TAXONOMIC STUDY OF ENTOMOPATHOGENIC NEMATODES (NEMATODA : STEINERNEMATIDAE, HETERORHABDITIDAE) FROM BENIN

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ABSTRACT

A study on the biodiversity of entomopathogenic nematodes was conducted during 2010 and 2011 in South Benin. Soil samples from eight sites production of annual and perennial crops were analysed. We obtained 13.21 % of positive soil samples out of 280. We here report on the identification of six of these isolates. Molecular, morphometrical and morphological observations classified the isolates within the genus *Heterorhabditis* ; one isolate was conspecific with *H. indica* and two other isolates with *H. sonorensis*. More information is needed for effective identification of the remaining three isolates. Phylogenic analysis based on sequences of ITS regions of rDNA grouped our isolates with *H. sonorensis* and *H. taysearae* with bootstrap support values of 94 and 99 % in Maximum Parsimony and Neighbour Joining trees, respectively. Morphological characters of the infective juveniles and males did not correspond to those of *H. taysearae*, but were close to *H. sonorensis*. In contrast, the female of the *H. sonorensis* populations did show some minor differences with the originally described one. No progeny was obtained from the crossbreeding of Beninese isolates and *H. taysearae*. Crossing with an isolate of *H. sonorensis* would have been more conclusive, but no isolates were available even for specimen's morphological comparison.

Keywords : Survey, identification, Molecular, cross-hybridization, *Heterorhabditis*.

RESUME

ETUDE TAXONOMIQUE DES NEMATODES ENTOMOPATHOGENES (NEMATODA : STEINERNEMATIDAE, HETERORHABDITIDAE) DU BENIN

*Une étude diagnostique réalisée sur les nématodes entomopathogènes (NEP) en 2010 et 2011 dans le Sud-Bénin sur huit sites de production de cultures annuelles et pérennes a conduit à 13,21 % d'échantillons de sols positifs sur 280. Le présent travail a porté sur l'identification de six des isolats de NEP extraits. Les études moléculaires, morphologiques, morphométriques et d'hybridation effectuées utilisant les stades développementaux des nématodes ont révélé un isolat d'*H. indica*, deux d'*H. sonorensis*. Pour les autres isolats, des informations complémentaires sont nécessaires pour une identification complète. Le séquençage et l'analyse phylogénétique de la région interspécifique de l'ADN ribosomal ont groupé nos isolats avec* H. sonorensis *et* H. Taysearae *dans les arbres de parcimonie maximale et de Neighbour Joining avec les supports respectifs 94 et 99 %. Les caractères morphologiques des juvéniles infectieux et des mâles ne correspondent pas à ceux de la première description d'*H. taysearae*, mais sont proches d'*H. sonorensis*. Cependant, certaines femelles avec bouchon copulatoire ne correspondent pas à* H. sonorensis*. Nos isolats n'ont pas été féconds avec* H. taysearae*. Un croisement avec* H. sonorensis *population type serait plus concluant, mais il n'y avait aucun isolat disponible dans la base de gènes.*

Mots-clés : *Etude, identification, moléculaire, hybridation-croisée,* Heterorhabditis.

INTRODUCTION

Entomopathogenic nematodes (EPN) belonging to the genera *Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976 are characterized by their symbiotic association with bacteria of the genera *Xenorhabdus* Thomas & Poinar, 1979 and *Photorhabdus* Boemare, Akhurst & Mourant, 1993, respectively (Ehlers, 2007). They are released by the nematode upon penetration into the insect. The bacteria cause insect death by septicaemia and function as food source for the nematodes in the insect (Ciche *et al.,* 2006). Due to the relatively rapid death of the insect host (within 24 - 48 hours) and their wide host range, considerable interest has been given to the study of EPNs and their use as biological control agents (Brusselman, 2011).

In Africa, several research activities have focused on EPN and are still going on. Quite a large number of EPN species have been found in some countries ; several new species were described from the continent (e.g. Kaya *et al.,* 2006 ; Malan *et al.,* 2008 ; Ngo Kanga *et al.,* 2012).

The diversity, distribution and pathogenicity of EPN from Benin are studied within the framework of the Flemish Interuniversity Council-University Development Co-operation (VLIR-UOS) Own Initiative project entitled «Ecologically sustainable *Citrus* Production in Benin» (ESCiP-Benin). The project is a co-operation between Ghent University (Belgium) and the University of Parakou (Benin) and aims at promoting the use of EPN as an environmental friendly tool for termite control in *Citrus*.

Because information on the identity and distribution of EPN in Benin was lacking, several surveys were conducted in Southern Benin during the period 2010 - 2011. We here report on the identification six isolates of the collected EPN isolates (as part of a Master dissertation research) of EPN. They were identified using an integrated approach based on their morphology and morphometrics (using light microscopy and scanning electron microscopy), data from molecular analysis and cross-hybridization tests.

MATERIALS AND METHODS

NEMATODE COLLECTION AND EXTRACTION

Soil samples were taken from *Acacia* spp., *Mangifera indica, Tectona grandis, Anacardium occidentiale*, *Musa* spp., *Elaeis guineensis* and *Citrus* spp. production areas in the Southern part of Benin (= main *Citrus* production area). Each sample (*ca* 1.5 kg soil) was composed of 3 - 4 sub-samples randomly taken around trees at 0 - 15 cm depth. The samples were taken to the laboratory in sealed polythene bags to prevent drying (Uribe-Lorío *et al.,* 2005).

Entomopathogenic nematodes were extracted from *ca* 350 ml soil of each sample by using the *Galleria mellonella* baiting method (Bedding & Akhurst, 1975) followed by the White trap (White, 1927) to collect juveniles emerging from the insect cadaver. After verifying Koch's postulate, *i.e.* checking the virulence of the extracted nematodes (Ngo Kanga *et al.,* 2012), the isolates were continuously mass-reared on *Galleria* larvae and stored in water at 15 °C. EPN obtained from the same insect cadaver were considered as one isolate. For the present work, the isolates used are : Ayogbe1 (from Ayogbe), Kpedekpo (from Kpedekpo), Akohoun (from Akohoun), Setto1 (from Setto), Ze1 (from Ze) and Ze3 (from Ze).

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSES

For each isolate, DNA was extracted from 10 to 15 third-stage infective juveniles (IJ) as described in Holterman *et al.* (2006). Both the polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region of rDNA and the purification of the PCR-product cut out of gel were performed according to the manufacturer's instructions[1]. All samples were sent to a sequencing service (Macrogen, South-Korea). Finally, the sequences were blasted against relevant sequences available in database of NCBI (Genbank).

