN, C.T. & BURNELL, A.M. (2001). Morphological characterization of three isolates of *Heterorhabditis* Poinar, 1976 from the "Irish group" (Nematoda: Rhabditida: Heterorhabditidae) and additional evidence supporting their recognition as a distinct species, *H. downesi* n. sp. *Systematic Parasitology* 51, 95-106.

SUBBOTIN, S.A., VIERSTRAETE, A., DE LEY, P., ROWE, J., WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R.

(2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution* 21, 1-16.

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

WHITE, G.F. (1927). A method for obtaining infective nematode larvae from cultures. *Science* 66, 302-303.