Entomopathogenic nematodes and their symbiotic bacteria to control fruit flies (*Bactrocera dorsalis*) in mango cultivation in Benin

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Anique GODJO

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List of abbreviations

A

ASECNA	Agency for aerial navigation safety in Africa and Madagascar
ANOVA	analysis of variance
API	analytical profile index

B

BLAST	basic local alignment search tool
BOF	Bijzonder Onderzoeksfonds

C

CFU	colony forming unit
CORAF/WECARD	West and Central African Council for Agricultural Research and Development

D

DNA	deoxyribonucleic acid
ddH2O	double distilled water
DJ	dauer juvenile

E

ESCIP-Benin	ecological sustainable citrus production in Benin (project)
EPN(s)	entomopathogenic nematode(s)

G

Gm	*Galleria mellonella*

I

IJ(s)	infective juvenile(s)
IPM	integrated pest management
ITS	internal transcribed spacer
ILVO	Instituut voor Landbouw- en Visserijonderzoek

J

J3	third stage juvenile

L

LaPAPP	laboratoire de phytotechnie, d'amélioration et de protection des plantes
L3	third instar larvae
LD50	letal dose 50
LM-UGent	laboratory of microbiology UGent

M

MALDI-TOF MS	matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry
MAT	male annihilation technique
MLSA	multilocus sequence analysis
MEGA	molecular evolutionary genetics analysis
$Mgcl_2$	magnesium chloride

N

NBTA	nutrient bromothymol agar
NCBI	national center for biotechnology information
NaOH	sodium hydroxide
NaCL	sodium chloride

P

PCR	polymerase chain reaction
PRIA	plan régional d'investissement agricole
PNIA	plans nationaux d'investissement agricole
PVC	polyvinyl chloride
PhD	doctor of philosophy
PFA	Paraformaldehyde

R

RH	relative humidity
rRNA	ribosomal ribonucleic acid
RMANOVA	repeated measures analysis of variance

S

SEM	standard error of the mean
SEM	scanning electron microscopy

SIT	sterile insect technique
SNK	Student-Newman Keul
T	
TSA	tryptone soya agar
TSB	tryptone soya broth
U	
UGent	Ghent University
UP	University of Parakou
UV	ultra violet

English summary

Mango is an economically important tropical fruit produced in West-Africa as well as in other regions of the world that share a similar tropical or sub-tropical climate. The production of mango is affected by several problems, including most importantly the attack of fruit flies of the family of Tephritidae. Among them, *Bactrocera dorsalis* represents the most invasive and damaging species which greatly affects the production qualitatively and quantitatively. In Benin, more than 89% of fruit fly populations captured from 2004 to 2010 on cucurbits farms and mango orchards, were *B. dorsalis*. The production loss induced by these flies can easily exceed 75% in Benin as was reported in 2006. The control of *B. dorsalis* is really difficult especially in case of severe orchard infestations. To efficiently reduce the damage caused by *B. dorsalis*, a sustainable management using Integrated Pest Management (IPM) methods is recommended and many approaches are under exploration at national, regional and international levels. In this study, the biocontrol potential of Beninese entomopathogenic nematodes (EPNs) towards *B. dorsalis* in mango orchards was investigated.

Fourteen selected mango orchards in North Benin, a region where mango is mostly produced in the country, were surveyed for natural occurrence of EPNs (Chapter 2). Out of 70 soil samples taken in total in the surveyed orchards, just 2 EPN isolates (KorobororouC2 and KorobororouF4) were retrieved from soil. The molecular, morphological and morphometric observations and cross-hybridization tests supported the classification of those two isolates as *Heterorhabditis taysearae*. These nematode isolates together with 10 available native strains (previously isolated from diverse vegetation in the country), were investigated for their pathogenicity against *B. dorsalis* third instar larvae and 1 to 3 day-old pupae under different laboratory conditions. Results indicated that *B. dorsalis* larvae were highly susceptible to EPNs that caused 7.03 to 96.09% mortality. At a dose of 32 IJs/ larva, *H. taysearae* strains Azohoue2 and Hessa1 induced the greatest insect mortality (96.09% and 94.53%, respectively). The tested *Steinernema* isolates induced lower insect mortality even at high IJ concentration. *Steinernema* sp. Thui caused less than 90% insect mortality at 300 IJs/ larva. In addition, at higher moisture content of the substrate (25% and 30%), larvae were less susceptible to EPNs. EPNs were able to kill the insects at pupal stage (up to three day-old), however, pupae were less susceptible to EPNs than the larvae.

Furthermore, EPNs were able to reproduce inside *B. dorsalis* third instar larvae or pupae and the *Heterorhabditis* isolates gave the greatest multiplication rate (59577.20 ± 14307.41 IJs in total from a single larva or pupa).

Next to the identification of the two EPN isolates newly isolated from mango orchards, the taxonomic study of Beninese *Steinernema* strains was conducted (Chapter 3). Two nematode strains, Bembereke157C and Thui168d, where previously retrieved from soil in North Benin and where included in the pathogenicity tests against *B. dorsalis*. Morphological, morphometric, molecular and cross-hybridization studies indicated that they belong to a new species which clustered within the *S. bicornutum* species group. They appeared to be most close to *S. abassi* based on molecular data (97.5% ITS nucleotide similarity). However, they failed to inter-breed with the latter and some other related species (*S. riobrave*, *S. yirgalemens*) within the same group. In addition, they display several morphological differences compared to *S. abbasi* such as the number and position of genital papillae of the first generation males (12 pairs and one single precloacal papilla) and the length of their spicules (average 67 µm). Therefore, we proposed their delineation into a new species, *Steinernema* n. sp., within the *bicornutum* clade.

In Chapter 4, we isolated forty-three bacterial isolates associated with the Beninese entomopathogenic nematodes and their biological diversity was investigated molecularly by analysing the 16S rRNA, recA and gyrB genes. Fifteen bacterial isolates were found to belong to the genus *Xenorhabdus*, 27 isolates to *Photorhabdus* and one to *Serratia*. The *Xenorhabdus* isolates were identified as *Xenorhabdus indica* based on their 16S rRNA gene and concatenated recA and gyrB sequence analyses. However, based on these three genes, the molecular study of the *Photorhabdus* isolates resulted in two separate sub-clusters (A) and (B) within the *Photorhabdus luminescens* group, both distinct from existing subspecies. They share low sequence similarities with nearest phylogenetic neighbors *Photorhabdus luminescens* subsp. *luminescens* Hb^T, *Photorhabdus luminescens* subsp. *caribbeanensis* HG29^T and *Photorhabdus luminescens* subsp. *noenieputensis* AM7^T. Further analyses (Chapter 5) based on more housekeeping genes (dnaN, gltX and infB genes) and phenotypic studies using the Biolog GN plates and the API 20NE, 20 E and 50CH systems confirmed that isolates in sub-cluster (A) represent a new subspecies of *P. luminescens*, sharing at most 96.8%, 96.2% and 95.6% nucleotide similarity with *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp.

noenieputensis and *P. luminescens* subsp. *luminescens*, respectively. Therefore, they were classified as *P. luminescens* susbp. *beninensis* subsp. nov.

As a first investigation of the potential of Beninese EPNs to control *B. dorsalis* under field conditions, three EPN isolates (two *H. taysearae* and one *Steinernema* sp.) were studied for their persistence in mango orchards and their virulence under semi-field conditions (Chapter 6). The results showed that all three EPN isolates were pathogenic to *B. dorsalis* in semi field assays, with *H. taysearae* Hessa1 being the most virulent (70.84% ± 10.46 mortality). These results confirmed our previous observations in the laboratory tests. Furthermore, insect mortality was higher when EPNs were applied 3 days before insect inoculation than when they were applied at the same moment as insect introduction in the experimental pots. This suggests that nematode establishment in soil is important in the virulence process against the insect pest. All three EPN isolates persisted in soil up to 30 weeks post nematode application in the mango orchard. However, the density of IJs, four weeks upon nematode application, decreased considerably and was lower than expected. We therefore suggest more nematode applications (2 to 3) to enhance their persistence and establishment in the soil during the mango season when abiotic conditions may sometimes be harsh. In addition, the application time should also be considered with care to ensure EPN efficiency in mango orchards as our results confirmed the presence of third instars larvae from the early fructification stage (March) of mango trees.

In conclusion, this study provides for the first time a platform for developing a sustainable management strategy of *B. dorsalis* using EPNs. The identity of the EPNs as well as their associated bacteria has been provided. In addition, the laboratory bioassays clearly demonstrated the potential of Beninese EPNs to control *B. dorsalis* population, and their virulence against the latter has been confirmed under semi-field conditions. However, further investigations including more mango cultivars under diverse field conditions are still required before the proposition of EPNs as new alternative method to control *B. dorsalis* in mango plantations and other host crops.

Nederlandstalige samenvatting

Mango is een economisch belangrijke tropische vrucht die gekweekt wordt in West Afrika en in andere delen van de wereld met een gelijkaardig tropisch of subtropisch klimaat. De teelt heeft te lijden onder verschillende problemen, waarvan een van de meeste ernstige de schade is veroorzaakt door fruitvliegen van de familie Tephritidae. Hiervan is *Bactrocera dorsalis* de meest invasieve en schadelijke soort die zowel kwalitatief als kwantitatief de productie beperkt. Meer dan 89% van de fruitvliegen die van 2004 tot 2010 in Benin werden aangetroffen in mango boomgaarden en op boerderijen waar men komkommerachtigen kweekt, waren *B. dorsalis*. In 2006 werd berekend dat het productieverlies veroorzaakt door deze fruitvliegen in Benin vaak oploopt tot meer dan 75%. Bestrijding van *B. dorsalis* is erg moeilijk, vooral bij zware invasies van boomgaarden. Om effectief de schade door *B. dorsalis* te beperken, is duurzaam beheer met Geïntegreerd Pest Management (Integrated Pest Management, IPM) aangewezen en gebeuren talrijke studies op nationaal, regionaal en internationaal niveau. In deze doctoraatsstudie werd het potentieel onderzocht van biocontrole van *B. dorsalis* in mango boomgaarden in Benin met behulp van lokale entomopathogene nematoden (EPNs).

In veertien boomgaarden in Noord Benin, de belangrijkste regio voor mango productie in dit land, werd het natuurlijk voorkomen van EPNs in kaart gebracht (Hoofdstuk 2). Van 70 bodemstalen die werden genomen, werden slechts 2 EPN isolaten bekomen (KoroborororouC2 en KoroborororouF4). Moleculaire, morfologische en morfometrische data en kruishybridisatietesten lieten toe deze te identificeren als *Heterorhabditis taysearae*. Deze twee isolaten werden samen met 10 andere isolaten die eerder waren bekomen uit diverse vegetatie in Benin, onderzocht voor pathogeniciteit tegen *B. dorsalis* derde instar larven en een tot drie dagen oude poppen en dit onder verschillende laboratorium condities. De resultaten toonden aan dat *B. dorsalis* larven zeer gevoelig zijn aan de EPN, die mortaliteit veroorzaakten van 7,03 tot 96,09 %. Bij een dosis van 32 infectieve juvenielen (IJs) per larve veroorzaakten de *H. taysearae* stammen Azohoue2 en Hessa1 de hoogste mortaliteit (96,09% en 94,53%, respectievelijk). De geteste *Steinernema* isolaten veroorzaakten, zelfs bij hogere dosis, een lagere mortaliteit. *Steinernema* sp. Thui gaf minder dan 90% insect mortaliteit bij 300 IJs/ larve. Verder werd gezien dat bij hoger vochtgehalte van het substraat (25 en 30%), larven minder gevoelig werden aan EPNs. EPNs

konden insecten doden in het pop stadium (tot 3 dagen oud), maar poppen waren wel minder gevoelig dan larven. Verder konden de EPNs zich vermenigvuldigen in *B. dorsalis* derde instar larven of poppen en de *Heterorhabditis* isolaten vertoonden de hoogste vermenigvuldigingsgraad (59577.2 ± 14307.41 IJs uit één larve of pop).

Naast de identificatie van de twee nieuwe EPN isolaten uit mango boomgaarden, werd een taxonomische studie van *Steinernema* stammen uit Benin uitgevoerd (Hoofdstuk 3). Twee nematode stammen, Bembereke157C en Thui168d, vroeger bekomen uit grond in Noord Benin, werden ingesloten bij de pathogeniciteitstesten tegen *B. dorsalis*. Moleculaire, morfologische en morfometrische data en kruishybridisatietesten toonden aan dat ze behoren tot een nieuwe soort die behoort tot de *S. bicornutum* soortengroep. Op basis van moleculaire gegevens (97.5% ITS nucleotide similariteit) zijn ze nauwst verwant met *S. abassi*, hoewel ze niet konden gekruist worden met deze soort, noch met andere verwante soorten (*S. riobrave*, *S. yirgalemense*) uit diezelfde soortengroep. Verder vertoonden ze verschillende morfologische verschillen met *S. abassi*, zoals het aantal en de locatie van de genitale papillae van de eerste generatie mannetjes (12 paren en 1 precloacale papilla) en de lengte van hun spicula (gem. 67 μm). Daarom werden ze als een nieuwe soort, *Steinernema* n. sp. voorgesteld in de *bicornutum* tak.

In Hoofdstuk 4 werden 43 bacteriële isolaten bekomen geassocieerd met Beninese entomopathogene nematoden en hun diversiteit werd onderzocht door studie van de 16S rRNA, recA en gyrB genen. Vijftien bacteriële stammen behoorden tot het genus *Xenorhabdus*, 27 tot *Photorhabdus* en een tot *Serratia*. De *Xenorhabdus* stammen werden op basis van 16S rRNA genen en de concatenatie van gyrB en recA genen, geïdentificeerd als *Xenorhabdus indica*. Gelijkaardige analyse resulteerde voor de *Photorhabdus* echter twee subclusters (A) en (B) binnen de *Photorhabdus luminescens* groep en verschillend van bestaande subspecies binnen die soort. Ze vertonen lage sequentiesimilariteiten met de dichtste fylogenetische buren *Photorhabdus luminescens* subsp. *luminescens* Hb^T, *Photorhabdus luminescens* subsp. *caribbeanensis* HG29^T en *Photorhabdus luminescens* subsp. *noenieputensis* AM7^T. Verder onderzoek (Hoofdstuk 5) met meer huishoudgenen (dnaN, gltX en infB) en fenotypische testen met Biolog GN platen en API 20NE, 20E en 50CH systemen, bevestigden dat subcluster (A) een nieuw subspecies van *P. luminescens* vertegenwoordigt, met ten hoogste 96,8%, 96,2% en 95,6% nucleotide similariteit met *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp.

noenieputensis en *P. luminescens* subsp. *luminescens*, respectievelijk. Daarom werd deze groep voorgesteld als *P. luminescens* susbp. *beninensis* subsp. nov.

Als een verkennend onderzoek naar het potentieel van Beninese EPNs voor de biocontrole van *B dorsalis* in het veld, werden voor drie EPN isolaten (twee *H. taysearae* en een *Steinernema* sp.) de persistentie in mango boomgaarden en virulentie onder semi-veld omstandigheden onderzocht (Hoofdstuk 6). De drie EPN isolaten bleken pathogeen voor *B. dorsalis* in semi-veld condities met *H. taysearae* als meest virulente (mortaliteit 70.84 ± 10.46%). Deze resultaten bevestigden onze eerdere waarnemingen in de laboratorium tests. De insectmortaliteit was bovendien hoger wanneer EPNs aangebracht werden 3 dagen voor de insecten werden geïntroduceerd, dan wanneer beiden gelijktijdig werden aangebracht in de potexperimenten. Dit toont aan dat de vestiging van de nematoden in de grond belangrijk is voor het virulentieproces tegen de pestinsecten. De drie geteste EPNs bleven aanwezig in de grond tot 30 weken na hun aanbrengen in de nematode mango boomgaard. De densiteit van de IJs daalde echter in belangrijke mate, vier weken na het aanbrengen en was lager dan verwacht. Daarom is het aangeraden om meer nematode applicaties (2 tot 3) uit te voeren om hun vestiging en persistentie in de grond te verbeteren gedurende het mangoseizoen wanneer omgevingscondities soms hard kunnen zijn. Verder moet het moment van toediening goed gekozen worden om de effectiviteit van de EPNs in de boomgaard te verzekeren, aangezien onze resultaten de aanwezigheid aantonen van derde instar larven van de vroege vruchtzetting van de mangobomen in maart.

Tot besluit kunnen we stellen dat dit onderzoek voor de eerste maal een basis aanreikt voor de ontwikkeling in Benin van een duurzame beheersingsstrategie voor *B. dorsalis* met behulp van EPNs. De identiteit van de EPNs en van hun geassocieerde bacteriën is nu gekend. Verder toonden de biologische laboratoriumtesten duidelijk het potentieel van de Beninese EPNs voor de beheersing van *B. dorsalis* populaties en hun virulentie tegen deze pest werd bevestigd in semi-veld condities. Verder onderzoek van meer mango cultivars onder diverse veldcondities is echter nodig voordat EPNs als een volwaardige alternatieve biocontrole methode voor *B. dorsalis* kunnen worden voorgesteld in mango plantages en voor andere gewassen.

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1 Chapter 1:

General introduction

1.1 Mango production and constraints

Mango (*Mangifera indica* L., Anacardiaceae) is a voluminous and rustic tropical tree which originates from South Asia (Kostermans 2012). In Africa, mango was reported since the 14th century in East Africa and the beginning of the 19th century in the West of the continent. Mango is important for sub-Saharan African populations as it is mainly cultivated for its juicy fruits, but also serves as protection in some cases when it is considered as a shade tree. Mango can be produced in almost any well-drained soil with pH between 5.5 and 7.5. Mango trees need a deep soil to accommodate their extensive root systems for good growth. In Benin, the production of mango is constantly growing (15067 tons of mango, mangosteens and guava produced in 2014 over 3186 ha, http://www.fao.org/faostat) and it is ranked sixth fruit exported worldwide (Vayssieres et al. 2008). In Northern Benin where more than 75% of the national production is grown, mango has a dual role as fruit and also as subsistence crop (Vayssieres et al. 2008). The fruits are equally of high importance in similar agro-ecological zones in the neighboring countries where they are grown. The mango ripening period falls in late dry season (October-April) and early wet season (May- September), and the fruits constitute a source of essential nutrients such as vitamin C, potassium, alpha-carotene and calcium for rural populations living in relatively poor areas in West Africa. Over one hundred mango cultivars exist worldwide. In Benin, an inventory of all present cultivars was previously made and twenty nine cultivars such as Gouverneur, Eldon, Zill, Dabshar, Kent, Smith, Keitt, Brooks and Ifac3 were reported to be locally produced (Vayssieres et al. 2008). However, mango production income is generally compromised due to many constraints mainly represented by pest problems and poor access to markets (Van Melle et al. 2008). Pest problems particularly involve fruit fly (Diptera, Tephritidae) infestations. Among numerous tephritid insect pests that attack mango fruits in Benin, *Ceratitis cosyra* (Walker), *Ceratitis silvestrii* (Bezzi), *Ceratitis quinaria* (Bezzi), and *Bactrocera invadens* (Drew Tsuruta and White) were reported to be of economic importance (Vayssieres et al. 2008). These quarantine insects can cause more than 75% loss of the national production. More importantly, *B. invadens* was later recognized as a junior synonym of *Bactrocera dorsalis* (Hendel) by Schutze et al. (2015) and is considered as the most invasive and economically important pest causing serious mango yield losses in Benin and other tropical

environments where they are grown (Goergen et al. 2011; Rousse and Quilici 2009; Vayssières et al. 2009b).

1.2 Generalities on *Bactrocera dorsalis* Hendel

1.2.1 Biology

The *Bactrocera dorsalis* complex is a group of about 75 species (Diptera, Tephriditae, Dacinae) of fruit flies which are known for their damage on a wide range of fruits and vegetables (Clarke et al. 2005). Among this group of tephritid insect pests, *B. dorsalis,* also known as oriental fruit fly, represents the most widely distributed species and the most destructive pest species to fruit crops (Clarke et al. 2005; Drew 1989). This insect pest originated from Asia and later was found in many countries with tropical or sub-tropical climate around the world. It can infest a large range of fruits and vegetable crops with the most common being: citrus, guava, mango, papaya and avocado. *B. dorsalis* was recently introduced in Africa and was first reported in Kenya as *Bactrocera invadens* in 2003 (Goergen et al. 2011; Lux et al. 2003). The adult oriental fruit fly is clearly larger than a house fly and is about 8 mm long, with clear wing membranes of 7.3 mm long. They display variable body color but generally bright yellow with dark markings on the abdomen sometimes forming a "T" shape (Figure 1.1). Generally, the abdomen has two horizontal black stripes and a longitudinal median stripe extending from the base of the third segment to the apex of the abdomen. Based on the similar molecular and some morphological features of *B. invadens* and *B. dorsalis*, the two species have recently been synonymized and *B. dorsalis* is considered as a senior synonym of *B. invadens* (Schutze et al. 2015).

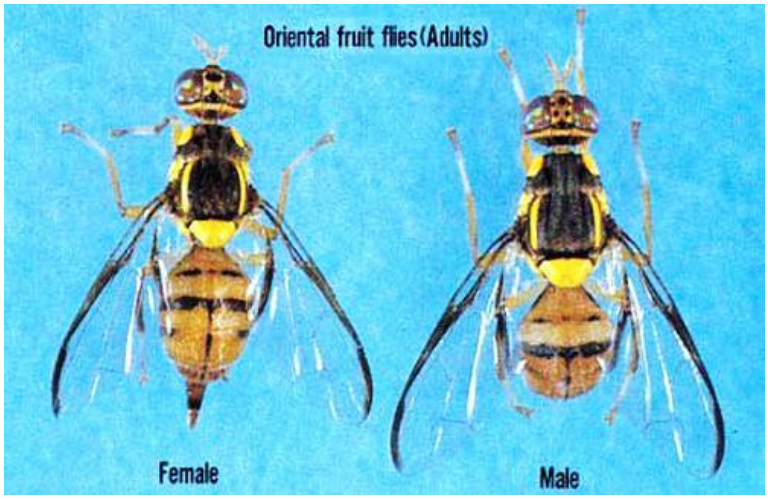

Figure 1.1 Adults of *Bactrocera dorsalis* (Weems et al. 2012)

1.2.2 *Bactrocera dorsalis* Life cycle

As most tephritid insect pests, *B. dorsalis* is a multivoltine species (Aluja and Mangan 2008; Zwolfer) with an average of 3 to 8 generations per year (Saeki et al. 1980). Females of *B. dorsalis* use their pointed slender ovipositor to lay eggs in batches under the skin of ripened host fruits (Figure 1.2, Figure 1.3), but also in young fruits which have fallen on the ground after abscission. These eggs hatch within a day (or may be delayed up to 20 days in cool conditions) and then develop into larvae (Figure 1.4) which can feed on the fruit for 6 to 35 days depending on the environmental conditions. These larvae are creamy-white, legless and undergo 3 different larval developmental stages. The last larval stage (L3) of the insect may reach about 10 mm in length inside the host fruit. The infested fruits rot quickly and become improper for human consumption and easily fall on the ground. Afterwards, the matured L3 larvae exit the infested fruit and jump on the ground in which they develop into pupae after a short dispersal period in the top 4 cm of the soil (Hou et al. 2006). The puparial stage can last in soil for 8-10 days at 25°C and 80% RH, or up to 90 days under cool conditions. Adults of *B. dorsalis* start mating at 8-12 days old and live for 2-3 months before they die. They can occur the whole year depending on the environmental conditions.

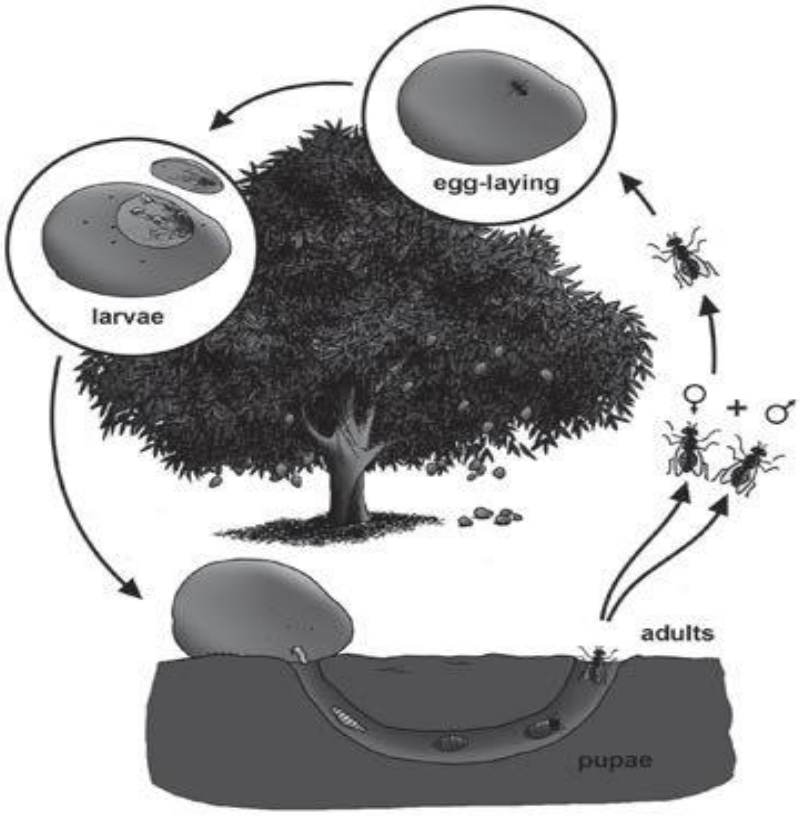

Figure 1.2 The *B. dorsalis* life cycle. Figure from the CTA Practical Guide Series, No. 14 http://en.calameo.com/read/003440269541b1c70088b

1.2.3 *Bactrocera dorsalis* damage

B. dorsalis causes huge economic losses to fruit crops in different ways. First of all, the quality of fruits is affected due to direct fruit attack generally seen as punctures by females through oviposition and subsequent larval development. In addition, part of the production is lost because young fruits are also susceptible to *B. dorsalis* puncture leading to their drop before ripening. Furthermore, fruit flies are classified as quarantine insects, therefore many international markets are lost for export of fruits and growers struggle to sell the consumable part of their harvest as soon as possible on local markets.

Punctures made by females on the attacked fruit present zones of discoloration which evolve later into spots of rot (Figure 1.3). The attack often results in the early ripening of the fruit and on the

most sensitive host, such as mango, the damage of *B. dorsalis* can lead to a complete destruction of the infested orchard. In Benin, it has earlier been reported that *B. dorsalis* and other tephritid species can cause up to 17% harvest losses at the beginning of the growing season (early April), over 50% at the mid-season and during the ripening period (mid-June), losses can exceed 70% (Vayssieres et al. 2008).

Figure 1.3 Tephritid - infested mango fruit

Figure 1.4 Mango infested with tephritid larvae

1.2.4 *Bactrocera dorsalis* management

B. dorsalis requires appropriate control methods for orchard protection because it represents such a huge threat to fruit and vegetable production, especially the mango sector. In case of severe fruit flies infestation, no method has so far been proven to be genuinely effective and beneficial for population management. To control *B. dorsalis* population, methods used include preventive measures, chemical treatments, genetic (Sterile Insect Technique) and biological control.

1.2.4.1 Preventive measures

Prevention of *B. dorsalis* infestation is crucial to preserve uninfested or less infested environments from pest introduction. In infested orchards, interrupting the fly's developmental cycle is important to maintain the population as low as possible in the orchard. Therefore, prophylactic measures are employed for orchard sanitation such as frequent weeding of the entire orchard, removing and destroying all fallen fruits, burying infested fruits deeply in a pit to prevent new generations of insects to fly away, tilling the top soil (5-10cm deep) in the orchard to

expose the pupae to the sun, avoiding growth of alternative host crops in the same orchard or in nearby orchards, sorting the harvested fruits and eliminating as quickly as possible any fruit with traces of fly puncture before their transportation to the local, regional or international markets. In addition, quarantine restrictions suggest post-harvest treatments such as the use of fumigants (Mille 2010) or hot water (46.5-51°C) treatment (Self et al. 2012) of mango fruits to eliminate eggs and larvae present in marketable fruits before their introduction in a non-infested area. Some of these prophylactic measures can only be applied by small-scale mango growers as their implementation on large scale orchards will require a lot of manual work and is therefore challenging.

1.2.4.2 Chemical treatments

1.2.4.2.1 Conventional pesticides treatment

Chemical applications have been used as traditional methods for many years to control fruit flies. In Benin, in a search of rapid solutions, desperate mango growers can sometimes turn to application of pesticides intended for treatment of cotton, *Gossypium hirsutum* L., (Sinzogan et al. 2008). Since egg and larval development occur inside the fruits, chemical treatment generally target the invasive stage of the insect, mainly constituted by male and female adults. Traditionally, organochlorine, carbamate, pyrethroid and organophosphate insecticides were used by fruit growers (Vayssieres et al. 2009a; Vontas et al. 2011). These compounds are usually combined with attractants (foodstuff) and applied as liquid bait spray or in bait stations in orchards (Ekesi 2016). In that case, insects are attracted by the foodstuff and killed by the insecticide. However, some pesticides are applied alone with conventional sprayer under the canopy of each tree (avoiding fruits) throughout the whole field. For instance, excellent results were reported when Proteus 170 O-TEQ (Thiaclopride + Deltamethrine) was applied in traps (N'depo et al. 2015; Vayssieres et al. 2009a) or even in a full orchard treatment in Côte d'Ivoire (N'depo et al. 2015). In the latter case, to prevent residue on harvested fruits, treatments were urged to occur 15 days before harvest, limiting their use to the growth phase of the fruit.

1.2.4.2.2 Male Annihilation Technique

The Male Annihilation Technique (MAT) is another example of bait stations which uses attractants for males in combination with an insecticide. This technique is based on trapping the males in a fly population using male lures together with an approved killing agent. It consists of installing, at the beginning of the season (at least two months before fruits mature), a device supplied with the male attractant and a contact insecticide (generally Malathion or Deltamethrin). The idea is to lower the number of successful matings in a fruit fly population by trapping as many male individuals as possible. Therefore, the population of flies will surely decrease if the concept is well implemented because mating will reduce due to the low number of males. The effectiveness of the technique depends on the density of bait stations inside the orchard and the type of lure used. It is recommended to install 10 male lure traps per hectare for effective results. Generally, cue-lure, methyl eugenol, trimedlure, and terpinyl-acetate are examples of attractants used to trap males (Ekesi 2016). In Africa, significant reduction of pupae and fly populations of *B. dorsalis* was reported in Benin (Hanna et al. 2008) and Kenya (Ndlela et al. 2016) when methyl eugenol was used in combination with Malathion and Deltamethrin, respectively.

Apart from their negative impact on public health, pesticides also affect the ecological sustainability and the resilience of the farming system. Furthermore, these chemical products, when applied as full orchard treatment, harm the majority of natural fruit fly enemies present in the orchards which are used to restrict pest development.

1.2.4.3 Bio pesticides

In the search of sustainable solutions for fly management, the use of bacteria based bio-pesticides has also been explored as alternative method for pest control. Spinosad is a neurotoxin compound derived from the bacterium *Saccharopolyspora spinosa* Mertz and Yao which operates as a contact and stomach poison to insects (Vayssieres et al. 2009a). Spinosad GF-120 (Success Appat) was formulated with Spinosad + foodstuff attractant, and is very well known around the world for its efficacy against tephritid flies (Adán et al. 1996; Pelz et al. 2005; Thomas and Mangan 2005). In West Africa and Benin particularly, the effectiveness of the Spinosad GF-120 on Tephritids flies was also demonstrated (Ekesi et al. 2011; N'depo et al. 2015; Vayssieres et al. 2009a).

1.2.4.4 Sterile Insect Technique

The Sterile Insect Technique (SIT) is a genetic method used to suppress or reduce the next generation population of an insect pest. The principle of the technique is to rear sterile males in great number using gamma radiation, and to release them in the target area in such a way they can compete with the wild males and mate with the wild females resulting in infertile eggs. This will allow the decline of the fly population as unfertile eggs will yield no progeny. The SIT was recently successfully used in the southwestern islands of Japan to eradicate the melon fruit fly, *Bactrocera cucurbitae* (McQuate and Teruya 2015). The drawback of the technique is that sterile males are not self-replicating so they cannot persist in the environment. Therefore a repeated release of sterile males is needed until the complete eradication of the targeted pest. The technique is very expensive and hardly affordable by local mango growers especially in developing countries where tephritid flies cause a lot of damage.

1.2.4.5 Biological control

Generally, every organism has natural enemies that diminish or suppress their population under certain conditions. Biological control is a component of an integrated pest management strategy which is defined as the reduction of pest populations by natural enemies and typically involves an active human role (https://biocontrol.entomology.cornell.edu/what.php). Regarding tephritid flies, their negative impact on fruit crops and the side-effects of the application of chemicals on the environment have motivated many researchers around the world to investigate the use of existing biological control agents such as predators, parasitoids, pathogens or competitors to control fruit flies. Several organisms have been studied and proposed to be used in classical, augmentative and conservative biological control methods. Indeed, the classical biological control strategy involves importation of new organisms into a new environment to control pests while the augmentative option aims the breeding of locally present organisms and their release into the same environment to achieve pest control. Contrary to the classical and augmentative strategies, the conservative method targets the use of environmental management practices to protect the existing beneficial fauna.

1.2.4.5.1 Parasitoids

A parasitoid is an insect that completes its larval developmental stages on/ or in an insect host and ultimately causes its death. Generally, females of parasitoids lay eggs within the body of the insect host, these eggs develop into larvae which feed on the internal vital organs of the host and eventually cause its death (Waage and Greathead 1992). Fruit flies of Tephritidae family have been linked to more than 200 parasitoids (belonging to the order Hymenoptera) which showed great potential to be used for their control (Rousse and Quilici 2009). Among them, species in the subfamily Opiinae (Braconidae) are mostly used in classical biological control programs of tephritids (Billah et al. 2008; Gnanvossou et al. 2016; Gomina 2015). Species of the genera *Fopius* Wharton, *Diachasmimorpha* Viereck, *Psyttalia* Walker, *Tetrastichus* Haliday and *Coptera* Say, currently gained substantial research attention around the world (Argov and Gazit 2008; Mohamed et al. 2006; Rousse and Quilici 2009) in controlling fruit flies. *Fopius arisanus* (Sonan) constitutes an example of an opiine parasitoid which has been tested in many environments where tephritid flies have a heavy economic impact. This species was initially reported in Asia as egg and first instars larvae parasitoid of fruit flies (Altuzar et al. 2004). Later, its potential in classical biological control was proven in many countries such as Hawaii where it was applied against the melon fruit fly (Harris et al. 2010), French Polynesia (Vargas et al. 2007), Kenya (Mohamed et al. 2010) and Benin (Gnanvossou et al. 2016) where the population of *B. dorsalis* was targeted. In Benin, *F. arizanus* annual (average) percentage parasitism ranged from 0.01 to 21.04% which corresponded to the reduction of 33 to 65% of *B. dorsalis* (Gnanvossou et al. 2016). Besides *B. dorsalis, F. arisanus* was also used to control other tephritids such as *Bactrocera tryoni, Bactrocera kirki, Ceratitis capitata, Ceratitis cosyra, Ceratitis anonae, Ceratitis rosa,* and *Ceratitis fasciventris* with promising results (Argov and Gazit 2008; Mohamed et al. 2010; Vargas et al. 2007). However, in Benin, the impact of *F. arizanus* on beneficial insects has not yet been investigated, nor their cost-effectiveness in biocontrol of fruit fly to decide whether they are good candidates to combat *B. dorsalis* in the country and the neigboring regions.

1.2.4.5.2 Predators

Predators are organisms that live by preying on other organisms. Considering tephritid insect pests, Arthropods such as ants (Hymenoptera) have been reported to be the most common predators of larvae and pupae of tephritids (Aluja et al. 2005; Urbaneja et al. 2006). Recently, *Oecophylla smaragdina has been* investigated for the control of fruit flies in Australia (Peng and Christian 2006). In Benin, the potential of weaver ants (*Oecophylla longinoda*) for biological control of fruit flies has been explored (Van Mele et al. 2007). These predators have been argued to represent good candidates for conservation biological control of fruit flies in Africa and Asia where they are of negative economic impact. Moreover, it has been demonstrated that *O. longinoda* secretes substances that affect oviposition behavior of *C. cosyra* and *B. dorsalis* on mango fruits (Van Mele et al. 2009). However, while these ants may be effective as generalist predators that continuously patrol the trees for prey, they are also perceived as a considerable nuisance during the harvest which is done through manual picking and they are thought to be associated with small black spots on the fruit (Sinzogan et al. 2008).

1.2.4.5.3 Entomopathogenic bacteria and fungi

The use of bacteria and fungi in the control of insect pests has been investigated in recent years. On one hand, *Bacillus thuringiensis* has been reported to be pathogenic to *C. capitata* and *B. olea* tephritids larvae and adult flies under laboratory and field conditions even though the bacteria did not significantly reduce adult fly populations as expected (Floris et al. 2007). This could represent a limit to their use in fly pest management as some bacterial strains may lack specificity and / or toxicity against a particular damaging pest (Rousse and Quilici 2009).

On the other hand, researchers invested in the use of entomopathogenic fungi to control fly pests. Among the known fungi used in biological control of pests, *Metarhizium anisopliae (*Metsch) and *Beauveria bassiana* (Balsamo) were most investigated. Significant insecticidal impact of these fungi was recorded on *C. capitata* (Castillo et al. 2000; Ekesi et al. 2002, 2003; Quesada-Moraga et al. 2006), *C. rosa* and *C. fasciventris* (Ekesi et al. 2002, 2003) and *C. cosyra* (Dimbi et al. 2004; Ekesi et al. 2003). The combination of *M. anisopliae* with GF-120 resulted in a remarkable decrease of *B. dorsalis* infestation in mango orchards in Kenya (Ekesi et al. 2011). These fungi were applied under the canopy of mango trees and buried into the soil to target larvae, pupae and

adults of the insect host. Developing spores of the fungus were able to penetrate the insect via their cuticle and eventually kill them. However, unfavorable environmental conditions (temperature and humidity) may affect the effectiveness of these living organisms against fruit flies which consequently limits their use in pest management program (Gomina 2015).

1.2.4.5.4 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) have efficiently been used as potential biocontrol agents against various crop pests (Ehlers 2001). Considering tephritids, significant mortality was reported upon *Steinernema* or *Heterorhabditis* species application on larvae and or pupae developmental stages of *Ceratitis capitata* (mediterranean fly) (Gazit et al. 2000; Kepenekci and Susurluk 2006; Lindegren et al. 1990; Lindegren and Vail 1986; Malan and Manrakhan 2009; Poinar and Hislop 1981), *Bactrocera tryoni* (Froggatt) (Langford et al. 2014), *Rhagoletis cerasi L*. (cherry fruit fly) (Herz et al. 2006; Kepenekci and Susurluk 2006; Köppler et al. 2005), *Rhagoletis indifference* (Yee and Lacey 2003); *Anastrepha serpentina* (Mexican fruit fly) (Toledo et al. 2006a; Toledo et al. 2006b); *Anastrepha ludens* (Toledo et al. 2005; Toledo et al. 2006a); *Dacus ciliatus* (Melon fruit fly) (Hussein et al. 2006); *Bactrocera oleae* (Rossi) (Sirjani et al. 2009); *Bactrocera dorsalis* (Hendel) (Lin et al. 2004; Lindegren and Vail 1986) and *Ceratitis rosa* (Malan and Manrakhan 2009). Most of these studies were performed in the laboratory and few experiments have been extended to field conditions. Indeed, the high susceptibility of *Rhagoletis cerasi* larvae to *S. feltiae* and *S. carpocapsae* (Herz et al. 2006; Yee and Lacey 2003) and *S. intermedium* (Yee and Lacey 2003) were previously demonstrated under semi-field and field conditions. In addition, investigation of *H. bacteriophora* virulence against *A. ludens* larvae in mango orchards resulted in high fly mortality even though a high nematode concentration (250 IJs/cm^2) was required (Toledo et al. 2005; Toledo et al. 2006a). Moreover, larvae and pupae of *C. capitata* have successively been eliminated by EPNs under semi-field conditions in Brazil (Minas et al. 2016).

1.3 Overview of entomopathogenic nematodes

1.3.1 Taxonomy of Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are a group of nematodes (roundworms) of the Phylum Nematoda that use their bacterial symbionts to cause the death of the insect host. Because the nematode was previousely not known to kill the insect itself, Nguyen and Smart (2004) proposed the name "Entomophilic nematodes" (entomo= insect, phile= friend) instead of "Entomopathogenic nematode" (entomo= insect, patho= desease, genic=producing) which is generally used up to now. EPNs were first described in 1923 by Steiner (1923) and *Steinernema glaseri* Steiner was the first species to be used in biological control of the Japanese beetle *Popillia japonica* Newman (Glaser et al. 1935). After many laboratory investigations and field trials, the use of EPNs has later on gained the attention of researchers for biological control of several insect pests. EPNs are distributed within three different genera of the Rhabditida order, *Heterorhabditis, Steinernema* and *Neosteinernema*. Heterorhabtids belong to the family Heterorhabditidae Poinar, 1976 (Rhabditomorpha, Strongyloidea) while Steinernematids and Neosteinernematids are members of the family Steinernematidae Filipjev, 1934 (Panagrolaimorpha, Strongyloidoidea) (Figure 1.5).