[1]Wizard SV Gel and PCR Clean-Up System, Promega Corporation, Madison USA, 2009

The sequences of ITS region of the isolates (Genbank accession numbers : Ayogbe1 (KF723816), Kpedekpo (KF723815), Akohoun (KF723818), Setto1 (KF723819), Ze1 (KF723827) and Ze3 : (KF723828)) were aligned using BioEdit Sequence Alignment Editor (ClustalW Multiple alignment) with rDNA sequences of 14 other *Heterorhabditis* species (since only *Heterorhabditis* species were found according to the *Galleria* baiting method and sequencing of ITS region of the rDNA of our isolates) obtained from GenBank (accession number) : i.e. *H. sonorensis* Caborca isolate (FJ477730), *H. taysearae* (EF043443), *H. mexicana* (EF043444), *H. amazonensis* (DQ665222), *H. floridensis* (DQ372922), *H. baujardi* (EU363039), *H. indica* (HQ225858), *H. bacteriophora* (JF358015), *H. georgiana* (EU099032), *H. safricana* (EF488006), *H. downesi* (AY321482), *H. zealandica* (AY321481), *H. megidis* (AY321480) and *Caenorhabditis elegans* (EU131007) as an out-group taxon.

Phylogeny reconstruction analysis was performed based on Maximum Parsimony (MP) using MEGA 5.05. For the MP tree, bootstrap analysis with 1000 replicates was conducted as a measure of support. MP search level was 1 and tree search method used was Close-Neighbour interchange on random trees and the number of the initial trees (random addition) was 10. A phylogenetic tree was made as condensed tree with topology ; cut-off value for consensus tree was equal to 50 %.

Neighbour-Joining test (tree) was also performed using default parameters.

MORPHOLOGICAL CHARACTERIZATION

First-generation hermaphrodites and second-generation adults were isolated from *G. mellonella* 4 - 5 and 7 - 8 days after infection, respectively (Stock *et al.,* 2009). Nematodes were heat killed and fixed using triethanolamine-formalin (Courtney *et al.,* 1955). They were transferred to glycerol-ethanol (Seinhorst, 1959). Permanent slides were prepared according to Cobb (1918). For the morphometric observations, 20 specimens of each developmental stage were used (Nguyen, 2007). Digital light microscopic pictures and all measurements were made using a Soft Imaging System GmbH («cell^D» software, Münster-Germany) installed on a computer connected to an Olympus BX51 light microscope (Olympus Company Belgium) equipped with differential interface contrast optics. The measurements of the tails of IJ included the ensheated second stage cuticle (Nguyen, 2007).

The following abbreviations and ratios were used based on Nguyen (2007) : n = number of specimens measured, L = body length, MBD = maximum body diameter, SP = secretory-excretory pore, SEP = distance from anterior end to the secretory-excretory pore, ES = pharynx length, NR = nerve ring, T = tail length, ABD = anal body diameter, SL = spicule length (arc length), GL = gubernaculum length, TR = testis reflexion, H% = (hyaline tail portion length/ T) x 100. Ratios a = L/MBD, b = L/T, c = L/T, D% = (SEP/ES) x 100, E% = (SEP/T) x 100, SW% = (SL/ABD) x 100, GS% = (GL/SL) x 100, V% = (distance from anterior end to vulva/L) x 100.

For electron microscopy observations, all stages of nematodes were directly isolated from insect cadavers in Ringer's solution. They were kept in anhydrous glycerine, transferred into a drop of glycerine in an embryo dish and further processed according to Steel *et al.* (2011). Finally, the nematodes were observed at 15 kV with a JSM-840 EM (JEOL, Tokyo, Japan).

CROSS-HYBRIDIZATION TESTS

Cross hybridization tests were carried out on 3.5 cm lipid agar plates (Wouts, 1981). These plates were pre-inoculated with 1-2 drops of primary phase of the bacteria (previously extracted from the haemolymph of infested *Galleria* larvae) and incubated for 48 h in the dark at 30 °C (Phan *et al.,* 2003). The following crossings were performed : Ayogbe1 × *H. indica,* Kpedekpo × *H. taysearae*, Akohoun × *H. taysearae*, Setto1 × *H. taysearae*, Ze1 × *H. taysearae* and Ze3 × *H. taysearae*. For each crossing, 20 males and 20 females of the appropriate isolate were added to the plate and incubated at 25 °C. Two other tests served as controls : a) a virginity/self fertility test in which 20 virgin females were incubated without males on pre-inoculated lipid agar plates (Stack *et al.,* 2000) and b) a self-cross where 20 males and 20 females of the same isolate were incubated together (Phan *et al.,* 2003). The results of crossing between different isolates were considered as valid only if there was no progeny in both the virginity test and the self-cross (Stack *et al.,* 2000). Mating between male and female

of the same species should produce fertile offspring (Nguyen, 2007).

Unfortunately, no isolates of *H. sonorensis* were available for crossbreeding tests. Also the Beninese specimens could not be morphologically compared with type material of *H. sonorensis* since type specimens were not available in the nematode collections indicated in the original description.

RESULTS

MOLECULAR OBSERVATIONS AND RELATIONSHIPS WITH OTHER SPECIES

The sequence length of the isolates varied from 688 (Ayogbe1) to 707 bp (Kpedekpo, Akohoun, Setto1, Ze1 and Ze3).

The BLAST (March 2012) against the sequence database available in NCBI (Genbank) indicated that all six isolates belonged to the genus *Heterorhabditis*. Isolates Kpedekpo and Ze1 shared 99 % similarity with *H. sonorensis* and *H. taysearae*, while isolates Akohoun, Setto1 and Ze3 shared 100 % similarity with *H. sonorensis* and 99 % similarity with *H. taysearae*. All isolates shared 98 % similarity with *H. mexicana* and *H. floridensis*, 97 % similarity with *H. amazonensis*, and 96 % similarity with *H. baujardi*. Isolate Ayogbe1 shared 99 % similarity with *H. indica*.

SEQUENCE ALIGNMENT AND RELATIONSHIPS WITH OTHER SPECIES

The pairwise divergence between the sequences of all isolates ranged from 0 to 172 nucleotides. The ITS sequence of Ayogbe1 differed from *H. indica* by a single nucleotide.

No difference in nucleotide composition was found between the other five Beninese isolates (Kpedekpo, Akohoun, Setto1, Ze1 and Ze3) and *H. sonorensis* (FJ477730) but they differed from *H. taysearae* (EF43443) by 1 nucleotide.

The phylogenetic trees MP (Figure 1) and NJ (Figure 2) revealed two main clades viz. the *indica*-clade and the *megidis*-clade. All Beninese isolates were members of the *H. indica*-clade with a high (MP) to moderate (NJ) bootstrap support. Within this clade of the MP tree, five Beninese isolates clustered with *H. sonorensis* and *H. taysearae* with a 94 % bootstrap support. In the NJ tree, *H. taysearae* appeared as a sister-group to the group composed by the five Beninese isolates and *H. sonorensis* with low bootstrap support (67 %) ; the clade including *H. taysearae* had a 99 % bootstrap support.

