FIRST RECORD OF ENTOMOPATHOGENIC NEMATODES (STEINERNEMATIDAE AND HETERORHABDITIDAE) FROM NIGERIAN SOIL AND THEIR MORPHOMETRICAL AND RIBOSOMAL DNA SEQUENCE ANALYSIS

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Summary. A survey on the occurrence of entomopathogenic nematodes (EPNs) was conducted in Niger, Kano and Plateau States of Nigeria. From the total of 200 soil samples, 13 (6.5%) tested positive for the presence of EPNs, containing the genera *Steinernema* and *Heterorhabditis*. Morphological characterization and sequence analysis of the ITS regions of ribosomal DNA allowed the identification of EPN isolates as *Heterorhabditis bacteriophora* and *Steinernema feltiae* representing the first report of these nematodes from Nigeria.

Key words: *Heterorhabditis bacteriophora*, identification, ITS, morphology, *Steinernema feltiae*.

Entomopathogenic nematodes (EPNs) belonging to the genera *Heterorhabditis* and *Steinernema* occur naturally in soils around the world and are excellent biocontrol agents for a wide range of insect pests (Georgis *et al*., 2006; Ansari *et al*., 2008, 2009). They offer a benign alternative to chemical insecticides, mainly because of their ability to locate insects in cryptic habitats, their high reproductive ability, the simplicity of mass producing them, and their safety to humans and other vertebrates (Gaugler, 2007).

Currently, over 60 species of *Steinernema* and 16 species of *Heterorhabditis* (Nguyen and Hunt, 2007) have been described, and the number of nominal species is increasing rapidly. Hominick (2002) reported that the number of EPN species described until 2002 was 34 of which 23 were described from 1989-2002. Researchers continue to search for new and better strains of EPNs, in part for commercial reasons. Particular attention has focused on indigenous EPNs as they are considered to be better adapted to local habitats. Furthermore, the use of native EPNs allays the fears of those concerned about release of exotic EPNs into the environment particularly with regard to their impact on non-target insects and possible displacement of native species (Ehlers, 2005).

Previously, several surveys seeking to identify endemic EPN species have been conducted in African countries including Kenya (Burnell and Stock, 2000), Egypt (Shamseldean *et al*., 1996), Ethiopia (Mekete *et al*., 2005), Tanzania (Mwaitulo *et al*., 2011), South Africa (Malan *et al*., 2011) and Cameroon (Kanga *et al*., 2012). However, information on the occurences and diversity of EPNs in Nigeria is currently non-existent, as no systematic survey has been conducted to document the presence of these nematodes in this country. Our recent survey of EPNs in Nigeria identified nematode isolates of the genera *Steinernema* and *Heterorhabditis* based on morphological characters, morphometrics, and molecular data. This is the first report of these nematodes in Nigeria.

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MATERIALS AND METHODS

Soil sampling and nematode isolation. A total of 200 soil samples were collected from three States in Nigeria (Fig. 1). The details of sampling are given in Table I. In all cases, each soil sample (2 kg) was a composite of 10 random sub-samples taken distantly located from each other in an area of 10 m^2 and to a depth of 0-20 cm. Samples were taken with a hand shovel, placed in polyethylene bags to prevent water loss, and kept in coolers during transit to the laboratory.

Each soil sample was gently shaken so the particles were fairly uniform and friable and then 0.5 kg of soil was transferred to 500 ml plastic containers (11 cm diameter; 6 cm height) and EPNs were isolated using the insect baiting method (Bedding and Akhurst, 1975). Ten last-instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were placed in each plastic container filled with the moistened soil obtained from each sample. Containers were covered with a lid, turned upside down and incubated in the dark at 20 ± 1 °C. Water was added to the samples if they appeared dry at any point during the baiting. *Galleria mellonella* larvae were checked every two to three days and dead larvae were replaced with fresh ones. After seven days, dead insects

Fig. 1. Occurence and distribution of entomopathogenic nematodes in Nigeria. * = *Heterorhabditis bacteriophora*; ▲ = *Steinernema feltiae*.

were collected and thoroughly rinsed in distilled water and placed in modified White traps (Kaya and Stock, 1997). All emerging nematodes were collected from single dead larvae and considered as one isolate. After that, each nematode isolate was cultured on *G. mellonella* larvae to produce nematodes for identification and establishment of cultures. These nematodes were stored at 10 ºC in tissue culture flasks containing distilled water.