Within the *Heterorhabditis* genus, 16 valid species have currently been recognized (Hunt and Subbotin 2016). The *Steinernema* genus comprises more than 96 valid species and *Neosteinernema* is a monotypic genus (Nguyen and Smart Jr 1994) with the type species lacking molecular information and its phylogenetic position therefore remains unresolved (Hunt and Subbotin 2016). *Heterorhabditis* and *Steinernema* species are associated with bacteria (*Enterobacteriaceae*) of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Ciche et al. 2006) which are used by the nematodes to kill their host.

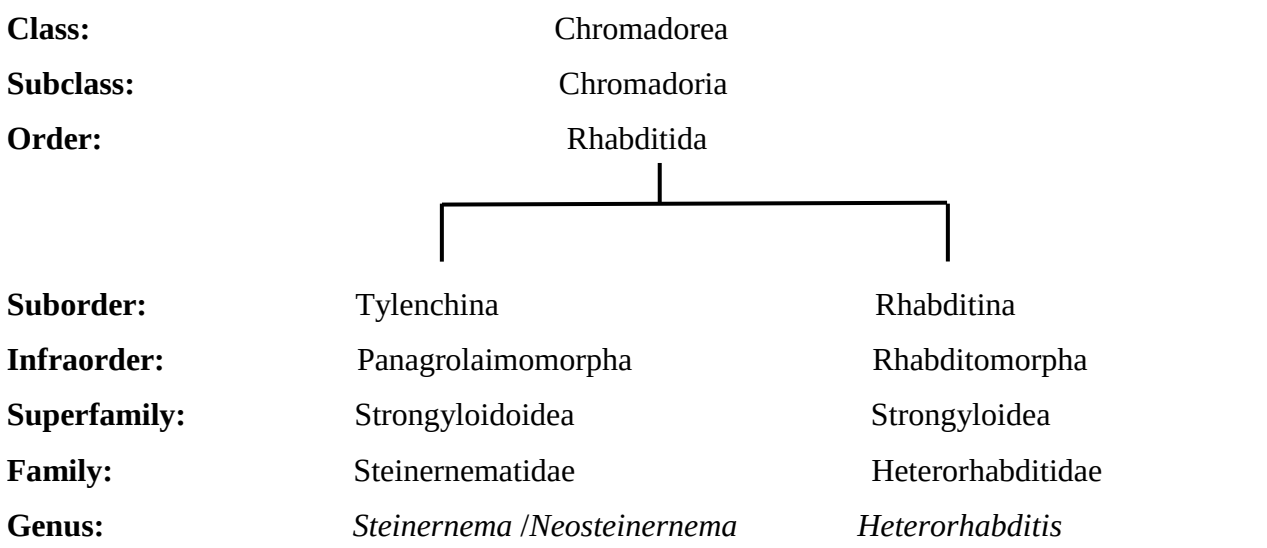

Figure 1.5 Taxonomic positions of EPNs according to the classification of De Ley and Blaxter (2002)

1.3.2 Life cycle of entomopathogenic nematodes

The life cycle of these nematodes (Figure 1.6) is composed of two main stages including a free-living stage in soil and a parasitic stage inside the insect host. During the free stage, the infective juvenile (IJ) also named Dauer Juvenile (DJ) that carries the symbiotic bacteria in its intestine, searches for a host to infect. The IJ represents a specialized developmental stage which does not feed and is adapted to survival in the unfavorable soil environment. The parasitic stage begins when the IJ penetrates the hemocoel of the new host, recovers from non-feeding stage and resumes development due to food signal (Emelianoff et al. 2007; Strauch and Ehlers 1998). IJs of both genera *Steinernema* and *Heterorhabditis* can enter the host body via natural opening such as anus, mouth or spiracles. In addition to these ways of penetration, *Heterorhabditis* IJs are equipped with an additional dorsal tooth that they can use to perforate the inter-segmental membrane of the cuticle (Bedding and Molyneux 1982; Griffin et al. 2005) and enter the hemocoel of the host. *Heterorhabditis* IJs, once inside the insect host, molt into a feeding third stage juvenile (J3). They release the bacterial symbionts which multiply quickly within the hemocoel of the host. The J3 individuals feed on the symbiotic bacteria and molt subsequently into J4 and self-fertilizing (automictic) hermaphrodites (Figure 1.7) with a female phenotype

(Adams and Nguyen 2002). The offspring coming from the hermaphrodites develop either into males or females, passing successively through J1, J2, J3 and J4 stages, or into IJs depending on the environmental conditions (Johnigk and Ehlers 1999; Strauch et al. 1994). Indeed, the development of EPNs is highly affected by food availability mainly constituted by bacterial symbionts inside the insect host (Ehlers 2001). When their food sources are depleted, the newly hatched offspring develop into IJs which exit the insect cadaver for new search of insect host to parasitize. *Steinernema* nematodes have a similar life cycle with the difference that sex differentiation occurs after recovery of the IJs inside the host. Therefore, in most case (exception with *S. hermaphroditum*), only amphimictic (cross-fertilizing) adults are produced after development of IJs subsequently into J3, J4 and males or females at the first generation. Reproduction continues by cross-fertilization inside the parasitized host. Both *Steinernema* and *Heterorhabditis* nematodes can develop by *endotokia matricida* when there is environmental or food stress (Ehlers 2001; Johnigk and Ehlers 1999). *Endotokia matricida* (intra-uterine birth, leading to maternal death) is a phenomenon that was first observed by Maupas (1899) with rhabditid nematodes and occurs when juveniles hatch and develop inside the mother's uterus by consuming her inner vital organs before they exit the cadaver (Johnigk and Ehlers 1999). EPNs are believed to complete 2 to 3 generations inside the insect host before they stop development at IJ stage when food resources are depleted. IJs then ingest and acquire bacterial cells before leaving the cadaver to seek new hosts (Adams and Nguyen 2002).

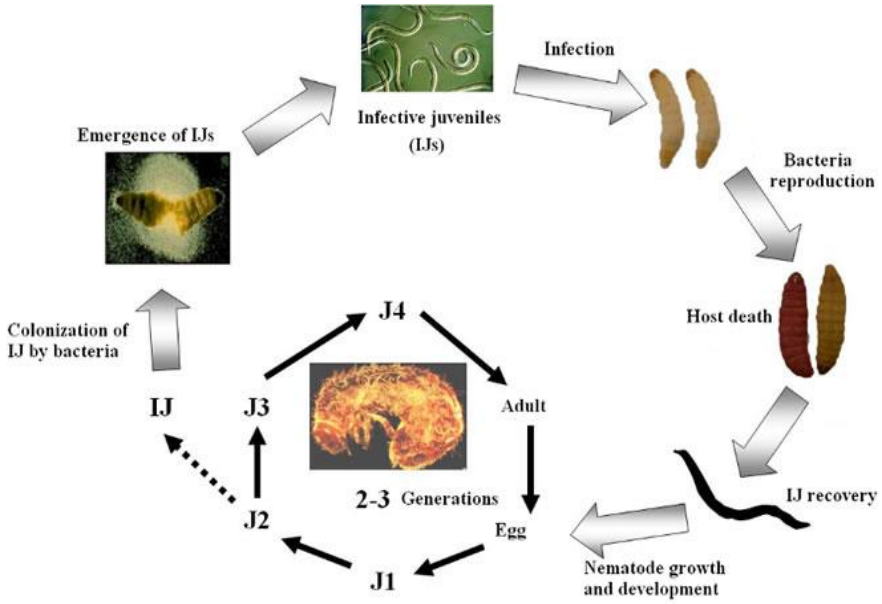

Figure 1.6 Entomopathogenic nematodes life cycle. (Modified figure based on Ffrench-Constant, 2003) (From Website: http://www.giabr.gd.cn/kxcb/kpdt/201405/t20140516_234014.html)

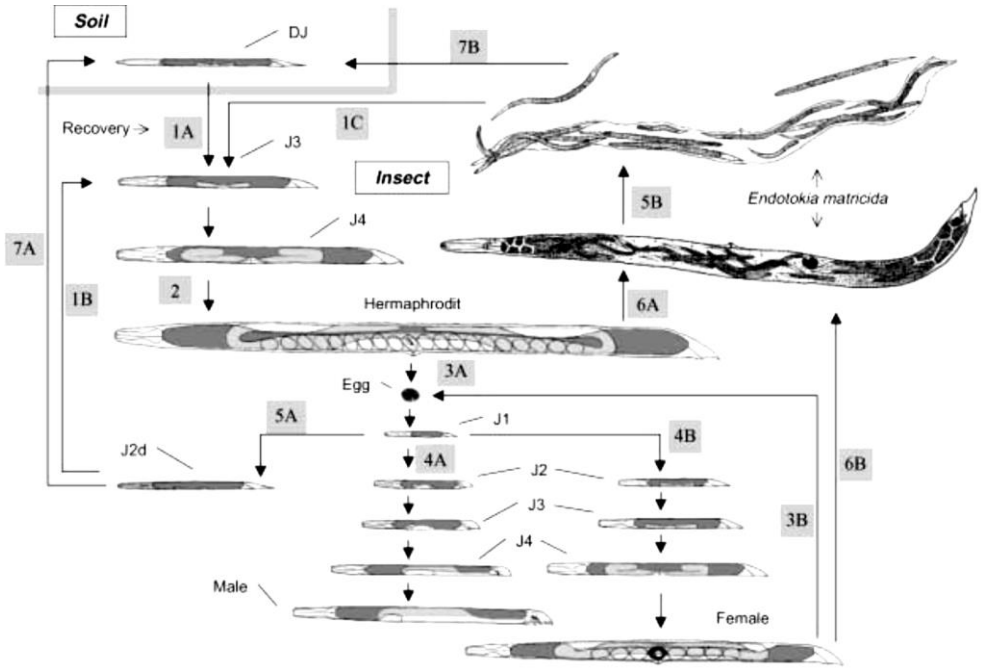

Figure 1.7 Detailed life cycle of a *Heterorhabditis* sp., with alternative developmental pathways. *Numbers* indicate the critical developmental steps during the process.

Figure 1.7 (legend continued) *1* Recovery of dauer juvenile (*DJ*) from free-living stage (*1A*), pre-dauer stage (*J2d*) originating from laid eggs (*1B*) or from *endotokia matricida* (*1C*). *2* Development of hermaphrodite. *3* Egg laying by automictic hermaphrodite (*3A*) or amphimictic female (*3B*). *4* Development to amphimictic male (*4A*) and female (*4B*). *5* DJ formation of J1 originating from eggs laid (*5A*) or from *endotokia matricida* (*5B*). *6 Endotokia matricida* of hermaphrodite (*6A*) or amphimictic female (*6B*). *7* DJ emigration of DJ originating from eggs (*7A*) or from *endotokia matricida* (*7B*). Figure by Ehlers (2001).

1.3.3 Entomopathogenic nematodes characterization and identification

Nowadays, diverse identification approaches mainly based on molecular information, morphometrics, morphology and biology data, are used to determine the appropriate taxonomical position of known or unknown nematode specimens. Considering *Steinernema* and *Heterorhabditis* nematodes, morphological and morphometrical features of males, females and IJs are traditionally used for specimen identification. Afterwards, molecular analysis has been introduced. For steinernematids, morphometrics and morphological data provide additional support for species differentiation as they rarely affect results inferred from molecular identification (Spiridonov et al. 2004). However, for *Heterorhabditis* nematodes, molecular results may contradict morphology and morphometric identification at species level. Therefore, in addition to morphological and morphometric data, molecular characterization is necessary for final confirmation of the nematode identity (Hunt and Subbotin 2016). Molecular phylogenies of *Heterorhabditis* and *Steinernema* nematodes have been reconstructed based on the analysis of partial sequence of diverse genes such as the 28S rRNA gene, 18S rRNA, Internal Transcribed Spacer (ITS) region, *nd*4 gene, *cox*I mtDNA gene and the 12S rRNA gene (Hunt and Subbotin 2016). In recent taxonomic studies of these nematodes, molecular analyses are commonly focused on the ITS region and the D2-D3 region of the 28S rRNA gene as they have successfully been used to resolve the phylogeny of a number of species unlike the 18S rRNA gene (Liu et al. 1997) which was found to be too conservative and therefore not useful for differentiating species within the two genera.

1.3.4 Symbiotic relation between entomopathogenic nematodes and their bacteria

As stated above, *Photorhabdus* and *Xenorhabdus* are symbiotically associated with *Heterorhabditis* and *Steinernema* nematodes respectively (Boemare 2002b; Forst and Clarke 2002). In this mutualistic relationship developed by EPNs and their symbionts, three phases of bacterial occurrence are involved. First of all, in the **phoretic phase,** the bacterial symbionts are well protected inside the intestine or in the anterior intestinal diverticulum of the free-living IJs during the search for an insect host. The second phase constitutes the **pathogenic phase** where the IJs penetrate the host, release the bacteria which in turn overcome the immune system of the insect host, proliferate and establish. The last stage is the **saprophytic phase** where the bacteria ultimately kill the insect host and constitute a food source for the nematodes favoring their development and reproduction (Ciche et al. 2006).

This relationship is beneficial for both bacteria and nematodes as the nematodes provide protection (by retaining the bacteria inside their gut) and transportation for the bacterial symbionts. The bacteria in turn help the nematodes by killing the insect hosts, and in addition by constituting their main food source, essential for development and reproduction (Boemare 2002b; Forst et al. 1997; Forst and Nealson 1996). However, it has earlier been demonstrated that the mortality of *Steinernema carpocapsae* IJs increases with higher number of the bacterial symbionts and high bacterial multiplication rates (Emelianoff et al. 2007). When the IJs find a suitable host, they penetrate the host hemocoel and release their intestinal symbionts that multiply quickly and the insect dies of septicemia within 24-48 hours. The penetration of IJs into the insect host does not go unnoticed by the latter, which uses its innate immune system to restrain the dissemination of the nematode and its bacterial symbiont within its body (Cooper and Eleftherianos 2016; Razia et al. 2011). Both humoral and cellular immune responses are used by the insect host (Jiang et al. 2010) to promote 1) the secretion of a range of antimicrobial peptides into the hemolymph and 2) and the proliferation of hemocytes within the insect host that initiates the encapsulation and phagocytosis mechanisms (Cooper and Eleftherianos 2016). However, in most cases, EPNs modulate the insect immune system to survive and release their bacterial symbiont by producing some protease compounds such as trypsin-like serine protease into the host (Balasubramanian et al. 2010) upon invasion. In addition, when the bacterial symbionts reach the stationary phase of their multiplication within insect host, they also produce several

extracellular products including lipases, phospholipases, proteases and different antibiotics in the host hemolymph. These enzymes break down the macromolecules of the host cadaver; the antibiotics suppress proliferation of other microorganisms within the host and provide suitable conditions for nematode development and reproduction (Couche and Gregson 1987; Sicard et al. 2003). After several nematode generations (2-3), food resources are depleted and nematode development stops at IJ stage. IJs then ingest and acquire bacterial cells before exiting the insect cadaver for new host search (Adams and Nguyen 2002). In case of *endotokia matricida* for *Heterorhabditis* nematodes, symbionts are maternally transmitted (Figure 1.8) to IJ offspring in seven different steps: i) following IJ development into female, bacterial symbionts adhere to the maternal posterior intestine, ii) they grow within the intestinal lumen, iii) they invade the rectal gland cells, iv) they are released into the maternal body cavity upon the lysis of rectal gland cells, v) they adhere to the pharyngeal intestinal valve cells, vi) they invade the pharyngeal intestinal valve cells, and vii) they colonize the intestinal lumen of IJs which are exclusively developed inside the maternal body cavity (Ciche et al. 2008; Somvanshi et al. 2010).

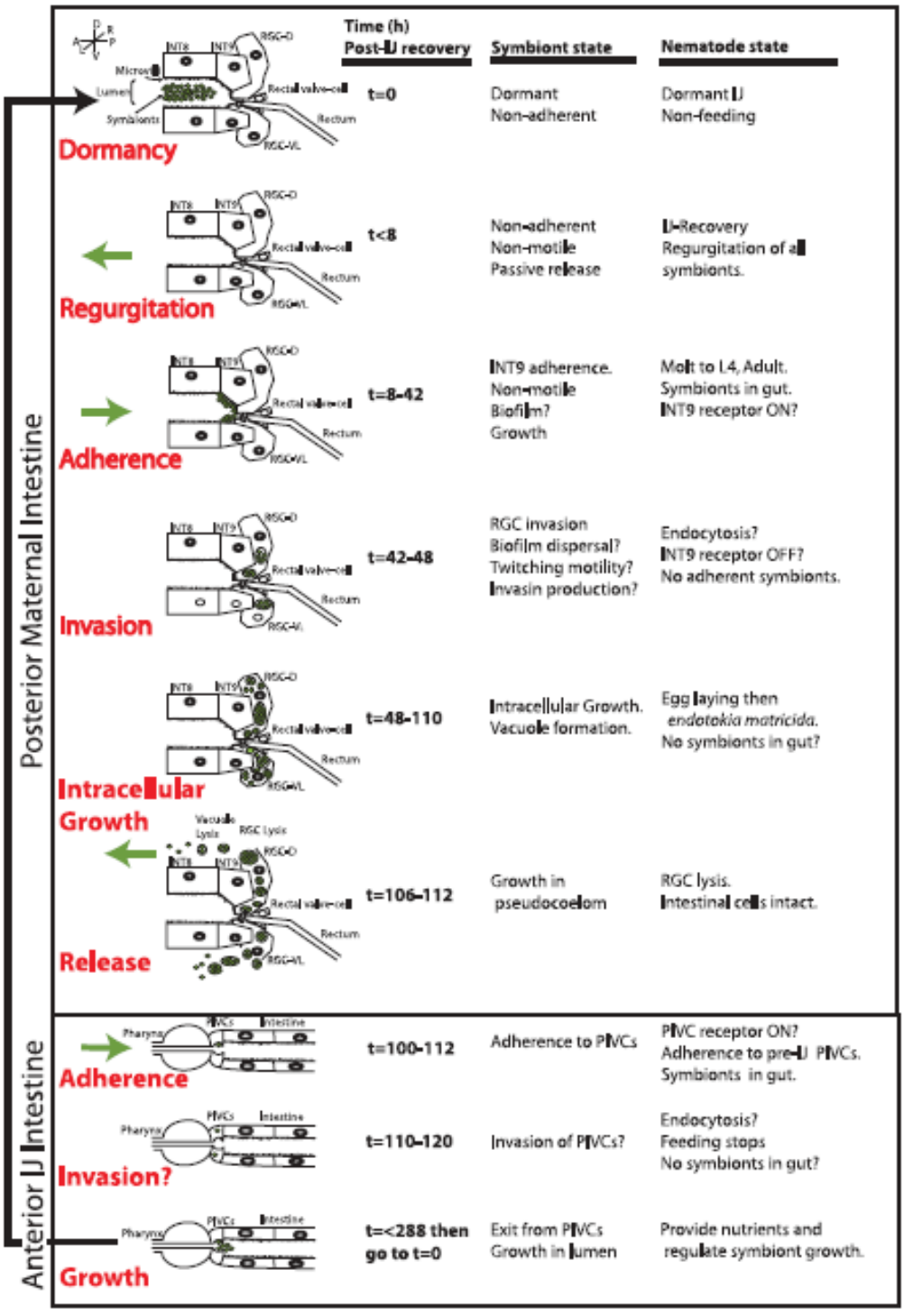

Figure 1.8 Model of the bacterial transmission cycle. Symbionts that have colonized the maternal intestine (top panel) or pre-IJs or IJs (bottom panel) are shown in the context of select nematode cells in the same orientation on the upper left, where the anterior (A) is on the left.

Figure 1.8 (legend continued) the posterior (P) is on the right, dorsal (D) is up, ventral (V) is down, left (L) is out, and right (R) is into the page. Nematode cells are abbreviated as follows: INT, intestinal cells; RGC-D, dorsal RGCs; RGC-VL, ventral-left RGCs; and PIVCs, pharyngeal intestinal valve cells. Colonized symbionts are indicated by green ovals, and the green arrows indicate regurgitation or ingestion of symbiont cells. The time (t) is the time (in hours) after IJ addition to lawns of symbiont bacteria (zero time). Dormant symbionts are not adherent to the IJ intestinal lumen. At 8 h intestinal symbionts are completely released during IJ recovery and regurgitation. At 8 to 42 h symbionts adhere to and grow within the maternal posterior intestine, corresponding to INT9L and INT9R cells. At 42 to 48 h adherent symbionts invade the RGCs and no longer adhere to the INT9 cells. At 48 to 110 h symbionts grow intracellularly in the RGCs and stimulate vacuole formation. At 106 to 112 h symbionts are released from RGCs after lysis and gain access to the pre-IJs developing in the maternal pseudocoelom. At 100 to 112 h symbionts adhere to the PIVCs of pre-IJs (L2) developing within the maternal pseudocoelom. At 110 to 120 h symbionts exit the pre-IJ intestinal lumen, possibly invade PIVCs, and multiply. At 120 to 288 h symbionts exit the PIVCs and colonize the IJ intestinal lumen. Note that vegetative progeny acquire symbionts 5 to 12 h after they hatch from laid eggs also by symbiont adherence to the INT9 cells but otherwise exhibit similar symbiont transmission. Figure by Ciche et al. (2008).

1.3.5 Nematode mass rearing, formulation and application

EPNs are commercially used to control insect pests around the world. *Steinernema glaseri* (formerly known as *Neoaplectana glaseri*) was the first nematode to be mass-produced and used for biological control of an insect pest under field conditions (Hunt and Subbotin 2016). Mass reproduction of EPNs includes two main approaches: *in vivo* and *in vitro* methods. The *in vivo* method is based on the white trap (White 1927) technique which consists of inoculating an insect host with a particular nematode species and harvesting the IJ progeny that would emerge out of the cadaver through natural migration when food is depleted inside the host. The device consists of a dish or tray on which the dead insects are deposited on top of a filter paper surrounded by water, which is contained by a larger dish (Shapiro-Ilan et al. 2012a). Emerged IJs are then

collected in water upon emergence. This method of EPN multiplication is appropriate for small scale field experiments or laboratory use even though the scale of the white trap can equally be increased for commercial purpose (Shapiro-Ilan et al. 2012a). The host size, inoculation method, nematode species and the environmental conditions can greatly influence the amount of harvested IJs. Indeed, depending on the host susceptibility and its immune response, nematode yield is proportional to host size (Flanders et al. 1996) and inversely proportional to nematode size (Shapiro-Ilan and Gaugler 2002). The greater wax moth, *Galleria mellonella* (Lepidopera, Piralidae), is an insect found throughout the world and commonly used in EPN mass rearing and also serves as their major trapping system from soil (Bedding and Akhurst 1975). These insects have a lot of advantages to be used in laboratory experiements (Mukherjee et al. 2013) including their easy and cheap rearing in laboratory conditions . *G. mellonella* is also used for isolating the nematode endosymbionts (explained in more detail under 1.4.3).

Considering *in vitro* mass production of EPNs, the production system is based on reproducing nematodes using a monoxenic bacterial culture containing exclusively the bacterial symbiont of the appropriate nematode to be cultured without any contaminant. Two ways of *in vitro* nematode mass reproduction exist: the *in vitro* solid culture and the *in vitro* liquid culture methods. In the first case, sterile foams are traditionally added to the culture system and emerged IJs are harvested by placing the foams on top of a sieve immersed with water (Bedding 1981, 1984). This approach was later improved by Gaugler and Han (2002) for large scale production. For *in vitro* liquid culture method, nematode reproduction is performed in a bioreactor containing a nutritive medium supplemented with the monoxenic bacterial culture adjusted to appropriate environmental conditions (Ehlers 2001). This method of nematode rearing was argued to represent a potential opportunity of EPN mass multiplication at low cost for pest control. Detailed description about EPN liquid culture is provided by Ehlers (2001). For storage purpose, IJs can be kept in liquid nitrogen for many years without great impact on their survival, pathogenicity and reproduction after storage (Popiel and Vasquez 1991).

Upon successful multiplication, formulation and application methods are determinant for EPN efficacy in biological control of insect pests (Shapiro-Ilan et al. 2012a). EPNs are commonly formulated and applied as aqueous suspension at large-scale by the use of conventional spraying equipment or irrigation system (Georgis 1990; Grewal 2002). In order to protect the applied

EPNs against external factors such as desiccation, UV radiation and natural enemies before they find suitable conditions to parasitize the targeted insect pest, EPNs can also be applied as EPN-killed insects (sometimes referred to as infected cadavers) to be dispersed in the field (Ansari et al. 2009; Shapiro-Ilan et al. 2001). In this case, the infected cadavers can equally be coated with protective material such as kaolin-based formulation to prevent desiccation and rupture of cadavers (Ansari et al. 2009) during storage and application. In addition, an elegant approach is to apply EPNs as biodegradable polymer-based capsules. The efficacy of such encapsulated EPNs have been explored (Cruz-Martinez et al. 2017; Goud et al. 2010) and recently reported to be effective against the major maize pest *Diabrotica vigifera vigifera* under field conditions (Hiltpold et al. 2012) even though EPNs were poorly retained inside the alginate capsules. Thereafter, an improved composition of the capsule has been recently proposed (Kim et al. 2015). To sum up, the success of EPNs in biological control of insect pests relies on their optimal mass production, formulation and application. At large scale, EPN multiplication in liquid culture requires the isolation, identification and multiplication of the bacterial symbiont for monoxenic medium preparation which represents an important component (food) for a successful multiplication. In addition using the right bacterial symbiont for EPN multiplication is important since there is a specific association between the nematode and its symbiont, and the use of non-symbiont bacteria may negatively impact their mass reproduction.

1.4 Symbiotic bacteria of entomopathogenic nematodes

1.4.1 Gut microbiota of entomopathogenic nematodes

It is very unlikely that the gut of EPNs is exclusively colonized by *Xenorhabdus* or *Photorhabdus* bacteria. An initial attempt to examine the microflora of *Steinernema carpocapsae* resulted in the isolation of several bacteria in addition to the known symbiont (*Xenorhabdus nematophila*) such as *Alcaligenes odorans*, *Pseudomonas fluorenscens*, *P. maltophilia*, *P. alcaligenes* and *Acinetobacter* sp. (Lysenko and Weiser 1974). Furthermore, *Ochrobactrum anthropi* and *O. intermedium* have been recovered together with the known symbiont *Photorhabdus luminescens* subsp. *akhurstii* from *Heterorhabditis indica* nematodes by Babic et al. (2000). In additon, *O. anthropi* and *Schineria larvae* were found to be associated with *H. indica* and *O. cytisi* with *Steinernema siamkayai* (Razia et al. 2011). These low diversity of EPN microbiome was later

confirmed by Koneru et al. (2016) who isolated from beetle-associated *S. carpocapsae* nematodes some *Echerichia coli*, *Pseudomonas* spp., *Xantomonas* spp. and *Alcaligenes* spp. Moreover, *Serratia nematodiphila* was found to be associated with *Heterorhabditidoides chongmingensis*, a new nematode species proposed to be part of the EPN group (Zhang et al. 2008). However, less research attention is given to EPN gut microbiota study compared to other free living bacterial feeder nematodes such as *Ceanorhabditis elegans* for which substantial data are available in the literature (Berg et al. 2016; Cabreiro and Gems 2013; Midha et al. 2017). The role of these other bacterial members of EPN gut microbiota is not yet elucidated to date, and these bacteria are sometimes referred to as "contaminants" or "non-symbionts". Most of the studies on EPN microbiology are usually focused on their main bacterial symbionts, *Xenorhabdus* and *Photorhabdus* species, due to their close association with *Steinernema* and *Heterorhabdus* EPNs, respectively, and their high relevance in the biological control of insect pests.

1.4.2 Taxonomy of *Xenorhabdus* and *Photorhabdus*

Xenorhabdus (Thomas and Poinar 1979) and *Photorhabdus* (Boemare et al. 1993) emend. (Fischer-Le Saux et al. 1999) are gram-negative bacteria that produce a range of substances that are toxic to insects. They are members of the Gammaproteobacteria as many other insect and vertebrate symbionts and they belong to the *Enterobacteriaceae* family (Forst et al. 1997). At the time of writing, the *Photorhabdus* genus contains 4 validly recognized species, *Photorhabdus luminescens* (Boemare et al. 1993; Thomas and Poinar 1979), *Photorhabdus temperata* (Fischer-Le Saux et al. 1999), *Photorhabdus heterorhabditis* (Ferreira et al. 2014) which are endosymbionts of *Heterorhabditis* spp.; and *Photorhabdus asymbiotica* (Fischer-Le Saux et al. 1999) which has been isolated from human wounds and also has *Heterorhabditis* as vector (Gerrard et al. 2006). The *Photorhabdus luminescens* species is divided into 11 subspecies, *Photorhabdus asymbiotica* into 2 subspecies, *Photorhabdus temperata* into 6 subspecies and *Photorhabdus heterorhabditis* which is the most recent described species contains no subspecies so far (Table 1.1). The *Xenorhabdus* genus comprises 25 described species (Table 1.1).

Table 1.1 List of described species of *Xenorhabdus* and *Photorhabdus* bacterial symbionts.

Genus	Species	Subspecies	References
Xenorhabdus	*Xenorhabdus bovienii*	-	(Akhurst and Boemare 1988)
	Xenorhabdus beddingii	-	(Akhurst and Boemare 1988)
	Xenorhabdus budapestensis	-	(Lengyel et al. 2005)
	Xenorhabdus cabanillasii	-	(Tailliez et al. 2006)
	Xenorhabdus doucetiae	-	(Tailliez et al. 2006)
	Xenorhabdus eapokensis	-	(Kämpfer et al. 2017)
	Xenorhabdus ehlersii	-	(Lengyel et al. 2005)
	Xenorhabdus griffiniae	-	(Tailliez et al. 2006)
	Xenorhabdus hominickii	-	(Tailliez et al. 2006)
	Xenorhabdus indica	-	(Somvanshi et al. 2006)
	Xenorhabdus innexi	-	(Lengyel et al. 2005)
	Xenorhabdus ishibashii	-	(Kuwata et al. 2013)
	Xenorhabdus japonica	-	(Nishimura et al. 1994)
	Xenorhabdus khoisanae	-	(Ferreira et al. 2013b)
	Xenorhabdus koppenhoeferi	-	(Tailliez et al. 2006)
	Xenorhabdus magdalenensis	-	(Tailliez et al. 2012)
	Xenorhabdus mauleonii	-	(Tailliez et al. 2006)
	Xenorhabdus miraniensis	-	(Tailliez et al. 2006)
	Xenorhabdus nematophila	-	(Poinar JR and Thomas 1965; Thomas and Poinar 1979)
	Xenorhabdus poinarii	-	(Akhurst and Boemare 1988)
	Xenorhabdus romanii	-	(Tailliez et al. 2006)
	Xenorhabdus stockiae	-	(Tailliez et al. 2006)
	Xenorhabdus szentirmaii	-	(Lengyel et al. 2005)
	Xenorhabdus thuongxuanensis	-	(Kämpfer et al. 2017)
	Xenorhabdus vietnamensis	-	(Tailliez et al. 2010)
Photorhabdus	*Photorhabdus heterorhabditis*	-	(Ferreira et al. 2014)
	Photorhabdus luminescens	*Photorhabdus luminescens* subsp. *luminescens*	(Fischer-Le Saux et al. 1999) (Thomas and Poinar 1979)
		Photorhabdus luminescens subsp. *sonorensis* Caborca	(Orozco et al. 2013)
		Photorhabdus luminescens subsp. *caribbeanensis*	(Tailliez et al. 2010)
		Photorhabdus luminescens subsp. *akhurstii*	(Fischer-Le Saux et al. 1999)
		Photorhabdus luminescens subsp. *hainanensis*	(Tailliez et al. 2010)
		Photorhabdus luminescens subsp. *kleinii*	(An and Grewal 2011)
		Photorhabdus luminescens subsp. *kayaii*	(Hazir et al. 2004)
		Photorhabdus luminescens subsp. *noenieputensis*	(Ferreira et al. 2013a)
		Photorhabdus luminescens subsp. *laumondii*	(Fischer-Le Saux et al. 1999)
		Photorhabdus luminescens subsp. *thracensis*	(Hazir et al. 2004)

Genus	Species	Subspecies	References
		Photorhabdus luminescens subsp. *namnaonensis*	(Glaeser et al. 2017)
	Photorhabdus temperata	*Photorhabdus temperata* subsp. *temperata*	(Fischer-Le Saux et al. 1999; Tóth and Lakatos 2008)
		Photorhabdus temperata subsp. *tasmaniensis*	(Tailliez et al. 2010)
		Photorhabdus temperata subsp. *stackebrandtii*	(An and Grewal 2010)
		Photorhabdus temperata subsp. *khanii*	(Tailliez et al. 2010)
		Photorhabdus temperata subsp. *cinerea*	(Tóth and Lakatos 2008)
		Photorhabdus temperata subsp. *thracensis*	(Tailliez et al. 2010)
	Photorhabdus asymbiotica	*Photorhabdus asymbiotica* subsp. *asymbiotica*	(Akhurst et al. 2004)
		Photorhabdus asymbiotica subsp. *australis*	(Akhurst et al. 2004)

1.4.3 *Photorhabdus* and *Xenorhabdus* general features

Bacterial symbionts *Xenorhabdus* and *Photorhabdu*s, carried by IJs of *Steinernema* and *Heterorhabditi*s respectively, are located within the whole intestine of *Heterorhabditis* or in a specialized anterior vesicle of the intestine of *Steinernema* nematodes (Bird and Akhurst 1983; Martens and Goodrich-Blair 2005). Even though Morgan et al. (1997) demonstrated that these bacteria might survive in soil for one week in the absence of nematodes, it is still clear that the nematodes provide hospitality and transport of these bacteria until they both find a suitable host for development and multiplication (Campos-Herrera et al. 2009), and this constitutes the only way of infection in natural conditions.

They are polymorphic and exist in two forms, the primary cells or phase I cells are preferentially carried by the IJs and they produce a range of antibiotics which inhibit the development of other microorganisms and therefore provide better conditions for EPN reproduction. The secondary cells or phase II cells are usually found in laboratory conditions (Boemare 2002b; Forst and Clarke 2002) or occasionally in the hemolymph of infested *G. mellonella* (Akhurst 1980). Phase I cells support better nematode reproduction by producing more progeny and bigger size adults than phase II cells (Akhurst 1980; Smigielski et al. 1994) and they can evolve into phase II cells in laboratory conditions, possibly caused by stress (Akhurst 1980).

Photorhabdus bacteria were initially placed in the genus *Xenorhabdus* and named as *Xenorhabdus luminescens*, but later on, based on the examination of phenotypic characters and molecular studies, this species was transferred to a new genus *Photorhabdus* (Boemare et al. 1993). It is the only known bioluminescent terrestrial bacterium, and lux genes are responsible for the production of light in these bacteria (Baldwin et al. 1989). All *Photorhabdus* strains are considered highly entomopathogenic, with a Lethal Dose 50 (LD50) lower than 100 cells per insect (Boemare 2002b).

The relationship between bacterial symbionts and nematodes are known to be specific, so initially it was assumed that each bacterial species is associated with a single nematode species (Akhurst 1982; Thomas and Poinar 1979). This high level of specificity was also confirmed when horizontal transmission of non-native *Xenorhabdus* sp. to *S. carpocapsae* was prevented and introduced bacteria were rather pathogenic to nematodes (Sicard et al. 2004). However, evidence from recent studies suggests that the specificity of this relationship seems to have expanded in such a way that in some cases the same bacterial species can be found in association with more than one nematode species of the same genus. For instance, *Xenorhabdus bovienii* was isolated from different *Steinernema* species such as *S. kraussei*, *S. feltiae*, *S. intermedium* (Tailliez et al. 2010). Moreover, *Xenorhabdus indica* was first reported to be associated to *Steinernema abbasi* (Somvanshi et al. 2006) and ten years later to *Steinernema yirgalemense* (Ferreira et al. 2016). Within the *Photorhabdus* genus, *Heterorhabditis bacteriophora* has been reported to be associated with several subspecies of *P. luminescens* including *P. luminescens* subsp. *kayai*, *P. luminescens* subsp. *laumondii*, *P. luminescens* subsp. *luminescens* and *P. luminescens* subsp. *carribeanensis* (Kazimierczak et al. 2017; Maneesakorn et al. 2011; Tailliez et al. 2010) and subspecies of *P. temperata* such as *P. temperata* subsp. *cinerea*, *P. temperata* subsp. *khanii* and *P. temperata* subsp. *stackebrandtii* (Kazimierczak et al. 2017; Maneesakorn et al. 2011). However, these new associations are in concordance with the specificity at genus level which assumes the association of *Xenorhabdus* to *Steinernema* and *Photorhabdus* to *Heterorhabditis* (Forst et al. 1997). So far, no *Xenorhabdus* has been reported to be isolated from a *Heterorhabditis* nematode and no *Photorhabdus* has been isolated from a *Steinernema* nematode.

Due to their importance in the infection process of EPNs, an increasing number of studies focus on the diversity and characterization of symbiotic bacteria which require at first point their isolation from the nematode host.

1.4.4 Isolation and DNA extraction

Bacterial symbionts of EPNs are usually isolated using the method described by Akhurst (1980) which involves bacterial extraction from infested *Galleria mellonella* hemolymph. First of all an infected mature *G. mellonella* larva is disinfected externally with 70% ethanol and a drop of its hemolymph is spread on a Nutrient Bromothymol Agar (NBTA) plate. After aerobic incubation at 28°C, bacterial isolates are purified and their monoxenic cultures can be stored at 4°C on shaker (20 rpm) for several months (Ehlers 2001) or in 10-20% glycerol at -80°C for long term storage. Besides this method, bacterial cells can also be isolated directly from IJs (Akhurst 1980; Yi et al. 2007). This method consists of surface sterilizing an amount of IJs with sodium hypochlorite and subsequently rinsing with Ringer's solution (Humason 1962). The sterile IJs are thereafter crushed in Ringer's solution and the homogenized solution is diluted with sterile distilled water and streaked onto a nutritive medium plate. A few days later, incubated plates will exhibit bacterial growth which may be purified for further use. A third method for bacterial isolation is the "hanging drop" method which consists of the use of a sterile drop of insect hemolymph incubated with surface sterilized IJs for 24 - 48h. After incubation, the microorganisms that develop in the hemolymph drop are plated on NBTA medium and incubated for bacterial development (Poinar and Thomas 1966). In most cases, results from the three methods are similar (Bonifassi et al. 1999). Purified bacterial colonies can be used to extract DNA using a simple alkaline lysis method (Baele et al. 2000) or a guanidinium thiocyanate based method (Pitcher et al. 1989).

1.4.5 *Photorhabdus* and *Xenorhabdus* identification and characterization

Traditional differentiation of *Photorhabdus* and *Xenorhabdus* is based on a differential NBTA medium and the catalase test (Thanwisai et al. 2012). NBTA medium is composed of Nutrient Agar, 2,3,5 Triphenyltetrazolium chloride and Bromothymol blue with the latter giving the opportunity to bacterial cells to take up the blue color and be easily recognizable among contaminants on the medium plate (Akhurst 1980). *Xenorhabdus* is morphologically

characterized based on a dark blue or dark red colony color with a convex or umbonate surface and swarming on NBTA plates after 3 to 4 days at room temperature (25°C) and is catalase negative whereas *Photorhabdus* is characterized by a dark green colony color with a convex or umbonate surface on NBTA in the same conditions, and is catalase positive (Thanwisai et al. 2012). Colonies of *Xenorhabdus* on NBTA agar can absorb the bromothymol blue from the medium and in combination with the pigments produced by the bacteria, these colonies change their initial red color and turn blue or shades of green or greenish blue with a clear zone in the agar around them. Therefore, it may be not easy to distinguish the two genera based exclusively on the colonies color. This aptitude of the bacterial cells to absorb color from the NBTA medium is also phase dependent. Phase I bacteria change color on NBTA medium while ageing but Phase II bacteria do not. Some *Photorhabdus* strains are bioluminescent, giving them the property to produce light in the darkness (Peat et al. 2010), this represents a main character that distinguishes *Photorhabdus* bacteria from *Xenorhabdus* bacteria. Moreover, *Xenorhabdus* is catalase negative, which is an unusual property for bacteria in the *Enterobacteriaceae* family (Boemare and Akhurst 1988).