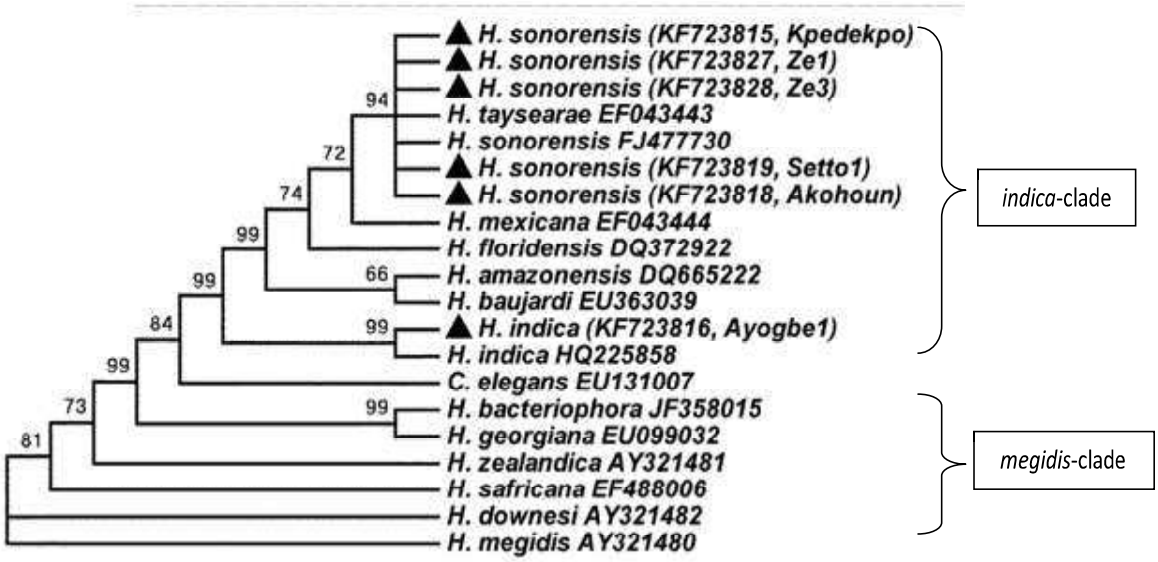

Figure 1 : Phylogenetic relationships using Maximum Parsimony method of the isolates used. ▲: Beninean isolates ; H. : *Heterorhabditis* ; C. : *Caenorhabditis* ; FJ477730 : GenBank accession number.

Arbre phylogénétique des isolats réalisé selon la méthode de Parcimonie Maximale. ▲: isolats béninois; H. : Heterorhabditis *; C. :* Caenorhabditis *; FJ477730 : numéro d'accession dans la Banque de Gène.*

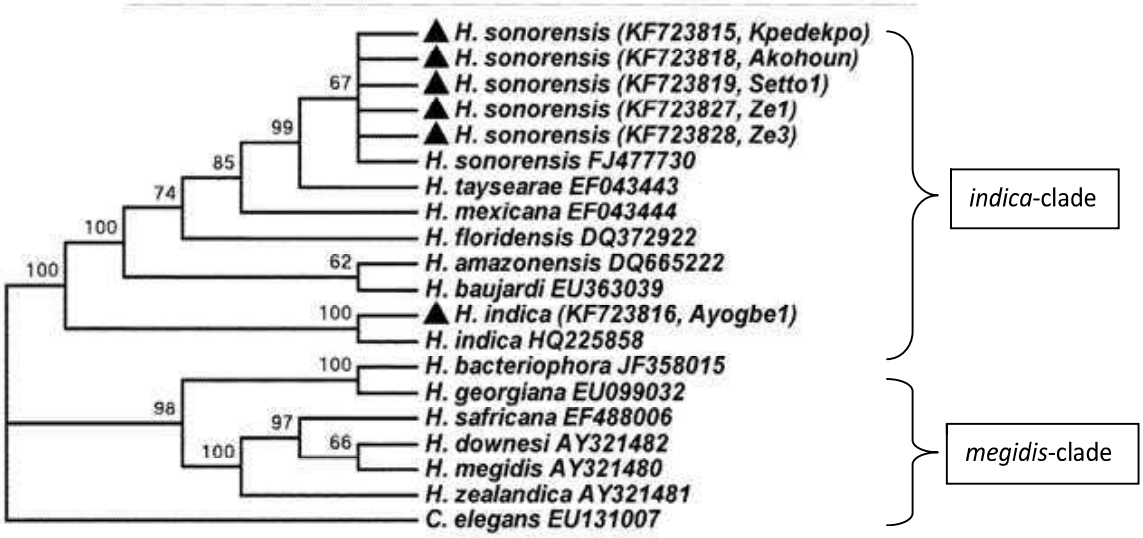

Figure 1 : Phylogenetic relationships using Neighbour-Joining method of the isolates used. ▲: Beninean isolates ; H. : *Heterorhabditis* ; C. : *Caenorhabditis* ; FJ477730 : GenBank accession number.

Arbre phylogénétique des isolats réalisé selon la méthode de Neighbour-Joining. ▲ : isolats béninois; H. : Heterorhabditis *; C. :* Caenorhabditis *; FJ477730 : numéro d'accession dans la Banque de Gène.*

MORPHOMETRICS AND MORPHOLOGY

Heterorhabditis sonorensis (Stock, Rivera-Orduño & Flores-Lara, 2009): isolates Kpedekpo, Akohoun, Setto1, Ze1 and Ze3

Infective juvenile

Body slender and long 480 - 651 µm (ensheated), with wider range than *H. sonorensis* type population (495 - 570 µm) but comparable with the *H. sonorensis* Arizona population (414 - 647 µm). Anterior region rounded. Nerve ring located at the pharyngeal isthmus level. EP located at the level of the pharyngeal basal bulb or just anterior to it. Bacteria present in the anterior intestine. Tail long (94 -130 µm) with pointed terminus, range very close to that of *H. sonorensis* (91 - 125 µm). E% and D% more or less comparable to range mentioned in original description, H% clearly higher (36 - 43 % vs. 29 - 33 % in original description) (Figure 3 and Table 1).

Male

Ventrally curved body when heat-killed. Total body length (635 - 984 µm) longer than in original description of *H. sonorensis* (500 - 761 µm). Head rounded, lip region slightly truncated with six labial papillae, four cephalic papillae and pore-like amphidial apertures. Pharynx with pyriform basal bulb separated from the corpus by a narrower isthmus surrounded by the nerve ring ; cardia small. SP usually at level of the basal bulb or shortly posterior to it. Spicules paired, without differentiation apart from a minor widened capitulum, blade slightly ventrally curved. Spicules somewhat longer (41 - 46 µm) than in original description (38 - 39 µm), also lower GW%. Gubernaculum slightly ventrally curved, about half the spicule length. Tail curved ventrally, with peloderan bursa. Nine pairs of genital papillae with the typical *Heterorhabditis* formula : 1-2-3-3. D% clearly higher (102 - 125 %) than in type description (78 - 79 %) due to more posterior position of EP with respect to pharynx. (Figure 3 and Table 2).