Morphological identifications. Ten *G. mellonella* larvae were exposed to about 300 infective juveniles (IJs) in a 9-cm-diameter Petri dish lined with two moistened filter papers (Whatman No. 1). For *Heterorhabditis* sp., the first generation hermaphrodites and second-generation females and males, were obtained by dissecting infected insects at four days and seven days, respectively, after the insect died. Third stage IJs were obtained during the first two days after emerging from insect cadavers.

For light microscope observations, twenty individuals of different life stages (IJs, males, females, and hermaphrodites) were examined alive. Additional specimens of the different stages were killed and fixed in lactophenol. These nematodes were used when more observations were needed to confirm the morphology or variation of some structures. At least twenty males, females, hermaphrodites and IJs of each isolate were observed, drawn, and measured. Measurements were made using a drawing camera plan Axiophoto attached to a Nikon light microscope (Nikon CoolPix. 995).

Molecular characterization. Total genomic DNA was extracted from a single female using a DNeasy Blood and Tissue Kit (Qiagen. Inc.). The entire internal transcribed spacer region (ITS) was PCR amplified using the primers TW81 (5'-GTTTCCDGTAGGTGAACCTGC-3' (forward) and AB28: 5'-ATATGCTTAAGTTCAGCGGGT-3' (reverse). All PCR reactions were conducted in a Gradient Thermocycler, PTC-200 (MJ Research, Inc., Watham, MA) with the cycle profile: one cycle of 94 °C for 15 minutes followed by 35 cycles of 94 °C for 60 seconds, 55 °C for 60 seconds and 72 °C for 60 seconds. The last step was 72 °C for 10 minutes. PCR products were sequenced bi-directionally in their entirety. Internal primers used for sequencing were: 58P = 5'-ACGAATTGCAGACGCTTAG-3' (forward) and H58R = 5'-GTGCGTTCAAAACTTCACC-3' (reverse) (Nguyen *et al*., 2004). A portion (10 µl) of the amplification product was electrophoresed on a 1.8% agarose gel and stained with ethidium bromide. The sizes of amplified products were determined by comparison with a 1 Kb molecular weight ladder (Invitrogen). For direct sequencing, PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced at the University of Florida Core DNA

Table I. Number of soil samples from different locations in three regions of Nigeria, frequency of samples positive for entomopathogenic nematodes, number of isolates recovered, commodities and soil types.

Location	Region	Total samples	Samples with nematodes	Nematode isolates recovered	Commodities	Soil types
Suleja. Abuja	Niger state	100	4	5	Natural and managed habitat	Sandy loam
Babaldu local government	Kano state	40	6	3	Maize. cowpea	Sandy loam
Bariki. Riyom local government	Plateau state	60	3	2	Potato	Sandy loam
Total		200	13	10		

sequencing facility. Sequences of the complete ITS region were aligned to previously published sequences of the ITS region (Nguyen *et al*., 2006) using the profile alignment option of Clustal X (Thompson *et al*., 1997), and then compared with those in Genbank by means of a BLAST search.

Phylogenetic analyses were performed by the neighbour-joining method, with bootstrap analysis based on 1000 re-samplings using PAUP software (Swofford, 2002).

RESULTS

From all of the 200 samples tested, 6.5% were positive for EPNs. A total of 100 samples came from Niger State, with 4 (4%) samples testing positive for EPN's; 40 samples from Kano State, with 6 (15%) testing positive; 60 samples from Plateau State, with 3 (5%) testing positive (Fig. 1). The baited *Galleria* larvae infected from these samples displayed the typical colouration characteristics typical to the respective species. The morphometric and

Table II. Morphometrics of *Heterorhabditis bacteriophora*. Measurements are in µm and are in the form: mean ± SD (range).