Next to morphological characters, molecular information is important when bacteria are to be taxonomically classified. Initially, DNA-DNA hybridization analysis was seen as a key method for bacterial species delineation (Wayne et al. 1987). Afterwards, the 16S rRNA gene became the marker of choice in studying the taxonomic status of symbiotic bacteria (Fischer-Le Saux et al. 1999; Fukushima et al. 2002; Liu et al. 2001; Rainey et al. 1995; Szállás et al. 1997). Bacterial 16S rRNA gene generally contains nine hypervariable regions (V1-V9) which show considerable nucleotide sites variation among bacterial species and therefore useful for species identification (Chakravorty et al. 2007; Van de Peer et al. 1996). Hypervariable regions are intercalated by conserved base pairs in most bacteria which help the implication of targeted fragment using universal primers (Baker et al. 2003). One of the main difference between *Photorhabdus* and *Xenorhabdus* bacteria is that the 16S rRNA gene sequences of *Photorhabdus* has TGAAAG while *Xenorhabdus* has TTCG at positions 208-211(*Escherichia coli* numbering) (Szállás et al. 1997). However, using both DNA-DNA hybridization and 16S rRNA gene analyses, *Photorhabdus* was reported as a heterogeneous genus with different genotypes within single species (Fischer-Le Saux et al. 1998; Fischer-Le Saux et al. 1999). In some cases, delineation of

strains into a new species is well supported by DNA-DNA hybridization and phenotypic characters whereas the 16S rRNA gene failed (Akhurst et al. 2004). For this reason, definition of subspecies within existing species appeared to be relevant for *Photorhabdus* genus (Akhurst et al. 2004; Fischer-Le Saux et al. 1999). For *Xenorhabdus* genus, the same practice was initially applied when four *Xenorhabdus* groups were described as subspecies of *Xenorhabdus nematophilus*: *X. nematophilus* subsp. *nematophilus*, Akhurst (1983), *X. nematophilus* subsp. *bovienii*, Akhurst (1983), *X. nematophilus* subsp. *poinarii*, Akhurst (1983) and *Xenorhabdus nematophilus* subsp. *beddingii*, Akhurst (1986). Later they were all elevated to species level by the same authors due to the close correspondence observed between the taxonomic groupings of *Xenorhabdus* and those of their associate nematodes. These subspecies are now known as *X. nematophilus*, *X. bovienii*, *X. poinarii* and *X. beddingii* (Akhurst and Boemare 1988).

Later, the inconsistent species-level grouping of some *Photorhabdus* strains (Akhurst et al. 2004) based on the 16S rRNA gene analysis, led to the use of more variable genes to provide complementary molecular information when evaluating bacterial phylogenies. Therefore, analyses of gyrB (Akhurst et al. 2004; Peat et al. 2010; Tóth and Lakatos 2008) and recA gene (Sergeant et al. 2006; Thanwisai et al. 2012) sequences have been used to complement 16S rRNA gene phylogeny, bacterial phenotypic and DNA-DNA hybridization studies to better characterize new *Photorhabdus* and *Xenorhabdus* isolates. Furthermore, potential lateral transfer of 16S rRNA genes was later demonstrated to exist in the *Photorhabdus* and *Xenorhabdus* clades (Tailliez et al. 2010) which may confound the classification of bacterial isolates, especially when only this gene is considered. Therefore, the evolution towards using multiple alternative markers, next to the 16S rRNA gene, to study nematode symbionts is part of a general trend. Using several phylogenetic markers has become very popular in many bacterial diversity studies in recent years because of the higher information content of more variable genes and increased reliability provided by using many markers. In this respect, a Multilocus Sequence Analysis (MLSA) approach involving several housekeeping genes such as recA, gyrB, gltX, dnaN and infB has been proposed (Tailliez et al. 2010; Tailliez et al. 2012) to increase the robustness of the phylogeny of *Photorhabdus* and *Xenorhabdus* bacteria. A threshold of 97% nucleotide identity of the concatenated recA, gyrB, gltX, dnaN and infB genes has been proposed (Tailliez et al. 2010) to distinguish species and subspecies in *Xenorhabdus* and *Photorhabdus* groups, respectively.

The **RecA** gene product plays a crucial role in genetic recombination and repair of the DNA (Horii et al. 1980). This gene has been first used by Sergeant et al. (2006) to identify *Xenorhabdus* isolates from the United Kingdom. The **GyrB** gene, encodes the subunit B protein of DNA gyrase (Peat et al. 2010). Previous studies have indicated that gyrB might prove to be more useful in identifying bacteria to the species level due to its higher rates of molecular evolution (Fukushima et al. 2002; Yamamoto and Harayama 1995). GyrB was previously analyzed to propose the delineation of *P. asymbiotica* into two subspecies (Akhurst et al. 2004). In addition, *P. temperata* subspecies *cinerea* has been proposed based on gyrB sequence analysis (Tóth and Lakatos 2008). The **gltX** gene encodes for the glutamate-tRNA ligase which catalyses the attachment of glutamate to tRNA (Glu). The **dnaN** is the gene that encodes for the DNA clamp (also known as β sliding clamp) of DNA polymerase III in prokaryotes. The **infB** gene encodes for the translation initiation factor IF-2, and was earlier proposed as one of the genes that reflect organismal phylogeny through vertical descent for the Gammaproteobacteria family (Lerat et al. 2003). It was used in combination with other protein coding genes to successfully resolve the phylogenetic position of *X. magdalensis* (Tailliez et al. 2012). Next to these genes, the **glnA** gene which encodes for the glutamine synthesize, is particularly useful in resolving specific and intra-specific relationships poorly resolved in other studies. It was concluded that a combination of gyrB and glnA, 16S rRNA genes analysis may be useful in resolving species delineation (Peat et al. 2010). A neighbor joining analysis using concatenated gyrB and glnA datasets was done by Gerrard et al. (2006) to successfully confirm the identity of a *P. asymbiotica* strain isolated from a nematode, though there was low support for many terminal nodes in their phylogeny relationship.

1.5 Research objectives

As stated above, many advantages qualify EPNs as commercially valuable biocontrol agents of soil born stages of insect pests. They are safe for both user and the environment; they are persistent and have the potential to recycle inside the host insect causing a long term and sustainable effect on the targeted pest (Peters 1996); they are cost effective and exempted from registration requirements in almost all countries aiding the development of small companies in production of nematode-based products for pest control (Ehlers et al. 1998). Applications of

EPNs to control fruit damaging pests, mainly aim for the suppression of insect population in a preventive way at the larval or pupae developmental stages which generally occur in soil (Herz et al. 2006; Yee and Lacey 2003). In addition, they can also demonstrate great performance in the galleries of boring insects or where resistance to chemicals has been developed (Ehlers 2001).

Mango is an important fruit for the West African population and for tropical countries around the world where it is grown. Mango production is constantly growing. In some agro-ecological zones like Nord and Center Benin where most of mango orchards are located, the fruit is believed to play a double role in the population diet, first as fruit, but also as subsistence crop (Vayssieres et al. 2008). In fact, as the fruits ripen in late dry season and early rainy season, mango plays a key role in food security and represents basically a daily diet of the population in that critical period of the year. However, the production of mango is confronted with several constraints including fruit flies damage which represents the major problem. These fruits flies belong to the Tephritidae family and *B. dorsalis* species is the most invasive and damaging in mango as well as some other important fruit crops. They have inflicted considerable yield losses in mango production in 2006-2007 evaluated at approximately 75% of the total production (Vayssières et al. 2009b; Vayssieres et al. 2008). Infested mangos with even a simple insect bite are refused on the markets especially at international level. Indeed, *B. dorsalis* and related species are on the quarantine list of many European countries where most of the export markets are located. Two developmental stages of the insect, the third instar larvae and pupae, occur in soil before adult fruit flies emergence. To manage these insect pests, growers mainly refer to the use of chemical compounds which may sometimes be applied with food attractants as baits (Ekesi 2016). Given the importance of fruit fly damage in the mango sector and alternative crops, and the side effects of chemical compound on the environment, human and wildlife health, it became urgent to look for sustainable solutions. To achieve this goal, investigation on natural enemies to be used in biological control is regarded as major component of Integrated Pest Management (IPM) of these insect pests. In this respect, EPNs may represent a potential natural enemy of *B. dorsalis* and can be valuable for the control of these insect pests in biological control. Therefore, this PhD project was initiated and received the financial support of the special research grant (BOF) of Ghent University, to investigate in collaboration with the University of Parakou in Benin, the potential

of local EPNs from Benin in association with their symbiotic bacteria, to reduce the population of *B. dorsalis* in mango orchards in the country. The overall aim of this doctoral research project was to contribute towards the development of indigenous EPNs as an effective control method against fruit flies, particularly for the cultivation of mango in Benin. Our specific objectives were:

- Isolation and characterization of EPNs from mango plantations in Northern Benin.
- Isolation, purification, identification and characterization of the symbiotic bacteria associated with these EPNs.
- Selection of the most effective isolates against *B. dorsalis* by virulence tests under laboratory and field conditions.

1.6 Outline

Chapter 1 presents a **General introduction** and the context of this research. It provides an overview on mango production in West Africa, on *Bactrocera dorsalis* as the main pest problem in mango cultivation and on EPNs as alternative to *B. dorsalis* management. The objectives of the study are also presented in this chapter.

Chapter 2-6 describe the experimental work of this research.

Chapter 2: Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*) under laboratory conditions. It presents the natural occurrence of EPNs in mango orchards in Nord-Benin, their isolation and identification. In addition, this chapter discusses the susceptibility of *Bactrocera dorsalis* larvae and pupae to Beninese EPNs under different environmental factors in laboratory conditions.

Chapter 3: *Steinernema* n. sp., a new *Steinernema* species (Rhabditida: Steinernematidae) from Northern Benin. In this chapter two *Steinernema* isolates from Benin are fully identified based on morphological/morphometrics, molecular and cross-hybridization analyses.

Chapter 4: Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin. Here, all bacterial symbionts of available Beninese EPN isolates were characterized.

Chapter 5: Description of *Photorhabdus luminescens* subsp. *beninensis* subsp. nov., a novel symbiotic bacterium associated with *Heterohabditis taysearae* (Nematoda, Heterorhabditidae) nematodes isolated from Benin. In this chapter, a new *Photorhabdus luminescens* subsp. *beninensis*, associated with isolates of native *H. taysearae* is described and its phylogenetic relationship with described subspecies is established using 16S rRNA, recA, gyrB, dnaN, gltX and infB genes.

Chapter 6: Evaluation of the ability of indigenous isolates of *Heterorhabditis taysearae* and *Steinernema* sp. to control mango fruit fly *Bactrocera dorsalis* under laboratory, semi-field and field conditions in Northern Benin. This chapter presents the results of the first implementation of the use of EPNs to control *B. dorsalis* under laboratory, semi-field and field conditions.

Chapter 7 presents a **General discussion** of the main findings of this PhD research and future perspectives are proposed.

1.7 References

Adams BJ, Nguyen KB (2002) Taxonomy and systematics. In: Gaugler R (ed) Entomopathogenic Nematology. CABI, Wallingford, UK, pp 1-33

Adán A, Estal PD, Budia F, González M, Viñuela E (1996) Laboratory evaluation of the novel naturally derived compound spinosad against *Ceratitis capitata*. Pest Management Science 48:261-268

Akhurst R (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. Journal of General Microbiology 121:303-309

Akhurst R (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061-3065

Akhurst R, Boemare N (1988) A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. Microbiology 134:1835-1845

Akhurst R, Boemare N, Janssen P, Peel M, Alfredson D, Beard C (2004) Taxonomy of Australian clinical isolates of the genus *Photorhabdus* and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. *australis* subsp. nov. International Journal of Systematic and Evolutionary Microbiology 54:1301-1310

Akhurst RJ (1983) Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. International Journal of Systematic and Evolutionary Microbiology 33:38-45

Akhurst RJ (1986) *Xenorhabdus nematophilus* subsp. *beddingii* (Enterobacteriaceae): a new subspecies of bacteria mutualistically associated with entomopathogenic nematodes. International Journal of Systematic and Evolutionary Microbiology 36:454-457

Altuzar A, Montoya P, Rojas JC (2004) Response of *Fopius arisanus* (Hymenoptera: Braconidae) to fruit volatiles in a wind tunnel. Florida Entomologist 87:616-618

Aluja M, Mangan RL (2008) Fruit fly (Diptera: Tephritidae) host status determination: critical conceptual, methodological, and regulatory considerations. Annual Review of Entomology 53:473-502

Aluja M, Sivinski J, Rull J, Hodgson PJ (2005) Behavior and predation of fruit fly larvae (*Anastrepha* spp.) (Diptera: Tephritidae) after exiting fruit in four types of habitats in tropical Veracruz, Mexico. Environmental Entomology 34:1507-1516

An R, Grewal PS (2010) *Photorhabdus temperata* subsp. *stackebrandtii* subsp. nov.(Enterobacteriales: Enterobacteriaceae). Current Microbiology 61:291-297

An R, Grewal PS (2011) *Photorhabdus luminescens* subsp. *kleinii* subsp. nov.(Enterobacteriales: Enterobacteriaceae). Current Microbiology 62:539-543

Ansari MA, Hussain MA, Moens M (2009) Formulation and application of entomopathogenic nematode-infected cadavers for control of *Hoplia philanthus* in turfgrass. Pest Management Science 65:367-374

Argov Y, Gazit Y (2008) Biological control of the Mediterranean fruit fly in Israel: Introduction and establishment of natural enemies. Biological Control 46:502-507

Babic I, Fischer-Le Saux M, Giraud E, Boemare N (2000) Occurrence of natural dixenic associations between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic *Heterorhabditis* spp.(Nematoda, Rhabditida). Microbiology 146:709-718

Baele M, Baele P, Vaneechoutte M, Storms V, Butaye P, Devriese LA, Verschraegen G, Gillis M, Haesebrouck F (2000) Application of tRNA intergenic spacer PCR for identification of Enterococcus species. Journal of Clinical Microbiology 38:4201-4207

Baker G, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. Journal of Microbiological Methods 55:541-555

Balasubramanian N, Toubarro D, Simoes N (2010) Biochemical study and in vitro insect immune suppression by a trypsin-like secreted protease from the nematode *Steinernema carpocapsae*. Parasite Immunology 32:165-175

Baldwin T, Devine J, Heckel R, Lin JW, Shadel G (1989) The complete nucleotide sequence of the lux regulon of Vibrio fischeri and the luxABN region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence. Luminescence 4:326-341

Bedding R (1981) Low cost in vitro mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. Nematologica 27:109-114

Bedding R (1984) Large scale production, storage and transport of the insect-parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. Annals of Applied Biology 104:117-120

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Bedding R, Molyneux A (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp.(Heterorhabditidae: Nematoda). Nematologica 28:354-359

Berg M, Stenuit B, Ho J, Wang A, Parke C, Knight M, Alvarez-Cohen L, Shapira M (2016) Assembly of the *Caenorhabditis elegans* gut microbiota from diverse soil microbial environments. The ISME Journal 10:1998

Billah M, Kimani-Njogu S, Wharton R, Woolley J, Masiga D (2008) Comparison of five allopatric fruit fly parasitoid populations (*Psyttalia* species) (Hymenoptera: Braconidae) from coffee fields using morphometric and molecular methods. Bulletin of Entomological Research 98:63-75

Bird A, Akhurst R (1983) The nature of the intestinal vesicle in nematodes of the family Steinernematidae. International Journal for Parasitology 13:599-606

Boemare N (2002) Systematics of *Photorhabdus* and *Xenorhabdus*. Entomopathogenic Nematology:35-56

Boemare N, Akhurst R (1988) Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). Journal of General Microbiology 134:751-761

Boemare N, Akhurst R, Mourant R (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249-255

Bonifassi E, Fischer-Le Saux M, Boemare N, Lanois A, Laumond C, Smart G (1999) Gnotobiological study of infective juveniles and symbionts of *Steinernema scapterisci:* a model to clarify the concept of the natural occurrence of monoxenic associations in entomopathogenic nematodes. Journal of Invertebrate Pathology 74:164-172

Cabreiro F, Gems D (2013) Worms need microbes too: microbiota, health and aging in *Caenorhabditis elegans*. EMBO Molecular Medicine 5:1300-1310

Campos-Herrera R, Tailliez P, Pagès S, Ginibre N, Gutierrez C, Boemare N (2009) Characterization of *Xenorhabdus* isolates from La Rioja (Northern Spain) and virulence with and without their symbiotic entomopathogenic nematodes (Nematoda: Steinernematidae). Journal of Invertebrate Pathology 102:173-181

Castillo M-A, Moya P, Hernández E, Primo-Yúfera E (2000) Susceptibility of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) to entomopathogenic fungi and their extracts. Biological Control 19:274-282

Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods 69:330-339

Ciche TA, Darby C, Ehlers R-U, Forst S, Goodrich-Blair H (2006) Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. Biological Control 38:22-46

Ciche TA, Kim K-s, Kaufmann-Daszczuk B, Nguyen KC, Hall DH (2008) Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. Applied and Environmental Microbiology 74:2275-2287

Clarke AR, Armstrong KF, Carmichael AE, Milne JR, Raghu S, Roderick GK, Yeates DK (2005) Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. Annual Review of Entomology 50:293-319

Cooper D, Eleftherianos I (2016) Parasitic nematode immunomodulatory strategies: recent advances and perspectives. Pathogens 5:58

Couche GA, Gregson RP (1987) Protein inclusions produced by the entomopathogenic bacterium *Xenorhabdus nematophilus* subsp. *nematophilus*. Journal of Bacteriology 169:5279-5288

Cruz-Martinez H, Ruiz-Vega J, Matadamas-Ortíz PT, Cortes-Martinez CI, Rosas-Diaz J (2017) Formulation of entomopathogenic nematodes for crop pest control: A review. Plant Protection Science 53:15-24

De Ley P, Blaxter M (2002) Systematic position and phylogeny. In: Lee D (ed) The Biology of Nematodes. Taylor and Francis, London and New York, pp 1-30

Dimbi S, Maniania NK, Lux SA, Mueke JM (2004) Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. BioControl 49:83-94

Drew RA (1989) The tropical fruit flies (Diptera: Tephritidae: Dacinae) of the Australasian and Oceanian regions. Memoirs of the Queensland Museum 26:1-521

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Ehlers R-U, Lunau S, Krasomil-Osterfeld K, Osterfeld KH (1998) Liquid culture of the entomopathogenic nematode-bacterium-complex *Heterorhabditis megidis*/*Photorhabdus luminescens*. BioControl 43:77-86

Ekesi S (2016) Baiting and male annihilation techniques for fruit fly suppression in Africa. In: Ekesi S, Mohamed SA, De Meyer M (eds) Fruit Fly Research and Development in Africa-Towards a Sustainable Management Strategy to Improve Horticulture. Springer, pp 275-292

Ekesi S, Maniania N, Lux S (2002) Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. Biocontrol Science and Technology 12:7-17

Ekesi S, Maniania N, Lux S (2003) Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. Journal of Invertebrate Pathology 83:157-167

Ekesi S, Maniania N, Mohamed S (2011) Efficacy of soil application of *Metarhizium anisopliae* and the use of GF-120 spinosad bait spray for suppression of *Bactrocera invadens* (Diptera: Tephritidae) in mango orchards. Biocontrol Science and Technology 21:299-316

Emelianoff V, Sicard M, Le Brun N, Moulia C, Ferdy J-B (2007) Effect of bacterial symbionts *Xenorhabdus* on mortality of infective juveniles of two *Steinernema* species. Parasitology Research 100:657-659

Ferreira T, Van Reenen C, Pagès S, Tailliez P, Malan AP, Dicks LM (2013a) *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov., a symbiotic bacterium associated with a

novel *Heterorhabditis* species related to *Heterorhabditis indica*. International Journal of Systematic and Evolutionary Microbiology 63:1853-1858

Ferreira T, Van Reenen C, Tailliez P, Pagès S, Malan A, Dicks L (2016) First report of the symbiotic bacterium *Xenorhabdus indica* associated with the entomopathogenic nematode *Steinernema yirgalemense*. Journal of Helminthology 90:108-112

Ferreira T, Van Reenen CA, Endo A, Spröer C, Malan AP, Dicks LM (2013b) Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae*. International Journal of Systematic and Evolutionary Microbiology 63:3220-3224

Ferreira T, van Reenen CA, Endo A, Tailliez P, Pagès S, Spröer C, Malan AP, Dicks LM (2014) *Photorhabdus heterorhabditis* sp. nov., a symbiont of the entomopathogenic nematode *Heterorhabditis zealandica*. International Journal of Systematic and Evolutionary Microbiology 64:1540-1545

Ffrench-Constant R, Waterfield N, Daborn P, Joyce S, Bennett H, Au C, Dowling A, Boundy S, Reynolds S, Clarke D (2003) *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. FEMS Microbiology Reviews 26:433-456

Fischer-Le Saux M, Mauléon H, Constant P, Brunel B, Boemare N (1998) PCR-Ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean Region in Relation to the Taxonomy and Geographic Distribution of Their Nematode Hosts. Applied and Environmental Microbiology 64:4246-4254

Fischer-Le Saux M, Viallard V, Brunel B, Normand P, Boemare NE (1999) Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49:1645-1656

Flanders KL, Miller JM, Shields EJ (1996) In vivo production of *Heterorhabditis bacteriophora* 'Oswego'(Rhabditida: Heterorhabditidae), a potential biological control agent for soil-inhabiting insects in temperate regions. Journal of Economic Entomology 89:373-380

Floris I, Ruiu L, Satta A, Delrio G, Ellar DJ, Rubino S, Paglietti B, Loru L, Verdinelli M (2007) Researches on entomopathogenic bacteria and applications for fly pest control in a Mediterranean region (Sardinia, Italy). IOBC WPRS BULLETIN 30:159

Forst S, Clarke D (2002) Bacteria-nematode symbiosis. In: Gaugler R (ed) Entomopathogenic Nematology. CABI, Wallingford, UK, pp 57-77

Forst S, Dowds B, Boemare N, Stackebrandt E (1997) *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annual Reviews in Microbiology 51:47-72

Forst S, Nealson K (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiological Reviews 60:21-43

Fukushima M, Kakinuma K, Kawaguchi R (2002) Phylogenetic analysis of *Salmonella*, *Shigella*, and *Escherichia coli* strains on the basis of the gyrB gene sequence. Journal of Clinical Microbiology 40:2779-2785

Gaugler R, Han R (2002) Production Technology. In: Gaugler R (ed) Entomopathogenic Nematology. CABI, Wallingford, UK pp 289–310

Gazit Y, Rossler Y, Glazer I (2000) Evaluation of entomopathogenic nematodes for the control of Mediterranean fruit fly (Diptera: Tephritidae). Biocontrol Science and Technology 10:157-164

Georgis R (1990) Formulation and application technology. In: R G, HK. aK (eds) Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, FL., pp 173-191

Gerrard JG, Joyce SA, Clarke DJ (2006) Nematode symbiont for *Photorhabdus asymbiotica*. Emerging Infectious Diseases 12:1562

Glaeser SP, Tobias NJ, Thanwisai A, Chantratita N, Bode HB, Kämpfer P (2017) *Photorhabdus luminescens* subsp. *namnaonensis* subsp. nov., isolated from *Heterorhabditis baujardi* nematodes. International Journal of Systematic and Evolutionary Microbiology 67:1046-1051

Glaser R, Farrell C, Gowen J (1935) Field experiments with the Japanese beetle and its nematode parasite. Journal of the New York Entomological Society 43:345-371

Gnanvossou D, Hanna R, Bokonon-Ganta AH, Ekesi S, Mohamed SA (2016) Release, establishment and spread of the natural enemy *Fopius arisanus* (Hymenoptera:

Braconidae) for control of the invasive oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) in Benin, West Africa. In: Ekesi S, Mohamed SA, Meyer MD (eds) Fruit Fly Research and Development in Africa-Towards a Sustainable Management Strategy to Improve Horticulture. Springer, pp 575-600

Goergen G, Vayssières J-F, Gnanvossou D, Tindo M (2011) *Bactrocera invadens* (Diptera: Tephritidae), a new invasive fruit fly pest for the Afrotropical region: host plant range and distribution in West and Central Africa. Environmental Entomology 40:844-854

Gomina M (2015) Contribution à la connaissance des mouches des fruits (Diptera: Tephritidae) et de leurs parasitoïdes au sud du Togo. Thèse de Doctorat, Universite de Lome, 190p.

Goud S, Hugar P, Prabhuraj A (2010) Effect of temperature, population density and shelf life of EPN *Heterorhabditis indica* (RCR) in sodium alginate gel formulation. Journal of Biopesticides 3:627-632

Grewal PS (2002) Formulation and application technology. In: Gaugler R (ed) Entomopathogenic Nematology. CABI, Wallingford, UK, p 265

Griffin C, Boemare N, Lewis E (2005) Biology and behaviour. In: Grewal PS, Ehlers R-U, Shapiro-Ilan DI (eds) Nematodes as Biocontrol Agents. CABI Publishing, pp 47-64

Hanna R, Gnanvossou D, Grout T (2008) Male annihilation technique (MAT) in eliminating *B. invadens* in Northern Bénin. Fighting fruit and vegetable flies regionally in Western Africa COLEACP/CIRAD, Information Letter 7

Harris EJ, Bautista RC, Vargas RI, Jang EB, Eitam A, Leblanc L (2010) Suppression of melon fly (Diptera: Tephritidae) populations with releases of *Fopius arisanus* and *Psyttalia fletcheri* (Hymenoptera: Braconidae) in North Shore Oahu, HI, USA. Biocontrol 55:593-599

Hazir S, Stackebrandt E, Lang E, Schumann P, Ehlers R-U, Keskin N (2004) Two new Subspecies of *Photorhabdus luminescens*, Isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. Systematic and Applied Microbiology 27:36-42

Herz A, Köppler K, Vogt H, Elias E, Katz P, Peters A (2006) Biological control of the cherry fruit fly, *Rhagoletis cerasi* L.(Diptera, Tephriti-dae) by use of entomopathogenic

nematodes: first experiences towards practi-cal implementation. In: Proceedings to the Conference Ecofruit-12th international conference on cultivation technique and phytopathological problems in organic fruit-growing, Weinsberg/Germany. Fördergemeinschaft Ökologischer Obstbau eV (FÖKO), Traubenplatz 5, D-74189 Weinsberg, pp 67-72

Hiltpold I, Hibbard BE, French BW, Turlings TC (2012) Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. Plant and Soil 358:11-25

Horii T, Ogawa T, Ogawa H (1980) Organization of the recA gene of *Escherichia coli*. Proceedings of the National Academy of Sciences 77:313-317

Hou B, Xie Q, Zhang R (2006) Depth of pupation and survival of the Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) pupae at selected soil moistures. Applied Entomology and Zoology 41:515-520

Humason GL (1962) Animal tissue techniques. 3rd edn Freeman, San Francisco, :307-308

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors) Advances in Taxonomy and Phylogeny of Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Hussein MA, El-Wakeil N, El-Sebai T (2006) Susceptibility of melon fruit fly, *Dacus ciliatus*, to entomopathogenic nematodes (Rhabditida) and to insecticides. International Journal of Nematology 16:13-18

Jiang H, Vilcinskas A, Kanost MR (2010) Immunity in lepidopteran insects. Invertebrate Immunity 708:181-204

Johnigk S-A, Ehlers R-U (1999) Endotokia matricida in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. Nematology 1:717-726

Kämpfer P, Tobias NJ, Ke LP, Bode HB, Glaeser SP (2017) *Xenorhabdus thuongxuanensis* sp. nov. and *Xenorhabdus eapokensis* sp. nov., isolated from *Steinernema species*. International Journal of Systematic and Evolutionary Microbiology 67:1107-1114

Kazimierczak W, Skrzypek H, Sajnaga E, Skowronek M, Waśko A, Kreft A (2017) Strains of *Photorhabdus* spp. associated with polish *Heterorhabditis* isolates: their molecular and

phenotypic characterization and symbiont exchange. Archives of Microbiology 199:979–989

Kepenekci I, Susurluk A (2006) Infectivity of two Turkish isolates of *Steinernema feltiae* (Rhabditida: Steinernematidae) against *Rhagoletis cerasi* and *Ceratitis capitata*. Nematologia Mediterranea 34:95-97

Kim J, Jaffuel G, Turlings TC (2015) Enhanced alginate capsule properties as a formulation of entomopathogenic nematodes. BioControl 60:527-535

Koneru SL, Salinas H, Flores GE, Hong RL (2016) The bacterial community of entomophilic nematodes and host beetles. Molecular Ecology 25:2312-2324

Köppler K, Peters A, Vogt H (2005) Initial results in the application of entomopathogenic nematodes against the European cherry fruit fly *Rhagoletis cerasi* L.(Diptera, Tephritidae). IOBC/WPRS Bulletin 28:13-18

Kostermans AJG (2012) The mangoes: their botany, nomenclature, horticulture and utilization. IBPGR Academic Press, London

Kuwata R, Qiu L-h, Wang W, Harada Y, Yoshida M, Kondo E, Yoshiga T (2013) *Xenorhabdus ishibashii* sp. nov., isolated from the entomopathogenic nematode *Steinernema aciari*. International Journal of Systematic and Evolutionary Microbiology 63:1690-1695

Langford EA, Nielsen UN, Johnson SN, Riegler M (2014) Susceptibility of Queensland fruit fly, *Bactrocera tryoni* (Froggatt)(Diptera: Tephritidae), to entomopathogenic nematodes. Biological Control 69:34-39

Lengyel K, Lang E, Fodor A, Szállás E, Schumann P, Stackebrandt E (2005) Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Systematic and Applied Microbiology 28:115-122

Lerat E, Daubin V, Moran NA (2003) From gene trees to organismal phylogeny in prokaryotes: the case of the γ-Proteobacteria. PLoS Biology 1:e 19 doi: 10.1371/journal.pbio.0000019

Lin J, Zeng L, Liang G, Lu Y, Bin S (2004) Effects of entomopathogenic nematodes on the oriental fruit fly, *Bactrocera dorsalis* (Hendel). Kun chong xue bao Acta Entomologica Sinica 48:736-741

Lindegren J, Wong T, McInnis D (1990) Response of Mediterranean fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema* feltiae in field tests in Hawaii. Environmental Entomology 19:383-386

Lindegren JE, Vail PV (1986) Susceptibility of Mediterranean fruit fly, melon fly, and oriental fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema feltiae* in laboratory tests. Environmental Entomology 15:465-468

Liu J, Berry RE, Blouin MS (2001) Identification of symbiotic bacteria *(Photorhabdus* and *Xenorhabdus*) from the entomopathogenic nematodes *Heterorhabditis marelatus* and *Steinernema oregonense* based on 16S rDNA sequence. Journal of Invertebrate Pathology 77:87-91

Liu J, Berry RE, Moldenke AF (1997) Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. Journal of Invertebrate Pathology 69:246-252

Lux SA, Copeland RS, White IM, Manrakhan A, Billah MK (2003) A new invasive fruit fly species from the *Bactrocera dorsalis* (Hendel) group detected in East Africa. International Journal of Tropical Insect Science 23:355-361

Lysenko O, Weiser J (1974) Bacteria associated with the nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. Journal of Invertebrate Pathology 24:332-336

Malan AP, Manrakhan A (2009) Susceptibility of the Mediterranean fruit fly (*Ceratitis capitata*) and the Natal fruit fly (*Ceratitis rosa*) to entomopathogenic nematodes. Journal of Invertebrate Pathology 100:47-49

Maneesakorn P, An R, Daneshvar H, Taylor K, Bai X, Adams BJ, Grewal PS, Chandrapatya A (2011) Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (*Heterorhabditis*: Rhabditida) and their symbiotic bacteria (*Photorhabdus*: Enterobacteriaceae). Molecular Phylogenetics and Evolution 59:271-280

Martens EC, Goodrich-Blair H (2005) The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. Cellular Microbiology 7:1723-1735

Maupas E (1899) La mue et l'enkystement chez les nématodes. Arch Zool exp gén,(3e Série) 7:563-628

McQuate GT, Teruya T (2015) Melon Fly, *Bactrocera cucurbitae* (Diptera: Tephritidae), infestation in host fruits in the southwestern islands of japan before the initiation of island-wide population suppression, as recorded in publications of japanese public institutions. International Journal of Insect Science 7:27

Midha A, Schlosser J, Hartmann S (2017) Reciprocal interactions between nematodes and their microbial environments. Frontiers in Cellular and Infection Microbiology 7:144

Mille C (2010) Les mouches des fruits de Nouvelle-Calédonie (Diptera, Tephritidae): systématique, comportement, dynamique et gestion des populations. Thèse de Doctorat, Université de la Nouvelle-Calédonie, 349p.

Minas RdS, Souza RM, Dolinski C, Carvalho RdS, Burla RdS (2016) Potential of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) to control Mediterranean fruit fly (Diptera: Tephritidae) soil stages. Nematoda 3:e02016 doi:10.4322

Mohamed SA, Ekesi S, Hanna R (2010) Old and new host-parasitoid associations: parasitism of the invasive fruit fly *Bactrocera invadens* (Diptera: Tephritidae) and five African fruit fly species by *Fopius arisanus*, an Asian opiine parasitoid. Biocontrol Science and Technology 20:183-196

Mohamed SA, Wharton RA, von Merey G, Schulthess F (2006) Acceptance and suitability of different host stages of *Ceratitis capitata* (Wiedemann) (Diptera : Tephritidae) and seven other tephritid fruit fly species to *Tetrastichus giffardii* Silvestri (Hymenoptera : Eulophidae). Biological Control 39:262-271 doi:10.1016/j.biocontrol.2006.08.016

Morgan J, Kuntzelmann V, Tavernor S, Ousley M, Winstanley C (1997) Survival of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* in water and soil. Journal of Applied Microbiology 83:665-670

Mukherjee K, Raju R, Fischer R, Vilcinskas A (2013) *Galleria mellonella* as a model host to study gut microbe homeostasis and brain infection by the human pathogen *Listeria monocytogenes*. Adv Biochem Eng Biotechnol 135:27-39 doi:10.1007/10_2013_203

N'depo OR, Hala N, N'Da Adopo A, Coulibaly F, Kouassi P, Vayssieres J, De Meyer M (2015) Effective chemical control of fruit flies (Diptera: Tephritidae) pests in mango orchards in

Northern Côte-d'Ivoire. International Journal of Biological and Chemical Sciences 9:1299-1307

Ndlela S, Mohamed S, Ndegwa P, Ong'Amo G, Ekesi S (2016) Male annihilation technique using methyl eugenol for field suppression of *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) on mango in Kenya. African Entomology 24:437-447

Nguyen KB, Smart GCJ (2004) Taxonomy of insect parasitic nematodes. In: Chen Z X, Chen S Y, Dickson D W (eds) Nematology Advances and Perspectives, vol 2. CABI Publishing, China, Tsinghua University Press, pp 795-878

Nguyen KB, Smart Jr GC (1994) *Neosteinernema longicurvicauda* n. gen., n. sp.(Rhabditida: Steinernematidae), a parasite of the termite *Reticuldermes flavipes* (Koller). Journal of Nematology 26:162-174

Nishimura Y, Hagiwara A, Suzuki T, Yamanaka S (1994) *Xenorhabdus japonicus* sp. nov. associated with the nematode *Steinernema kushidai*. World Journal of Microbiology and Biotechnology 10:207-210

Orozco RA, Hill T, Stock SP (2013) Characterization and phylogenetic relationships of *Photorhabdus luminescens* subsp. *sonorensis* (γ-Proteobacteria: Enterobacteriaceae), the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae). Current Microbiology 66:30-39

Peat SM, Waterfield NR, Marokházi J, Fodor A, Adams BJ (2010) A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: a case for 16S, gyrB, and glnA. Molecular Phylogenetics and Evolution 57:728-740

Pelz KS, Isaacs R, Wise JC, Gut LJ (2005) Protection of fruit against infestation by apple maggot and blueberry maggot (Diptera: Tephritidae) using compounds containing spinosad. Journal of Economic Entomology 98:432-437

Peng R, Christian K (2006) Effective control of Jarvis's fruit fly, *Bactrocera jarvisi* (Diptera: Tephritidae), by the weaver ant, *Oecophylla smaragdina* (Hymenoptera: Formicidae), in mango orchards in the Northern Territory of Australia. International Journal of Pest Management 52:275-282

Peters A (1996) The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations. Biocontrol Science and Technology 6:389-402

Pitcher D, Saunders N, Owen R (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters in Applied Microbiology 8:151-156

Poinar G, Hislop R (1981) Mortality of mediterranean fruit-fly adults (*Ceratitis capitata*) from parasitic nematodes (*Neoaplectana* and *heterorhabditis* spp). IRCS Medical Science-Biochemistry 9:641-641

Poinar GO, Thomas GM (1966) Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (Neoaplectana sp. Steinernematidae). Parasitology 56:385-390

Poinar JR GO, Thomas GM (1965) A new bacterium, *Achromobacter nematophilus* sp. nov.(Achromobacteriaceae: Eubacteriales) associated with a nematode. International Journal of Systematic and Evolutionary Microbiology 15:249-252

Popiel I, Vasquez EM (1991) Cryopreservation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. Journal of Nematology 23:432-437

Quesada-Moraga E, Ruiz-García A, Santiago-Alvarez C (2006) Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae). Journal of Economic Entomology 99:1955-1966

Rainey F, Ehlers R-U, Stackebrandt E (1995) Inability of the polyphasic approach to systematics to determine the relatedness of the genera *Xenorhabdus* and *Photorhabdus*. International Journal of Systematic and Evolutionary Microbiology 45:379-381

Razia M, Raja RK, Padmanaban K, Chellapandi P, Sivaramakrishnan S (2011) 16S rDNA-based phylogeny of non-symbiotic bacteria of entomopathogenic nematodes from infected insect cadavers. Genomics, Proteomics and Bioinformatics 9:104-112

Rousse P, Quilici S (2009) Recent advances in biological control of fruit fly pests. In: Bandeira RR (ed) Current Trends in Fruit Fly Control on Perennial Crops and Research Prospects. Transworld Research Network, Kerala, India, pp 91-115

Saeki S, Katayama M, Okumura M (1980) Effect of temperatures upon the development of the oriental fruit fly and its possible distribution in the mainland of Japan. Research Bulletin of the Plant Protection Service, Japan 16:73-76

Schutze MK, Mahmood K, Pavasovic A, Bo W, Newman J, Clarke AR, Krosch MN, Cameron SL (2015) One and the same: integrative taxonomic evidence that *Bactrocera invadens* (Diptera: Tephritidae) is the same species as the Oriental fruit fly *Bactrocera dorsalis*. Systematic Entomology 40:472-486

Self G, Ducamp M-N, Thaunay P, Vayssières J-F (2012) The effects of phytosanitary hot water treatments on West African mangoes infested with *Bactrocera invadens* (Diptera: Tephritidae). Fruits 67:439-449

Sergeant M, Baxter L, Jarrett P, Shaw E, Ousley M, Winstanley C, Morgan JAW (2006) Identification, typing, and insecticidal activity of *Xenorhabdus* isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the xpt toxin loci. Applied and Environmental Microbiology 72:5895-5907

Shapiro-Ilan D, Gaugler R (2002) Production technology for entomopathogenic nematodes and their bacterial symbionts. Journal of Industrial Microbiology and Biotechnology 28:137-146

Shapiro-Ilan DI, Han R, Dolinksi C (2012) Entomopathogenic nematode production and application technology. Journal of Nematology 44:206

Shapiro-Ilan DI, Lewis EE, Behle RW, McGuire MR (2001) Formulation of entomopathogenic nematode-infected cadavers. Journal of Invertebrate Pathology 78:17-23

Sicard M, Ferdy JB, Le Brun N, Godelle B, Boemare N, Moulia C (2004) When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). Journal of Evolutionary Biology 17:985-993

Sicard M, Le Brun N, Pages S, Godelle B, Boemare N, Moulia C (2003) Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. Parasitology Research 91:520-524

Sinzogan AAC, Van Mele P, Vayssières J-F (2008) Implications of on-farm research for local knowledge related to fruit flies and the weaver ant *Oecophylla longinoda* in mango production. International Journal of Pest Management 54:241-246

Sirjani FO, Lewis EE, Kaya HK (2009) Evaluation of entomopathogenic nematodes against the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). Biological Control 48:274-280

Smigielski AJ, Akhurst RJ, Boemare NE (1994) Phase variation in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: differences in respiratory activity and membrane energization. Applied and Environmental Microbiology 60:120-125

Somvanshi VS, Kaufmann-Daszczuk B, Kim Ks, Mallon S, Ciche TA (2010) *Photorhabdus* phase variants express a novel fimbrial locus, mad, essential for symbiosis. Molecular Microbiology 77:1021-1038

Somvanshi VS, Lang E, Ganguly S, Swiderski J, Saxena AK, Stackebrandt E (2006) A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. Systematic and Applied Microbiology 29:519-525

Spiridonov S, Krasomil-Osterfeld K, Moens M (2004) *Steinernema jollieti* sp n.(Rhabditida: Steinernematidae), a new entomopathogenic nematode from the American midwest. Russ J Nematol 12:85-95

Steiner G (1923) *Aplectana kraussei* n. sp., eine in der Blattwespe *Lyda sp*. parasitierende Nematodenform, nebst. Bemerkungen über das Seitenorgan der parasitischen Nematoden. Zentralblatt fuer Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 59

Strauch O, Ehlers R-U (1998) Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Applied Microbiology and Biotechnology 50:369-374

Strauch O, Stoessel S, Ehlers R-U (1994) Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. Fundamental and Applied Nematology 17:575-582