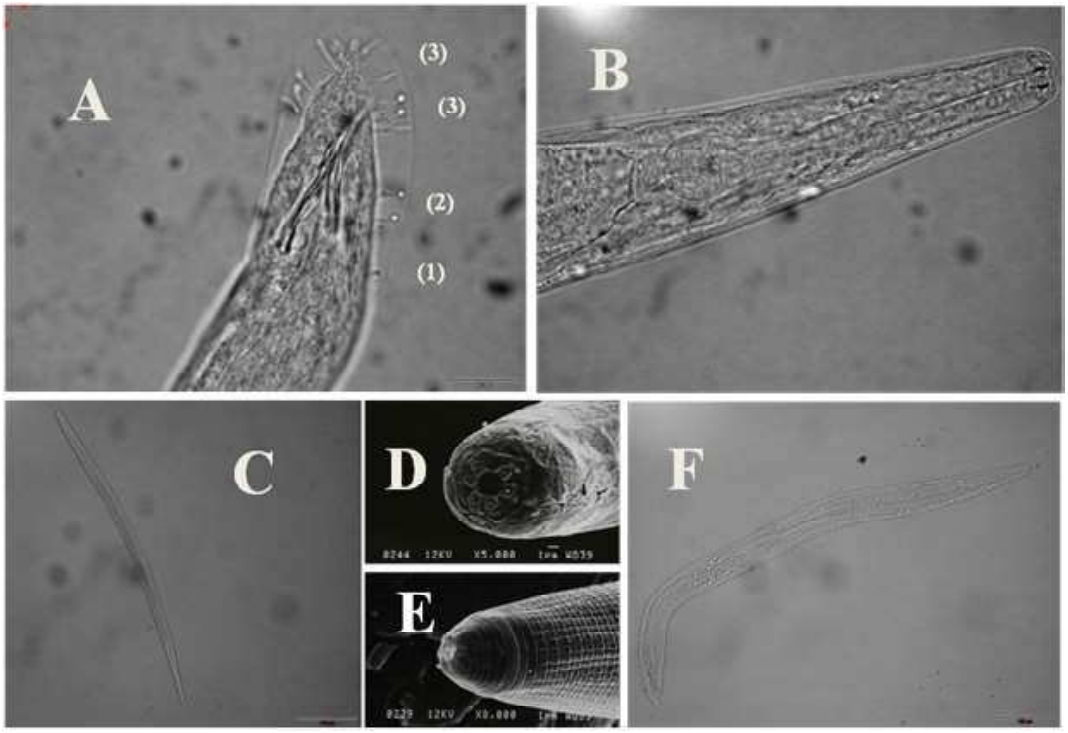

Figure 3 : Light (A,B,C,F) and scanning electron microscopy (D,E) photographs of male (A,B,D,F) and infective juvenile (C,E) specimen of Kpedekpo, Akohoun, Setto1, Ze1 and Ze3.

Spécimens mâle (A,B,D,F) et juvénile infectieux (C,E) des isolats Kpedekpo, Akohoun, Setto1, Ze1 et Ze3.

A : Tail in ventral view showing peloderan bursa and the 1 2 3 3 pattern of genital papillae. B : Pharyngeal region. D : Anterior region. F : Entire view. C : Entire body. E : Anterior region following by tessellate pattern of the cuticle. Scale bars : A,B,C, = 20 µm ; D,E = 1 µm ; F = 100µm.

A : Vue ventrale de la queue montrant la bourse pélodérane et le model 1 2 3 3 des papilles génitales. B : Pharynx. D,E : Région antérieure. F,C : Vue entière. Barres d'échelle : A,B,C, = 20 µm, D,E = 1 µm, F = 100 µm.

Table 1 : Comparative table of morphometrics of infective juvenile of the isolates Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 from Benin and their relative species (in µm, mean ± standard error and range in parenthesis).

Tableau comparatif des mesures morphométriques de juvénile infectieux des isolats Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 et leurs espèces associées (en µm, moyenne ± erreur standard et les variations entre parenthèses).

Characters	Kpedekpo	Akohoun	Setto1	Ze1	Ze3	*H. sonorensis**	*H. taysearae***	Ayogbe1	*H. indica****
n	20	20	20	20	20	20	30	20	25
L	530 ± 21 (480-557)	586 ± 18 (557-623)	565 ± 25 (498-607)	613 ± 12 (579-631)	606 ± 27 (554-651)	557 ± 28 (495-570)	418 ± 38 (332-499)	541 ± 18 (497-561)	528 ± 26 (479-573)
ES	117 ± 6 (96-129)	114 ± 8 (96-127)	120 ± 10 (104-140)	125 ± 3 (120-135)	125 ± 3 (120-130)	119 ± 7 (110-131)	110 ± 8.4 (96-130)	119 ± 5 (112-130)	117 ± 3 (109-123)
SEP	100 ± 8 (81-113)	99 ± 7 (87-108)	106 ± 12 (85-133)	107 ± 2 (103-114)	109 ± 4 (102-118)	99 ± 4.5 (97-116)	90 ± 9.1 (74-113)	95 ± 5 (89-110)	98 ± 7 (88-107)
NR	91 ± 5.7 (73-99)	95 ± 5.3 (83-107)	93 ± 4.1 (79-95)	93 ± 2.2 (89-98)	94 ± 3.4 (89-105)	93 ± 4 (87-98)	64 ± 6.8 (58-87)	87 ± 5 (79-100)	82 ± 4 (72-85)
MBD	23 ± 1 (20-25)	24 ± 2 (23-27)	24 ± 1 (23-26)	25 ± 1 (23-28)	25 ± 3 (22-31)	25.5 ± 4 (19-32)	20 ± 1.9 (17-23)	22 ± 1 (21-24)	20 ± 6 (19-22)
T	104 ± 4 (94-111)	118 ± 5 (109-127)	117 ± 8 (101-130)	115 ± 5 (106-123)	116 ± 7 (103-128)	105 ± 7 (91-125)	55 ± 6.6 (44-70)	102± 4 (96-109)	101 ± 6 (93-109)
ABD	14 ± 1 (12-15)	18 ± 1 (16-19)	17 ± 1 (15-19)	16 ± 1 (14-17)	17 ± 1 (14-21)	16 ± 2.0 (13-16)	-	14 ± 0.7 (13-15)	-
a	23 ± 1 (21.7-25.2)	24 ± 1.9 (23.9-27)	24 ± 1.8 (24-26.3)	24 ± 1.4 (22.4-26.8)	24 ± 2.1 (19.9-27)	23 ± 1.5 (19-26)	21 ± 2.2 (18-27)	24 ± 0.8 (23-25)	26 ± 4 (25-27)
b	4.5 ± 0.3 (4-5.5)	5.2 ± 0.4 (4.6-6.4)	5.4 ± 0.4 (4.6-6.3)	4.9 ± 0.1 (4.6-5.1)	4.9 ± 0.2 (4.5-5.2)	4.8 ± 0.4 (4.4-5.4)	3.8 ± 0.2 (3.4-4.2)	4.6 ± 0.2 (3.8-4.8)	4.5 ± 0.34 (4.3-4.8)
c	5.1 ± 0.2 (4.9-5.4)	5.0 ± 0.2 (4.6-5.2)	4.8 ± 0.3 (4.2-5.4)	5.3 ± 0.2 (5.1-5.7)	5.2 ± 0.3 (4.8-6.2)	5.5 ± 1.0 (4.0-6.5)	7.7 ± 0.7 (6.5-8.7)	5 ± 0.2 (5-6)	5.3 ± 0.5 (4.5-5.6)
D%	85 ± 4 (79-93)	87 ± 5 (75-92)	88 ± 4 (81-97)	86 ± 2 (80-91)	87 ± 3 (83-93)	90 ± 8.5 (78-110)	82 ± 6 (71-96)		84 ± 5 (79-90)
E%	96 ± 9 (76-113)	84 ± 6 (69-98)	91 ± 11 (69-110)	93 ± 3 (88-100)	94 ± 6 (82-110)	99 ± 8 (81-111)	180 ± 27 (110-230)		94 ± 7 (83-103)
H%	38 ± 4 (29-45)	40 ± 3 (35-42)	43 ± 3 (38-47)	36 ± 2 (33-40)	38 ± 2 (33-41)	29 ± 1 (28-31)	-		-