Character	Male		Female		Hermophrodite 20	Infective Juvenile 20
	First generation 20	Second generation 20	First generation 20	Second generation 20		
Body length (L)	924.6±213.9 (655.4-1553.7)	739.5±109.0 (536.7-898.3)	1552.9±179.8 (1314.3-1942.9)	901.3±159.5 (700.5-1497.0)	4492.9±476.3 (3714.3-5428.6)	575.5±31.9 (522.8-665.7)
a	16.3±2.7 (11.4-26.1)	17.3±2.2 (13.1-20.7)	15.2±1.4 (12.0-17.2)	11.5±1.5 (8.9-15.6)	21.3±2.4 (17.8-26.5)	24.5±2.3 (20.2-28.7)
b	8.0±1.8 (5.7-13.1)	6.6±0.9 (4.6-8.0)	10.7±0.9 (9.3-13.0)	7.0±1.0 (5.7-10.6)	22.1±2.3 (18.6-27.8)	4.4±0.2 (3.9-5.0)
c	25.9±5.5 (20.8-44.1)	23.2±3.9 (16.6-31.5)	18.3±1.7 (14.6-20.3)	13.5±1.5 (11.3-18.3)	42.6±5.8 (33.5-53.3)	6.0±0.4 (5.1-6.5)
Greatest body diam. (D)	57.3±13.3 (39.5-96.0)	42.8±4.1 (35.2-56.3)	103.8±18.5 (81.7-143.7)	79.2±12.8 (56.3-111.3)	212.8±28.0 (162.9-257.1)	23.8±3.5 (21.1-32.4)
EP	136.4±15.0 (115.5-169.0)	97.5±7.8 (84.5-119.7)	100.6±9.8 (77.5-126.8)	132.4±9.0 (116.9-149.3)	148.9±17.0 (120.0-177.1)	105.1±6.6 (95.8-119.7)
NR	87.1±7.6 (73.2-102.8)	85.7±4.9 (76.1-95.8)	100.8±10.0 (87.3-122.5)	97.0±5.8 (83.1-108.5)	154.4±18.6 (128.6-201.4)	98.2±6.5 (90.1-114.1)
ES	115.3±8.3 (101.4-139.4)	112.4±4.6 (102.8-119.7)	145.2±10.3 (133.8-166.2)	128.4±5.3 (112.7-140.8)	203.5±17.1 (171.4-237.1)	130.7±7.8 (115.5-147.9)
Tail length with sheath (T)	35.7±3.4 (31-42.3)	32.2±3.7 (26.8-39.4)	85.3±9.7 (70.4-112.7)	66.5±4.7 (60.6-81.7)	107.1±16.2 (85.7-137.1)	95.5±7.0 (83.1-111.3)
Anal body diam. (ABD)	23.1±2.2 (18.3-26.8)	20.9±2.2 (18.3-25.4)	27.8±2.7 (23.9-33.8)	25.5±3.5 (16.9-35.2)	55.4±8.3 (45.7-73.4)	15.2±2.0 (12.7-21.1)
Spicule length (SL)	41.3±3.2 (33.8-46.5)	37.3±3.8 (28.2-42.3)				
Gubernaculum length(GL)	21.5±1.9 (18.3-23.9)	18.5±1.5 (15.5-21.1)				
Stoma length	26.9±2.8 (22.5-35.2)	23.9±2.2 (19.7-28.2)	35.6±3.5 (28.2-40.8)	28.0±2.5 (23.9-31.0)	46.0±6.9 (34.3-62.9)	
Stoma diam.	21.3±3.1 (18.3-31)	18.3±2.1 (14.1-22.5)	27.5±2.5 (22.5-32.4)	21.0±2.3 (14.1-23.9)	33.1±2.8 (28.6-40.0)	
D% = EP/ES × 100	118.4±10.8 (104.8-150.7)	86.8±6.4 (77.9-107.6)	69.4±5.9 (56.7-80.0)	103.1±5.8 (92.2-116.7)	73.2±6.2 (57.5-86.0)	80.4±2.6 (77.3-87.0)
SW% = SP/ABD × 100	179.8±16.9 (150.2-220.4)	180.6±28.1 (117.6-230.8)				
GS% = GU/SP × 100	52.3±3.9 (43.7-57.7)	49.8±5.2 (42.3-65.0)				
E=EP/Tx100			119.0±14.9 (91.2-145.1)	199.5±12.9 (180.0-233.3)	141.5±22.8 (87.5-193.8)	110.5±9.8 (91.1-125.1)
H%						61.3±7.1 (50.7-77.5)
V%			49.4±3.2 (40.3-53.8)	49.8±3.8 (39.6-54.7)		

EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of pharynx; a = body length/greatest body diameter; b = body length/tail length; c = body length/ES

Table III. Morphometrics of *Steinernema feltiae*. Measurements are in µm and are in the form: mean ± SD (range).