Szállás E, Koch C, Fodor A, Burghardt J, Buss O, Szentirmai A, Nealson KH, Stackebrandt E (1997) Phylogenetic evidence for the taxonomic heterogeneity of *Photorhabdus*

luminescens. International Journal of Systematic and Evolutionary Microbiology 47:402-407

Tailliez P, Laroui C, Ginibre N, Paule A, Pagès S, Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60:1921-1937

Tailliez P, Pagès S, Edgington S, Tymo LM, Buddie AG (2012) Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. International Journal of Systematic and Evolutionary Microbiology 62:1761-1765

Tailliez P, Pages S, Ginibre N, Boemare N (2006) New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. International Journal of Systematic and Evolutionary Microbiology 56:2805-2818

Thanwisai A, Tandhavanant S, Saiprom N, Waterfield NR, Long PK, Bode HB, Peacock SJ, Chantratita N (2012) Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. PLoS One 7:e43835

Thomas DB, Mangan RL (2005) Nontarget impact of spinosad GF-120 bait sprays for control of the Mexican fruit fly (Diptera: Tephritidae) in Texas citrus. Journal of Economic Entomology 98:1950-1956

Thomas GM, Poinar GO (1979) *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. International Journal of Systematic Bacteriology 29:352-360

Toledo J, Ibarra JE, Liedo P, Gómez A, Rasgado MA, Williams T (2005) Infection of *Anastrepha ludens* (Diptera: Tephritidae) larvae by *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) under laboratory and field conditions. Biocontrol Science and Technology 15:627-634

Toledo J, Rasgado MA, Ibarra JE, Gómez A, Liedo P, Williams T (2006a) Infection of *Anastrepha ludens* following soil applications of *Heterorhabditis bacteriophora* in a mango orchard. Entomologia Experimentalis et Applicata 119:155-162

Toledo J, Rojas R, Ibarra JE (2006b) Efficiency of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) on *Anastrepha serpentina* (Diptera: Tephritidae) larvae under laboratory conditions. Florida Entomologist 89:524-526

Tóth T, Lakatos T (2008) *Photorhabdus temperata* subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes. International Journal of Systematic and Evolutionary Microbiology 58:2579-2581

Urbaneja A, Marí FG, Tortosa D, Navarro C, Vanaclocha P, Bargues L, Castañera P (2006) Influence of ground predators on the survival of the Mediterranean fruit fly pupae, *Ceratitis capitata*, in Spanish citrus orchards. BioControl 51:611-626

Van de Peer Y, Chapelle S, De Wachter R (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Research 24:3381-3391

Van Mele P, Vayssières J-F, Van Tellingen E, Vrolijks J (2007) Effects of an African weaver ant, *Oecophylla longinoda*, in controlling mango fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 100:695-701

Van Mele P, VAYSSIERES JF, Adandonon A, Sinzogan A (2009) Ant cues affect the oviposition behaviour of fruit flies (Diptera: Tephritidae) in Africa. Physiological Entomology 34:256-261

Van Melle C, Arinloye D, Coulibaly O, Vayssières J-F, Hell K (2008) Contribution to mango value chain development in Benin-A producer perception survey. In: IV International Symposium on Tropical and Subtropical Fruits 975. pp 607-613

Vargas RI, Leblanc L, Putoa R, Eitam A (2007) Impact of introduction of *Bactrocera dorsalis* (Diptera: Tephritidae) and classical biological control releases of *Fopius arisanus* (Hymenoptera: Braconidae) on economically important fruit flies in French Polynesia. Journal of Economic Entomology 100:670-679

Vayssières J-F, Korie S, Ayegnon D (2009b) Correlation of fruit fly (Diptera Tephritidae) infestation of major mango cultivars in Borgou (Benin) with abiotic and biotic factors and assessment of damage. Crop Protection 28:477-488

Vayssieres J-F, Korie S, Coulibaly O, Temple L, Boueyi SP (2008) The mango tree in central and northern Benin: cultivar inventory, yield assessment, infested stages and loss due to fruit flies (Diptera Tephritidae). Fruits 63:335-348

Vayssieres J-F, Sinzogan A, Korie S, Ouagoussounon I, Thomas-Odjo A (2009a) Effectiveness of spinosad bait sprays (GF-120) in controlling mango-infesting fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 102:515-521

Vontas J, Hernández-Crespo P, Margaritopoulos JT, Ortego F, Feng H-T, Mathiopoulos KD, Hsu J-C (2011) Insecticide resistance in tephritid flies. Pesticide Biochemistry and Physiology 100:199-205

Waage J, Greathead D (1992) Protection Intégrée contre les ravageurs. In: RH: Manuel de lutte biologique. Premier tome: Principes et application de la lutte biologique PNUD/FAO, pp 5-15

Wayne L, Brenner D, Colwell R, Grimont P, Kandler O, Krichevsky M, Moore L, Moore W, Murray R, Stackebrandt E (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. International Journal of Systematic and Evolutionary Microbiology 37:463-464

Weems H, Heppner J, Nation J, Fasulo T (2012) Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Insecta: Diptera: Tephritidae). University of Florida Extension Services Publication 21:EENY-083/IN240 doi:http://edis.ifas.ufl.edu/in240

White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Applied and Environmental Microbiology 61:1104-1109

Yee WL, Lacey LA (2003) Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of *Steinernema* nematodes. Biological Control 27:349-356

Yi Y, Park HW, Shrestha S, Seo J, Kim Y-O, Shin CS, Kim Y (2007) Identification of two entomopathogenic bacteria from a nematode pathogenic to the oriental beetle, *Blitopertha orientalis*. Journal of Microbiology and Biotechnology 17:968

Zhang C, Liu J, Xu M, Sun J, Yang S, An X, Gao G, Lin M, Lai R, He Z (2008) *Heterorhabditidoides chongmingensis* gen. nov., sp. nov.(Rhabditida: Rhabditidae), a novel member of the entomopathogenic nematodes. Journal of Invertebrate Pathology 98:153-168

Zwolfer H (1983) Life systems and strategies of resource exploitation in tephritids. In: Fruit flies of economic importance. Proceedings of the CEC/IOBC International Symposium, Athens, Greece, 16-19 November 1982. AA Balkema, pp 16-30

2 Chapter 2:

Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*) under laboratory conditions

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Author's contribution:

AG designed and performed the experiments. AG and LZ analyzed the data. AG wrote the manuscript. LZ, WD, AW and LA revised the manuscript.

2.1 Abstract

Bactrocera dorsalis fruit fly is the economically most significant tephritid pest species on Mango, *Mangifera indica L.*, in Benin, and entomopathogenic nematodes (EPNs) represent good candidates for its control in the soil. In this study, the susceptibility of larvae and pupae of *B. dorsalis* to 12 EPN isolates originating from Benin was investigated. The effect of nematode concentrations (20, 50, 100, 200 and 300 Infective Juveniles (IJs)/ *B. dorsalis* larva) and of different substrate moisture content (10, 15, 20, 25 and 30% v/w) on *B. dorsalis* mortality at the larval stage was studied. Also, the reproduction potential inside *B. dorsalis* larvae was assessed. Our results revealed that the susceptibility of *B. dorsalis* larvae was significantly different among the 12 tested nematode isolates with *H. taysearae* isolate Azohoue2 causing the greatest insect mortality (96.09±1.44%). The lowest insect mortality (7.03±4.43%) was recorded with *Steinernema* sp. strain Bembereke. Significant differences in insect mortality were recorded when EPNs were applied at varying IJ concentrations. A concentration of 100 nematodes of either *H. taysearae* Azohoue2 or *H. taysearae* Hessa1 per *B. dorsalis* larva was enough to kill at least 90% of *B. dorsalis* larvae. Larvae were less susceptible to nematodes at higher moisture content (25% and 30%). In addition, pupae were less susceptible to nematodes than larvae. Furthermore, the tested nematode isolates were able to reproduce inside *B. dorsalis* third instar larva or pupa with the *Heterorhabditis* isolates giving the greatest multiplication rate (59577.20 IJs ± 14307.41).

2.2 Introduction

Mango (*Mangifera indica* L., Anacardiaceae) is one of the most important tropical fruits produced in West Africa, a region most favorable for fruit production and export (Gerbaud 2007; Vannière et al. 2004; Vayssieres et al. 2009a). Mango fruit constitutes a very important source of nutrition for rural populations living in Northern Benin (Vayssieres et al. 2008). In Africa and particularly in Benin, the production of this fruit is confronted with several problems including quality loss due to fruit flies (Tephritidae, Diptera), especially *Ceratitis capitata, Ceratitis cosyra* and *B. dorsalis* (Vayssières et al. 2009b). The latter, formerly known as *Bactrocera invadens* (Schutze et al. 2015), is the most important pest causing serious damage in orchards of mango as well as in other important tropical fruit crops including guava and citrus (Goergen et al. 2011; Vayssières et al. 2009b). Chemical applications have been used as traditional methods to control these fruit flies for many years. For example, Spinosad GF-120 (Spinosad + foodstuff attractant) and Proteus 170 O-TEQ (Thiaclopride + Deltamethrine) showed great performance for control of flies (N'depo et al. 2015; Vayssieres et al. 2009a). However, the environmental side- effects have led to interest in other, environmental friendly, cost effective and locally available control strategies to inhance mango production and export. In this respect, several control methods have recently been developped including the sterile insect technique (Clarke et al. 2011) and the biological control based on the use of weaver ants, *Oecophylla smaragdina* and *Oecophylla longinoda*, (Anato et al. 2015a; Offenberg et al. 2013; Wargui et al. 2015). Unfortunatly, the latter method is associated with some constraints as the ants delay the labor during harvest and are responsible for small black spots left on the fruit (Sinzogan et al. 2008).

EPNs of the genera *Steinernema* (Panagrolaimomorpha: Steinernematidae) and *Heterorhabditis* (Rhabditomorpha: Heterorhabditidae) are effective biocontrol agents (Grewal et al. 2005). They have been found in most countries and are successfully used to control many insect pests around the world (Ehlers 2001). Several strains of *Heterorhabditis taysearae*, *Heterorhabditis indica* and *Steinernema* sp. have been isolated from Benin and all demonstrated a cruiser type insect search strategy (Zadji et al. 2014c). *H. taysearae* Shamseldean, Abou El-Sooud, Abd-Elgawad and Saleh, 1996, has been recently considered as a senior synonym of *Heterorhabditis sonorensis* Stock, Rivera-Orduño and Flores-Lara, 2009 by Hunt and Subbotin (2016).

The Infective Juvenile (IJ) represents the only free-living developmental stage of EPNs that occurs naturally in the soil. They are symbiotically associated with bacteria of the family *Enterobacteriaceae* which belong to the genera *Xenorhabdus* (*Steinernema*) or *Photorhabdus* (*Heterorhabditis*) (Ciche et al. 2006). IJs of both genera *Steinernema* and *Heterorhabditis* can infect the insect larvae via body openings such as anus, mouth or spiracles (Campbell and Lewis 2002). In addition to these ways of penetrating the insect host, *Heterorhabditis* species are able to actively enter the hemocoel through the host cuticle by the use of their additional dorsal tooth to perforate the inter-segmental membrane of the cuticle (Bedding and Molyneux 1982; Griffin et al. 2005). Inside the host they release intestinal bacteria into the insect hemocoel. These bacteria reproduce and produce metabolites that kill the insect within 1-2 days (Dowds and Peters 2002) and serve at the same time as food source for the nematode. An effective sustainable *B. dorsalis* management approach could be the use of EPNs to control insect pests at soil-borne stages of the insect life cycle. Indeed, the late larval instar of *B. dorsalis* leaves the infested fruit and falls on the ground where it burrows in the top 4 cm of the soil prior to pupating after a short dispersal period (Hou et al. 2006). Adult flies emerge from pupae after 1-2 weeks (longer in cool conditions). This offers an opportunity to EPN IJs present in the soil to invade *B. dorsalis* larvae or pupae even if the exposure time to the larvae is relatively short. Many studies have been conducted on the Mediterranean fruit fly *Ceratitis capitata* (Gazit et al. 2000; Lindegren et al. 1990; Lindegren and Vail 1986; Malan and Manrakhan 2009; Minas et al. 2016; Poinar and Hislop 1981), the Queensland fruit fly *Bactrocera tryoni* (Froggatt) (Langford et al. 2014), the cherry fruit fly *Rhagoletis cerasi L*. (Herz et al. 2006) (Herz et al. 2006) (Herz et al. 2006) (Herz et al. 2006), *Bactrocera oleae* (Sirjani et al. 2009), *Bactrocera cucurbitae*, *B. dorsalis* (Lindegren and Vail 1986) and the Natal fruit fly *Ceratitis rosa* (Malan and Manrakhan 2009) and have demonstrated that the flies were highly susceptible to *Steinernema* and *Heterorhabditis* nematodes.

Based on these previous studies and their known biocontrol abilities, EPNs of the families Heterorhabditidae and Steinernematidae in association with their symbiotic bacteria *Photorhabdus* and *Xenorhabdus* respectively, are considered to be promising biocontrol candidates against *B. dorsalis* on mango trees in Benin.

Several studies have revealed that indigenous EPNs are well adapted to local environmental conditions and therefore considered as good biological agents to control insect pests (Bedding 1990; Grewal et al. 1994; Noujeim et al. 2015; Zadji et al. 2014c). To our knowledge, the susceptibility of *B. dorsalis* to Beninese EPNs has not yet been investigated. The current study is one of a series anticipated for the implementation of cost-effective *B. dorsalis* management using EPNs in mango orchards in Benin. It aimed to: (i) investigate the occurrence of EPNs in mango orchards in Northern Benin, (ii) identify the recovered EPN isolates, (iii) test their pathogenicity against mango fruit fly (*B. dorsalis*) under laboratory conditions. Specifically, 12 EPN isolates from Benin were screened for their virulence against the third instar larvae of *B. dorsalis* and the most virulent isolates were selected to investigate the susceptibility of larvae and pupae of *B. dorsalis* under different abiotic laboratory conditions.

2.3 Materials and methods

2.3.1 Source of insects

B. dorsalis used in this study were obtained from laboratory rearing initiated from *B. dorsalis* pupae provided by IITA-Benin (International Institute of Tropical Agriculture-Benin). The original colony of *B. dorsalis* used at the IITA- Benin institute was established from naturally infested mango fruits collected in Northern Benin. Flies were fed with a mixture of brown sugar and yeast extract at 3:1 proportion (Vayssières et al. 2015a). Cages were supplied with water. Ripened papaya fruits were exposed to 10 day old female flies to allow them laying eggs into the ripened papaya used as host. The infested papaya was incubated at 28°C and 60–80% relative humidity (RH) during 7 days, after which the third instar of *B. dorsalis* larvae started to exit the fruit. We used in our assays the third instar larvae collected approximately 1 hour after they had jumped from infested papaya to pupate. Larvae that were not used in assays were left in sand with 10% humidity to pupate and become adults within approximately eight days.

2.3.2 Source of nematodes

Most of the nematodes used in this study were provided by the Laboratoire de Phytotechnie, d'Amélioration et de Protection des Plantes (LaPAPP), Benin. They were collected from soil in several vegetations (Table 2.1) in Benin (Zadji et al. 2013). Other nematodes were newly

collected from a local soil sampling (January- February 2015) exclusively in several mango orchards located in Northern Benin. Seventy soil samples in total were collected from fourteen mango orchards (each at least 1 hectare of area) selected at random in eight villages of Parakou, Borgou department located in Northern Benin. In each orchard, 5 samples of approximately 1.5 kg each were taken randomly at ≤ 15 cm depth. Each soil sample was individually processed for nematode extraction using the *Galleria mellonella* (Lepidoptera, Piralidae) baiting method (Bedding and Akhurst 1975) and white trap (White 1927). Pathogenicity of the isolated nematodes was confirmed by re-infesting fresh *G. mellonella* larvae as described above and newly emerged IJs collected from white trap were kept at 13°C for further study.

The nematode species, sample number, origin, vegetation and accession numbers of all EPN isolates included in this study are presented in Table 2.1. Nematodes used for the assays were acclimated for 2 hours at room temperature (25°C) after removal from incubator (13°C) to help them adjust to the new temperature and allow better performance. Nematode viability (based on their movement) was checked under a stereomicroscope. The concentrations of nematodes were calculated by volumetric dilutions in tap water using the formula of Navon and Ascher (2000).

2.3.3 Nematode identification

The identity of most of the nematode isolates provided by the LaPPAP laboratory was described by Zadji et al. (2013). However, new nematode isolates retrieved from soil samples collected in mango orchards were identified in this study (Table 2.1).

2.3.3.1 Molecular identification

For each nematode isolate, DNA was extracted from a single specimen in an Eppendorf tube (250 µl) containing 1 µl of double distilled water. Ten µl of 0.05 N NaOH was added plus 1 µl of 4.5% Tween 20 solution (Janssen et al. 2016). The tube was heated at 95°C for 15 min and cooled at room temperature prior to storage at 4°C for use within next month or at -20°C for later use. The ITS region was amplified and sequenced using the primers pair AB28 (ATATGCTTAAGTTCAGCGGGT) and TW81 (GTTTCCGTAGGTGAACCTGC). ITS sequences were aligned with their closest BLAST search matches (obtained from GenBank database) using ClustalW Multiple alignment. Afterwards, a phylogenetic tree was generated in

Mega-6 software using the Neighbor-Joining method (Saitou and Nei 1987). *Caenorhabditis elegans* EU131007 was used as outgroup.

2.3.3.2 Morphological/morphometric identification

Light microscopic pictures were taken using a Soft Imaging System GmbH (Cell^D software, Münster-Germany) connected to an Olympus BX51 microscope. The same system was used to measure 20 IJs and, 20 F1 males of the studied nematode isolates. Juveniles were heat killed and mounted on temporal slides while males were fixed and mounted on permanent slide for measurements.

2.3.3.3 Cross breeding

To confirm the reproduction compatibility of the new *Heterorhabditis* isolates with described ones, cross-hybridization tests were performed on lipid agar (Wouts 1981) according to the method of Phan et al. (2003). Indeed, it was assumed that mating between male and female of the same species should produce fertile offspring (Nguyen 2007). Crossings were restricted to the newly isolated *Heterorhabditis* nematode isolates and *H. taysearae* Hessa1, described by Zadji et al. (2013). Twenty males and 20 virgin females of the appropriate nematode strains were crossed. Controls consisted of incubating 20 virgin females without males (virginity test) and 20 males x 20 females of the same isolate (self-cross test). Plates were incubated at 25°C for 2-4 days after which the presence of juveniles was examined. Results were considered valid only when the self-cross test was positive and the virginity test negative.

2.3.4 Pathogenicity tests

2.3.4.1 Screening of insect mortality induced by nematode isolates

Twenty four well plates were used. Each well (3.14 cm^2 surface area) was filled with 1 ml of heat-treated (80°C, 72 h) sand (grain size < 2 mm). One hundred IJs suspended in 200µl of tap water were transferred into each well in order to obtain 20% (v/w) moisture content. Controls received only 200 µl tap water (without nematodes). Thereafter, one third instar *B. dorsalis* larva was placed on top of the sand in each well to allow them to burrow in the sand naturally. Plates were arranged in a completely randomized block design with three replications (a plate with 24 wells represented one replicate for each EPN isolate or the control), and kept in dark at 28°C. A

replicate (bloc) was consisted of 13 treatments (12 isolates plus one control). After 48 hours of incubation, insects were retrieved from the sand of individual wells. The number of dead larvae was recorded and pupae from the same twenty four well plate were transferred into a small plastic container (7 cm diameter x 5.5 cm height). The plastic container was covered with a perforated lid to allow aeration before being kept at 28°C. After 14 days, emerged flies as well as unemerged pupae were recorded. We hypothesized that after 14 days the pupae that had not developed into adult (flies) had been killed by nematodes. Therefore, at most five dead insects (larvae and unemerged pupae) were randomly selected and individually dissected after being kept at room temperature for 48 hours to ascertain their infection by nematodes.

The number of dead larvae was added to that of unemerged pupae to determine insect mortality. The experiment was repeated twice with different batches of nematodes. All nematode isolates in Table 2.1 were involved in this study.

2.3.4.2 Effect of nematode concentrations on *Bactrocera dorsalis* mortality

H. taysearae isolates Azohoue2 and Hessa1 and *Steinernema* sp. isolate Thui were selected to examine the effect of their concentration on *B. dorsalis* mortality because they induced higher insect mortality among isolates of their species in the screening experiment (2.4.1). The isolate *H. indica* Ayogbe1 which also induced a higher insect mortality was not included because it was contaminated by fungi during the experiment and has been discarded. The experiment arena consisted of a 24-well plate as described above. Different nematode concentrations of 20, 50, 100, 200 and 300 IJs/ well corresponding to 6, 16, 32, 64 and 95 IJs/cm2 respectively, were tested at 20% (v/w) moisture content. Controls received only 200 µl tap water (without nematodes). Three plates (replicates) were used per treatment (isolate x concentration). They were arranged in a completely randomized block design with all plates of the same replicate representing each bloc. The experiment was repeated twice with different batches of the three nematode isolates. Insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes.

2.3.4.3 Effect of soil moisture on the pathogenicity of EPNs to *Bactrocera dorsalis*

Two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* sp. (Thui) were used to examine the effect of soil moisture on insect mortality. Different soil moistures were tested to determine the optimal soil moisture content that is conducive to the nematode isolates to control *B. dorsalis.* Sandy soil was heat-treated as above mentioned and wetted to reach the final moisture content (v/w) of 10, 15, 20, 25 and 30% including the water added with the nematode suspension. Nematodes were applied at 100 IJs per *B. dorsalis* larva as described above and plates were incubated in dark at 28°C for 48 hours. The experiment was repeated twice with different batches of the three nematode isolates. Insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes.

2.3.4.4 Comparative susceptibility of larvae and pupae of *Bactrocera dorsalis* to entomopathogenic nematodes

Two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* sp. (Thui) were used to examine the susceptibility to EPNs of *B. dorsalis* pupae compared to that of the larvae. Third instar pupating *B. dorsalis* larvae were collected and put on sand adjusted to 10% moisture content (v/w). Pupae were collected after 24h, 48h and 72h from the same larvae batch, and pupae collected at each time were considered to be of the same age (less than one, two and three days, respectively) as we cannot know exactly when the larvae have pupated. The experiment arena consisted of a 24-well plate and nematodes were applied in the same conditions as described above at 10% moisture content with 100 IJs per pupa or larva of *B. dorsalis*. Plates were arranged in a completely randomized block design and incubated in the dark at 28°C for 48 hours. Insect mortality (larvae and pupae) was determined in the same conditions as described above.

2.3.4.5 Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

H. taysearae (Azohoue2 and Hessa1) and *Steinernema* sp. (Thui) isolates were used to evaluate their ability to find *B. dorsalis* in sand substrate. Pieces of PVC tubing (diameter 4 cm) of different length (5 cm, 10 cm, 15 cm and 20 cm) were filled with sterile sand adjusted to 10%

humidity. Third instar larvae of *B. dorsalis* were placed individually at one end of each piece of PVC tubing as described by (Zadji et al. 2014c) and nematodes (100 IJs) were inoculated at the other end of the PVC tubing. Per combination PVC tube length × isolate, three replicates were performed, and ten PVC tubings were assigned for each replicate.

Water evaporation was controlled by closing both ends of the PVC tubings with plastic lids to maintain constant humidity during the experiment. PVC tubings were maintained vertically, with EPNs on top and the larvae at the bottom, at 28°c during 48 h after which insect mortality (larvae and pupae) was determined in the same conditions as described above. At most five dead insects were randomly selected and individually dissected to confirm death by nematodes. This assay was repeated twice with different batches of nematodes.

2.3.4.6 Nematode reproduction in *Bactrocera dorsalis* third instar larvae

Five dead *B. dorsalis* larvae of approximately the same size were randomly selected from the previous experiment (2.4.5) for each nematode isolate (*Heterorhabditis*: Azohoue2, Hessa1; and *Steinernema*: Thui) and placed individually on white trap to evaluate nematode reproduction potential in *B. dorsalis* larvae. Traps were incubated at 28°c. After approximately seven or five days for *Heterorhabtidis* and *Steinernema* isolates respectively, IJs were collected daily until no nematode was observed in the white trap. The total number of nematodes produced by a single *B. dorsalis* larva was evaluated as described by Navon and Ascher (2000).

2.4 Data Analysis

Insect mortality data were corrected for control mortality according to the formula of Abbott (1925). Data obtained for all experiments were analyzed using SAS (version 16). To stabilize the variance of means, mortality percentages were arcsine transformed and subjected to a General Linear Model analysis. Student-Newman Keul's (SNK) test when $P<0.05$ was carried out to assess efficacy differences among nematode isolates. One-way analysis of variance (ANOVA) tests were conducted to determine if concentration, moisture content and migration distance had an effect on the mortality caused by EPN isolates, while two-way ANOVAs were conducted to determine whether mortality was influenced by nematode isolates, by the treatments (concentration of IJs, moisture content, host status and migration distance), or an interaction

between the two. Probit regression analysis was performed in SPSS (16.0) software to calculate the LC_{50} of the tested nematode isolates.

2.5 Results

2.5.1 Nematode occurrence in mango orchards and identification

Two nematode isolates (KoroborororouC2 and KoroborororouF4) were retrieved from the 70 soil samples taken in mango orchards. This means that 2.86% of soil samples were positive. The two nematode isolates were isolated from two different mango orchards (KoroborororouC2: 09°22.356'N/02°41.175'E; KoroborororouF4: 09°22.287'N / 02°40.233'E) in the same village. They share 100% ITS sequence similarity with each other and with *H. taysearae* FJ477730 and 99% similarity with *H. taysearae* EF043443. Molecular identification based on the ITS regions showed that the two new nematode isolates grouped with *H. taysearae* with relatively high bootstrap value and no difference in nucleotides could not be observed with *H. taysearae* FJ477730 (Figure 2.1).

Morphological (data not shown) as well as morphometrics (Supplementary material 2.1) information confirmed the identification of the two nematodes isolates as *H. taysearae*. They share all morphological characters with *H. taysearae* previously described by Stock et al. (2009) and Zadji et al. (2013). Cross-hybridization test yielded in fertile progeny when the isolates KoroborororouC2 or KoroborororouF4 and *H. taysearae* (KF723802, Hessa1) were crossed.

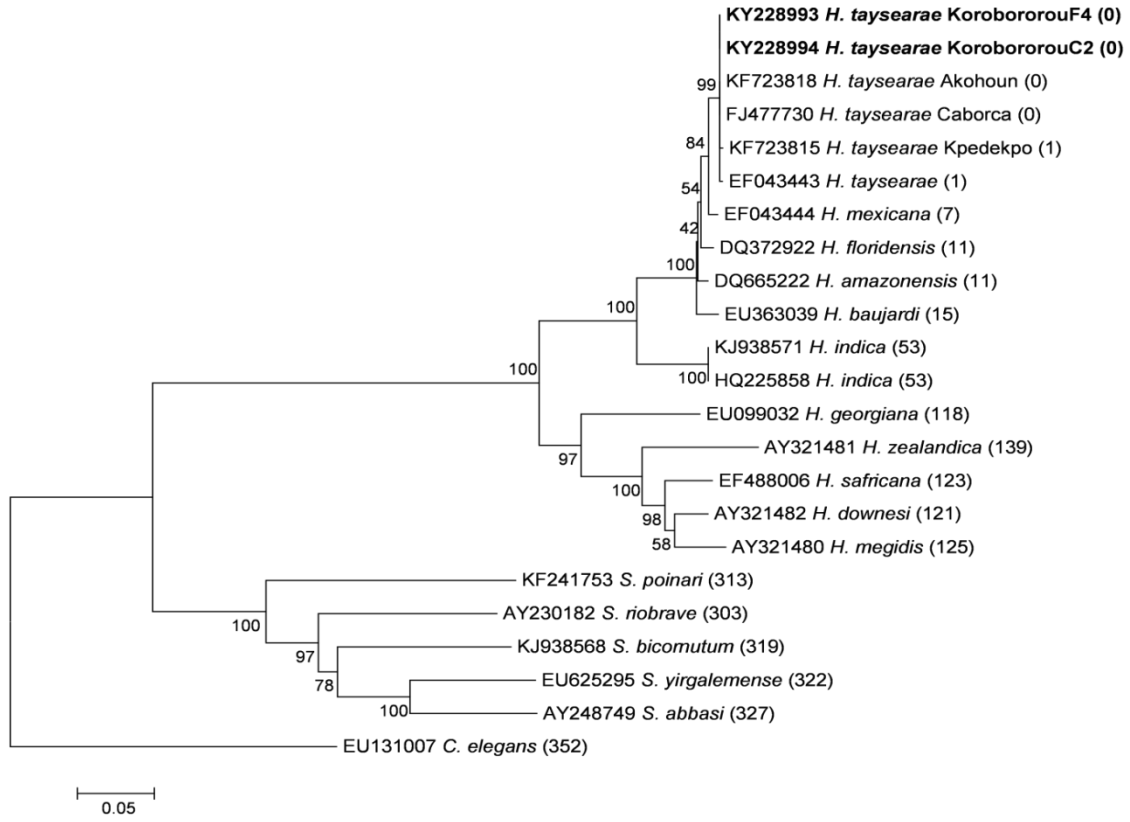

Figure 2.1 Phylogenetic relationships based on Neighbor Joining clustering of *Heterorhabditis* ITS sequences showing the position of both nematode isolates retrieved from mango orchards in Northern Benin (shown in bold). *H: Heterorhabditis*, *C: Caenorhabditis*, *S: Steinernema*. Numbers at the nodes indicate bootstrap value (1000 replicates) and numbers after species in parentheses represent nucleotide differences between ITS sequences of described species and the newly isolated *Heterorhabditis* isolates from mango orchards.

2.5.2 Screening of insect mortality induced by nematode isolates

All 12 tested nematode isolates infected *B. dorsalis* larvae. However, we recorded only some dead larvae (which died before pupating) and most *B. dorsalis* died as pupae (larvae which have pupated despite nematode infection). Susceptibility of *B. dorsalis* larvae was significantly different among the 12 tested nematode isolates ($F= 62.03$; $df= 11, 60$; $P<0.001$). The percentages of insect mortality varied between 7.03% and 96.09% (Table 2.1). The greatest insect mortality was recorded for the *H. taysearae* isolate Azohoue2 (96.09%) followed by *H. taysearae*

Hessa1 (94.53%) and *H. indica* Ayogbe1 (93.75%) (Table 2.1). The two latter isolates were not significantly different in causing *B. dorsalis* mortality. *Steinernema* isolates Thui and Bembereke induced lower insect mortalities (69.53% and 7.03% respectively) with the latter causing the lowest mortality percentage to *B. dorsalis* among all tested nematode isolates (Table 2.1).

2.5.3 Effect of nematode concentrations on *Bactrocera dorsalis* mortality

Difference in *B. dorsalis* insect mortality was significant among nematode isolates ($F= 98.89$; $df= 2, 75$; $P < 0.0001$) and among IJ concentrations ($F= 31.60$; $df= 4, 75$; $P< 0.0001$). However, the interaction insect mortality x IJ concentration was not significantly different ($F= 1.49$; $df= 8, 75$; $P= 0.1736$). Detailed analysis showed that the three EPN isolates induced different levels of mortality for all tested IJ concentrations (Figure 2.2). The isolate *Steinernema* sp. Thui was the least virulent compared to the *H. taysearae* isolates (Azohoue2 and Hessa1) (Figure 2.2).

A concentration of 100 IJs/*B. dorsalis* larva corresponding to 32 IJs / cm^2 was enough to kill at least 90% of *B. dorsalis* larvae (Figure 2.2) for the *H. taysearae* isolates Azohoue2 (96.09%) and Hessa1 (94.53%) while the *Steinernema* isolate Thui could not induce the same level of mortality even when applied at high concentration (300 IJs/*B. dorsalis* larva or 95 IJs/ cm^2).

Based on the 95% confidence limits of the LC_{50} (Table 2.2), significant differences were observed among isolates, the highest LC_{50} was recorded with *Steinernema* sp. Thui. No significant difference was observed between the *H. taysearae* isolates, Azohoue2 and Hessa1 (Table 2.2).

Table 2.1 Characteristics of the 12 studied EPN isolates from Benin and mortality (%±SEM) caused to *B. dorsalis* with 100 IJs/insect

Sampling number	Nematode species	ITS accession number	Vegetation	Origin in Benin	References	% Mortality of *B. dorsalis* (±SEM)*
32b	*H. taysearae*	KF723809	Mandarin	Azohoue2	Zadji et al., 2013	96.09±1.44 a
9a	*H. taysearae*	KF723802	Lemon	Hessa1	Zadji et al., 2013	94.53±2.82 ab
51a	*H. indica*	KF723816	Mango	Ayogbe1	Zadji et al., 2013	93.75±2.32 ab
83a	*H. taysearae*	KF723828	Palm	Ze3	Zadji et al., 2013	90.62±3.42 abc
59a	*H. taysearae*	KF723818	Teak	Akohoun	Zadji et al., 2013	85.93±1.71 abcd
9d	*H. taysearae*	KF723803	Lemon	Hessa2	Zadji et al., 2013	82.81±0.99 bdc
44a	*H. taysearae*	KF723813	Orange	Kemondji	Zadji et al., 2013	76.56±4.01 de
F4**	*H. taysearae*	KY228993	Mango	Korobororou F4	This study	79.69±4.47 dec
168d	*Steinernema* sp.	KY228996	Eucalyptus	Thui	Unpublished	69.53±2.00 ef
118c	*H. taysearae*	KY228995	Cashew	Gouka	Unpublished	64.06±3.12 f
C2**	*H. taysearae*	KY228994	Mango	Korobororou C2	This study	51.56±4.63 g
157c	*Steinernema* sp.	KY228997	Gallery forest	Bembereke	Unpublished	7.03±4.43 h

SEM: Standard Error of the Mean.

*Means with the same letter are not significantly different. ** EPN isolates retrieved from soil sampled in mango orchards during January-February 2015 survey.

2.5.4 Effect of soil moisture on the pathogenicity of EPNs to *Bactrocera dorsalis*

For each nematode isolate, significant differences of *B. dorsalis* mortality were observed (F= 3.74; df= 2, 75; P= 0.0283). Also, at each soil moisture, IJs induced significantly different levels of *B. dorsalis* mortality (F=42.89; df=4, 75; P<0.0001). Furthermore, insect mortality was significantly influenced by the interactions between nematode isolates and levels of moisture content (F=7.37; df= 8, 75; P< 0.0001). Therefore, effect of soil moisture levels for each nematode isolate, and differences in mortality caused by nematode isolates at each soil moisture

level were assessed. Significant differences in mortality caused to *B. dorsalis* by the three nematode isolates were observed only at 15% (F= 5; df= 2, 15; P< 0.0217) and 25% (F= 16.66; df= 2, 15; P < 0.0002) moisture levels. Lower insect mortalities were recorded at 25-30% moisture levels for all tested nematode isolates (Figure 2.3). All the three tested nematode isolates induced similar insect mortality at 10% and 15% moisture levels for which higher mortality percentages were recorded. *H. taysearae* Azohoue2 induced higher mortality percentage at 10% (99.21%) and 15% (96.76%) than at 25% (52.7%) soil moisture, while *H. taysearae* Hessa 1 and *Steinernema* sp. Thui caused lower mortalities at 30% (70.31% and 68.75% respectively) compared to 10% (95.24% and 91.27% respectively) and 15% (96.78% and 90.32% respectively) moisture levels.

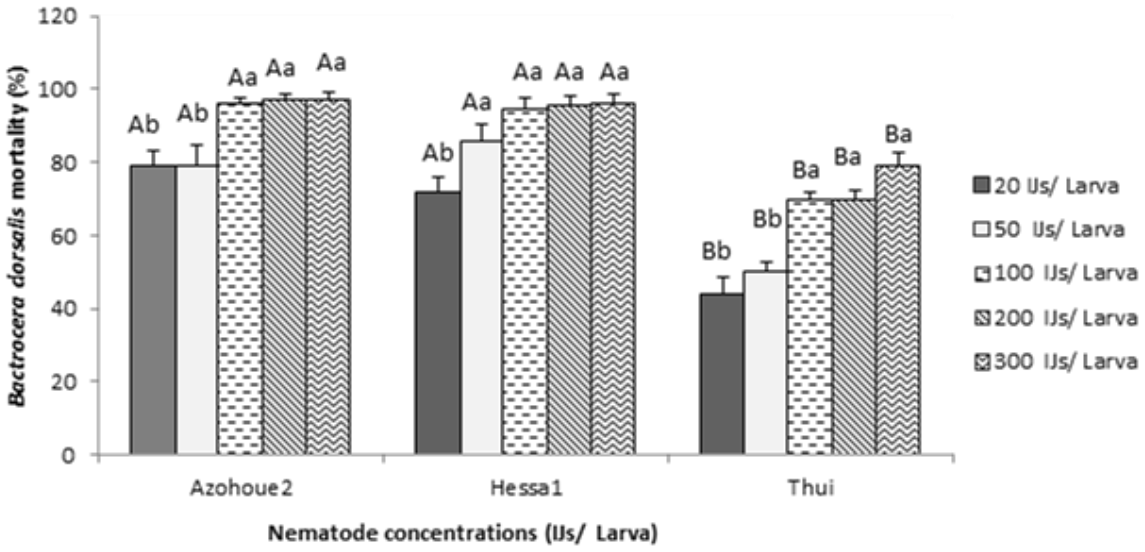

Figure 2.2 Effect of different concentrations (20, 50, 100, 200, and 300 IJs/*B. dorsalis* larva) of two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* n. sp. (Thui) on *B. dorsalis* mortality (% ±SEM). Vertical bars are standard error of the means. Bars with the same uppercase letter stand for non-significant differences existing among nematode isolates causing *B. dorsalis* mortality at the same concentration level. Bars with the same lowercase letter stand for non-significant differences existing among nematode concentrations causing *B. dorsalis* mortality for the same nematode isolate (SNK's test at P<0.05).

Table 2.2 Comparison of lethal concentrations (LC_{50}) of two *Heterorhabditis* isolates (Azohoue2 and Hessa1) and one *Steinernema* isolate (Thui) against *B. dorsalis* larvae.

Nematode isolates	Origin of nematode isolates	Probit equation[a]	Chi-square	LC_{50}[b]	95% CL[c]
H. taysearae	Azohoue2	Y= - 0.710+1.071C	8.113	4.603 b	0-18.785
H. taysearae	Hessa1	Y= - 0.803+1.097C	1.642	5.396 b	1.488-10.511
***Steinernema* n. sp.**	Thui	Y= - 1.253+0.810C	2.973	35.205a	19.503-50.820

[a] General responses of insect mortality (Y) as a function of nematode concentration (C).

[b] Nematode concentration (number of IJs per *B. dorsalis* larva) required for killing 50% of treated larvae; LC_{50} values followed by the same letter are not significantly different, based on non-overlapping 95% CL.

[c] 95% confidence limits (CL) for the LC_{50}.

2.5.5 Comparative susceptibility of larvae and pupae of *Bactrocera dorsalis* to EPNs

Results showed that both larvae and pupae (up to three days old) were susceptible to nematodes. Insect mortality recorded with infected larvae and 1-3 day old infected pupae revealed significant differences ($F= 400.13$; $df= 3, 60$; $P< 0.0001$) and the greater mortality level (99.21%) was recorded for infected larvae. In addition, no significant differences in insect mortality were observed among tested nematode isolates ($F= 2.40$; $df= 2, 60$; $P= 0.0993$). Fully formed *B. dorsalis* pupae were less susceptible to nematodes than the third instar larvae (Figure 2.4). Furthermore, susceptibility of *B. dorsalis* pupae to EPNs decreased with age (Figure 2.4). We recorded up to 99.21% insect mortality when nematodes were applied on *B. dorsalis* third instar larvae while insect mortality induced on 1-3 day old pupae did not exceed 23% with any of the 3 tested EPN isolates (Figure 2.4).