* : Stock, Rivera-Orduño and Flores-Lara, 2009, Carboca isolate ; **: Sham-seldean *et al.,* 1996 ; *** Poinar, Karunakar and David, 1992.

Table 2 : Comparative table of morphometrics of male specimens of the isolates Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 from Benin and their relative species (in µm, mean ± standard error and range in parenthesis).

Tableau comparatif des mesures morphométriques du spécimen mâle des isolats Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 et leurs espèces associées (en µm, moyenne ± erreur standard et les variations entre parenthèses).

Characters	Kpedekpo	Akohoun	Setto1	Ze1	Ze3	*H. sonorensis**	*H. taysearae***	Ayogbe1	*H. indica****
n	20	20	20	20	20	20	20	20	12
L	764 ± 61 (635-877)	856 ± 40 (778-934)	926 ± 82 (747-984)	814 ± 44 (725-905)	809 ± 20 (768-841)	725 ± 31 (500-750)	703 ± 23 (648-736)	822 ± 49 (740-918)	721 ± 64 (573-788)
ES	107 ± 6 (96-116)	114 ±4 (106-121)	113 ± 3 (109-112)	113 ± 4 (105-118)	114 ±4 (107-121)	93 ± 7 (80-100)	112 ± 12.5 (85-123)	95 ± 5 (88-105)	101 ± 4 (93-109)
SEP	128 ± 11 (111-148)	142 ± 7 (131-159)	140 ± 14 (107-145)	133 ± 11 (100-146)	116 ± 9 (100-137)	73 ± 5 (60-84)	95 ± 12.3 (78-120)	113 ± 8 (103-125)	123 ± 7 (109-138)
NR	80 ± 5 (71-89)	90 ± 4 (83-98)	86 ± 3 (80-85)	86 ± 4 (77-91)	88 ± 4 (79-94)	71 ± 5 (60-80)	65 ± 11.6 (54-88)	75 ± 4 (66-84)	75 ± 4 (72-85)
T	35 ± 3 (27-38)	38 ± 2 (34-41)	41 ± 4 (32-44)	35 ± 3 (31-40)	38 ± 4 (30-45)	34 ± 5 (25-45)	25 ± 5.4 (20-29)	33 ± 3 (26-39)	28 ± 2 (24-32)
ABD	23 ± 3 (19-29)	24 ± 1 (22-26)	25 ± 2 (21-27)	24 ± 1 (21-27)	24 ± 2 (22-28)	25 ± 2 (20-30)	25 ± 2.9 (21-30)	25 ± 2 (21-30)	23 ± 8 (19-24)
SL	41 ± 4 (33-47)	45 ± 2 (37-48)	46 ± 2 (43-49)	44 ± 3 (39-48)	44 ± 2 (40-46)	39 ± 2.5 (31-45)	39 ± 4.8 (30-42)	39 ± 2 (35-42)	43 ± 3 (35-44)
GL	22 ± 2 (17-27)	23 ± 2 (19-25)	25 ± 1 (22-25)	24 ± 2 (20-29)	23 ± 1 (21-25)	22 ± 3 (20-31)	18 ± 3.2 (14-21)	22 ± 2 (20-26)	21 ± 3 (18-23)
TR	102 ± 17 (48-140)	111 ± 9 (94-123)	92 ± 23 (45-118)	94 ± 13 (72-117)	115 ± 11 (96-134)	-	122 ± 14.4 (100-146)	133 ± 16 (97-156)	91 ± 26 (35-144)
SW%	297 ± 41 (216-362)	188 ± 16 (145-220)	183 ± 15 (154-182)	181 ± 16 (158-212)	179 ± 11 (162-198)	150 ± 20 (110-180)	156 -	159 ± 15 (127-194)	187 -
GS%	54 ± 4 (47-59)	51 ± 4 (43-58)	53 ± 3 (47-52)	55 ± 4 (45-65)	52 ± 3 (48-57)	60 ± 10 (40-75)	46 -	56 ± 4 (50-68)	49 -
D%	119 ± 6 (105-128)	125 ± 4 (116-134)	124 ± 12 (92-129)	117 ± 9 (90-129)	102 ± 7 (89-119)	79 ± 6 (72-91)	88 -	120 ± 6 (111-133)	121 -

*: Stock, Rivera-Orduño and Flores-Lara, 2009, Carboca isolate ; **: Sham-seldean *et al.,* 1996 ; *** Poinar, Karunakar and David, 1992.

Hermaphrodite

Body C-shaped after heat-killed or ventrally curved ; longer, with longer tail compared to original description (4756 - 5412 vs. 4175 - 4356 µm). Head region slightly truncate. Pharynx with a cylindrical corpus, isthmus surrounded by nerve ring, basal bulb pyriform or slightly rounded. Vulva located more anteriorly (V = 41 - 43 %) compared to original description (53.5 - 55 %). Vulva lips slightly to clearly protruding. Tail conoid with prominent, post-anal swelling. (Figure 4 & Table 3)

Female

Body C-shaped after heat-killed, or more ventrally curved posteriorly; body length as in original description and much smaller than in hermaphrodites. Head region and pharyngeal region similar to hermaphrodites. Reproductive system didelphic, amphidelphic. Vulva, a transverse slit, located at mid-body (V = 46 - 49 %). Copulatory plug present or absent, while no plug was observed in type specimens ; vulva lips non-protruding. Tail conical with weak post-anal swelling. (Figure 4 and Table 4)

Heterorhabditis indica **(Poinar, Karunakar & David, 1992) : isolate Ayogbe1**

The morphology of all developmental stages (IJ, male, female and hermaphrodite) of isolate Ayogbe1 corresponds largely to the original description of *H. indica*. However, some minor differences were observed in morphometrics (slightly longer males with longer tail and lower SW%, and longer hermaphrodites with longer tails) (Tables 1 - 4).