Character	Male (1st generation) 10	Infective Juvenile 10
Body length (L)	1231± 114.5 (916-1542)	736.8.6±81.5 (652.1-902.5)
a	13.1±1.9 (9-15.3)	29.7±2.3 (25.4 -35.2)
b	9.8±2.0 (7.2-14.1)	6.8±0.4 (5.4-8.0)
c	50.8 ±4.6 (45.2-69.8)	9.6±0.8 (7.5-11.1)
Greatest body diameter (D)	101.2±19.9 (84.7-105.9)	21.8±4.3 (19-23.5)
EP	99.6±7.8 (78.5-104.3)	65.3±10.2 (58.9-72.1)
NR		93.7±8.6 (8.5-102.1)
ES	139±11.9 (114.1-158.3)	122.7±10.5 (118.3-134.8)
Tail length (T)	32.2±7.3 (27.1-35.2)	69.6±6.8 (64.2-85.2)
Anal body diameter (ABD)	33.2±5.6 (29.8-38.8)	

EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = distance from anterior end to end of pharynx; a = body length/greatest body diameter; b = body length/tail length; c = body length/ES

morphological characters of the IJs, females, and males of the isolates from Nigeria resemble descriptions from isolates originating in different geographical regions. Morphological characters included for identification were: distance from anterior end to excretory pore, distance from anterior end to nerve ring, distance from anterior end to end of pharynx, *a* (body length/greatest body diameter), *b* (body length/tail length), *c* [body length/ES (distance from anterior end to end of pharynx)]. All morphological and morphometric characters agree with descriptions by Nguyen and Smart (1995) and Hominick *et al*. (1997) (Tables II and III).

PCR of the entire ITS region amplified a single band of 970 base pairs (bp) composed of the partial 18S, ITS1, 5.8S, ITS2 and partial 28S. A BLAST search of GenBank revealed that the Nigerian isolates of *S. feltiae* sequences were identical to those of *S. feltiae* isolates from different geographical regions (NCBI accession # JF728858.1, AB243439.1, AY 171257.1, and others). The sequences obtained have been deposited in GenBank under the accession number JX403718 for *H. bacteriophora* and JX403719 for *S. feltiae*. Phylogenetic analysis based on maximum parsimony (MP) sequence of the ITS rDNA region confirmed that the Nigerian isolate grouped with other isolates of *S. feltiae*, when compared with other species of the genus (Fig. 2).

The second species identified molecularly was *H. bacteriophora*. Analysis of the entire ITS rDNA region composed of the partial 18S, ITS1, 5.8S, ITS2 and partial 28S was characterized by a sequence length of 1001 base pairs (bp). Phylogenetic analysis based on maximum parsimony (MP), placed the isolate with the respective group. The analysis sequences of known *Heterorhabditis* species from GenBank with those generated during the survey, based on MP, are indicated in Fig. 3. Sequence similarity of Nigerian isolates identified as known species with previously published sequences of other populations of the same species varied between 99-100% (Fig. 3). The neighbour-joining method was used to construct a phylogenetic tree based on the full length of the ITS region and showed the close relationship of the respective species.