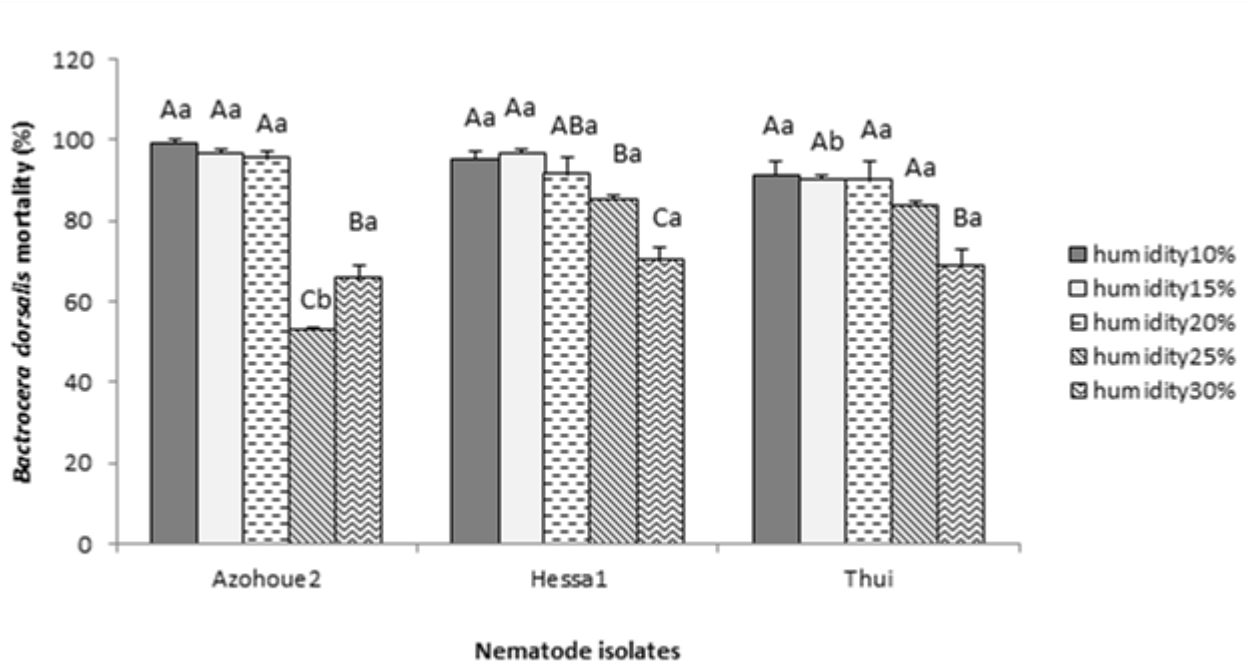

Figure 2.3 Effect of moisture content (10%; 15%; 20%; 25% and 30%) of the substrate (sterile sand) on *B. dorsalis* mortality (% ±SEM) exposed to two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* n. sp. (Thui). Vertical bars are standard error of the means. Bars with the same uppercase letters stand for non-significant differences existing among levels of moisture content for the same nematode isolate. Bars with the same lowercase letters stand for non-significant differences among nematode isolates for the same level of moisture content (SNK's test at $P<0.05$).

2.5.6 Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

The three tested nematode isolates were capable of causing *B. dorsalis* mortality at all tested migration distances up to 20 cm except *H. taysearae* Hessa1 which induced no mortality at 20 cm. In addition, low levels of *B. dorsalis* mortality were recorded in general for all tested distances (Table 2.3). Results revealed that larvae/pupae mortality varied significantly with migration distance ($F= 10$; $df= 3, 228$; $P<0.0001$), with greater mortality levels recorded at 5 cm (Table 2.3). However, no significant difference was found in *B. dorsalis* mortality according to nematode isolates used ($F= 1.42$; $df= 2, 228$; $P= 0.2431$).

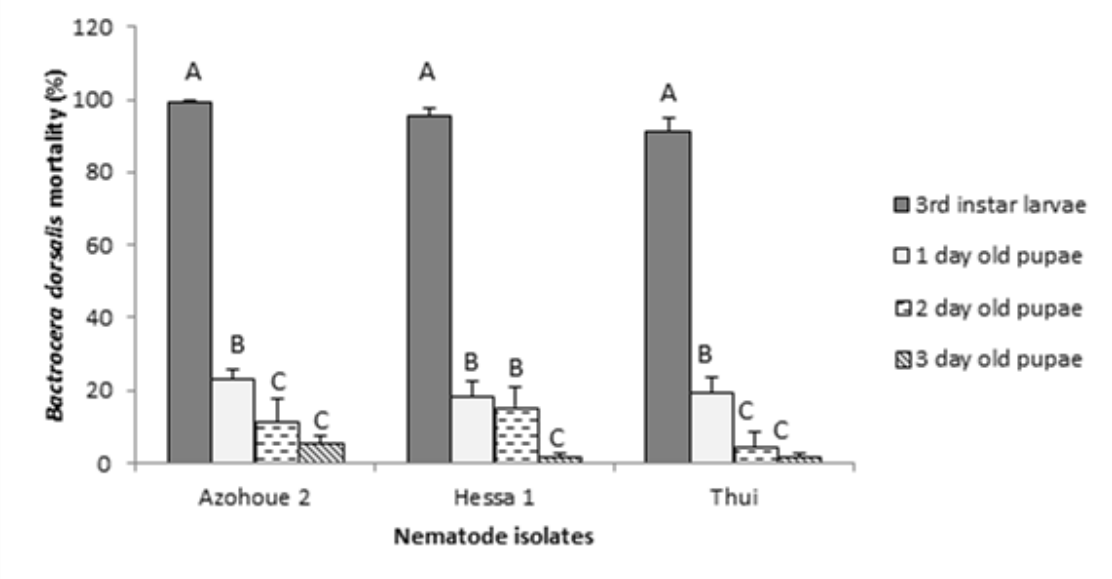

Figure 2.4 Comparison of susceptibility of *B. dorsalis* pupae and third instar larvae (% ± SEM) to two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* n. sp. (Thui) (100 IJs/ pupa or larva). Vertical bars are standard error of the means. Bars with different letters stand for significant differences among insect developmental stages (SNK's test at P<0.05).

Table 2.3 Mortality of *B. dorsalis* (Means ± SEM) caused by different nematodes isolates (100 IJs/larva) at 5, 10, 15 and 20 cm depth (distance between insect and nematode inoculation point).

Nematode isolates	Mortality (% ± SEM)			
	5 cm distance	10 cm distance	15 cm distance	20 cm distance
H. taysearae Azohoue2	45 ± 11.41 Aa	20 ± 9.17 Ba	5 ±5.00 Ba	5± 5.00 Ba
H. taysearae Hessa1	40 ± 11.24 Aa	10 ± 6.88 Ba	5 ±5.00 Ba	0.00 Ba
Steinernema sp. Thui	15 ± 8.19 Aa	10 ± 6.88 Aa	10 ± 6.88 Aa	5 ± 5.00 Aa

SEM: Standard Error of the Mean. Means (% ± SEM) with the same uppercase letter are not significantly different for the same nematode isolate. Means with the same lowercase letters stand for non-significant differences among nematode isolates for the same migration distance (SNK's test; P< 0.05).

2.5.7 Nematodes reproduction in *Bactrocera dorsalis* third instar larvae

The reproduction potential of EPNs inside third instar larvae of *B. dorsalis* varied significantly according to nematodes isolates (F=9.26; df= 2, 12; P=0.0037). It was possible to yield up to 59577.2 ±14307.41 IJs from larvae infested with the *Heterorhabditis* isolates (Figure 2.5) which showed the greatest multiplication rate compared to the *Steinernema* one (4858.2 ±890.28 IJs per *B. dorsalis* third instars larva).

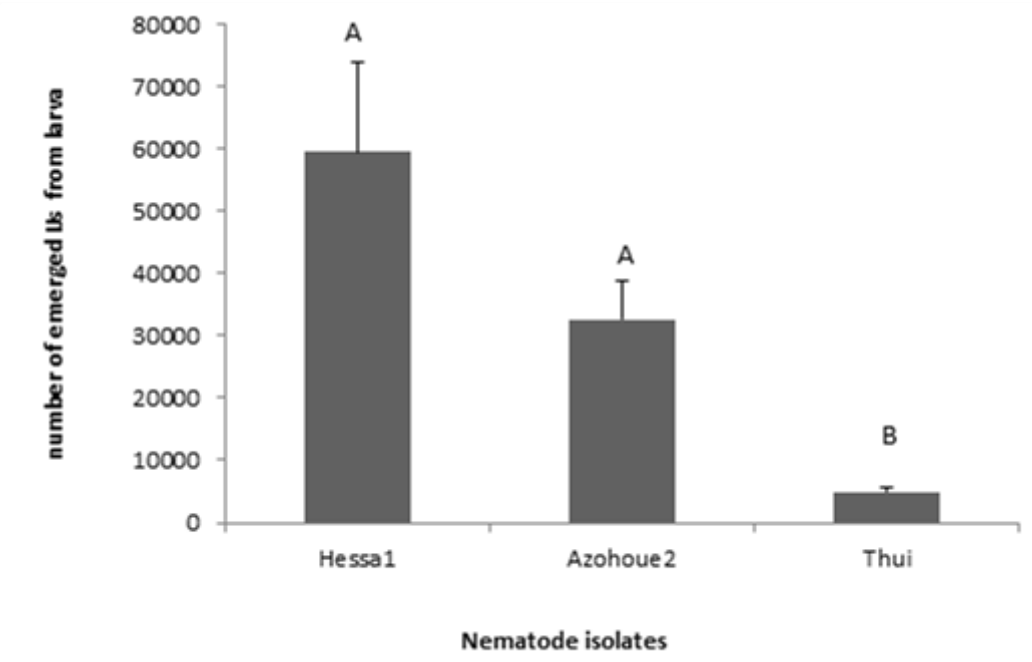

Figure 2.5 Reproduction potential of two isolates of *H. taysearae* (Azohoue2 and Hessa1) and one of *Steinernema* n. sp. (Thui) in third instar larvae of *B. dorsalis*. Vertical bars are standard errors of the means. Bars with the same letters stand for non-significant differences of EPN reproduction among tested nematode isolates (SNK's test at P<0.05).

2.6 Discussion

In this study, we investigated the susceptibility of *B. dorsalis*, a serious mango pest in Benin, to indigenous EPN isolates recovered from soil samples collected in mango orchards and other vegetations in Benin. EPNs are, in most cases, more effective in their natural environment than exotic ones (Bedding 1990). Therefore, exploring the natural occurrence of EPNs in mango

orchards in Northern Benin was a first step towards their application in biocontrol. Laboratory investigations to screen available isolates for effectiveness on *B. dorsalis* in variable abiotic conditions were then required before conducting field assays on a reduced number of isolates. This knowledge will help us to optimize their application in mango orchards and other environments they may be applied to. In this way, their possibilities to be introduced in the commercial market will be increased and economical losses due to *B. dorsalis* will be reduced (Gazit et al. 2000; Ma et al. 2013).

2.6.1 Nematode occurrence in mango orchards and identification

A prospection in several mango orchards in Northern Benin revealed only 2.86% of positive samples. This percentage of positive samples is lower than reported (11.43%) in southern Benin during the rainy season by Zadji et al. (2013), but still fits the range (2% to 45%) of EPN occurrence specified by Hominick (2002). However, the number of EPN isolates retrieved in our study from mango orchards might represent an underestimation since the prospection was done during the dry season (when nematode activity is limited) over a reduced number of sites (14 mango orchards).

The identification of the two nematode isolates as *Heterorhabditis taysearae* constitutes a confirmation of the wide occurrence of that species in Benin as was reported for the first time by Zadji et al. (2013). *H. taysearae* were originally reported by Shamseldean et al. (1996) and later isolated in Mexico (Stock et al. 2009) from the Sonoran desert which shares a tropical climate with Benin.

The effective presence of EPNs in surveyed mango orchards is a promising result for any future application of EPNs as this ensures that they can establish and persist in this ecological environment. Also an eventual EPN application could take into account their natural initial population in the orchard.

2.6.2 Screening of insect mortality induced by nematode isolates

Our laboratory experiments demonstrated the susceptibility of *B. dorsalis* larvae to all twelve tested nematode isolates. Great larval mortality (up to 96.09%) was caused by the *Heterorhabditis* isolates with *H. taysearae* isolates Azohoue2 and Hessa1 being highly

pathogenic to *B. dorsalis*. These results confirm earlier findings of (Zadji et al. 2014a) who demonstrated the same EPN isolates causing the highest mortality (98.6%) to *Macrotermes bellicosus* in citrus orchards. Furthermore, low insect mortality percentages were recorded with the two tested *Steinernema* isolates with *Steinernema* n. sp. strain Bembereke inducing the lowest insect mortality (7.03±4.43) among all tested EPN isolates. This could be explained by the fact that IJs of *Steinernema* species penetrate an insect host only via natural openings, while *Heterorhabditis* species are equipped with a dorsal tooth (Griffin et al. 2005) that they can use to puncture the cuticle of the insect pest to penetrate their body through the integument. *Heterorhabditis* and *Steinernema* nematodes are known to live in close association with different symbiotic bacteria (Boemare 2002a). Even though other virulence factors are involved in the death of the insect host induced by EPNs (Ensign and Ciche 2000; Zadji et al. 2014c), the toxicity of the associated bacterial symbiont could also be a contributing factor.

2.6.3 Effect of nematode concentration on *Bactrocera dorsalis* mortality

The three selected nematode isolates caused different *B. dorsalis* mortality at varying nematode concentrations with *Heterorhabditis* strains causing the highest mortality level. These results confirm those obtained above with the initial screening test where *H. taysearae* strains Azohoue2 and Hessa1 were highly pathogenic to *B. dorsalis* larvae. The highest LC_{50} (95% confidence limit) was recorded with *Steinernema* n. sp. Thui isolate which confirms its lower performance in killing *B. dorsalis* larvae compared to the two *H. taysearae* isolates. For all isolates, there was no significant increase in larval mortality as nematode concentration augmented beyond 100 IJs/ larvae (or 32 IJs /cm^2) meaning that a concentration of 32 IJs /cm^2 was enough to obtain the optimal *B. dorsalis* mortality in our experimental conditions. This optimal EPN concentration is much lower than reported in the literature by Minas et al. (2016) and Gazit et al. (2000) who conducted similar work on other tephritid pests. For example, Minas et al. (2016) reported 87% mortality of *C. capitata* when a *H. baujardi* strain was applied at much higher concentration (237 IJs/cm^2). In addition, Gazit et al. (2000) demonstrated that 100 IJs/cm^2 of *S. riobrave* could induce 82.5% of *C. capitata* mortality.

2.6.4 Effect of soil moisture on the pathogenicity of EPNs to *Bactrocera dorsalis*

We recorded significant differences in insect mortality when nematodes where applied at different moisture content (10%-30%). This means that sand moisture level influenced nematode activity in causing *B. dorsalis* mortality under our experimental conditions. Langford et al. (2014) reported significant differences in *B. tryoni* mortality when EPNs were applied at 10-25% substrate moisture. However, Gazit et al. (2000) stated that soil moisture does not affect *S. riobrave* activity in controlling *C. capitata* at larval stages. More interestingly, we observed that nematode performance in killing *B. dorsalis* at the late larval stage is reduced at high levels (25% and 30%) of soil moisture while higher mortalities were recorded at 10-15% soil moistures. This is in contrast to Langford et al. (2014) who reported low insect mortality when nematode were applied at 10% substrate moisture while higher mortality percentages were observed at 25% soil moisture. Basically, nematodes are aquatic animals that require water to maintain their activity. However, some nematode species including bacterial feeding nematodes like EPNs have the ability to be active in soil even when water films are thin (Gaugler and Bilgrami 2004). In water saturated substrate, oxygen diffusion rate may be compromised (Kaya 1990), thus inhibiting nematode locomotion and persistence (Kung et al. 1990; Patel et al. 1997). Moreover, under conditions of high substrate moisture, nematodes are more active (Kable and Mai 1968) and quickly lose their stored energy. Therefore their pathogenicity potential is reduced (Kung et al. 1991). The contrast between our results and those of Langford et al. (2014) may be due to the effect of soil moisture content on the pest itself which, could die of suffocation (Hulthen and Clarke 2006). In this respect, Shapiro-Ilan et al. (2006) reported no nematode effect (though high mortality level >90%) on *Cucurlio caryae* (Pecan weevil) at 23.6% soil moisture because of the sensitivity of the latter to high moisture level. In our case, *B. dorsalis* larvae successfully complete their development at soil moisture ranging from 10 to 70% (Hou et al. 2006) which means that nematodes were the most responsible for insect mortality at that moisture level of soil.

According to (Vayssières et al. 2015a), *B. dorsalis* occurred in mango orchard from April to May, corresponding to the beginning of the rainy season when soil moisture is still relatively low. We therefore hypothesize based on our results that in these relatively low humidity conditions of the

soil, nematodes are active and control of *B. dorsalis* soil-borne stages will be enhanced, reducing then future populations of the insects in the orchard.

2.6.5 Comparative susceptibility of larvae and different developmental stages of *Bactrocera dorsalis* pupae to EPNs

All three EPN isolates were able to induce *B. dorsalis* larval and pupal mortality. Late instar larvae of *B. dorsalis* were more susceptible than pupae to all nematode isolates tested. Furthermore, older pupae were less susceptible to nematodes than younger ones. We could obtain 22.8% mortality of 1 day old pupae treated with *H. taysearae* Azohoue2 using 32 IJs / cm^2 while 5.56% mortality was recorded for 3 days old pupae treated with the same nematode isolate at the same concentration. These findings agree with earlier work of Gazit et al. (2000) who reported that *S. riobrave* could cause up to 20% mortality in young pupae of *C. capitata*. In addition, recent work of Minas et al. (2016) revealed that up 100% mortality of *C. capitata* 1 day old pupae could be achieved when applying *H. baujardi* LPP7 at higher concentration (1079 IJs/ cm^2). However, this is in contrast to Langford et al. (2014), Malan and Manrakhan (2009), and Yee and Lacey (2003) who observed no pupal susceptibility of *B. tryoni*, *C. capitata/C. rosa* and *R. indifferens*, respectively, to EPNs. It is known that the body of pupae is much harder (due to the sclerotization) than that of the larvae, making nematode penetration through the insect cuticle (for *Heterorhabditis* species) much easier in larvae compared to pupae.

This result of positive (though sometimes low) susceptibility of *B. dorsalis* pupae to both EPN genera constitutes an opportunity for future control of this pest in field conditions as larvae which will escape parasitism by nematodes in soil could still be caught at pupal stage. We recommend further tests on older pupae (above 3 day- old pupae) to assess their susceptibility to nematodes. In addition, timing of EPN application should be considered so as to prioritize targeting of third instar larvae.

2.6.6 Ability of nematodes to find larva/pupa of *Bactrocera dorsalis*

The three tested EPN isolates were able to parasitize *B. dorsalis* larvae present at 15 cm distance. *H. taysearae* Azohoue2 and *Steinernema* n. sp. Thui could even induce *B. dorsalis* mortality at 20 cm distance. These results confirm the cruiser type insect search strategy of the Beninese EPN

isolates described by Zadji et al. (2014c). In addition, the overall low insect mortality recorded for all tested distances could be explained by the fact that third instar larvae spend relatively short time at that stage before they switch to pupal stage which is less susceptible to EPNs than larvae as documented above. Thus, before nematodes can migrate from the inoculation point to the insect host, the latter could have pupated explaining the low mortality levels recorded. In nature, third instar larvae of *B. dorsalis* leave the host fruit and migrate in soil where they pupate in the top 4 cm (Hou et al. 2006). This means that they could still be reached while pupating in soil in case of future nematode application under field conditions.

2.6.7 Nematodes reproduction in *Bactrocera dorsalis* third instar larvae

All three tested nematode isolates were able to reproduce in *B. dorsalis* larvae. This result has great importance for nematode establishment and persistence in mango orchards as nematode populations in the orchard could be increased upon the presence of *B. dorsalis* hosts. In addition, the higher reproduction potential of *Heterorhabditis* isolates in *B. dorsalis* host compared to the *Steinernema* could be explained by differences in multiplication rate of the associated bacterial symbiont and their number released inside the host by IJs (Grewal et al. 1997). We were able to obtain up to 59577.2 ±14307.41 IJs of *H. taysearae* produced per larval host. This value of *Heterorhabditis* isolates is considerably higher than reported by Malan and Manrakhan (2009) when *Ceratitis rosa* larvae were infested with *H. zealandica*. A number of 6171.43 ± 814.66 IJs were counted after 19- 21 days of incubation. This large difference may reside in the variability in host size which relates to food availability for nematode reproduction. Indeed, the third instar larvae or pupae of *B. dorsalis* are naturally larger than larvae or pupae of *Ceratitis rosa* as reported by Ekesi and Mohamed (2011) when those two tephritids were fed with several diets. In reality, a bigger host represented by *B. dorsalis* infected with small size IJs like *H. taysaerae* (418 µm IJ body length) should yield more progeny than a smaller host represented by *C. rosa* infected with bigger size IJs like *H. zealendica* (685 µm IJ body length).

Overall, our results showed that the susceptibility of *B. dorsalis* to *H. taysearae* was persistent under different tested abiotic conditions which, *B. dorsalis* could encounter in nature. *H. taysearae* isolates (Azohoue2 and Hessa1) therefore represent potential biological agents that may be used in the control of *B. dorsalis* in mango orchards. The concentration of 100 IJs / larva

applied at 10-15% soil moisture provided optimal results in laboratory tests. Fields trials are now required to test the effectiveness of *B. dorsalis* to these *Heterorhabditis* isolates under natural environmental conditions.

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2.8 References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18:265-267

Anato FM, Sinzogan AAC, Adandonon A, Hounlidji X, Offenberg J, Kossou DK, Vayssières J-F (2015) Impact of African weaver ant nests [*Oecophylla longinoda* latreille (Hymenoptera: Formicidae)] on mango [*Mangifera indica* L.(Sapindales: Anacardiaceae)] leaves. Sociobiology 62:39-45

Bedding R (1990) Logistics and strategies for introducing entomopathogenic nematode technology into developing countries. In: Gaugler R, Kaya HK (eds), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, pp 233-246

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Bedding R, Molyneux A (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp.(Heterorhabditidae: Nematoda). Nematologica 28:354-359

Boemare N (2002) Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler R (ed), Entomopathogenic Nematology CABI Wallingford, UK, pp 35-56

Campbell J, Lewis E (2002) Entomopathogenic nematode host-search strategies. In: Lewis EE, Campbell JF, Sukhdeo MVK (eds), The Behavioural Ecology of Parasites. CABI, Wallingford, UK, pp 13-38

Ciche TA, Darby C, Ehlers R-U, Forst S, Goodrich-Blair H (2006) Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. Biological Control 38:22-46

Clarke AR, Powell KS, Weldon CW, Taylor PW (2011) The ecology of *Bactrocera tryoni* (Diptera: Tephritidae): what do we know to assist pest management? Annals of Applied Biology 158:26-54

Dowds BC, Peters A (2002) Virulence mechanisms. In: Gaugler R (ed), Entomopathogenic Nematology. CABI, Wallingford, UK, pp 79-98

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Ekesi S, Mohamed SA (2011) Mass rearing and quality control parameters for tephritid fruit flies of economic importance in Africa. In: Akyar I (ed), Wide spectra of quality control. InTech, Rijeka, Croatia, pp 387-410

Ensign J, Ciche T (2000) Release of *Photorhabdus luminescens* cells from infective juvenile nematodes of *Heterorhabditis*. In: 2nd US/Hungarian EPN/EPB Annual Meeting, Hungary.

Gaugler R, Bilgrami AL (2004) Nematode Behaviour. CABI Publishing, UK

Gazit Y, Rossler Y, Glazer I (2000) Evaluation of entomopathogenic nematodes for the control of Mediterranean fruit fly (Diptera: Tephritidae). Biocontrol Science and Technology 10:157-164

Gerbaud P (2007) European market: mango. Fruitrop 143:11–26

Goergen G, Vayssières J-F, Gnanvossou D, Tindo M (2011) *Bactrocera invadens* (Diptera: Tephritidae), a new invasive fruit fly pest for the Afrotropical region: host plant range and distribution in West and Central Africa. Environmental Entomology 40:844-854

Grewal P, Matsuura M, Converse V (1997) Mechanisms of specificity of association between the nematode *Steinernema scapterisci* and its symbiotic bacterium. Parasitology 114:483-488

Grewal PS, Ehlers R-U, Shapiro-Ilan DI (2005) Nematodes as Biocontrol Agents. Grewal PS, Ehlers R-U, Shapiro-Ilan DI (series editors). CABI Publishing, Wallingford, UK

Grewal PS, Selvan S, Gaugler R (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. Journal of Thermal Biology 19:245-253

Griffin C, Boemare N, Lewis E (2005) Biology and behaviour. In: Grewal PS, Ehlers R-U, Shapiro-Ilan DI (eds), Nematodes as Biocontrol Agents. CABI Publishing, pp 47-64

Herz A, Köppler K, Vogt H, Elias E, Katz P, Peters A (2006) Biological control of the cherry fruit fly, *Rhagoletis cerasi* L.(Diptera, Tephriti-dae) by use of entomopathogenic nematodes: first experiences towards practi-cal implementation. In: Proceedings to the Conference Ecofruit-12th international conference on cultivation technique and phytopathological problems in organic fruit-growing, Weinsberg/Germany. Fördergemeinschaft Ökologischer Obstbau eV (FÖKO), Traubenplatz 5, D-74189 Weinsberg, pp 67-72

Hominick W (2002) Biogeography. In: Gaugler R (ed), Entomopathogenic Nematology, vol 10. CABI Wallingford, UK, pp 115-143

Hou B, Xie Q, Zhang R (2006) Depth of pupation and survival of the Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) pupae at selected soil moistures. Applied Entomology and Zoology 41:515-520

Hulthen AD, Clarke AR (2006) The influence of soil type and moisture on pupal survival of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). Australian Entomology 45:16-19

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors), Advances in Taxonomy and Phylogeny of Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Janssen T, Karssen G, Verhaeven M, Coyne D, Bert W (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. Scientific Reports 6 doi:10.1038/srep22591

Kable P, Mai W (1968) Influence of soil moisture on *Pratylenchus penetrans*. Nematologica 14:101-122

Kaya HK (1990) Soil ecology. In: Gaugler R, Kaya HK (eds), Entomopathogenic Nematodes in Biological Control. CRC, Boca Raton, FLorida, pp 93-115

Kung S-P, Gaugler R, Kaya HK (1990) Soil type and entomopathogenic nematode persistence. Journal of Invertebrate Pathology 55:401-406

Kung S-P, Gaugler R, Kaya HK (1991) Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. Journal of Invertebrate Pathology 57:242-249

Langford EA, Nielsen UN, Johnson SN, Riegler M (2014) Susceptibility of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), to entomopathogenic nematodes. Biological Control 69:34-39

Lindegren J, Wong T, McInnis D (1990) Response of Mediterranean fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema* feltiae in field tests in Hawaii. Environmental Entomology 19:383-386

Lindegren JE, Vail PV (1986) Susceptibility of Mediterranean fruit fly, melon fly, and oriental fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema feltiae* in laboratory tests. Environmental Entomology 15:465-468

Ma J, Chen S, Moens M, De Clercq P, Li X, Han R (2013) Characterization in biological traits of entomopathogenic nematodes isolated from North China. Journal of Invertebrate Pathology 114:268-276

Malan AP, Manrakhan A (2009) Susceptibility of the Mediterranean fruit fly (*Ceratitis capitata*) and the Natal fruit fly (*Ceratitis rosa*) to entomopathogenic nematodes. Journal of Invertebrate Pathology 100:47-49

Minas RdS, Souza RM, Dolinski C, Carvalho RdS, Burla RdS (2016) Potential of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) to control Mediterranean fruit fly (Diptera: Tephritidae) soil stages. Nematoda 3:e02016 doi:10.4322

N'depo OR, Hala N, N'Da Adopo A, Coulibaly F, Kouassi P, Vayssieres J, De Meyer M (2015) Effective chemical control of fruit flies (Diptera: Tephritidae) pests in mango orchards in Northern Côte-d'Ivoire. International Journal of Biological and Chemical Sciences 9:1299-1307

Navon A, Ascher K (2000) Bioassays of entomopathogenic microbes and nematodes. CABI Publishing, Wallingford, UK

Nguyen KB (2007) Methodology, morphology and identification. In: Nguyen KB, Hunt DJ (eds), Hunt DJ, Perry RN (series editors), Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Nematology Monographs and Perspectives, vol 5. Leiden, The Netherlands, pp 59-119

Noujeim E, Rehayem M, Nemer N (2015) Comparison of indigenous and exotic entomopathogenic nematode strains for control of the cedar web-spinning sawfly, *Cephalcia tannourinensis* in vitro. Biocontrol Science and Technology 25:843-851

Offenberg J, Cuc NTT, Wiwatwitaya D (2013) The effectiveness of weaver ant (*Oecophylla smaragdina*) biocontrol in Southeast Asian citrus and mango. Asian Myrmecology 5:139-149

Patel MN, Perry RN, Wright DJ (1997) Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp.(Rhabditida: Steinernematidae). International Journal for Parasitology 27:61-70

Phan KL, Subbotin SA, Nguyen NC, Moens M (2003) *Heterorhabditis baujardi* sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations. Nematology 5:367-382

Poinar G, Hislop R (1981) Mortality of mediterranean fruit-fly adults (*Ceratitis capitata*) from parasitic nematodes (*Neoaplectana* and *Heterorhabditis* spp). IRCS Medical Science-Biochemistry 9:641-641

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425

Schutze MK, Mahmood K, Pavasovic A, Bo W, Newman J, Clarke AR, Krosch MN, Cameron SL (2015) One and the same: integrative taxonomic evidence that *Bactrocera invadens* (Diptera: Tephritidae) is the same species as the Oriental fruit fly *Bactrocera dorsalis*. Systematic Entomology 40:472-486

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

Shapiro-Ilan DI, Cottrell TE, Brown I, Gardner WA, Hubbard RK, Wood BW (2006) Effect of soil moisture and a surfactant on entomopathogenic nematode suppression of the pecan weevil, *Curculio caryae*. Journal of Nematology 38:474-482

Sinzogan AAC, Van Mele P, Vayssières J-F (2008) Implications of on-farm research for local knowledge related to fruit flies and the weaver ant *Oecophylla longinoda* in mango production. International Journal of Pest Management 54:241-246

Sirjani FO, Lewis EE, Kaya HK (2009) Evaluation of entomopathogenic nematodes against the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). Biological Control 48:274-280

Stock SP, Rivera-Orduño B, Flores-Lara Y (2009) *Heterorhabditis sonorensis* n. sp. (Nematoda: Heterorhabditidae), a natural pathogen of the seasonal cicada *Diceroprocta ornea* (Walker) (Homoptera: Cicadidae) in the Sonoran desert. Journal of Invertebrate Pathology 100:175-184

Vannière H, Didier C, Rey J-Y, Diallo TM, Kéita S, Sangaré M (2004) La mangue en Afrique de l'Ouest francophone: les systèmes de production et les itinéraires techniques. Fruits 59:383-398

Vayssières J-F, De Meyer M, Ouagoussounon I, Sinzogan A, Adandonon A, Korie S, Wargui R, Anato F, Houngbo H, Didier C (2015) Seasonal abundance of mango fruit flies (Diptera: Tephritidae) and ecological implications for their management in mango and cashew orchards in Benin (Centre & North). Journal of Economic Entomology 108:2213-2230

Vayssières J-F, Korie S, Ayegnon D (2009b) Correlation of fruit fly (Diptera Tephritidae) infestation of major mango cultivars in Borgou (Benin) with abiotic and biotic factors and assessment of damage. Crop Protection 28:477-488

Vayssieres J-F, Korie S, Coulibaly O, Temple L, Boueyi SP (2008) The mango tree in central and northern Benin: cultivar inventory, yield assessment, infested stages and loss due to fruit flies (Diptera Tephritidae). Fruits 63:335-348

Vayssieres J-F, Sinzogan A, Korie S, Ouagoussounon I, Thomas-Odjo A (2009a) Effectiveness of spinosad bait sprays (GF-120) in controlling mango-infesting fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 102:515-521

Wargui R, Offenberg J, Sinzogan A, Adandonon A, Kossou D, Vayssières J-F (2015) Comparing different methods to assess weaver ant abundance in plantation trees. Asian Myrmecology 7:159-170

White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

Wouts W (1981) Mass production of the entomogenous nematode *Heterorhabditis heliothidis* (Nematoda: Heterorhabditidae) on artificial media. Journal of Nematology 13:467-469

Yee WL, Lacey LA (2003) Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of *Steinernema* nematodes. Biological Control 27:349-356

Zadji L, Baimey H, Afouda L, Houssou FG, Waeyenberge L, de Sutter N, Moens M, Decraemer W (2013) First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Southern Benin. Russ J Nematol 21:117-130

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014a) Characterization of biocontrol traits of heterorhabditid entomopathogenic nematode isolates from South Benin targeting the termite pest *Macrotermes bellicosus*. BioControl 59:333-344

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014b) Effectiveness of different *Heterorhabditis* isolates from Southern Benin for biocontrol of the subterranean termite, *Macrotermes bellicosus* (Isoptera: Macrotermitinae), in laboratory trials. Nematology 16:109-120

2.9 Supplementary material 2.1

Comparative table of morphometrics of infective juveniles of *H. taysearae* isolates KoroborororouC2 and KoroborororouF4 from Benin and described *H. taysearae* species (in µm, ± standard error and range in parenthesis).

Characters	***H. taysearae* (this study) Koroborororou C2 isolate**	***H. taysearae* (this study) Koroborororou F4 isolate**	***H. taysearae* (Stock et al., 2009) Caborca isolate**	***H. taysearae* (Shamseldean et al., 1996)**
n	20	20	20	30
L	**570.39 ± 38.36 (512-615)**	**501.67 ± 35 (459-580)**	**557 ± 28 (495-570)**	**418 ± 38 (332-499)**
a	24,76 ± 2.35 (19-28)	21.60 ± 2.43 (17-27)	23 ± 1.5 (19-26)	21 ± 2.2 (18-27)
b	4.59 ± 0.31 (4-5)	4.30 ± 0.33 (3-5)	4.8 ± 0.4 (4.4-5.4)	3.8 ± 0.2 (3.4-4.2)
c	**9.48 ± 1.07 (7-10)**	**8.66 ± 0.7 (7-9)**	**5.5 ± 1.0 (4.0-6.5)**	**7.7 ± 0.7 (6.5-8.7)**
MBD	23.18 ± 2.25 (20-27)	23.41 ± 2.4 (20-29)	25.5 ± 4 (19-32)	20 ± 1.9 (17-23)
EP	**102.95 ± 7 (91-114)**	**102.1 ± 8.96 (82-115)**	**99 ± 4.5 (97-116)**	**90 ± 9.1 (74-113)**
NR	**93.24 ± 5.19 (87-108)**	**91.35 ± 7.74 (79-105)**	**93 ± 4 (87-98)**	**64 ± 6.8 (58-87)**
ES	**124.33 ± 6.63 (111-144)**	**117.13 ± 11.59 (95-136)**	**119 ± 7 (110-131)**	**110 ± 8.4 (96-130)**
T	**60.69 ± 6.5 (49-77)**	**58.42 ± 7.52 (48-70)**	**105 ± 7 (91-125)**	**55 ± 6.6 (44-70)**
ABD	11.77 ± 1.47 (9-16)	12.89 ± 1.7 (10-16)	16 ± 2 (13-16)	-
D%	**82.85 ± 4.73 (73 - 89)**	**87.32 ± 3.87 (81-97)**	**90 ± 8.5 (78-110)**	**82 ± 6 (71-96)**
E%	**171.51 ± 22.07 (135-212)**	**174.75 ± 17.05 (137-205)**	**99 ± 8 (81-111)**	**180 ± 27 (110-230)**

L= Body length, MBD= Maximum Body Diameter, EP= distance from anterior end to secretory-excretory pore, ES= pharynx length, NR = Nerve Ring, T=tail length, ABD= Anal Body Diameter, a= L/MBD, b= L/T, c= L/T, D%= (EP/ES) x 100, E%= (EP/T) x 100

3 Chapter 3:

Steinernema n. sp., a new *Steinernema* species (Rhabditida: Steinernematidae) from Northern Benin

Redrafted from:

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Author's contribution:

AG, WD designed the experiments. AG performed the experiments. LZ, BH, HG and LA collected the nematode isolates. CM took and arranged the SEM pictures. AG, CM and WD took the light microscopic pictures. WD did the drawing plate. AG, AW, WD, LA and WB analyzed the data and wrote the manuscript.

3.1 Abstract

Two nematode isolates from the genus *Steinernema* were isolated in Northern Benin. Morphological, morphometric, molecular and cross-hybridization studies placed these nematodes into a new species, *Steinernema* n. sp., within the *S. bicornutum* species group. Phylogenetic analyses based on both ITS and D2-D3 regions of 28S rDNA revealed that *Steinernema* n. sp. is different from all known *Steinernema* species and sister to *S. abbasi* (97.3-97.6% ITS nucleotide similarity) and within a clade of *S. bifurcatum* (98.3-98.4% D2-D3 similarity). *Steinernema* n. sp. can be separated from other members of the *S. bicornutum* species group by their greater IJ maximum body diameter (average 34.6 µm, type strain). They differ from *S. abbasi* by their greater IJ body length (average 707µm, type strain), EP distance (average 55 µm, type strain), their spicule length (average 67 µm, type strain) and the occurrence of one pair of genital papillae at the cloacal aperture.

3.2 Introduction

Entomopathogenic nematodes (EPNs) of the family Steinernematidae (Rhabditida) have been found in many places around the world. They have been considered in several research programs for the biological management of several insect pests (Ehlers 2001; Kaya et al. 2006). According to Hunt and Subbotin (2016), 95 *Steinernema* species have been officially recognized. Afterwards, new species have been described including *Steinernema bidulphi* (Cimen et al. 2016a). In the classification of *Steinernema* species, the *S. bicornutum* species group is characterized by the presence of two horn-like structures on the labial region of IJs. This group includes at present ten described species: *S. bicornutum* Tallosi, Peters and Ehlers, 1995 (from Yugoslavia); *S. riobrave* Cabanillas, Poinar and Raulston, 1994 (from Texas, USA); *S. abbasi* Elawad, Ahmad and Reid, 1997 (from Oman); *S. ceratophorum* Jian, Reid and Hunt, 1997 (from north- east China); *S. pakistanense* Shahina, Anis, Reid, Rowe and Maqbool, 2001 (from Pakistan); *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler and Adams, 2004 (from Ethiopia); *S. bifurcatum* Fayyaz, Yan, Qui, Han, Gulsher, Khanum and Javed, 2014 (from Pakistan); *S. papillatum* San-Blaz, Portillo, Nermut, Puza and Morales-Montero, 2015 (from Venezuela); *S. biddulphi* Cimen, Puza, Nermut, Hatting, Ramakuwela and Hazir, 2016 (from South Africa) and *S. goweni* San-Blaz, Morales-Montero, Portillo, Nermut and Puza, 2016 (from Zulia State of Venezuela).

The effective performance of the association *Steinernema* - *Xenorhabdus* on insect pests in laboratory and field assays has encouraged research investigations on these organisms in several countries (San-Blas 2013). Soil sampling performed in recent years in Northern Benin, yielded four nematode isolates identified as *Steinernema* sp. (Zadji et al. 2013). In this work, we focus on the identification of two isolates (Bembereke157c and Thui168d) which have recently been investigated for their effectiveness on the termites *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Baimey et al. 2015; Zadji et al. 2014b) and on mango fruit fly *Bactrocera dorsalis* (Godjo et al. 2018b). These two nematode isolates are here described as *Steinernema* n. sp. on the basis of the molecular, morphological, morphometric and cross-hybridization information.

3.3 Material and methods

3.3.1 Nematode strains collection and isolation

Two nematode isolates, Bembereke157c and Thui168d were isolated from soil samples collected during the dry season 2012 in a gallery forest (10°11.472'N 02°39.266'E) and an *Eucalyptus* vegetation (11°21.993'N 03°03.959'E) in Northern Benin, respectively. At each of the two locations, 5 subsamples of 1 kg each, were randomly collected and combined together to constitute a soil sample from which, nematodes were extracted using the *Galleria* baiting method (Bedding and Akhurst 1975). Insect cadavers were afterwards placed on white traps (White 1927) to extract Infective Juveniles (IJs), which were used to re-infect *Galleria mellonella* and thus multiply the nematode population to have enough material for their identification.

In this work the two nematode isolates are characterized and the isolate Thui168d was used as the type strain for species description.

3.3.2 Molecular characterization

For each of the two nematode isolates, DNA was extracted from a single IJ placed in an Eppendorf tube (250 µl) containing 1 µl of double distilled water. Ten µl of NaOH (0.05N) and 1 µl of tween 20 (4.5%) were added to the tube which was heated at 95°C for 15 min and immediately cooled at room temperature afterwards as described by Janssen et al. (2016). Before utilization, DNA was stored at -20°C in a freezer. DNA extraction was performed in five replicates (5 different IJs randomly selected per EPN isolate) to ensure the homogeneity of each nematode population.

A fragment of the 28S rDNA gene containing the D2-D3 region was amplified using primers D2F: 5'-CCTTAGTAACGGCGAGTGAAA-3' (Forward) and 536: 5'-CAGCTATCCTGAG GAAAC-3' (Reverse) (Nguyen 2007). The PCR mixture of each sample consisted of 2.5 µl of 10xPCR buffer, 2.5 µl dNTPs (2mM), 1.25 µl Taq Polymerase (1u/µl), 2 µl MgCl2 (25mM), 1 µl of each forward and reverse primers (10 µM), 14.75 µl ddH_2O and 2.5 µl of DNA extract. The thermal cycling programs for PCR consisted of 4 min at 94°C; 5 cycles of 30s at 94°C, 30s at 55°C, 2 min at 72°C; 35 cycles of 30s at 94°C, 30s at 50°C, 2 min 72°C followed by a final elongation step of 10 min at 72°C. In addition to the D2-D3 fragments, the Internal Transcribed Regions (ITS) were amplified as previously described (Godjo et al. 2018a). The amplicons were

cleaned with the Nucleofast 96 PCR membrane in a Tecan Genesis Workstation 200. The near complete ITS (Partial ITS1, 5.8S, partial ITS2) and partial D2-D3 regions of the 28S rDNA were sequenced using the same primers as specified above. The sequencing was performed by using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 3130xl DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). Sequences were assembled in Bionumerics 7 and they were first identified by the means of a Basic Local Alignment Search Tool (BLAST) in NCBI.