Infective juvenile

Body slender, 497 - 561 µm long. Head region annulated. Ensheated J2 cuticle with tessellate pattern, longitudinal ridges present posteriorly. Hemizonid distinct, located just anterior the secretory-excretory pore. Symbiotic bacteria well observed in the anterior intestine and along the lumen. Tail long, with pointed terminus. All measurement ranges are either within the range of the type population or very close. (Figure 5 and Table 1)

Male

Body curved. Head slightly rounded with six labial papillae. Pore-like amphidial aperture. Cylindrical corpus; isthmus present and surrounded by the nerve ring. SP located posterior to the basal pharyngeal bulb. Spicules paired and separate, with pointed distal tips as in the original description. Gubernaculum straight, length about half the spicule length. Peloderan bursa. Nine pairs of genital papilla as in the typical pattern of *Heterorhabditis* spp. (Figure 5 and Table 2)

Hermaphrodite

Cephalic region slightly rounded. SE- pore located at the level of the basal pharyngeal bulb or slightly posterior. Vulva located near mid-body. No vulva flap present. Tail conoid with prominent post-anal swelling. (Figure 6 and Table 3)

Female

Morphology as in hermaphrodite. The main difference is the presence of a copulatory plug. Slit-like vulva opening. (Figure 6 and Table 4)

CROSSBREEDING TESTS

Crossbreeding of Ayogbe1 and *H. indica* yielded fertile progeny. No progeny was obtained from the crossing between Kpedekpo, Akohoun, Setto1, Ze1, Ze3 and *H. taysearae.*

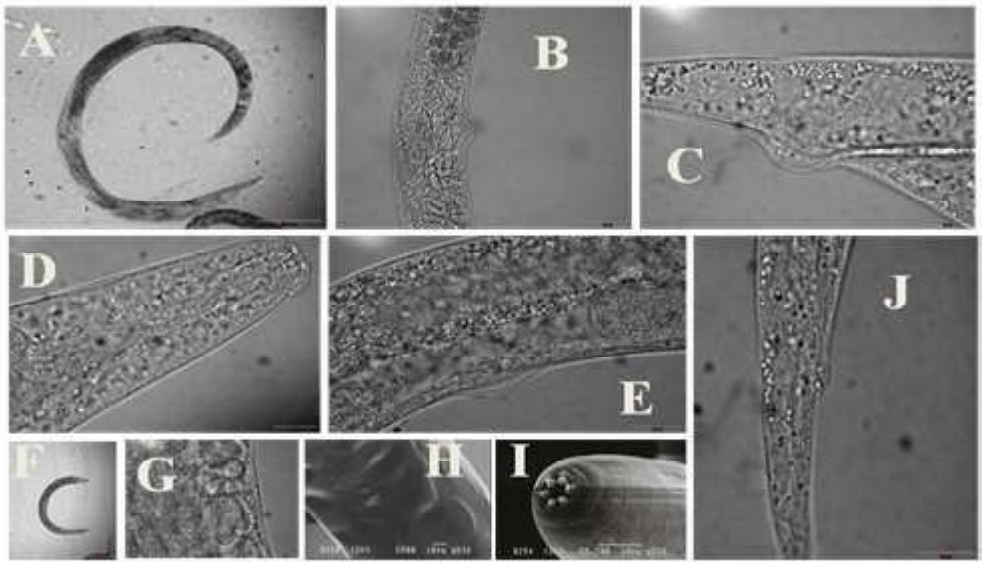

Figure 4 : Light (A,B,C,D,E,F,G,J) and scanning electron microscopy (H,I) photographs of hermaphrodite (A,B,C) and female (D,E,F,G,H,I,J) of Kpedekpo, Akohoun, Setto1, Ze1 and Ze3.

Spécimens femelle (D,E,F,G,H,I,J) et hermaphrodite (A,B,C) des isolats Kpedekpo, Akohoun, Setto1, Ze1 et Ze3.

A,F : Entire body. B : Protruding vulva. C : Post-anal swelling. D : Pharyngeal region. E : Vulva region with copulatory plug. G : Vulva without copulatory plug. H : Vulva in ventral view. I : head region. J : Post-anal region, slight swelling. Scale bars : A,F = 500 µm, B = 100 µm, C,D,E,G,J : 20 µm, H,I = 10 µm.

A,F : Vue entière. B : Vulve protubérante. C : Enflure post-anale. D : Pharynx. E : Vulve avec bouchon copulatoire. G : Vulve sans bouchon copulatoire. H : Vulve, vue ventrale. I : Tête. J : région post-anale. Barres d'échelle : A,F = 500 µm, B = 100 µm, C,D,E,G,J : 20 µm, H,I = 10 µm.

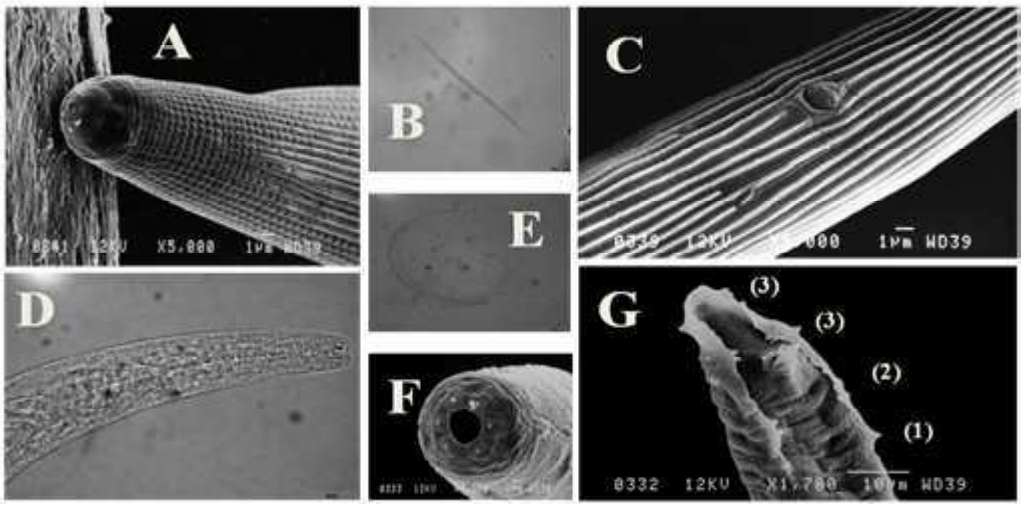

Figure 5 : Light (B,E,D) and scanning electron microscopy (A,C,F,G) photographs of infective juvenile (A,B,C) and male (D,E,F,G) specimen of H. indica (Ayogbe1).

Spécimens mâle et juvénile infectieux de H. indica (Ayogbe1).