DISCUSSION

Heterorhabditis bacteriophora and *S. feltiae* are the two most geographically widespread species of EPNs and have been reported from different parts of the world, including Africa, Asia, Australia, Europe and North and South America (Hominick, 2002). Several surveys for EPNs have been documented with a number of new species and strains from Africa. These species have widespread abundance and are associated with several types of habitats in South Africa, Ethiopia, Kenya and Egypt (Shamseldean *et al*., 1996; Burnell and Stock, 2000; Nguyen *et al*., 2004; Mekete *et al*., 2005; Mwaitulo *et al*., 2011; Malan *et al*., 2011; Kanga *et al*., 2012). Based on the previous surveys undertaken in different parts of the world, including Africa, the presence of *H. bacteriophora* and *S. feltiae* should be expected in Nigeria. How-

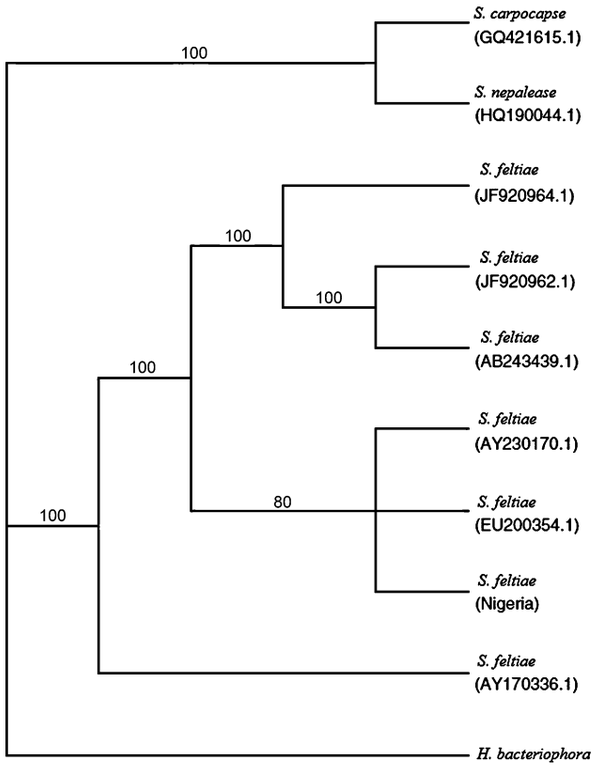

Fig. 2. Phylogenetic relationships of *Steinernema feltiae* isolate from Nigeria based on the sequence alignment of the ITS regions composed of the partial 18S, ITS1, 5.8S, ITS2, and partial 28S. The phylogenetic tree was generated by neighbour-joining analysis with 1000 bootstrap replication. Bootstrap values (100%) are indicated at the nodes.

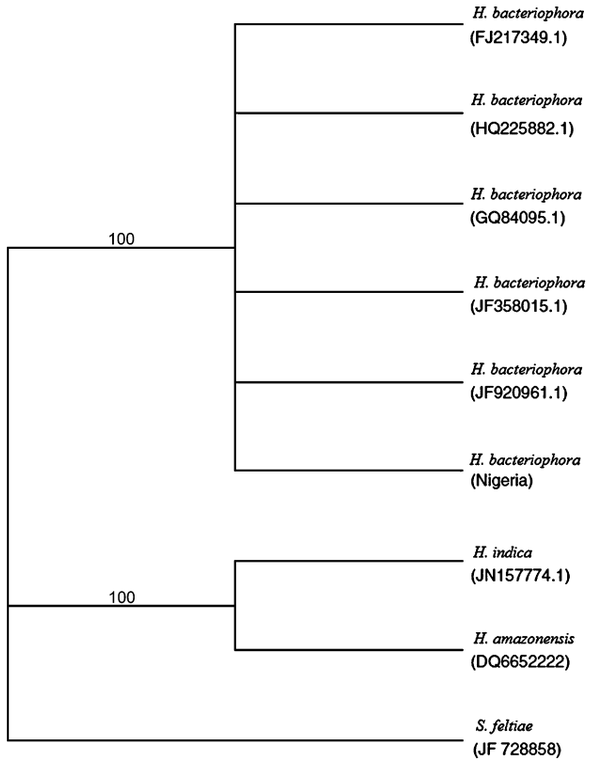

Fig. 3. Phylogenetic relationships of *Heterorhabditis bacteriophora* isolate from Nigeria based on the sequence alignment of the ITS regions composed of the partial 18S, ITS1, 5.8S, ITS2, and partial 28S. The phylogenetic tree was generated by neighbour-joining analysis with 1000 bootstrap replication. Bootstrap values (100%) are indicated at the nodes.

ever, no species of these genera has been reported from Nigeria until now. Therefore, this work represents the first report from Nigeria of EPNs that may have potential to be developed as bio-control agents for the control of important agricultural insect pests.