Multiple sequence alignments of D2-D3 and ITS sequences together with isolates of *bicornutum*-group retrieved from GenBank were made using MUSCLE (Edgar 2004) followed by postalignment trimming with G-Blocks as implemented in SeaView Version 4 (Gouy et al. 2010). Bayesian phylogenetic analysis was carried out in MrBayes v. 3.2.1 (Ronquist and Huelsenbeck 2003) using the GTR +G model as selected by the Akaike information criterion using MEGA7 (Kumar et al. 2016). Analyses were run under default settings for 3 × 106 generations, 25% of the converged runs were regarded as burn-in and discarded. To test distinctiveness of putative species, generated trees were imported into Geneious where the species delimitation plugin (Masters et al. 2011) was used to calculate Rosenberg's PAB, which tests the probability for reciprocal monophyly of the clusters (Rosenberg 2007). The pairwise distances were calculated using the p-distance method (Nei and Kumar 2000).

3.3.3 Morphological/morphometric characterization

To obtain all developmental stages of the nematode for taxonomic studies, *G. mellonella* larvae were infected with IJs of each nematode isolate at the ratio of 100 IJs/ larva and incubated in the darkness at 28°C. First generation males and females were obtained by dissecting *G. mellonella* cadavers in Ringer's solution 3 to 4 days after infection. Second generation adults were obtained 3 days later. Fresh IJs were obtained upon emergence from *G. mellonella* cadavers on a white trap device as explained above. Twenty females and twenty males of first and second generations were heat-killed and fixed as previously described by Singh et al. (2018). Fixed specimens were transferred to anhydrous glycerin and mounted on permanent slides in a drop of glycerin according to Seinhorst (1959). Light microscopic pictures were taken using an Olympus BX51 DIC microscope (Olympus Optical) and morphological vouchers were made using photomicrographs using a Nikon DS-Fi1. Measurements of morphometric characters were

directly made using NIS-Elements D measuring software. Adults were measured as fixed specimens; IJs were measured alive on temporal glass slides in a drop of water (Nguyen 2007). The morphometric features considered were: Body length (L), Maximum Body diameter (MBD), distance secretory-excretory pore from anterior end (EP), distance from anterior end to the Nerve Ring (NR), Pharynx length (ES), Tail Length (T), Anal Body Diameter (ABD), Testis Reflexion (TR), Spicule Length (SL), Spicule width (SW), Gubernaculum Length (GL), Gubernaculum Width (GW). Other parameters were calculated according to Nguyen (2007), a= L/MBD, b= L/ES, c= L/T, c'= T/ABD, D%= EP/ESx100, E%= EP/Tx100, GS%= GL/SLx100, SW%= SL/ABDx100, H%= H/Tx100.

Scanning Electron Microscopy observations (SEM pictures) were made according to Singh et al. (2018) on all developmental stages directly isolated from infested *G. mellonella*.

3.3.4 Cross-hybridization

Cross-hybridization tests were performed on lipid agar (Wouts 1981) according to the method of Phan et al. (2003). Crossings were performed between the nematode isolates Bembereke157c or Thui168d and three reference strains, *Steinernema abbasi*, *Steinernema riobrave* and *Steinernema yirgalemense*. These *Steinernema* species are members of the *S. bicornutum* species group and were obtained from the e-nema company (Gesellschaft für Biotechnologie und biologischen Pflanzenschutz mbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany). Twenty virgin females and males of the first generation of the appropriate nematode isolates were crossed. Two different kind of controls were considered, incubation of 20 virgin females without males (virginity test) and 20 males x 20 females of the same isolate (self-cross test). Plates were incubated at 28°C for 3-4 days after which the presence of juveniles was examined. Results were considered valid only when the self-cross test was positive and the virginity test negative.

3.4 Results and discussion

Steinernema n. sp.

(Figures 3.1-3.5)

3.4.1 Measurements

All measurements are presented in Tables 3.1 and 3.2

3.4.2 Description of type population (isolate Thui168d)

3.4.2.1 Life cycle

The life cycle of *Steinernema* n. *sp.* is similar to other *Steinernema* species. When *G. mellonella* was infested with 100 juveniles per insect at 28°C, most insects were dead 24 – 48 hours post infection. Three to four days after insect infection, first generation amphimictic males and females could be seen inside *G. mellonella* cadavers. The second generation adults were obtained 6-7 days insect post infection. By placing the infected cadavers on white trap, IJs were obtained 8 to 9 days after insect infection.

3.4.2.2 First generation male

Body curved ventrally in a G-shape when heat killed (Figures 3.1A; Figures 3.5A). The secretory-excretory pore (EP) located anterior to the nerve ring i.e. posterior to mid-pharynx. Genital system monorchic with reflexed testis, spicules yellowish, paired and 67 (57-75) µm long; Spicule head (manubrium) rounded, shaft (calomus) not pronounced and blade (lamina) ventrally curved with pointed end. Gubernaculum boat shaped in lateral view, cuneus sharply pointed, straight (Figures 3.1D; 3.5E). Twenty-five genital papillae were visible including 1 large mid ventral precloacal papilla, a pair of sublateral papillae, 1 pair of adanal papillae located at the level of cloacal aperture, 2 pairs of postcloacal subterminal papillae, a pair of postcloacal subdorsal papillae, 6 pairs of genital precloacal sublateral to lateral papillae and 1 pair occurring at the edge of cloacal aperture (Figure 3.3N, O; 3.4O, P). Tail short and rounded; mucron absent (Figures 3.1D).

3.4.2.3 Second generation male

Similar to males of first generation, but smaller in many features and calculated proportions. (Table 3.1). The cuneus of the gubernaculum can sometimes be slightly ventrally directed (Figures 3.1G; 3.5F)

3.4.2.4 First generation female

Body length greatly variable (3900 ±2473 µm) with very long individuals (giant form) and small sized individuals, but bigger than males. SEM in face view showing an anterior circle of protruding papillae around a marked perioral rim, four cephalic papillae and two small lateral amphideal apertures, oral aperture triangular (Figures 3.3B; 3.4B). EP located anterior to the

nerve ring and anterior to mid-pharynx (Figure 3.1I, 3.5I). Genital system amphidelphic didelphic with reflexed ovaries. Vulva protruding and a transverse slit in ventral view, located slightly posterior to the mid-body, V=60±17% (Table 3.1). Tail conoid presenting post anal swelling with presence of mucron (Figures 3.1K; 3.3M; 3.4K, L), the latter not always obvious.

3.4.2.5 Second generation female

Similar to first generation female but smaller in body length. Secretory-excretory pore more anterior than for the first generation female. Tail shorter.

3.4.2.6 Infective juvenile

Body elongated and nearly straight when heat killed (Figure 3.2A). Head continuous with body. Exsheathed IJs with two horn-like structures in lip region. Stoma closed. Anterior sensilla (6 labial papillae and 4 cephalic papillae) and amphideal apertures not visible beyond doubt in light microscopy. Body cuticle marked with transverse striations. Differentiated lateral field starting as a single line at level of the third annule and changing into three lines or two ridges at annule 14. A maximum of eight ridges could be counted in the lateral field (Figure 3.3F). Secretory-excretory pore (EP) located anterior to the nerve ring, and at the mid pharynx, D%= 50.6% (Figure 3.2D). Tail elongated and slightly ventrally curved (Figure 3.2I), hyaline portion occupying 51% (average) of the tail length.

3.4.2.7 Type locality

The type population (isolate Thui168d) was harvested by baiting soil collected in an *Eucalyptus* vegetation (11°21.993'N 03°03.959'E) located in the city of Kandi (Alibori department) in Northern Benin.

3.4.2.8 Other locality

Another population (isolate Bembereke157c), which is also described in this study, was found in a gallery forest (10°11.472'N 02°39.266'E) located in the city of Bembereke (Borgou department) in Northern Benin.

3.4.2.9 Type material

Ten slides containing the holotype male (first generation), 5 paratypes (2 males first generation, 1 male second generation and 2 females first generation), 6 juveniles (type strain Thui168d) and

specimens of the non-type population (Bembereke157c) were deposited at Ghent University, Institute for Zoology, K.L. Ledeganckstraat 35, 9000, Gent, Belgium (Accession numbers: from UGMD_104349 to UGMD_104358). Additional paratypes are available in the UGent Nematode Collection (slides UGnem179-186) of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium; And in the Laboratoire de Phytotechnie, d'Amélioration et de Protection des Plantes (LaPAPP), Faculty of Agronomy, University of Parakou, Benin (Accession Numbers: UP-ESCiPNem01-10). Living material is available at the LaPAPP laboratory in Benin.

3.4.2.10 Remark on the other population (isolate Bembereke157c)

The morphology of IJs and adults (both generations) were similar to the type strain but the morphometric of several features in the adults was somewhat larger (Table 3.4), extending their range: the body length in adults (both generations) were larger than in the type strain as well as the spicule and gubernaculum length in male; the D% in first generation male, on the contrary, was smaller in the non-type strain. These variations are considered as intraspecific variations and are included in the species diagnosis and in the discussion on its relationship.

3.4.3 Diagnosis and relationship

3.4.3.1 Morphology

Steinernema n. sp. belongs to the *S. bicornutum* species group because of the presence of the horn-like structures on the IJ labial region. It is characterized by the morphometrics of IJ which has a rather small body length of 707±55 µm (type strain) and 646±62 µm (non-type strain) and a body diameter (34.6±5 µm type strain, 31±4 µm non-type), the position of the secretory-excretory pore (55±2 µm type strain, 52±5.7 µm non-type) and in 1st generation males by the morphology and length of the spicules (67±5 µm type strain, 73±6 µm non-type) and gubernaculum (36±5 µm type strain, 42±4.6 µm non-type) and the number (25) and arrangement of the genital papillae i.e. 11 pairs and one mid-ventral arranged as in *Steinernema* species and one additional pair at the edge of the cloacal aperture. Females of the 1st generation have protruding vulval lips.

Morphology and morphometrics of IJs and males have been reported to provide more useful taxonomical characters for species discrimination (Hominick et al. 1997; Stock and Kaya 1996).

More specifically, the IJ body diameter, spicule length and gubernaculum length of males are important characters to consider in species delineation (Phan et al. 2003). IJ body length of *Steinernema* n. sp. in the type strain Thui168d (707±55 µm) and non-type strain Bembereke157c (646±62 µm) are in the range of IJ body length of *S. ceratophorum* (706±62 µm), *S. pakistanense* (683±21 µm), *S. biddulphi* (663±38.8 µm) and *S. papillatum* (652±39.06 µm), but they are longer than of *S. bifurcatum* (521±27.3 µm), *S. abbasi* (541±24 µm), *S. riobrave* (622±39.5 µm) and *S. yirgalemense* (635±36 µm) (Table 3.3). The maximum IJ body diameter of *Steinernema* n. sp. (34.6±5 µm type strain, 31±4 µm non-type) is greater than for all species in the *S. bicornutum* species group. The IJ EP distances (from anterior end to the secretory-excretory pore) of *Steinernema* n. sp. (55±2 µm type strain, and 52±5.7 µm non-type) are in the range of *S. ceratophorum* (55±5 µm), *S. biddulphi* (55±2.7 µm) and *S. pakistanense* (54±2.2 µm). However, both populations showed greater EP distance than *S. bifurcatum* (45±2.7 µm), *S. abbasi* (40-48±1.5 µm including *S. thermophilum* which is regarded as a junior synonym of *S. abbasi*), *S. papillatum* (50±3.3 µm) and *S. yirgalemense* (51±3.4 µm), but smaller than *S. riobrave* (56.2±3.2 µm) (Table 3.3).

The spicule length of *Steinernema* n. sp. (67±5 µm type strain, 73±6 µm non-type) are in the range of *S. riobrave* (67±4.2 µm), *S. pakistanense* (68 ±3.6 µm), *S. bifurcatum* (69±7.7 µm), *S. ceratophorum* (71±7 µm), *S. biddulphi* (72±3.5 µm), but greater than *S. abbasi* (61-65±4.2 µm including *S. thermophilum*) and *S. yirgalemense* (64±8 µm). *Steinernema* n. sp. possesses a similar gubernaculum length (36±5 µm type strain, 42 ±4.6 µm non-type) as *S. abbasi* (36-45±4.3 µm, including *S. thermophilum*) but smaller than *S. riobrave* (51±2.6 µm) and *S. yirgalemense* (48±6.9 µm). Male D% values of *Steinernema* n. sp. (63±9 µm type strain, 56.6±7.8 µm non-type) are in the range of *S. abbasi* (60-63 µm, including *S. thermophilum*) and *S. yirgalemense* (58±7.6 µm) but smaller than *S. riobrave* (71±8 µm) (Table 3.4).

In addition to these morphometrics, the number and location of male's genital papillae are also important (Nguyen et al. 2004). Males of *Steinernema* n. sp. totalize 25 genital papillae which include 11 pairs arranged in usual position for *Steinernema* species as described above next to one single mid-ventral papilla and one additional pair at the edge to the cloacal aperture. These number and position of genital papillae are shared with *S. goweni*, *S. riobrave* and *S. yirgalemense*. Moreover, the number of genital papillae is in the range of *S. abbasi* (23-27)

(including *S. thermophilum*) (Nguyen and Hunt 2007), which displays some intraspecific variation as was reported for *S. goweni* i.e. 12 or 13 pairs (San-Blas et al. 2016) (Table 3.4). However, the 12th pair of papillae found at the cloacal aperture of *Steinernema* n. sp. is missing in the description of *S. abbasi.* These morphological and morphometrics differences among the strains Thui168d and Bembereke157c with all described *S. bicornutum* species support the delineation of *Steinernema* n. sp. into a new species.

3.4.3.2 Cross-hybridization

Cross-hybridisation tests revealed no progeny in between *Steinernema* n. sp. Thui168d or *Steinernema* n. sp. Bembereke157c and *S. riobrave*, *S. abbasi* and *S. yirgalemense*. The fact that the Beninese isolates could not breed with any of the tested EPN isolates suggested that they do not belong to the same *Steinernema* species. This result supports our proposal to classify the Beninese isolates in a separate species *Steinernema* n. sp. within the *S. bicornutum* group.

3.4.3.3 Molecular characteristics

Sequences obtained for the five replicates of each nematode isolate per gene appeared to be identical and only one replicate was considered to reconstruct the phylogenies. The molecular analyses showed that *Steinernema* n. sp. Bembereke157C and Thui168d have virtual identical partial ITS and D2-D3 sequences with only maximum 2 nucleotide differences (Supplementary tables 3.1 and 3.2). The phylogenetic analyses based on the ITS (Figure 3.6) and the D2-D3 (Figure 3.7) regions confirmed the belonging of isolates Bembereke 157c and Thui168d to the *S. bicornutum* species group with nucleotide similarities varying from 97.6-72.8% (ITS region analysis) and 98.4-88.1% (D2-D3 region analysis) with the described species in the *S. bicornutum* species group. According to ITS analysis, *Steinernema* n. sp. is sister to *S. abbasi* with maximal support and differs 18-20 nucleotides (2.4-2.7%) with *S. abbasi* (Figure 3.6, Supplementary table 3.2). Lineage exclusivity of the new species was demonstrated, as 13 and 6 autapomorphic character states were present in the ITS sequences for the new species and its sister clade, respectively. Hence, our new species fulfils the requirements of an amalgamation of evolutionary and phylogenetic species concepts (Adams, 1998). Moreover, the species delimitation plugin of GENEIOUS 9.15, based on the ITS tree topology, strongly supports reciprocal monophyly of *Steinernema* n. sp. in respect to its sister clade (Rosenberg's PAB: 1.36E^{-3}). According to the partial D2-D3 region analysis, the new species is in an unresolved

position within a maximally supported clade with *S. bifurcatum*, *S. abbasi* and some undescribed species. Despite its unresolved position, *Steinernema* n. sp. differs 9-10 nucleotides (1.6-1.7%) with *S. bifurcatum* and *S. abbasi* in the D2D3 region (Figure 3.7, Supplementary table 3.1) including 8 autapomorphic character states out of 575 nucleotides compared. Verification of the origins of described *S. abbasi* isolates used for ITS phylogeny revealed that they do not necessarily cluster according to their isolation source (Figure 3.6, Supplementary figure 3.1). This suggests that differences displayed by Beninese isolates compared to the described *S. abbasi* do not necessarily relate to the origin of EPN isolation.

Table 3.1 Morphometrics of *Steinernema* n. sp. isolate Thui168d (type strain). All measurements are in µm in the form of "mean ±standard deviation (range)" and are based on fixed specimens except for IJs.

Characters	First generation			Second generation		Infective juveniles
	Male		Female	Male	Female	
	Holotype	Paratypes	Paratypes	Paratypes	Paratypes	Paratypes
n	1	20	20	20	20	20
L	1514	1445±205 (1147-1764)	3900±2473 (1400-7409)	1008±94 (822-1220)	1113±124 (892-1266)	707±55 (632-833)
MBD	156	139±35 (80-193)	168±56 (102-250)	68±8 (56-82)	103±24 (74-145)	34.6± 5 (27-48)
EP	101	87±10 (56-103)	87±14 (71±128)	83±6 (73-95)	55±7 (41-65)	55±2 (52-60)
NR	112	99±8 (81-118)	117±17 (93-145)	108±11 (86-129)	96±10 (83-112)	86±5 (76-100)
ES	146	139±12 (120-164)	168±24 (101-194)	145±10 (124-171)	125±10 (106-139)	108±8 (95-127)
TR	274	316 ± 38 (188-390)	–	162±43 (110-259)	–	–
T	34	34±7 (24-47)	51±13 (31±74)	44±5.7 (36-58)	37±7 (27-49)	63±4 (54-74)
ABD	52	53±8 (36-66)	67±19 (45-114)	44±5 (37-59)	44±10 (31-61)	21±3 (18-30)
SL	75	67±5 (57-75)	–	53±4.6 (43.6-60)	–	–
SW	7	8±1 (5.9-9.9)	–	6.8±0.8 (5.6-8.7)	–	–
GL	39	36±5 (26-46)	–	30±3 (20-36)	–	–
GW	7	7±0.8 (6.0-8.9)	–	5±0.6 (4-7)	–	–
D%	69	63±9 (38-77)	47±4.88 (38-55)	57±5.7 (45-72.8)	44±6 (35-53)	50.6± 3.6 (43-59)
E%	297	254±49 (193-343)	–	187± 3 (145-8)	–	87±5 (75-98)
SW%	154	129±23 (96-175)	–	123±18 (81-152)	–	–
GS%	73	55±7 (41-65)	–	56±8 (35-73)	–	–
V%	–	–	60±17 (39-126)	–	56±2 (52-60)	–
a	–	–	–	–	–	21±2 (17-24)
b	–	–	–	–	–	6.5±0.5 (5.5-7)
c	–	–	–	–	–	11±1 (9-13.6)
c'	–	–	–	–	–	3±1.3 (2-3.4)
H%	–	–	–	–	–	51±3 (45-56)

L = Body length, MBD = Maximum Body diameter, EP = distance secretory-excretory pore from anterior end, NR = distance from anterior end to the Nerve Ring, ES = Pharynx length, T = Tail Length, ABD = Anal Body Diameter, TR = Testis Reflexion, SL = Spicule Length, SW = Spicule width, GL = Gubernaculum Length, GW = Gubernaculum Width, a = L/MBD, b = L/ES, c = L/T, c' = T/ABD, D% = EP/ESx100, E% = EP/Tx100, GS% = GL/SLx100, SW% = SL/ABDx100, H% = H/Tx100.

Table 3.2 Morphometrics of *Steinernema* n. sp. isolate Bembereke 157c. All measurements are in µm in the form of: mean ±standard deviation (range) and are based on fixed specimens except for IJs.

Characters	First generation		Second generation		Infective juveniles
	Male	Female	Male	Female	
n	20	20	20	15	20
L	1661±188 (1275-1917)	4057 ±2150 (1132-7072)	1451 ±154 (1149-1678)	1511±133 (1348-1759)	646±62 (495-756)
MBD	133±18 (102-168)	137± 64 (112-305)	127±24 (87-170)	147±16 (115-171)	31±4 (25-43)
EP	85±11 (61-104)	76±8 (63-88)	90±6 (80-103)	57±5 (53-73)	52±5.7 (35-59)
NR	112±11 (91-132)	110±10 (98-133)	93±9 (72-113)	98±5 (89-105)	73±10 (55-88)
ES	151±11 (126-167)	150±13 (124-175)	127±5 (120-138)	133±6 (123-143)	107±7.4 (92-117)
TR	209±51 (150-260)	_	382±46 (287-513)	_	_
T	37±6 (25-47)	44± 10 (26-66)	31±3 (27-37)	46±9 (34-72)	62±6.8 (46-76)
ABD	57±5 (48-68)	83±25 (40-122)	49±4 (43-55)	58±10 (45-84)	22 ± 3 (18-30)
SL	73±6 (61-84)	_	74±4 (66-82)	_	_
SW	8±1 (6-10)	_	8±0.9 (7-10)	_	_
GL	42±4.6 (33-50)	_	40±3 (30-45)	_	_
GW	7.8±0.9 (6-9.6)	_	8±1 (6-10)	_	_
D%	56±8 (41-72)	51±5 (42-64)	71±5 (61-81)	43±5 (37-58)	49±4.6 (38-57)
E%	228± 42 (179-348)	_	290± 28	_	85±12 (60-110)
SW%	129±15 (104-157)	_	153±17	_	_
GS%	58±8 (43-73)	_	55±5 (39-66)	_	_
H%	_	_	_	_	53±8 (30-67)
V%	_	50±3 (43-58)	_	53±2 (48-56)	
a	_	_	_	_	21±1.5 (17-24)
b	_	_	_	_	6±0.7 (4-7)
c	_	_	_	_	10±0.9 (9-13)
c'	_	_	_	_	2.8±0.3 (2-3.2)

See Table 3.1 for the abbreviations of the studied characters

Table 3.3 Comparative morphometrics of infective juveniles of *Steinernema* n. sp. and related *Steinernema* species from the *bicornutum*-group. All measurements are in µm and in the form: mean ± standard deviation (range)

Species	L	MBD	EP	NR	ES	T	a	b	c	D%	E%	References
S. bicornutum	769 ± 52 (648-873)	29 (25-33)	61 (53-65)	92 (88-100)	124 (113-135)	72 ± 5 (63-78)	26.5 ± 1.5 (23-29)	6.2 ± 0.3 (5.6-6.9)	10.7 ± 0.66 (9.7-12)	50 ± 3 (40-60)	80 ± 6 (80-100)	(Tallosi et al. 1995)
Thui168d	**707±55 (632-833)**	**34.6± 5 (27-48)**	**55±2 (52-60)**	**86±5 (76-100)**	**108±8 (95-127)**	**63±4 (54-74)**	**21±2 (17-24)**	**6.5 ±0.5 (5.5-7)**	**11±1 (9-13.6)**	**50.6± 3.6 (43-59)**	**87±5 (75-98)**	This study
S. ceratophorum	706 ± 62 (591-800)	27 ± 3 (23-34)	55 ± 5 (47-70)	92 ± 6 (79-103)	123 ± 7 (108-144)	66 ± 5 (56-74)	25.9 (23.7-27.9)	–	10.6 (8.8-12.9)	45 ± 3 (40-56)	84 ± 6 (74-96)	(Jian et al. 1997)
S. pakistanense	683 ± 21 (649-716)	27 ± 1.2 (24-29)	54 ± 2.2 (49-58)	80 ± 2.1 (76-83)	113 ± 4.2 (108-122)	58 ± 2.1 (53-62)	24 ± 1.5 (21-27)	6 ± 0.3 (5-6)	11 ± 0.5 (10-12)	47 ± 2.7 (42-53)	91 ± 5 (87-102)	(Shahina et al. 2001)
S. biddulphi	663 ± 38.8 (606-778)	27 ± 1.5 (24-30)	55 ± 2.7 (51-64)	92 ± 5 (84-103)	118 ± 3.9 (111-126)	58 ± 2.4 (53-62)	25 ± 1.7 (21-28)	5.6 ± 0.3 (5.1-6.2)	12± 0.8 (10-13)	46 ± 2.2 (42-51)	95 ±5.7 (84-108)	(Cimen et al. 2016a)
S. papillatum	652 ±39.06 (572-720)	24 ±3.2 (21-31)	50 ± 3.3 (44-58)	88 ± 4.1 (81-96)	110 ± 4.9 (103-121)	54 ±6.7 (40-78)	27 ± 2.5 (22-30)	5.9 ± 0.4 (5.0-6.4)	12.1 ± 1.3 (8.3-15)	46 ± 3.3 (40-52)	93 ± 10.8 (66-121)	(San-Blas et al. 2015)
Bembeke157c	**646±62 (495-756)**	**31 ± 4 (25-43)**	**52 ± 5.7 (35-59)**	**73 ± 10 (55-88)**	**107 ± 7.4 (92-117)**	**62 ± 6.8 (46-76)**	**21 ± 1.5 (17-24)**	**6 ± 0.7 (4-7)**	**10 ± 0.9 (9-13)**	**49 ± 4.6 (38-57)**	**85 ± 12 (60-110)**	This study
S. goweni	640 ± 52.3 (531-719)	25 ± 2.5 (21-29)	51 ± 4.8 (32-58)	81 ± 7.4 (69-94)	119 ± 4.3 (109-126)	67 ± 7.8 (59-89)	25 ± 2 (22-29)	5.4 ±0.4 (4-6)	9 ± 1 (6-11)	43 ± 4 (27-49)	77 ± 10.4 (48-94)	(San-Blas et al. 2016)
S. yirgalemense	635 ± 36 (548-693)	29 ± 2.2 (24-33)	51 ± 3.4 (45-59)	88 ± 3.6 (82-93)	121 ± 3.7 (115-128)	62 ± 2.7 (57-67)	21 ± 1.3 (20-25)	5.2 ± 0.3 (4.8-5.9)	10.3 ± 0.6 (9.2-11.2)	42 ± 2.7 (38-48)	83 ± 7 (67-98)	(Nguyen et al. 2004)
S. riobrave	622 ± 39.5 (361-701)	27.6 ± 1.7 (25.6-30.0)	56.2 ± 3.2 (51.2-63.7)	77.2 ± 1.4 (83.7-88.7)	113.5 ± 2.1 (108.7-116.2)	53.5 ± 3.5 (46.2-58.7)	22 ± 1.1 (19.9-23.5)	5.4 ± 0.3 (4.9-6.0)	11.6 ± 0.7 (10.1-12.4)	49 ± 2 (45-55)	105 ± 5 (93-111)	(Cabanillas et al. 1994)
S. thermophilum	555 (510-620)	21 (21-23)	40 (37-46)	71 (65-79)	87 (80-100)	45 (40-52)	26 (24-28)	6.4 (5.8-7.1)	12.3 (11.5-12.8-	46 (42-53)	90 (81-102)	(Sudershan and Singh 2000)
S. abbasi	541 ± 24 (496-579)	29 ± 1 (27-30)	48 ± 1.5 (46-51)	68 ± 2.4 (64-72)	89 ± 1.8 (85-92)	56 ± 3.2 (52-61)	18 ± 0.9 (17-20)	6 ± 0.3 (5.5-6.6)	9.8 ± 0.8 (8.1-10.8)	53 ± 2 (51-58)	86 ± 5 (79-94)	(Elawad et al. 1997)
S. bifurcatum	521 ± 27.3 (460-590)	22 ± 1.48 (20-24)	45± 2.7 (40-49)	69± 2.63 (66-80)	114 ± 8 (102-130)	54± 2.45 (51-59)	24± 1.14 (22-25)	4.5 ± 0.46 (3.8-5.6)	9.6 ± 0.4 (9.2-10.5)	39.7 ± 4.2 (33-47)	85 ± 5.3 (77-94)	(Fayyaz et al. 2014)

See Table 3.1 for the abbreviations of the studied characters

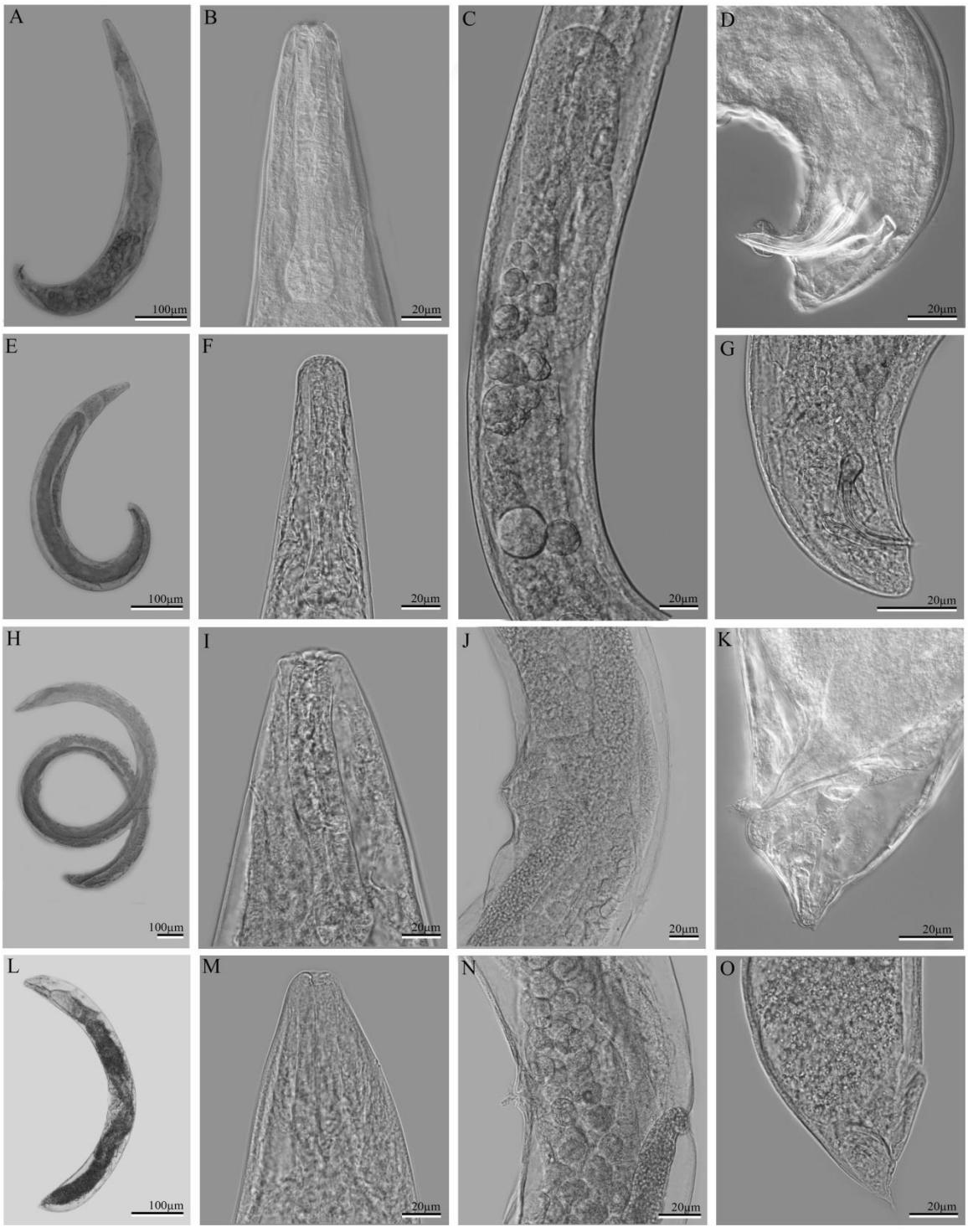

Figure 3.1 Light microscopic photographs of *Steinernema* n. sp. specimen. A-D and E-G indicate features of first and second generation male respectively, A, E: entire body view, B, F: pharyngeal region, C: Testis reflexion, D, G: posterior end showing spicule and gubernaculum; H-K and L-O show features of first and second generation female repectively, H, L: entire body view, I, M: pharyngeal region, J, N: vulva region, K-O: tail region with mucron.

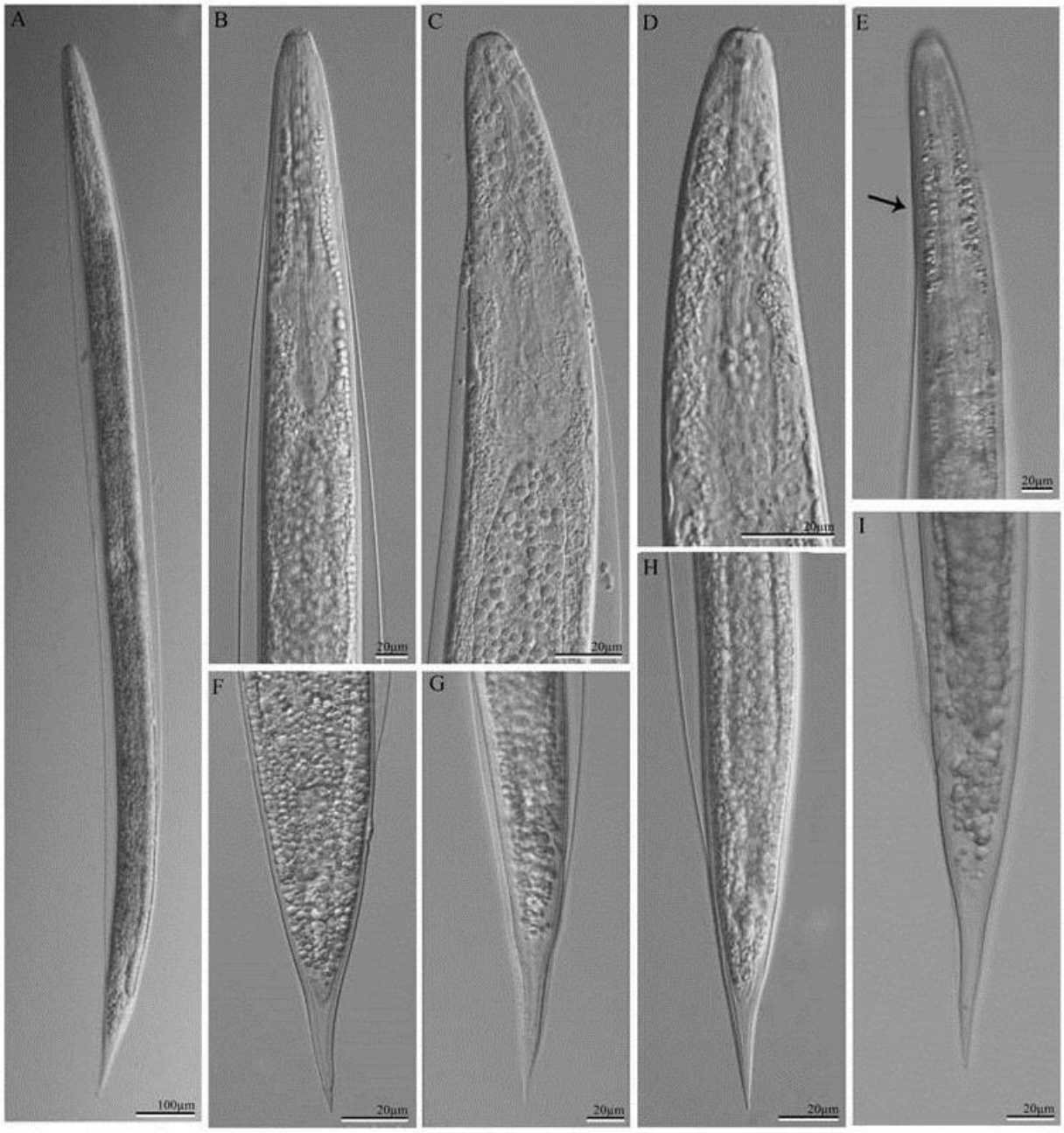

Figure 3.2 Paratypes light microscopic photographs of IJs of *Steinernema* n. sp. A: entire body view; B-E: pharyngeal region, the arrow on (E) indicates the SE pore position; C-I: tail region, (I) indicates the position of the anus.

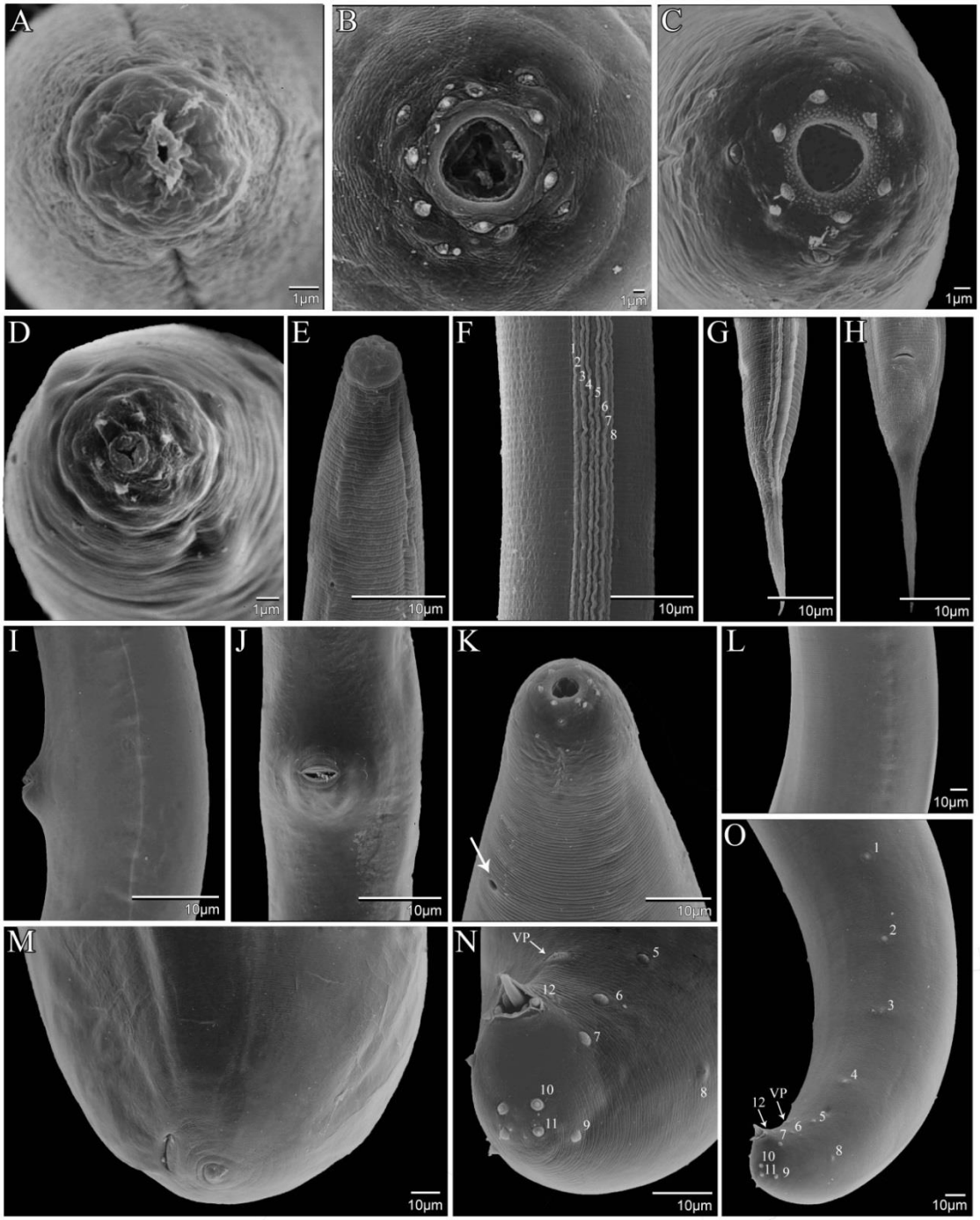

Figure 3.3 SEM pictures of *Steinernema* n. sp. isolate Bembereke157c. A: anterior region of infective juvenile (IJ) showing two horn like structures in labial region. B: anterior region of female showing mouth, labial and cephalic papillae. C: anterior region of male showing mouth, labial and cephalic papillae. D: anterior end of exsheathed IJ showing labial region lacking horn-like structures. E: anterior end of exsheathed IJ showing secretory excretory pore. F: IJ body showing lateral field with eight ridges. G: posterior region of IJ showing the change of ridges number from eight to two. H: elongate tail showing anal aperture. I, J: Vulva region. K: male anterior region showing SE pore. L: lateral field of male. M: posterior end of female showing the presence of mucron. N, O: posterior end of male showing genital and precloacal papillae and the absence of mucron. Arrows indicates the ventral papillae (VP) and the additional twelfth pair of papillae located at the edge of the cloacal aperture.