A : head region. B,E : Entire view. C : Tail in ventral view. D : Pharynx. F : en face view of head region. G : 1 2 3 3 pattern of the genital papillae, oblique ventral view. Scale bars : A,C,F = 1 µm, B = 500 µm, D = 20 µm, E = 100 µm, G = 10 µm.
A : Tête. B,E : Vue entière. C : Vue ventral de la queue. D : Pharynx. F : vue d'en face de la la région antérieure. G : model 1 2 3 3 des papilles génitales, vue ventrale oblique. Barres d'échelle : A,C,F = 1 µm, B = 500 µm, D = 20 µm, E = 100 µm, G = 10 µm.

Table 3 : Comparative table of morphometrics of hermaphrodites of the isolates Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 from Benin and their relative species (in µm, mean ± standard error and range in parenthesis).

Tableau comparatif des mesures morphométriques des hermaphrodites des isolats Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 et leurs espèces associées (en µm, moyenne ± erreur standard et les variations entre parenthèses).

Characters	Kpedekpo	Akohoun	Setto1	Ze1	Ze3	*H. sonorensis* *	*H. taysearae***	Ayogbe1	*H. indica****
n	20	20	20	20	20	20	20	20	12
L	5412 ± 806 (4238-6488)	4756 ± 657 (3700-5738)	5278 ± 601 (4404-5991)	5169 ± 979 (3310-7074)	4970 ± 539 (3970-5862)	4356 ± 854 (2856-5799)	2510 ± 49 (2200-2800)	4787 ± 395 (4213-5807)	2700 ± 1000 (2300-3100)
ES	186 ± 6 (174-201)	204 ± 11 (181-222)	202 ± 2 (199-206)	207 ± 13 (185-232)	215 ± 11 194-239()	180 ± 18 (133-215)	176 ± 14.5 (161-200)	219 ± 12 (192-238)	172 ± 6 (163-179)
SEP	217 ± 21 (191-249)	203 ± 19 (166-237)	197 ± 18 (164-217)	204 ± 25 (176-263)	201 ± 20 (173-239)	149 ± 19 (115-203)	163 ± 13.2 (137-182)	222 ± 14 (196-254)	173 ± 8 (163-187)
NR	126± 6 (110-132)	151 ± 9 (134-167)	147 ± 9 (134-158)	148 ± 10 (132-166)	157 ± 9 (139-171)	135 ± 15 (105-180)	92 ± 9.2 (83-120)	158 ± 11 (134-181)	115 ± 5 (104-123)
MBD	228 ± 19 (201-268)	214 ± 21 (173-241)	239 ± 25 (204-273)	239 ± 36 (175-294)	242 ± 19 (206-275)	175 ± 33 (150-200)	134 ± 21 (116-170)	238 ± 13 (210-259)	132 ± 9 (107-145)
T	150 ± 14 (128-182)	169 ± 13 (143-189)	149 ± 16 (123-176)	168 ± 22 (116-209)	152 ± 18 (122-192)	138 ± 19 (122-178)	85 ± 13.4 (72-100)	135 ± 19 (99-176)	92 ± 11 (72-110)
ABD	59 ± 5 (52-65)	59 ± 5 (47-68)	61 ± 7 (53-73)	68 ± 8 (55-83)	63 ± 6 (53-76)	55 ± 8 (40-75)	50 ± 6.9 (41-67)	62 ± 7 (53-78)	44 ± 9 (38-51)
V%	42 ± 2 (36-46)	42 ± 1 (39-45)	41 ± 2 (37-44)	42 ± 2 (38-46)	43 ± 1 (40-46)	55 ± 3 (50-58)	49 ± 5.3 (40-64)	45 ± 1 (42-48)	47 ± 3 (45-50)

*: Stock, Rivera-Orduño and Flores-Lara, 2009, Carboca isolate; **: Sham-seldean *et al.*, 1996; *** Poinar, Karunakar & David, 1992

Table 4 : Comparative table of morphometrics of females of the isolates Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 from Benin and their relative species (in µm, mean ± standard error and range in parenthesis).

Tableau comparatif des mesures morphométriques des femelles des isolats Kpedekpo, Akohoun, Setto1, Ze1 and Ze3 et leurs espèces associées (en µm, moyenne ± erreur standard et les variations entre parenthèses)

Characters	Kpedekpo	Akohoun	Setto1	Ze1	Ze3	*H. sonorensis**	*H. taysearae***	Ayogbe1	*H. indica****
n	20	20	20	20	20	20	30	20	12
L	2002 ± 198 (1699-2299)	1968 ± 200 (1689-2524)	2380 ± 226 (2055-2666)	2386 ± 151 (2125-2804)	1889 ± 186 (1626-2236)	1950 ± 300 (1500-2500)	1200 ± 16 (830-1400)	1679 ± 169 (1498-2121)	1600 ± 12 (1200-1800)
ES	142 ± 5 (133-151)	145 ± 6 (131-154)	152 ± 6 (144-161)	161 ± 7 (151-178)	151 ± 5 (144-161)	131 ± 10 (129-215)	151 ± 13 (129-179)	128 ± 8 (117-144)	131 ± 4 (120-139)
SEP	144 ± 13 (126-165)	164 ± 20 (115-186)	146 ± 14 (128-162)	178 ± 10 (159-201)	134 ± 15 (107-159)	115 ± 15 (95-140)	137 ± 14 (120-166)	133 ± 15 (106-164)	127 ± 4 (118-138)
NR	101 ± 4 (91-109)	110 ± 4 (100-117)	116 ± 6 (108-127)	113 ± 6 (103-126)	107 ± 4 (101-117)	95 ± 6 (85-105)	82 ± 6.8 (76-109)	94 ± 6 (86-110)	92 ± 4 (88-96)
MBD	123 ± 15 (104-150)	88 ± 12 (73-120)	130 ± 10 (114-141)	147 ± 14 (128-179)	112 ± 15 (107-159)	129 ± 11 (85-210)	69 ± 15 (42-96)	113 ± 23 (83-161)	95 ± 15 (76-113)
T	91 ± 7 (80-110)	112 ± 11 (93-139)	98 ± 6 (92-105)	110 ± 7 (100-121)	102 ± 7 (91-116)	86 ± 5 (75-99)	71 ± 7.4 (62-80)	84 ± 7 (68-95)	76 ± 9 (66-88)
ABD	34 ± 4 (28-42)	29 ± 2 (26-33)	38 ± 5 (30-45)	33 ± 2 (30-36)	33 ± 4 (28-42)	39 ± 3 (36-46)	23 ± 2.5 (19-28)	31 ± 4 (27-39)	26 ± 11 (22-32)
V%	47 ± 2 (43-51)	46 ± 2 (42-49)	47 ± 3 (42-50)	48 ± 4 (44-62)	49 ± 2 (45-53)	51 ± 2 (49-53)	57 ± 8.3 (44-73)	47 ± 2 (41-51)	48 ± 9 (40-53)

*: Stock, Rivera-Orduño & Flores-Lara, 2009, Carboca isolate ; **: Sham-seldean *et al.,* 1996 ; *** Poinar, Karunakar & David, 1992.