LITERATURE CITED

Ansari M.A., Shah F.A. and Butt T.M., 2008. Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) control. *Entomologia Experimentalis et Applicata*, *129*: 340-247.

Ansari M.A., Evans M. and Butt T.M., 2009. Identification of pathogenic strains of entomopathogenic nematodes and fungi for wireworm control. *Crop Protection*, *28*: 269-22.

Bedding R.A. and Akhurst R.J., 1975. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica*, *21*: 109-110.

Burnell A.M. and Stock S.P., 2000. *Heterorhabditis*, *Steinernema* and their bacterial symbionts - lethal pathogens of insects. *Nematology*, *2*: 31-42.

Ehlers R.-U., 2005. Forum on safety and regulation 2005. Pp. 107-114. *In*: Nematodes as Biological Control Agents (Grewal P.S., Ehlers R.-U. and Shapiro-Ilan D.I., eds). CABI Publishing, Wallingford, UK.

Gaugler R., 2007. *Entomopathogenic Nematology*. CABI Publishing, Wallingford, UK, 388 pp.

Georgis R., Koppenhofer A.M., Lacey L.A., Belair G., Duncan L.W., Grewal P.S., Samish M., Tan L., Torr P. and Van Tol R.W.H.M., 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biological Control*, *38*: 103-123.

Hominick W.M., 2002. Biogeography. Pp. 115-143. *In*: Entomopathogenic Nematology (Gaugler R., ed.). CABI Publishing, Wallingford. UK.

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. and Yoshida M., 1997. Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology*, *71*: 271-298.

Kanga F.N., Waeyenberge L., Hauser S. and Moens M., 2012. Distribution of entomopathogenic nematodes in Southern Cameroon. *Journal of Invertebrate Pathology*, *106*: 41-51.

Kaya H.K. and Stock S.P., 1997. Techniques in insect nematology. Pp. 281-324. *In*: Manual of Techniques in Insect Pathology (Lacey L.A., ed.). Academic Press, London, UK.

Malan A.P., Knoetze R. and Moore S.D., 2011. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology*, *8*: 115-125.

Mekete T., Gaugler R., Nguyen K.B., Mandefro W. and Tessera M., 2005. Biogeography of entomopathogenic nematodes in Ethiopia. *Nematropica*, *35*: 31-36.

Mwaitulo S., Haukeland S., Saethre M.-C., Laudisoit A. and Maerere A.P., 2011. First report of entomopathogenic nematode from Tanzania and their virulence against larvae and adults of the banana weevil *Cosmopolites sordidus* (Coleoptera: Curculionidae). *International Journal of Tropical Insect Science*, *31*: 154-161.

Nguyen K.B. and Hunt D.J., 2007. Heterorhabditidae: species descriptions. Pp. 611-692. *In*: Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Nematology Monographs and Perspectives 5 (Hunt D.J. and Perry R.N. series editors). Brill, Leiden, The Netherlands.

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

Nguyen K.B., Shapiro-Ilan D.I., Stuart R.J., McCoy C.W., James R.R. and Adams B.J., 2004. *Heterorhabditis mexicana* n. sp. (Rhabditida: Heterorhabditidae) from Tamaulipa, Mexico, and morphological studies of the bursa of *Heterorhabditis* spp. *Nematology*, *6*: 231-244.

Nguyen K.B., Gozel U., Koppenhöfer H.S. and Adams B.J., 2006. *Heterorhabditis floridensis* n. sp. (Rhabditida: Heterorhabditidae) from Florida. *Zootaxa*, *1177*: 1-19.

Shamseldean M.M., Abou El-Sooud A., Abd-Elgawad M.M. and Saleh M.M., 1996. Identification of a new heterorhabditid species from Egypt. *Heterorhabditis taysearae* n. sp. (Rhabditida: Heterorhabditidae). *Egyptian Journal of Biological Pest Control, 6*: 129-138.

Swofford D.L., 2002. *PAUP* Phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates, Sunderland, Massachusetts, USA, 77 pp.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research*, *25*: 4876-4882.