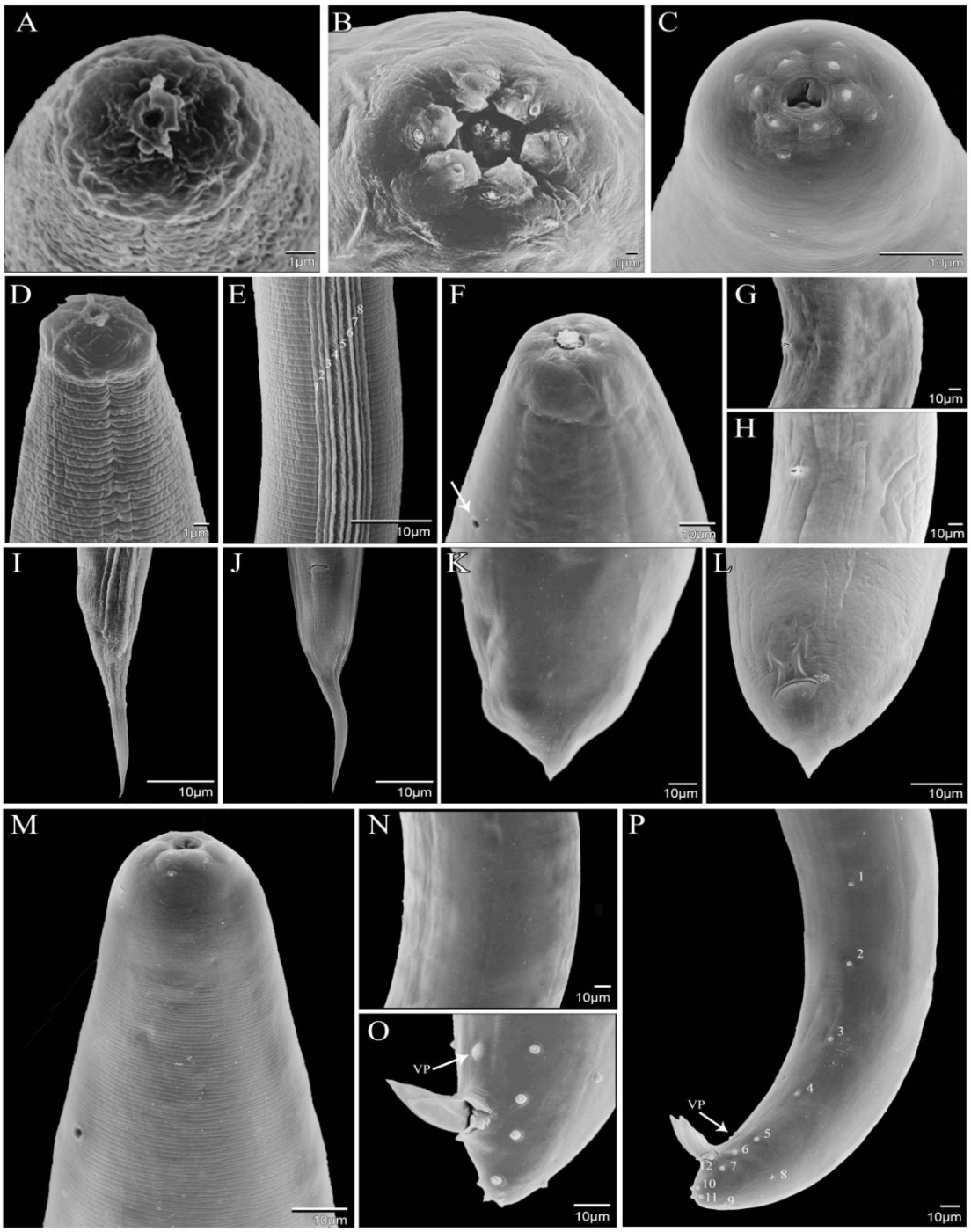

Figure 3.4 SEM pictures of *Steinernema* n. sp. isolate Thui168d. A: anterior region of infective juvenile (IJ) showing two horn-like structures in labial region. B: anterior region of female showing mouth, labial and cephalic papillae. C: anterior region of male showing the amphideal opening. D: anterior end of exsheathed IJ showing lateral field starting from 2 ridges at the level of annule 14. E: IJ lateral field with eight ridges. F: anterior end of female showing position of SE pore. G, H: Vulva region of female. I, J: posterior region of IJ showing anal opening and the change of ridges number from eight to two. K, L: posterior end of female showing post anal swelling and the presence of mucron. M: anterior end of male. N: male's body. O, P: posterior end of male showing genital and precloacal papillae and the absence of mucron.

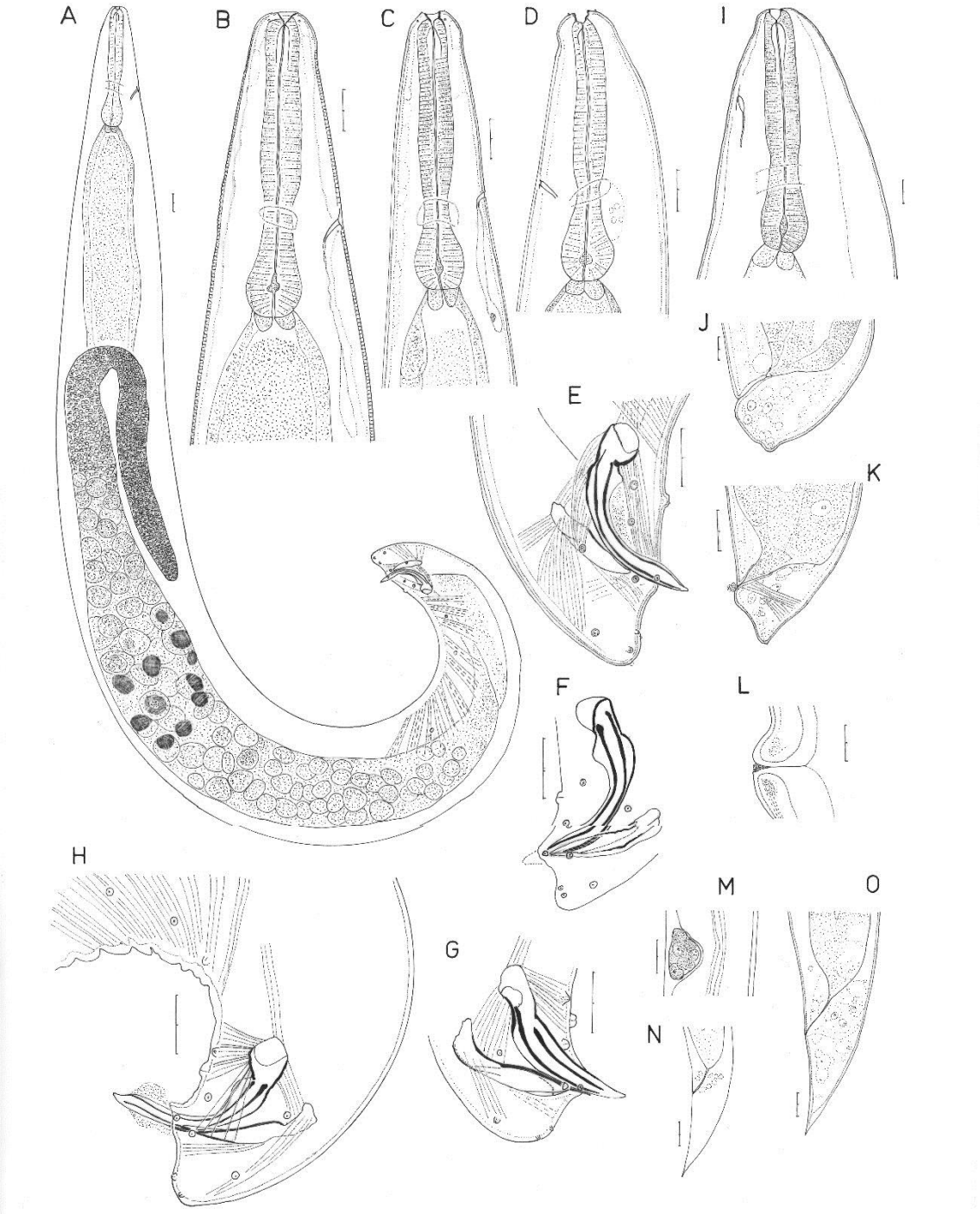

Figure 3.5 *Steinernema* n. sp. drawing plate. (A, B, E) Holotype male: A, total view; B, neck region; E, copulatory apparatus and tail. (C, F, G) Paratype males: C, neck region; F, G: copulatory apparatus. (I, J, K, L) Paratype females: I: head region; J, K: tail region; L, vulval region 168d. (D, H) male strain Bembereke 157c: D, neck region; H, copulatory apparatus and tail. (M-O) Juvenile strains Thui168d: M, genital primordium J3; N, O, tail region. Scale bars= 20 µm.

Table 3.4 Comparative morphometrics of first generation males of *Steinernema* n. sp. and related *Steinernema* species from the *bicornutum*-group. All measurements are in µm and in the form: mean ± standard deviation (range)

Species	SL	GL	ABD	D%	SW%	GS%	Number and location of genital papillae	References
Bembereke157c	**73 ± 6 (61-84)**	**42 ± 4.6 (33-50)**	**57 ± 5 (48-68)**	**56.6 ± 7.8 (41-72)**	**129 ± 15 (104-157)**	**58±8 (43-73)**	12 pairs plus one mid-ventral; the 12th pair located at the edge of the cloacal aperture	This study
S. biddulphi	72 ± 3.5 (65-78)	44 ±2.2 (41-48)	47 ±4.9 (40-59)	59 ±5.0 (52-69)	153 ± 19 (126-192)	62 ± 4.2 (54-70)	11 pairs and one unpaired preanal papilla	(Cimen et al. 2016a)
S. ceratophorum	71 ± 7 (54-90)	40 ± 4 (25-45)	52 ± 5 (45+70)	51 ± 7 (33-69)	140 ± 20 (100-200)	60 ± 10 (40-80)	11 pairs plus one unpaired preanal papilla	(Jian et al. 1997)
S. bifurcatum	69 ± 7.7 (60-85)	39 ± 5.4 (30-49)	48 ± 4.8 (45-56)	48 ± 4.3 (42-58)	1.4 ± 0.16 (1.2-1.7)	0.59 ± 0.08 (0.51-0.74)	11 pairs plus one unpaired preanal papilla	(Fayyaz et al. 2014)
S. pakistanense	68 ± 3.6 (62-73)	41 ± 3.2 (36-45)	36 ± 2.3 (32-40)	60 ± 3 (50-60)	189 (100-220)	60 (50-60)	11 pairs plus one unpaired preanal papilla	(Shahina et al. 2001)
Thui168d	**67 ± 5 (57-75)**	**36 ± 5 (26-46)**	**53 ± 8 (36-66)**	**63 ± 9 (38-77)**	**129 ± 23 (96-175)**	**55 ± 7 (41-65)**	12 pairs plus one mid-ventral; the 12th pair located at the edge of the cloacal aperture	This study
S. riobrave	67 ± 4.2 (63-75)	51 ± 2.6 (48-56)	59 ± 4.7 (50-640)	71 ± 8 (60-80)	114	76	12 pairs plus one mid-ventral; the 12th pair located at the edge of the cloacal aperture	(Cabanillas et al. 1994)
S. bicornutum	65 ± 4.3 (53-70)	47 ± 3.5 (38-50)	109 (80-127)	52 ± 3 (50-60)	222 (218-226)	72	12 pairs plus one mid-ventral	(Tallosi et al. 1995)
S. abbasi	65 ± 5.7 (57-74)	45 ± 4.3 (35-50)	43 ± 5 (37-55)	60 ± 5 (51-68)	156 ± 22 (107-187)	70 ± 7 (58-85)	11 pairs plus one single located in the ventral preanal position	(Elawad et al. 1997)
S. yirgalemense	64 ± 8 (51-77)	48 ± 6.9 (42-54)	38 ± 6 (32-45)	58 ± 7.6 (50-66)	171 ± 13 (121-213)	74 ± 8 (65-85)	12 pairs plus one mid-ventral; the 12th pair located at the edge of the cloacal aperture	(Nguyen et al. 2004)
S. thermophilum	61 (44-72)	36 (30-42)	77 (60-100)	63 (50-87)	170 (120-280)	60 (50-70)	12 or 13pairs of genital papillae with 1 single prominent ventral preanal papilla	(Sudershan and Singh 2000)
S. goweni	55 ± 2.3 (50-57)	35 ± 2.9 (30-40)	40 ± 5.8 (31-48)	42 ± 8.1 (28-59)	146 ± 25.4 (105-208)	64 ± 8 (49-79)	12 or 13 pairs plus one single ventral precloacal papilla. One pair at the edge of the cloacal aperture	(San-Blas et al. 2016)
S. papillatum	52 ±4.3 (42-62)	31 ±3.2 (23-36)	34 ± 4.7 (26-44)	54 ±5.3 (43-65)	156 ± 21.2 (125-194)	59 ±6.5 (48-70)	11 pairs and single mid-ventral precloacal papilla	(San-Blas et al. 2015)

See Table 3.1 for the abbreviations of the studied characters

3.4.3.4 Bacterial symbionts

The bacterial symbiont associated with *Steinernema* n. sp. Bembereke157C and Thui168d have been previously isolated and identified as *Xenorhabdus indica* (Godjo et al. 2018a). This bacterial symbiont is shared by many members of the *S. bicornutum* group including *S. abbasi* (Somvanshi et al. 2006), *S. yirgalemense* (Ferreira et al. 2016), *S. bifurcatum* (Fayyaz et al. 2014),and *Steinernema biddulphi* (Cimen et al. 2016a). It therefore suggests that *X. indica* is not host (vector) specific. The fact that the two Beninese isolates, representing a novel species, possess *X. indica* as symbiont represents an additional nematode-bacteria association and confirms the non-host specificity of the bacteria within the *S. bicornutum* group.

3.4.3.5 Conclusion

Based on molecular analyses of the ITS and D2-D3 regions, *Steinernema* n. sp. displayed 97.3% and 98.3% nucleotides similarities with its closely related species *S. abbasi*, respectively. Based on the ITS data *Steinernema* n. sp. is clearly a new species following of an amalgamation of evolutionary and phylogenetic species concepts as proposed by Adams (1998) and according GENEIOUS species delimitation plugin. Based on the partial D2D3 sequences *Steinernema* n. sp. is in an unresolved position in respect to *S. abbasi* and *S. bifurcatum*. However, eight autapomorphic character states for *Steinernema* n. sp. compared to virtually no variation among D2-D3 sequences of *S. abbasi, S. bifurcatum* and some undescribed species, indicates *Steinernema* n. sp. as different to all known species. Next to the relatively low nucleotide similarity between *Steinernema* n. sp. and *S. abbasi*, morphology/morphometric data revealed dissimilarities among the two species. The position of genital papillae, spicules length (SL) and D% of males; the body length and EP distance of IJs, which are important morphological and morphometric characters. Finally, the absence of progeny after cross-hybridization test, confirmed the appointment of *Steinernema* n. sp. as a new valid species within *S. bicornutum* species group.

3.5 Acknowledgements

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nematode materials needed for cross-hybridyzation test, and Leen Dierick and Patrick De Clercq for providing us *G. mellonella* larvae for nematode reproduction.

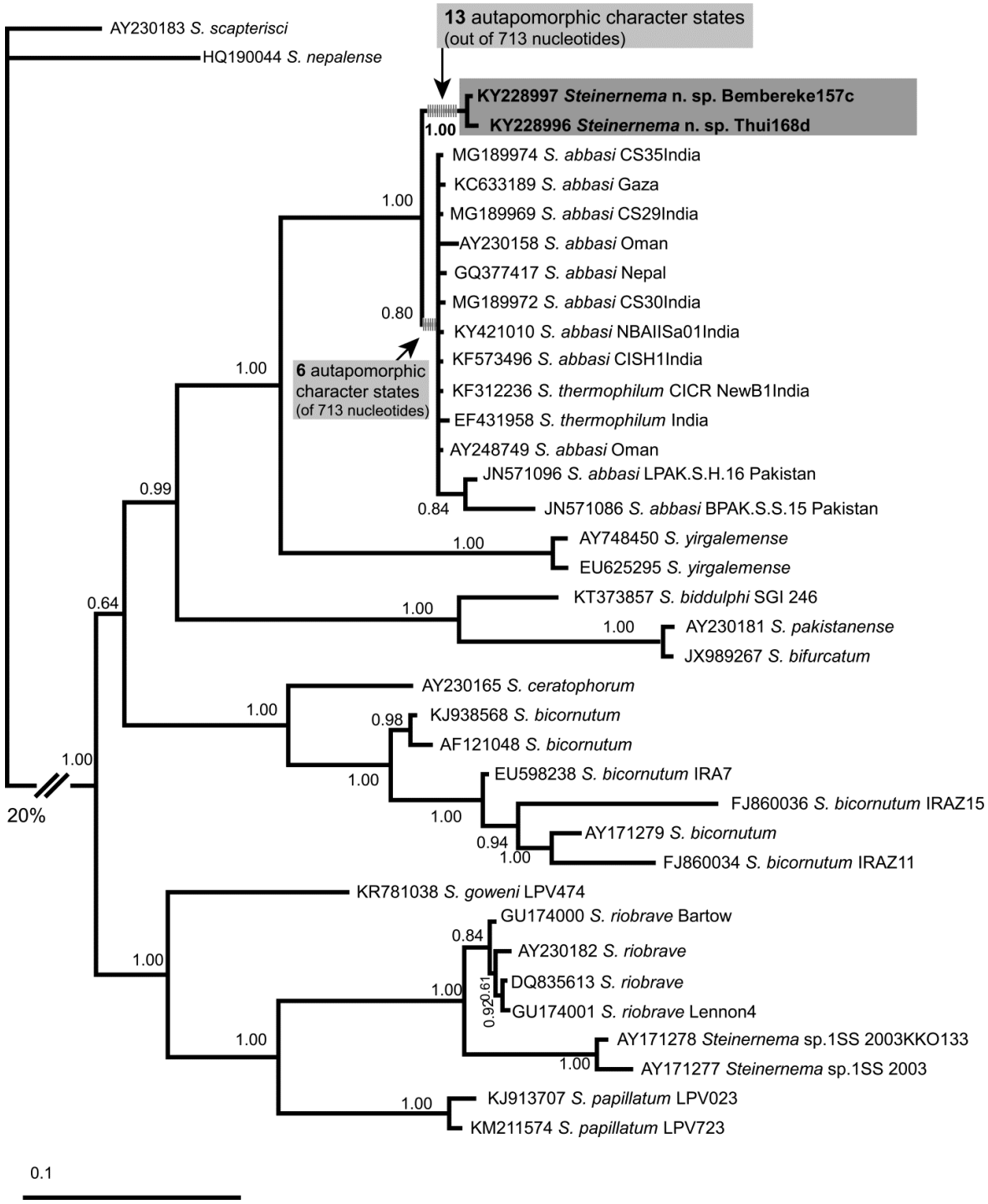


Figure 3.6 Phylogenetic relationships of *Steinernema* n. sp. with described species in the *Bicornutum*-group based on analysis of the ITS fragments as inferred from Bayesian analysis. Support values are shown at the nodes of each branch. Numbers before reference species name correspond to Genbank accession numbers. *S. nepalense* and *S. scapterisci* were used as outgroup.

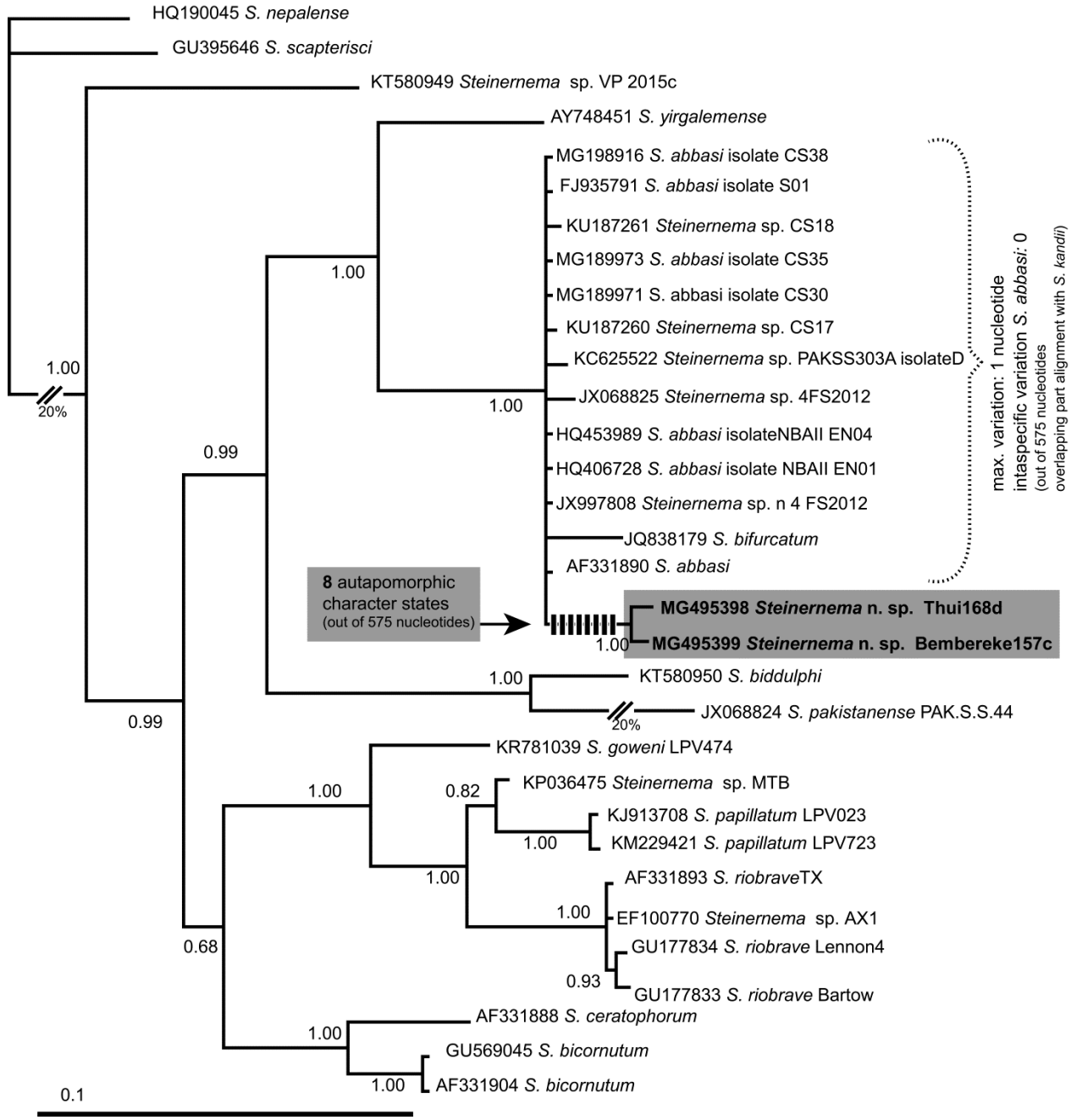


Figure 3.7 Phylogenetic relationships of *Steinernema* n. sp. with described species in the *Bicornutum*-group based on analysis of the D2-D3 fragments as inferred from Bayesian analysis. Support values are shown at the nodes of each branch. Numbers before reference species name correspond to Genbank accession numbers. *S. nepalense* and *S. scapterisci* were used as outgroup.

3.6 References

Adams BJ (1998) Species concepts and the evolutionary paradigm in modem nematology. Journal of Nematology 30:1-21

Baimey H, Zadji L, Afouda L, Moens M, Decraemer W (2015) Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from Southern Benin and control of underground termite nest populations. Nematology 17:1057-1069

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Cabanillas H, Poinar Jr G, Raulston J (1994) *Steinernema riobravis* n. sp.(Rhabditida: Steinernematidae) from Texas. Fundamental and Applied Nematology 17:123-131

Cimen H, Půža V, Nermut' J, Hatting J, Ramakuwela T, Hazir S (2016) *Steinernema biddulphi* n. sp., a New Entomopathogenic Nematode (Nematoda: Steinernematidae) from South Africa. Journal of Nematology 48:148-158

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Elawad S, Ahmad W, Reid A (1997) *Steinernema abbasi* sp. n. (Nematoda: Steinernematidae) from the Sultanate of Oman. Fundamental and Applied Nematology 20:435-442

Fayyaz S, Yan X, Qiu L, Han R, Gulsher M, Khanum TA, Javed S (2014) A new entomopathogenic nematode, *Steinernema bifurcatum* n. sp. (Rhabditida: Steinernematidae) from Punjab, Pakistan. Nematology 16:821-836

Ferreira T, Van Reenen C, Tailliez P, Pagès S, Malan A, Dicks L (2016) First report of the symbiotic bacterium *Xenorhabdus indica* associated with the entomopathogenic nematode *Steinernema yirgalemense*. Journal of Helminthology 90:108-112

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2018a) Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin. Archives of Microbiology doi:10.1007/s00203-017-1470-2

Godjo A, Zadji L, Decraemer W, Willems A, Afouda L (2018b) Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*)

under laboratory conditions. Biological Control 117:68-77 doi:10.1016/j. biocontrol.2017.10.009

Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27:221-224

Hominick W, Briscoe B, del Pino FG, Heng J, Hunt D, Kozodoy E, Mracek Z, Nguyen K, Reid A, Spiridonov S (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. Journal of Helminthology 71:271-298

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors), Advances in Taxonomy and Phylogeny of Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Janssen T, Karssen G, Verhaeven M, Coyne D, Bert W (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes (*Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. Scientific Reports 6 doi:10.1038/srep22591

Jian H, Reid A, Hunt D (1997) *Steinernema ceratophorum* n. sp.(Nematoda: Steinernematidae), a new entomopathogenic nematode from north-east China. Systematic Parasitology 37:115-125

Kaya HK, Aguillera M, Alumai A, Choo HY, De la Torre M, Fodor A, Ganguly S, Hazır S, Lakatos T, Pye A (2006) Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biological Control 38:134-155

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874

Masters BC, Fan V, Ross HA (2011) Species delimitation–a geneious plugin for the exploration of species boundaries. Molecular Ecology Resources 11:154-157

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University press

Nguyen K, Hunt D (2007) Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts vol 5. Nematology Monographs and Perspectives. Brill, Leiden. 816pp.

Nguyen KB (2007) Methodology, morphology and identification. In: Nguyen KB, Hunt DJ (eds), Hunt DJ, Perry RN (series editors), Entomopathogenic Nematodes: Systematics,

Phylogeny and Bacterial Symbionts. Nematology Monographs and Perspectives, vol 5. Leiden, The Netherlands, pp 59-119

Nguyen KB, Tesfamariam M, Gozel U, Gaugler R, Adams BJ (2004) *Steinernema yirgalemense* n. sp. (Rhabditida: Steinernematidae) from Ethiopia. Nematology 6:839-856

Phan KL, Subbotin SA, Nguyen NC, Moens M (2003) *Heterorhabditis baujardi* sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations. Nematology 5:367-382

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574

Rosenberg NA (2007) Statistical tests for taxonomic distinctiveness from observations of monophyly. Evolution 61:317-323

San-Blas E (2013) Progress on entomopathogenic nematology research: A bibliometric study of the last three decades: 1980–2010. Biological Control 66:102-124

San-Blas E, Morales-Montero P, Portillo E, Nermuť J, Puza V (2016) *Steinernema goweni* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from Zulia State, Venezuela. Zootaxa 4067:200-214

San-Blas E, Portillo E, Nermuť J, Půža V, Morales-Montero P (2015) *Steinernema papillatum n.* sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from Venezuela. Nematology 17:1081-1097

Seinhorst J (1959) A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica 4:67-69

Shahina F, Anis M, Reid A, Rowe J, Maqbool M (2001) *Steinernema pakistanense* sp. n.(Rhabditida: Steinernematidae) from Pakistan. International Journal of Nematology 11:124-133

Singh PR, Nyiragatare A, Janssen T, Couvreur M, Decraemer W, Bert W (2018) Morphological and molecular characterization of a new *Pratylenchus rwandae* n. sp. (Tylenchida: Pratylenchidae) associated with maize from Rwanda. under review

Somvanshi VS, Lang E, Ganguly S, Swiderski J, Saxena AK, Stackebrandt E (2006) A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. Systematic and Applied Microbiology 29:519-525

Stock SP, Kaya HK (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. The Journal of Parasitology 82:806-813

Sudershan G, Singh L (2000) *Steinernema thermophilum* sp. n. (Rhabditida: Steinernematidae) from India. International Journal of Nematology 10:183-191

Tallosi B, Peters A, Ehlers RU (1995) *Steinernema bicornutum* sp. n. (Rhabditida: Steinernematidae) from Vojvodina, Yugoslavia. Russ J Nematol 3:71-80

White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

Wouts W (1981) Mass production of the entomogenous nematode *Heterorhabditis heliothidis* (Nematoda: Heterorhabditidae) on artificial media. Journal of Nematology 13:467-469

Zadji L, Baimey H, Afouda L, Houssou FG, Waeyenberge L, de Sutter N, Moens M, Decraemer W (2013) First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Southern Benin. Russ J Nematol 21:117-130

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014) Comparative susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Isoptera: Termitidae) to entomopathogenic nematodes from Benin. Nematology 16:719-727

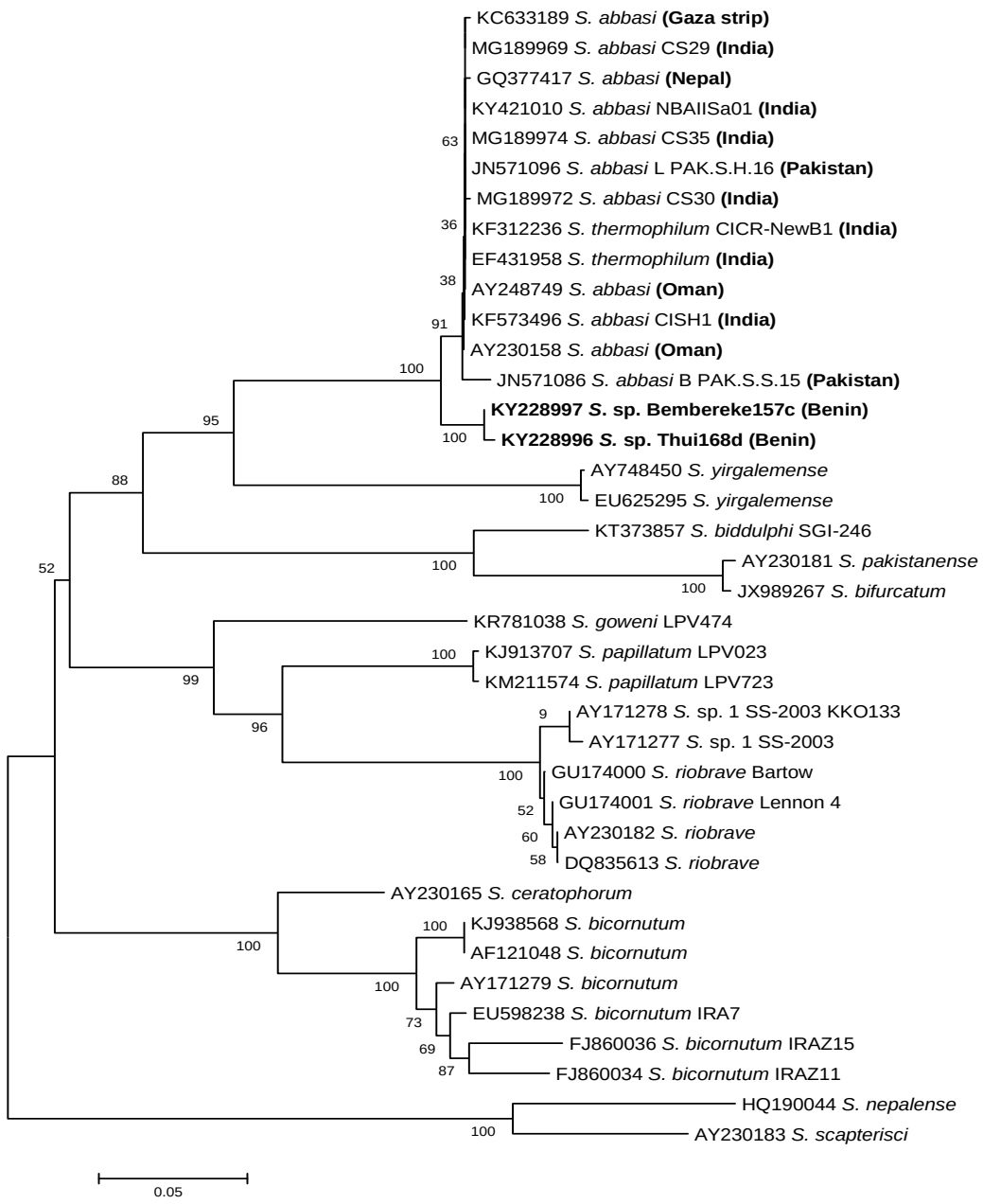
3.7 Supplementary materials

Supplementary table 3.1 Pairwise comparison of the D2-D3 region between species of the *bicornutum*-group and *Steinernema* n. sp. (above diagonal numbers represent the total nucleotides differences and below diagonal numbers indicate percentage of nucleotide similarity) Data of the described species are in bold.

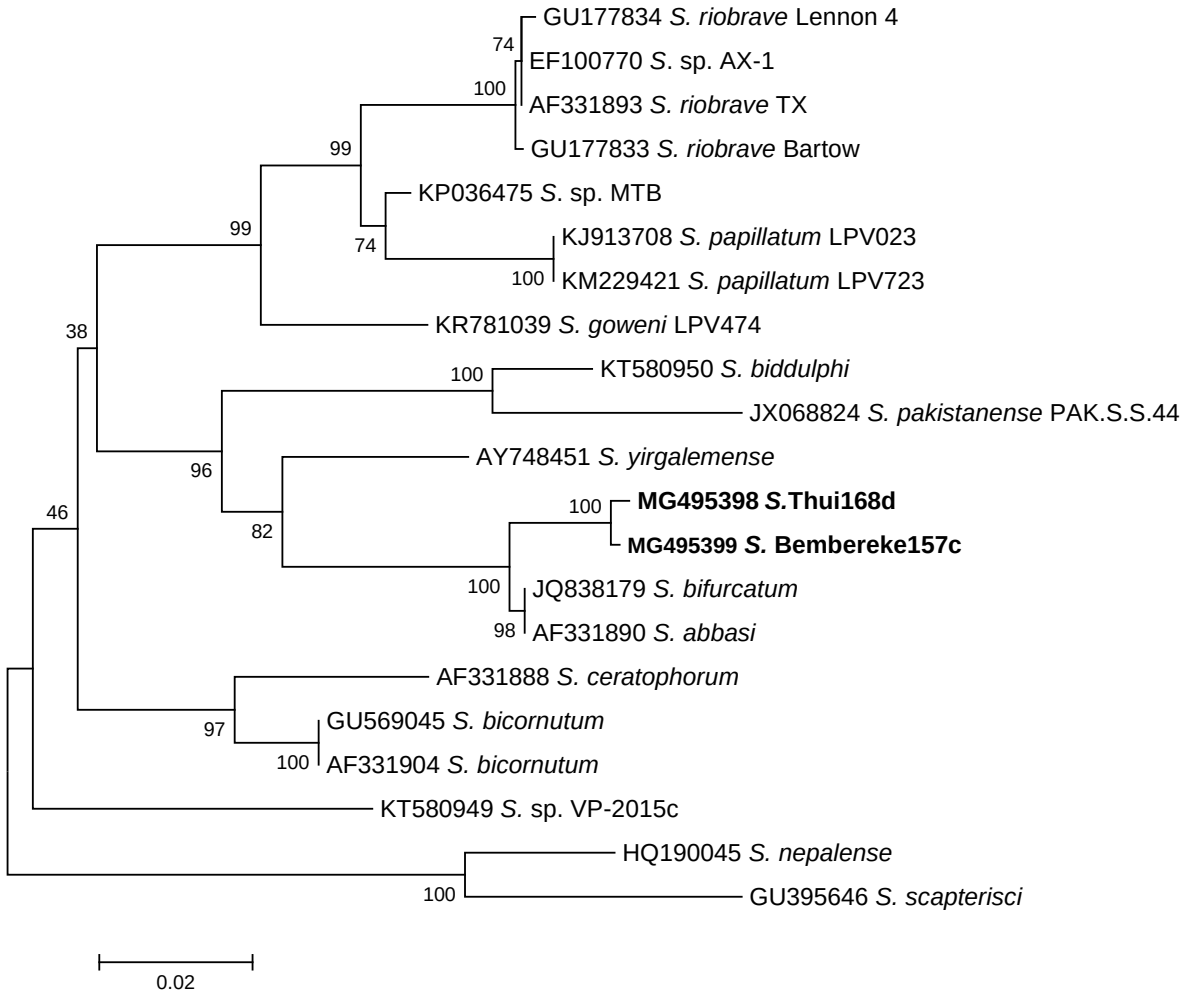
D2-D3 Regions	*168d*	*157c*	*bif*	*abb*	*yir*	*bid*	*bic*	*gow*	*cer*	*pak*	*pap*	*rio*
MG495398 *S.* n. sp. Thui168d		**2**	**10**	**10**	**40**	**51**	**52**	**58**	**60**	**62**	**66**	**68**
MG495399 *S.* n. sp. Bembereke157c	**99.7**		**9**	**9**	**39**	**51**	**50**	**57**	**59**	**62**	**66**	**68**
JQ838179 *S. bifurcatum*	**98.3**	**98.4**		**0**	32	48	47	52	52	61	60	60
AF331890 *S. abbasi*	**98.3**	**98.4**	100.0		32	48	47	52	52	61	60	60
AY748451 *S. yirgalemense*	**93.0**	**93.2**	94.4	94.4		50	49	49	53	54	59	54
KT580950 *S. biddulphi*	**91.1**	**91.1**	91.6	91.6	91.3		54	61	62	26	64	63
AF331904 *S. bicornutum*	**90.9**	**91.3**	91.8	91.8	91.4	90.6		40	20	58	54	46
KR781039 *S. goweni* LPV474	**89.9**	**90.1**	90.9	90.9	91.4	89.4	93		46	71	32	30
AF331888 *S. ceratophorum*	**89.5**	**89.7**	90.9	90.9	90.8	89.2	96.5	92		68	62	55
JX068824 *S. pakistanense*	**89.2**	**89.2**	89.4	89.4	90.6	95.5	89.9	87.6	88.1		76	70
KJ913708 *S. papillatum* LPV023	**88.5**	**88.5**	89.5	89.5	89.7	88.8	90.6	94.4	89.2	86.7		27
AF331893 *S. riobrave* TX	**88.1**	**88.1**	89.5	89.5	90.6	89.0	92.0	94.8	90.4	87.8	95.3	

Supplementary table 3.2 Pairwise comparison of the ITS region between species of the *bicornutum*-group and *Steinernema* n. sp. (above diagonal numbers represent the total nucleotides differences and below diagonal numbers indicate percentage of nucleotide similarity) Data of the described species are in bold.

ITS regions	*157c*	*168d*	*abb*	*yir*	*bid*	*cer*	*pap*	*gow*	*pak*	*bif*	*rio*	*bic*
KY228997 *S.* n. sp. Bembereke157c		**2**	**18**	**128**	**135**	**144**	**146**	**150**	**155**	**156**	**165**	**173**
KY228996 *S.* n. sp. Thui168d	**99.7**		**20**	**130**	**137**	**144**	**146**	**151**	**156**	**157**	**167**	**173**
AY230158 *S. abbasi*	**97.6**	**97.3**		124	134	138	147	148	159	160	163	167
AY748450 *S. yirgalemense*	**79.9**	**79.6**	80.5		160	156	158	151	175	176	163	177
KT373857 *S. biddulphi* SGI-246	**78.8**	**78.5**	79.0	74.9		156	165	160	71	69	162	173
AY230165 *S. ceratophorum*	**77.4**	**77.4**	78.3	75.5	75.5		133	128	171	169	134	78
KJ913707 *S. papillatum* LPV023	**77.1**	**77.1**	76.9	75.2	74.1	79.1		100	178	176	78	157
KR781038 *S. goweni* LPV474	**76.5**	**76.3**	76.8	76.3	74.9	79.9	84.3		173	172	112	141
AY230181 *S. pakistanense*	**75.7**	**75.5**	75.0	72.5	88.9	73.2	72.1	72.8		**6**	173	197
JX989267 *S. bifurcatum*	**75.5**	**75.4**	74.9	72.4	89.2	73.5	72.4	73.0	99.1		171	195
DQ835613 *S. riobrave*	**74.1**	**73.8**	74.4	74.4	74.6	79.0	87.8	82.4	72.8	73.2		155
AY171279 *S. bicornutum*	**72.8**	**72.8**	73.8	72.2	72.8	87.8	75.4	77.9	69.1	69.4	75.7	



Supplementary figure 3.1 Phylogenetic relationships of *Steinernema* n. sp. with described species in the *Bicornutum*-group based on analysis of the ITS fragments (592 positions compared). A neighbor joining tree constructed in Mega 6 using Kimura two parameters. Bootstrap values based on 10000 replicates are shown at the nodes of each branch. Numbers before reference species name correspond to Genbank accession numbers. Names in Parenthesis are the isolation origin of EPNs. *S. nepalense* and *S. scapterisci* were used as outgroup.