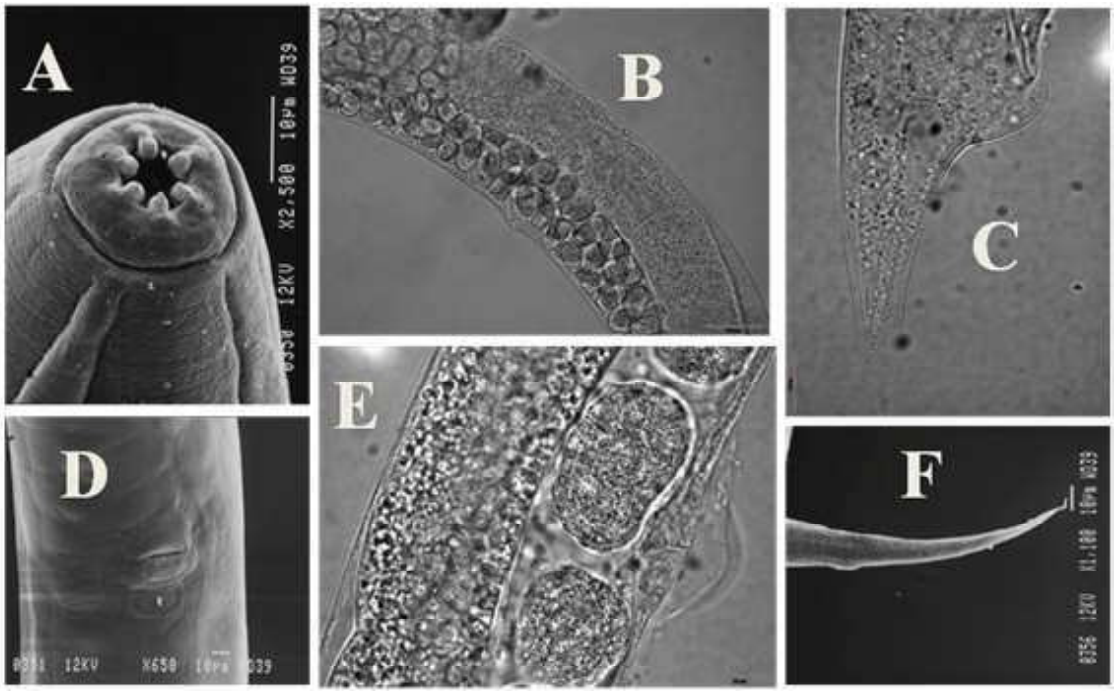

Figure 6 : Light (B,C,E) and scanning electron microscopy (A,D,F) photographs of hermaphrodite (A,B,C,D) and female (E,F) specimen of H. indica (Ayogbe1).

Spécimens femelle et hermaphrodite de H. indica (Ayogbe1).

A : Head region, B : Vulva region, lateral view. C : Tail in ventral view, post-anal swelling. D : Slit-like vulva opening. E : Vulva. F : Tail. Scale bars : A,D,F = 10 µm, C,E = 20 µm, B = 100 µm.

A : Tête, B : Vulve, vue latérale. C : Queue en vue latérale avec enflure post-anale. D : Ouverture de la vulve, en forme de fente. E: Vulve. F: Queue. Barres d'échelle : A,D,F = 10 µm, C,E = 20 µm, B = 100 µm.

DISCUSSION

For the identification of EPN, the combination of morphological, morphometric, molecular observations with cross hybridization tests is very important for identification beyond doubt (Nguyen, 2007). Unfortunately our investigations were hampered by the non-availability of *H. sonorensis* strains for the hybridization tests whereas the lack of type specimens did not allow a proper morphological comparison.

Both the analysis of the ITS sequences and the BLAST analysis showed that all six isolates from Benin belong to the *H. indica* clade (*sensu* Stock *et al.,* 2008). Five isolates (Kpedekpo, Akohoun, Setto1, Ze1 and Ze3) out of the six strongly resembled in ITS sequence composition with *H. sonorensis* and *H. taysearae* (99 - 100 %) while isolate Ayogbe1 shared 99 % similarity with *H. indica.*

The alignment of the ITS region showed 100 % similarity of the aforementioned five isolates.

Compared to the MP tree, the NJ tree seemed more informative by differentiating *H. taysearae* as a sister-group of the weakly supported grouping of the five Beninese isolates along with *H. sonorensis*. This result corresponds with the morphological and morphometric observations which also distinguished these isolates clearly from *H. taysearae*. The phylogenetic analyses do not provide resolution on the relationships within the *H. sonorensis* clade.

For the morphological support of the five isolates conspecific with *H. sonorensis*, we took into account all features considered in the literature. However, there is no general agreement on the diagnostic value of some of these features. Stock and Kaya (1996) considered body length and tail length of IJ as well as body length and length of testis reflexion of male, as important differentiating features. However, according to Phan *et al.* (2003) former characters do not contribute much to the discrimination of *Heterorhabditis* spp. These latter authors proposed the use of the spicule length,

gubernaculum length and SW% as discriminating features for the species of the genus. Finally, Nguyen (2007) considered body length, position of SE-pore, tail length and the ratios D% and E% as more informative for IJ whereas he suggested using spicule length and D% for the male. Based on the features used for the identification of our isolates, we entirely agree with Nguyen (2007) who suggested to use as much IJ features as possible ; the more information that can be obtained, the more reliable will be the species characterization.

Most of the morphometric differences observed between the Beninese isolates and both isolates used for the originally description of *H. sonorensis* (Stock *et al.,* 2009) fall within the range of intraspecific variation. The observation of copulatory plug in the hermaphrodites of the type population could indicate that they have a preference for male sperm for copulation as was shown for *H. bacteriophora* (Strauch *et al.,* 1994). The absence of mating plugs suggests that females do not mate repeatedly.

Based on the combination of the morphology, morphometrics and molecular data we conclude the five Beninese isolates to be conspecific with *H. sonorensis*. This is the first record of this species outside Mexico and the United States. A wide transcontinental geographic range is not uncommon within the genus *Heterorhabditis*, where speciation is known to be rather low. A broad host range and comparable tropical climate conditions in the Sonoran Dessert and in Benin might explain this wide dispersal.

The morphological characters of all developmental stages of the isolate Ayogbe1 fitted very well with the original description of *H. indica.* The crossbreeding test between *H. indica* and isolate Ayogbe1 yielded fertile progeny. As a consequence we concluded this isolate to be conspecific with *H. indica*, which is the first record of the species from Africa.

CONCLUSION

In the best of our knowledge, this study is the second study on EPN in West Africa after the work of Akyazi *et al.* in 2012. The present work represents the first characterization survey of indigenous EPNs species in Benin. It demonstrates the presence of EPN in Benin. This is an interesting starting point to explore the use of local EPN species for biological control of insects, more in particular of termites as foreseen in the project ESCiP-Benin.

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