Supplementary figure 3.2 Phylogenetic relationships of *Steinernema* n. sp. with described species in the *Bicornutum*-group based on analysis of the D2-D3 fragments (569 positions compared). A neighbor joining tree constructed in Mega 6 using Kimura two parameters. Bootstrap values based on 10000 replicates are shown at the nodes of each branch. Numbers before reference species name correspond to GenBank accession numbers. *S. nepalense* and *S. scapterisci* were used as outgroup.

4 Chapter 4:

Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin

Redrafted from:

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2018) Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin. Archives of Microbiology doi:10.1007/s00203-017-1470-2

Author's contribution:

AG, AW designed the experiments. AG and HB performed the experiments. AG and AW analyzed the data. AG, AW, WD and LA wrote the manuscript.

4.1 Abstract

The diversity of forty-three bacterial isolates recovered from *Galleria mellonella* infected with Beninese entomopathogenic nematodes was investigated molecularly by analyzing the 16S rRNA, recA and gyrB genes. Based on 16S rRNA sequence analysis, 15 bacterial isolates were identified as *Xenorhabdus* sp., 27 isolates as *Photorhabdus* sp. and one as *Serratia* sp. The *Xenorhabdus* isolates were recovered from *G. mellonella* infected with *Steinernema* nematodes and identified as *Xenorhabdus indica* based on 16S rRNA gene and concatenated recA and gyrB sequence analysis. However, analysis of 16S rRNA and concatenated recA and gyrB gene sequences of the *Photorhabdus* isolates, all isolated from hemoplymph of infected *Galleria mellonella* with *Heterorhabditis* nematodes, resulted in two separate sub-clusters (A) and (B) within the *Photorhabdus luminescens* group, distinct from existing subspecies. They share low sequence similarities with nearest phylogenetic neighbors *Photorhabdus luminescens* subsp. *luminescens* Hb^T, *Photorhabdus luminescens* subsp. *caribbeanensis* HG29^T and *Photorhabdus luminescens* subsp. *noenieputensis* AM7^T.

4.2 Introduction

Photorhabdus sp. and *Xenorhabdus* sp. are bacterial symbionts of entomopathogenic nematodes (EPNs) which are used around the world for biological control of insect pests (Ehlers 2001). The life cycle of EPNs is composed of a free living stage in soil and a parasitic stage inside an insect host. During the free stage, the Infective Juveniles (IJs), which represent the only stage of the nematode that is capable of infecting insects, live in a mutualistic relationship with symbiotic bacteria of the genera *Photorhabdus* and *Xenorhabdus*. These bacteria help the IJs once inside the insect host, to kill the latter by septicemia. The parasitic stage begins when the IJs enter the hemocoel of the insect host via body pores (*Steinernema*) or penetrate the cuticle (*Heterorhabditis*). There, they resume development and start feeding to become amphimictic adult males or females (*Steinernema*) or self- fertile hermaphrodites (*Heterorhabditis*) of the first generation. The subsequent generations of both *Steinernema* and *Heterorhabditis* are generally amphimictic. In most cases, 2 to 3 generations are completed inside the insect host before food is depleted (Emelianoff et al. 2007; Strauch and Ehlers 1998). In fact, the availability of food, mainly in the form of bacteria, is crucial for EPN development (Ehlers 2001).

Bacterial symbionts of EPNs have been described around the world, and so far, *Photorhabdus* (Boemare et al. 1993) and *Xenorhabdus* (Thomas and Poinar 1979) species have only been reported to be associated with EPNs belonging to the genera *Heterorhabditis* and *Steinernema*, respectively (Akhurst 1982; Boemare 2002a; Torres-Barragan et al. 2011). These bacterial symbionts are carried by the IJs, within the whole intestine of *Heterorhabditis* or in a specialized vesicle of the intestine in *Steinernema* nematodes (Bird and Akhurst 1983; Martens and Goodrich-Blair 2005). *Photorhabdus* and *Xenorhabdus* bacterial symbionts play an important role in the infectivity of the associated EPNs to insect pests. For use in biological insect control, EPNs need to be grown at large scale under *in vitro* conditions, with bacteria as a main component of their diet (Ehlers 2001). Knowing the identity of the symbiotic bacteria may help in choosing the most suitable bacteria to maintain the natural association of nematode-bacteria which is a requirement for a successful EPNs rearing and biocontrol.

EPNs have been retrieved from soil in many places around the world which suggests their worldwide presence. In general, local EPNs are considered more suitable for use as biological weapons against insect pests as they are better adapted to the indigenous environmental

conditions than the exotic introduced EPNs (Grewal et al. 1994). In Benin, several surveys were carried out to evaluate the presence of EPNs in soil. Thirty-two nematode isolates were recovered from soil in the Southern part of the country including twenty nine isolates identified as *Heterorhabditis sonorensis* and three as *Heterorhabditis indica* (Zadji et al. 2013). Several EPN strains were also retrieved from the Center and Northern regions of Benin and so far only few *Steinernema* strains have been reported without further characterization (Baimey et al. 2015; Zadji et al. 2013; Zadji et al. 2014b). These EPNs are currently being investigated for biological control study of mango fruit flies in Benin. In this work, we looked at the molecular diversity of symbiotic bacteria associated with nematodes collected in the Northern and Center Benin. To our knowledge, no published work has been carried out on the bacterial symbionts of Beninese EPNs despite their important role in the virulence process of the nematode against insect pests. Therefore the main objective of this study was to investigate the biological diversity of *Photorhabdus* and *Xenorhabdus* bacteria associated with EPNs from Benin.

In the past, analysis of 16S rRNA gene sequences was traditionally used to characterize *Photorhabdus* and *Xenorhabdus* bacteria at subspecies and species level (Fischer-Le Saux et al. 1999; Liu et al. 2001; Rainey et al. 1995; Szállás et al. 1997). The inconsistent species-level grouping of some *Photorhabdus* strains (Akhurst et al. 2004) based on the 16S rRNA gene analysis, led to the use of more variable genes to provide complementary molecular information when evaluating bacterial phylogenies. Therefore, analysis of gyrB (Akhurst et al. 2004; Peat et al. 2010; Tóth and Lakatos 2008) and recA gene (Sergeant et al. 2006; Thanwisai et al. 2012) sequences have been used to complement 16S rRNA gene phylogeny, bacterial phenotypic and DNA-DNA hybridization studies to better characterize new *Photorhabdus* and *Xenorhabdus* strains. Furthermore, potential lateral transfer of 16S rRNA genes was later demonstrated to exist in the *Photorhabdus* and *Xenorhabdus* clades (Tailliez et al. 2010) which may confound the classification of bacterial isolates, especially when only this gene is considered. In the study of new isolates from EPNs from Benin, gyrB and recA gene sequences were therefore used in addition to 16S rRNA genes.

4.3 Material and methods

4.3.1 Isolation and identification of EPNs and their phylogenetic position

EPNs considered in this study were isolated from soil samples collected in the North and Center of Benin (Table 4.1) as previously described (Zadji et al. 2013) using the *Galleria mellonella* (Gm) baiting method (Bedding and Akhurst 1975). After isolation, EPNs were multiplied in the laboratory to have enough material for molecular identification and bacteria isolation. In a Petri dish, IJs were used to infect Gm larvae at the ratio 100 IJs/Gm larva, at room temperature (22°C). After approximately 7 days, dead Gm larvae were individually transferred onto a modified white trap (White 1927) to collect the new generation of IJs that would exit the Gm cadaver approximately 10 days later. Harvested IJs were used to infect new Gm or stored in the incubator (15°C) for further use. To identify the nematodes, partial ITS regions were amplified and sequenced using primers proposed by Vrain et al. (1992).

4.3.2 Isolation of *Photorhabdus* and *Xenorhabdus* bacteria from nematodes

Mature Gm larvae were infected with IJs of pure culture of each nematode isolate at the dose of 100 IJs per Gm larva. After 48 hours, a single moribund insect was washed 5-10 min in a glass staining block with 70% ethanol. A drop of the insect's hemolymph was then streaked onto a Nutrient Bromothymol Agar (NBTA) plate containing 2,3,5 Triphenyltetrazolium chloride and Bromothylmol blue as described by Akhurst (1980) prior to incubation at 28°C during 48 h. Bacterial isolates were purified by picking a single colony and plating it onto a successive new NBTA plate until homogenous colonies were observed.

4.3.3 Morphological examination of bacterial colonies

Visual characteristics such as colony diameter, shape and color after 48 hours of incubation at 28°C on NBTA plates were recorded. In addition, the capacity for bioluminescence of some isolates was visually assessed in darkness (Kazimierczak et al. 2017)

4.3.4 Molecular characterization of symbiotic bacteria

Bacterial genomic DNA was extracted following the protocol of Pitcher et al. (1989) from a single colony of each bacterial isolate (Table 4.1).

The near-complete 16S rRNA gene was amplified using primers: forward 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGTGATCCAGCCGCA-3 as previously described (Cleenwerck et al. 2002). PCR products were cleaned with Nucleofast 96 PCR membrane in the Tecan Genesis Workstation 200 machine. Partial 16S rRNA gene sequencing was achieved on all recovered bacterial isolates by using primer 5'-TATTACCGCGGCTGCTGGCA-3' (Cleenwerck et al. 2007) producing a fragment of 427 nucleotides that covers V1-V2 variable regions. Sequencing was performed with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 3130xl DNA sequencer, using the protocols of the manufacturer (Applied Biosystems).

Partial sequences were identified by blasting them in NCBI and based on their grouping, representatives for near-complete 16S rRNA gene sequencing were selected so that different bacterial clusters and at least one bacterial isolate from each nematode isolate were represented. Full sequencing of the 16S rRNA gene was performed on the selected isolates using six sequencing primers described previously (Coenye et al. 1999). In addition, amplification and partial sequencing of two housekeeping genes, the recA and gyrB, was also achieved using primers and thermal cycling conditions as previously published (Tailliez et al. 2006).

Sequences were assembled in Bionumerics 7 (Applied Maths) and deposited in GenBank (accession numbers provided in Supplementary Table 4.1). Using Clustal W included in Mega 6.06 (Tamura et al. 2013), sequences of each gene were first aligned with reference type strains of *Photorhabdus* and *Xenorhabdus* retrieved from GenBank. Afterwards, non-overlapping reference sequences were removed and alignments were trimmed to the most common size of all sequences and visually inspected. Remaining sequences were realigned and used to construct Neigbor Joining (Saitou and Nei 1987) phylogenetic trees, in Mega 6.06, using the Kimura two parameter model (Kimura 1980). Aligned recA and gyrB gene sequences were exported from Mega 6.06 and concatenated using an in-house Python script. Concatenated sequences were re-aligned and used to reconstruct a Neighbor joining phylogeny in Mega 6.06. Bootstrap percentages (1000 replicates) more than 50% were shown at the nodes of branches on the trees.

4.4 Results

4.4.1 Phylogenetic assignment of nematodes

The phylogenetic analysis of the ITS fragments showed that collected nematode isolates (Table 4.1) were distributed within *Heterorhabditis* and *Steinernema* clusters. Ten nematode isolates shared 99.1 - 100% sequence similarity with *Heterorhabditis sonorensis* (junior synonym of *Heterorhabditis taysearae*) (Supplementary Figure 4.1) while two isolates clustered with *Heterorhabditis indica* (99.6 – 99.8%). In addition, 4 isolates formed a separate cluster within the *Steinernema* group with *Steinernema abbasi* representing their closest phylogenetic relative (97.3 - 97.7%) with a 98% bootstrap value.

4.4.2 Morphological examination of bacterial colonies

In total, we obtained 43 bacterial isolates from the 16 nematode isolates considered in this study (Table 4.1). These bacterial isolates were assigned an R-number (accession number) and were stored in the research collection of the Laboratory of Microbiology at the University of Ghent (LM-UGent) at -80°C. All bacterial isolates were able to grow after 48 hours with maximum colony diameter of 3 millimeters. Color of the colonies varied. They appeared on NBTA plate as either green or blue and sometimes reddish. Most of the bacterial isolates were able to absorb dye on NBTA plates and bioluminescence was observed for some *Photorhabdus* isolates and not for *Xenorhabdus* isolates after 48h of incubation (Table 4.1).

4.4.3 Molecular characterization of symbiotic bacteria

4.4.3.1 16S rRNA gene

Comparison of partial 16S rRNA gene sequences (414 bp) of the 43 isolated bacterial isolates with the type strains of references species showed that 27 and 15 isolates were respectively distributed within *Photorhabdus* and *Xenorhabdus* groups. One bacterial strain, isolated from Gm infected with *Steinernema* sp., formed a distinct cluster (D) together with *Serratia nematodiphila* (Supplementary Figure 4.2). All bacterial isolates recovered from Gm infected with *Heterorhabditis* nematodes, grouped with *Photorhabdus* reference strains and bacteria isolated from Gm infected with *Steinernema* nematodes clustered within *Xenorhabdus* species. The 27 *Photorhabdus* isolates were all distributed within the *Photorhabdus luminescens* group with 24 of them forming a separate sub-cluster (A) (Supplementary Figure 4.2). The 3 other isolates grouped

in a sub-cluster (B) together with *P. luminescens subsp. akhurstii* and *P. luminescens* subsp. *noenieputensis*. All *Xenorhabdus* isolates shared identical partial 16S rRNA and were grouped together in cluster (C) with *Xenorhabdus indica*. Based on the partial 16S rRNA gene grouping, 7 and 18 representative isolates from *Xenorhabdus* and *Photorhabdus* groups, were selected for further molecular analysis.

Analysis of full 16S rRNA gene sequences (1427 bp) revealed that the *Xenorhabdus* isolates were highly similar (100% sequence similarity) and confirmed their highest sequences similarity (99.7%) with *X. indica* DSM 17382^{T} (Figure 4.1). In addition, as noted with the partial 16S rRNA gene analysis above, *Photorhabdus* isolates formed two separate sub-clusters (A) and (B) (Figure 4.2) based on analysis of full sequences of 16S rRNA gene. Isolates of sub-cluster (A) showed 7 to 14 nucleotide differences of the 16S rDNA (1349 positions compared) sequences with isolates of sub-cluster (B). A cluster containing *P. luminescens* subsp. *luminescens* Hb and *P. luminescens* subsp. *sonorensis* Carbora and CH35 appeared to be a sister group of sub-cluster (A), and they all together formed a sister group of sub-cluster (B) (Figure 4.2). *P. luminescens* subsp. *luminescens* Hb^{T} shared 98.7-99.3% and 98.7 - 98.8% sequence similarities with isolates in sub-cluster (A) and (B), respectively, while *P. luminescens* subsp. *sonorensis* Carbora had 98.8 - 99.4% and 98.7% nucleotide identity with Beninese isolates in sub-cluster (A) and (B), respectively. The *Serratia* strain R-52436 shared 99.7% 16S rRNA nucleotide identity with *S. nematodiphila*.

Table 4.1 Morphological features of recovered *Photorhabdus* and *Xenorhabdus* isolates. Isolates selected for full 16S rRNA, recA and gyrB genes analyses (GenBank accession numbers provided in Supplementary Table 4.1) are shown in bold.

Bacterial isolates	Identification based on partial 16S rRNA	Biolumi-nescence feature	Colony shape	Colony size (mm)	Colony color on NBTA	Associated EPNs isolate	EPN identification based on ITS region*	EPN isolation source (Vegetation)	Origin of EPNs in Benin (GPS coordinates)
R-66822	*Photorhabdus* sp.	+	irregular	<1	Dark-green	F4	*H. taysearae*	Mango	09°22.287'N 02°40.233'E
R-66823	*Photorhabdus* sp.	+	irregular	2	Green-reddish	F4	*H. taysearae*	Mango	09°22.287'N 02°40.233'E
R-66825	*Photorhabdus* sp.	w	irregular	2	Green-reddish	C2	*H. taysearae*	Mango	09°22.356'N 02°41.175'E
R-52429	*Photorhabdus* sp.	+	irregular	2	Dark-green	138a	*H. taysearae*	Cashew	08°59.467'N 02°35.347'E
R-52373	*Photorhabdus* sp.	+	irregular	2	Green-reddish	138a	*H. taysearae*	Cashew	08°59.467'N 02°35.347'E
R-52403	*Photorhabdus* sp.	+	round	3	Green-reddish	138a	*H. taysearae*	Cashew	08°59.467'N 02°35.347'E
R-52416	*Photorhabdus* sp.	+	round	3	Green	138a	*H. taysearae*	Cashew	08°59.467'N 02°35.347'E
R-52415	*Photorhabdus* sp.	+	irregular	1	Green-reddish	139a	*H. taysearae*	Grassland	09°04.251'N 02°33.538'E
R-52402	*Photorhabdus* sp.	+	irregular	2	Red	139a	*H. taysearae*	Grassland	09°04.251'N 02°33.538'E
R-52380	*Photorhabdus* sp.	NT	irregular	1	Red	139a	*H. taysearae*	Grassland	09°04.251'N 02°33.538'E
R-52425	*Photorhabdus* sp.	+	irregular	3	Dark-green	152b	*H. taysearae*	Shea	09°48.441'N 02°39.275'E
R-52362	*Photorhabdus* sp.	NT	irregular	1	red	98c	*H. taysearae*	Cashew	08°07.263'N 02°14.534'E
R-52404	*Photorhabdus* sp.	+	irregular	1	Transparent	98c	*H. taysearae*	Cashew	08°07.263'N 02°14.534'E
R-52412	*Photorhabdus* sp.	+	irregular	1	Transparent	98c	*H. taysearae*	Cashew	08°07.263'N 02°14.534'E
R-52410	*Photorhabdus* sp.	+	irregular	1,5	Reddish	98c	*H. taysearae*	Cashew	08°07.263'N 02°14.534'E
R-52390	*Photorhabdus* sp.	NT	irregular	3	Blue	130d	*H. taysearae*	Cashew	08°10.223'N 02°37.173'E
R-52411	*Photorhabdus* sp.	NT	irregular	2	Red	130d	*H. taysearae*	Cashew	08°10.223'N 02°37.173'E
R-52383	*Photorhabdus* sp.	NT	irregular	2	Red	130d	*H. taysearae*	Cashew	08°10.223'N 02°37.173'E
R-52368	*Photorhabdus* sp.	+	irregular	3	Green-reddish	111b	*H. taysearae*	kpai (Tchabe)	08°35.847'N 01°41.202'E
R-52361	*Photorhabdus* sp.	NT	round	1	Red	111b	*H. taysearae*	kpai (Tchabe)	08°35.847'N 01°41.202'E
R-52427	*Photorhabdus* sp.	+	irregular	2	Red	111b	*H. taysearae*	kpai (Tchabe)	08°35.847'N 01°41.202'E
R-52389	*Photorhabdus* sp.	NT	irregular	1,5	Red	111b	*H. taysearae*	kpai (Tchabe)	08°35.847'N 01°41.202'E
R-66820	*Photorhabdus* sp.	w	irregular	2	Green	118c	*H. taysearae*	Cashew	08°07.520'N 01°57.912'E

Bacterial isolates	Identification based on partial 16S rRNA	Biolumi-nescence feature	Colony shape	Colony size (mm)	Colony color on NBTA	Associated EPNs isolate	EPN identification based on ITS region*	EPN isolation source (Vegetation)	Origin of EPNs in Benin (GPS coordinates)
R-52391	*Photorhabdus* sp.	NT	irregular	2	Dark-green	125a	*H. taysearae*	Grassland	07°59.256'N 02°16.428'E
R-52434	*Photorhabdus* sp.	+	round	2	Green	150d	*H. indica*	Forest	09°49.556'N 02°42.837'E
R-52366	*Photorhabdus* sp.	+	irregular	3	Green	150d	*H. indica*	Forest	09°49.556'N 02°42.837'E
R-52363	*Photorhabdus* sp.	NT	irregular	2	Dark-green	114c	*H. indica*	Cashew	08°25.904'N 01°51.900'E
R-52428	*Xenorhabdus* sp.	-	irregular	3	Green	168d	*Steinernema* n. sp.	Eucalyptus	11°21.993'N 03°03.959'E
R-52437	*Xenorhabdus* sp.	-	irregular	3	Red	168d	*Steinernema* n. sp.	Eucalyptus	11°21.993'N 03°03.959'E
R-52382	*Xenorhabdus* sp.	-	irregular	2	Blue	168d	*Steinernema* n. sp.	Eucalyptus	11°21.993'N 03°03.959'E
R-52377	*Xenorhabdus* sp.	-	irregular	3	Blue	168d	*Steinernema* n. sp.	Eucalyptus	11°21.993'N 03°03.959'E
R-52417	*Xenorhabdus* sp.	-	irregular	2	Red	168d	*Steinernema* n. sp.	Eucalyptus	11°21.993'N 03°03.959'E
R-52367	*Xenorhabdus* sp.	-	irregular	3	Green	157c	*Steinernema* n. sp.	Forest	10°11.472'N 02°39.266'E
R-52379	*Xenorhabdus* sp.	-	round	3	Green	157c	*Steinernema* n. sp.	Forest	10°11.472'N 02°39.266'E
R-52435	*Xenorhabdus* sp.	-	irregular	1	Green	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52364	*Xenorhabdus* sp.	-	irregular	3	Green	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52365	*Xenorhabdus* sp.	-	round	2	Red	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52430	*Xenorhabdus* sp.	-	irregular	2	Green	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52405	*Xenorhabdus* sp.	-	round	1	Green	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52426	*Xenorhabdus* sp.	-	irregular	3	Red	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52406	*Xenorhabdus* sp.	-	round	2	Green	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52372	*Xenorhabdus* sp.	-	round	3	Green-reddish	111c	*Steinernema* n. sp.	Baobab	08°35.847'N 01°41.202'E
R-52436	*Serratia*. sp.	-	irregular	2	Transparent	138d	*Steinernema* n. sp.	Cashew	08°59.467'N 02°35.347'E

+ means the bacterial isolate produced light in the darkness/ - means the bacterial isolate did not produce light in the darkness/w means the bacterial isolate produced weak light in the darkness/ NT means bioluminescence was not tested.

* *H.* stands for *Heterorhabditis*. If ITS nucleotide differences with reference type strains was lower than 9 seqeunces, the nematode isolate was considered to be a new species.

4.4.3.2 GyrB and recA genes

To increase the resolution of the molecular identification, gyrB and recA genes were explored. We were unable to sequence recA and gyrB gene sequences of the *Serratia* bacterial isolate (R-52436), because unexpectedly the strain lost its viability and could not be recovered. The neighbor joining trees reconstructed based on concatenated sequences of these two protein coding genes for *Photorhabdus* and *Xenorhabdus* strains are presented in Figures 4.3 and 4.4. Sequences of individual genes were also used to build neighbor joining phylogenetic trees which are presented in Supplementary Figures 4.3 to 4.6.

For *Photorhabdus*, the two sub-clusters (A) and (B) observed in the 16S rRNA phylogeny were recovered in the concatenated phylogeny reconstructed based on recA and gyrB genes (Figure 4.3) with high bootstrap value (100%). Strains in sub-cluster (B) shared a similar level of sequence similarity (94.8-96.1%) with the type strains of several subspecies of *P. luminescens*, including *P. luminescens* subsp. *luminescens* Hb, *P. luminescens* subsp. *caribbeanensis* HG29 and *P. luminescens* subsp. *noenieputensis* AM7 and *P. luminescens* subsp. *sonorensis* Caborca and CH35. For the strains of subcluster (B), concatenated recA and gyrB gene sequences showed a similar range of similarity (94.7-97.0%) to *P. luminescens* subsp. *luminescens* Hb, *P. luminescens* subsp. *caribbeanensis* HG29, *P. luminescens* subsp. *noenieputensis* AM7, and *P. luminescens* subsp. *sonorensis* Caborca and CH35. In the gyrB phylogeny, *Photorhabdus* strains were clearly separated in sub-clusters (A) and (B) and showed the closest similarity (95.9%) to *P. luminescens* subsp. *noenieputensis* (Supplementary Figure 4.3). In the recA phylogeny, sub-cluster (B) was clearly delineated but sub-cluster (A) strains were more dispersed and some grouped with different *P. luminescens* subspecies (Supplementary Figure 4.4).

For the *Xenorhabdus* isolates, comparison of concatenated sequences of recA and gyrB (Figure 4.4) with the reference type strains in GenBank revealed that they shared 98.5% nucleotide identity with *X. indica*. Furthermore, analysis of gyrB (Supplementary Figure 4.5) and recA (Supplementary Figure 4.6) sequences confirmed their closest similarity with *X. indica* (98.3 – 98.4% and 98.8 – 98.9%, respectively).

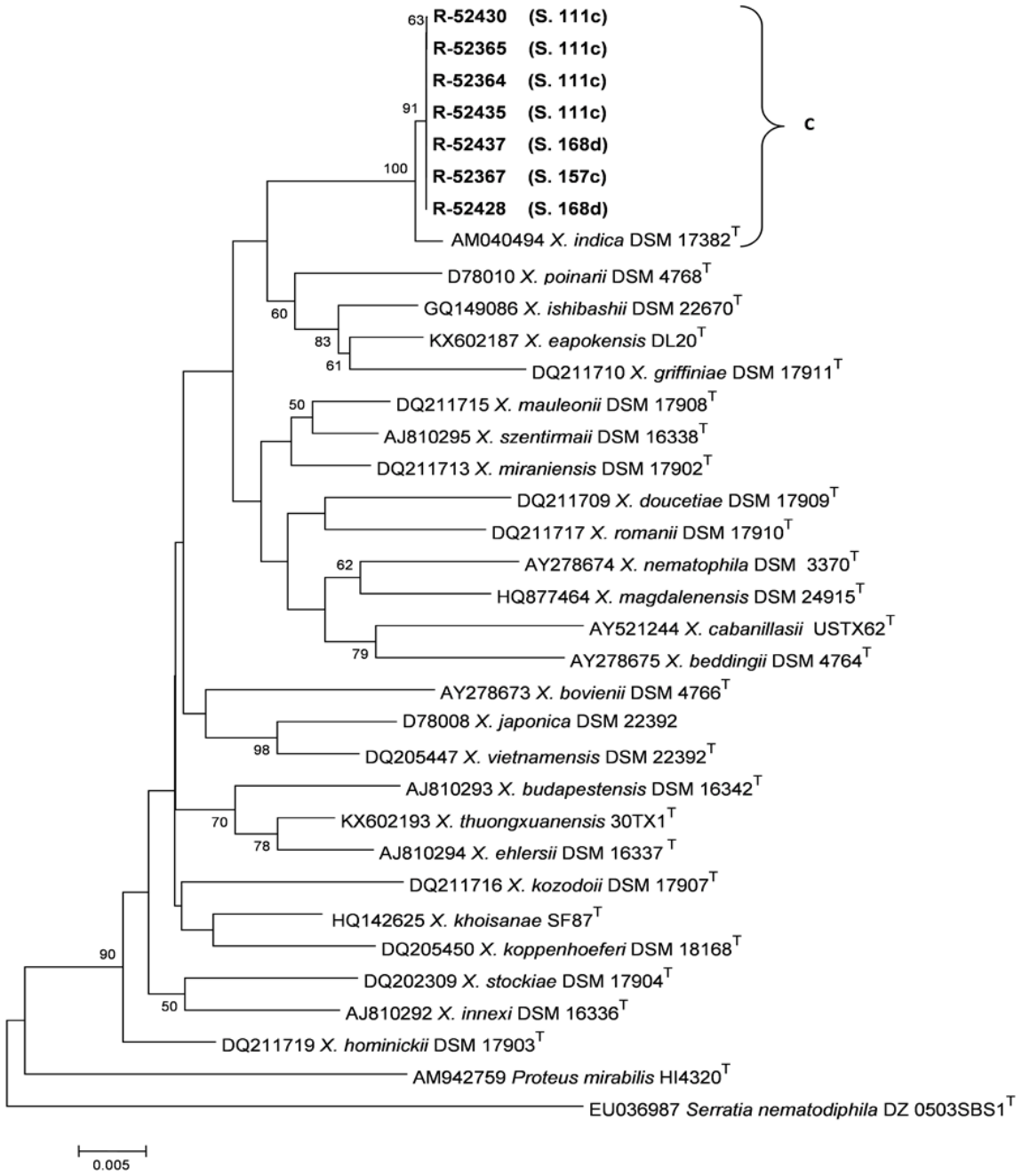


Figure 4.1 Neighbor joining tree based on 1,427 kb 16S rRNA sequences for 7 *Xenorhabdus* isolates (indicated in bold), from entomopathogenic nematodes (EPN isolates in brackets next to the bacterial isolate) recovered from Beninese soils and reference strains. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* and *Serratia nematodiphila* were used as outgroups. **S**. stands for *Steinernema* isolate

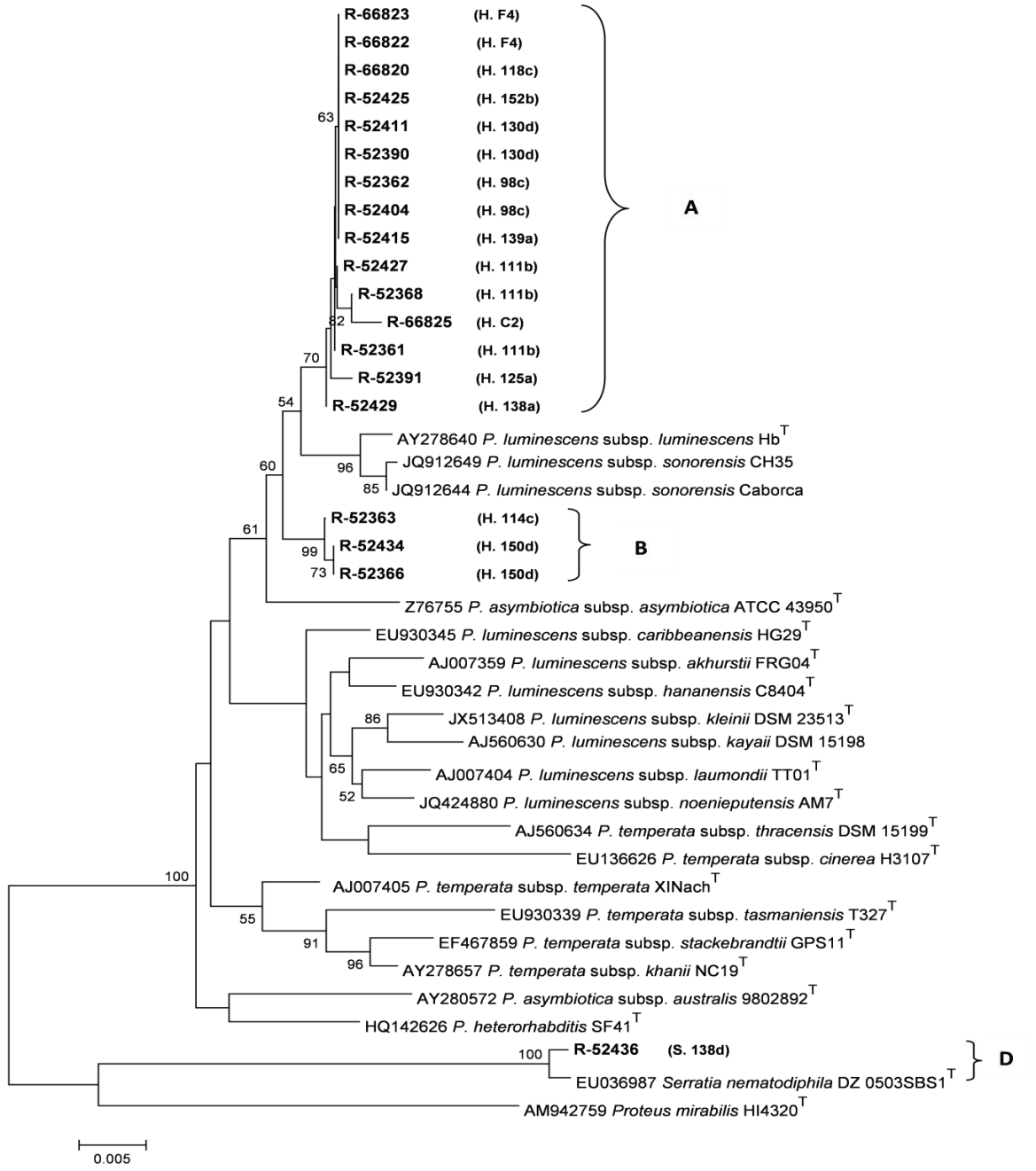


Figure 4.2 Neighbor joining tree based on 1,349 kb 16S rRNA sequences for 18 *Photorhabdus* and 1 *Serratia* strain (indicated in bold), from entomopathogenic nematodes (EPN isolates in brackets next to the bacterial isolate) recovered in Beninese soils and reference strains. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup. **H.** stands for *Heterorhabditis* isolate, **S**. stands for *Steinernema* isolate

4.5 Discussion

Bacterial symbionts of EPNs play an important role in the nematode's virulence against insect pests (Liu et al. 2001).

The main objective of this study was to investigate the biological diversity of *Photorhabdus* and *Xenorhabdus* bacteria associated with EPNs from Benin.

Bacterial colonies isolated from hemolymph of Gm infected with Beninese *Steinernema* and *Heterorhabditis* nematodes, turned blue, dark green or red on NBTA plates after 48h of incubation. This indicates they are able to absorb the Bromothymol blue dye contained in the NBTA medium. The variation of colony color has been attributed to bacterial phase variation. Indeed, *Xenorhabdus* and *Photorhabdus* bacteria have been described as occurring in two forms: phase I, occurring in IJs, and phase II, occurring under *in vitro* conditions (Akhurst 1980; Smigielski et al. 1994). Phase II bacteria have been demonstrated to have less ability to absorb dye from NBTA medium compared to phase I bacteria (Boemare and Akhurst 1988). Some isolates identified as *Photorhabdus* species showed bioluminescence in the darkness which is a typical characteristic of *Photorhabdus* species (Forst and Nealson 1996).

Phylogenetic analysis of the 43 bacterial symbionts isolated from Gm infected with Beninese EPNs indicated that most bacterial symbionts isolated from Gm infected with *Steinernema* nematodes were identified as *Xenorhabdus* species, and *Photorhabdus* species were isolated from Gm infected with *Heterorhabditis* EPNs. This result confirmed the assumption established over the last 20 years that *Xenorhabdus* is present in *Steinernema* and *Photorhabdus* in *Heterorhabditis* (Forst et al. 1997). However, the bacterial strain (R-52436), identified as *Serratia nematodiphila* based on 16S rRNA gene analysis, was isolated from Gm infected with a nematode strain (138d) which appears to be a *Steinernema* species based on the ITS fragments analysis. *Serratia nematodiphila* has been isolated from *Heterorhabditidoides chongmingensis* (Zhang et al. 2008), a newly described nematode proposed to be part of the EPN group. The present study is the first report of a *Serratia* strain isolated from Gm larvae infected with *Steinernema* nematodes. However, despite the surface sterilization of the *G. mellonella* cadaver with 70% alcohol prior to bacterial isolation and the fact that we did not pick up members of other bacterial genera, it cannot be totally excluded that R-52436 might be a contaminant. In previous studies, *Steinernema* have occasionally been found to be associated with bacteria other

Figure 4.3 Neighbor joining tree based on concatenated recA and gyrB gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* was used as outgroup

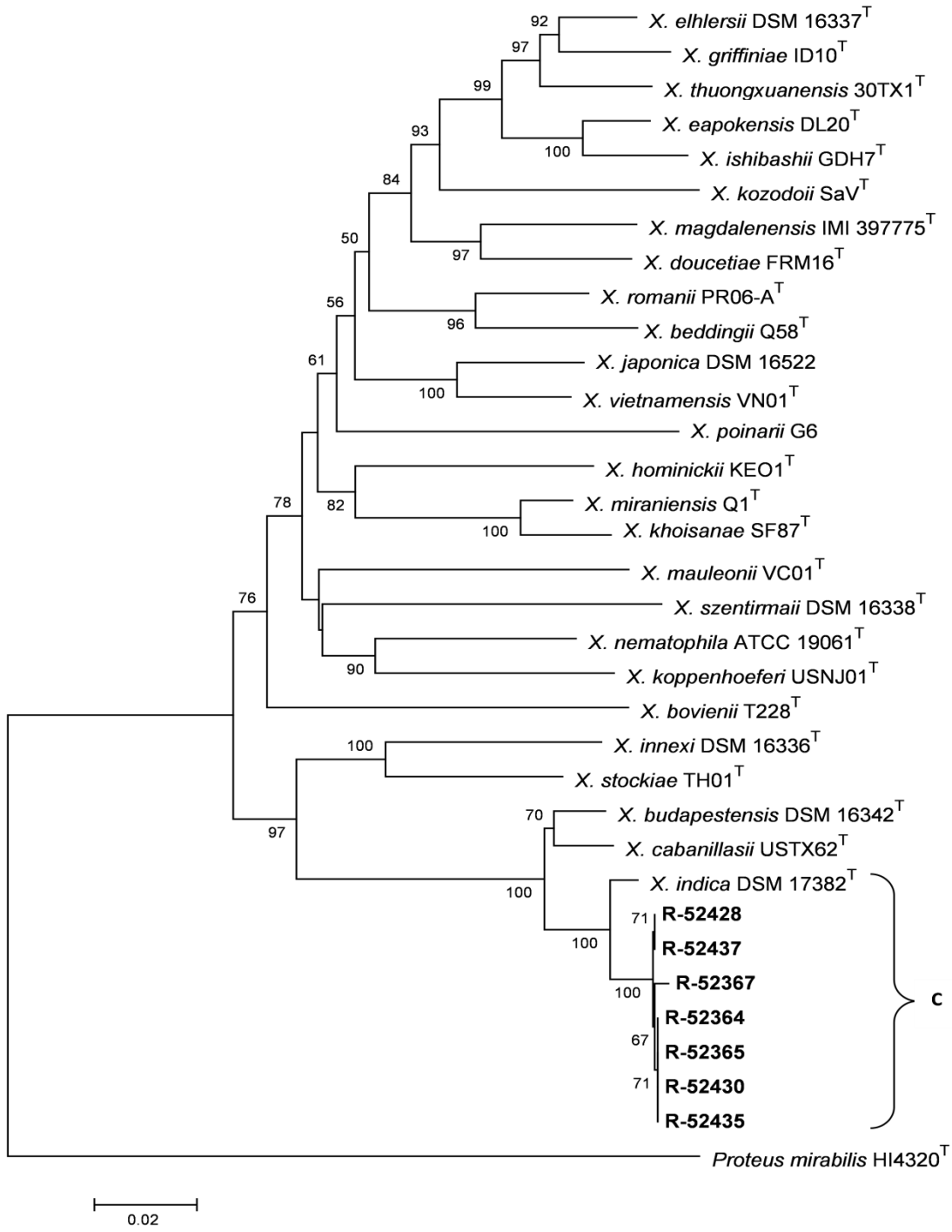


Figure 4.4 Neighbor joining tree based on concatenated recA and gyrB gene sequences showing the phylogenetic position of Beninese *Xenorhabdus* isolates (in bold) among type strains of than described *Xenorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* was used as outgroup

than *Xenorhabdus* (Aguillera et al. 1993; Elawad et al. 1999; Lysenko and Weiser 1974) although those bacteria were demonstrated to originate from the cuticle (Bonifassi et al. 1999). Also for EPNs of *Heterorhabditis indica*, Babic et al. (2000) reported in addition to the typical symbiotic bacteria (*Photorhabdus luminescens* subsp. *akhurstii*), the presence of *Ochrobactrum* spp. bacteria.

The 16S rRNA gene sequence analysis for the *Xenorhabdus* isolates showed their high similarity (99.7%) with *X. indica* DSM 17382^T. In addition, concatenated recA and gyrB sequences confirmed their identification as *X. indica* (98.5% similarity with strain DSM 17382^T). This species has been reported the first time by Somvanshi et al. (2006) and found to be associated to *Steinernema thermophilum*, junior synonym of *S. abbasi* (Hunt and Subbotin 2016; Nguyen and Hunt 2007; Tailliez et al. 2006). In our case these bacterial isolates were indeed isolated from Gm infected with nematodes clustering with *S. abbasi* based on ITS regions sequences analysis (Supplementary Figure 4.1). Recently, *X. indica* has also been reported to be associated to *Steinernema yirgalemense* (Ferreira et al. 2016). It can therefore be assumed that *X. indica* may not be specifically associated to a single nematode species although *S. yirgalemense* forms a sister clade to *S. abbasi/ S. thermophilum* based on the ITS phylogeny (Supplementary Figure 4.1). The association of a single bacterial species to different nematode species within and in between clades is increasingly reported in recent years. More than 17 host switches have been reported by Lee and Stock (2010). Furthermore, Dreyer et al. (2017) have recently demonstrated three new *Xenorhabdus-Steinernema* associations with *X. khoisanae* found in association with *Steinernema jeffreyense* and *Steinernema sacchari* which belong to distantly related EPN clades V an III, respectively. The same authors reported the association of *Steinernema nguyeni* with *X. bovieni*, initially reported (Stock 2015) to be associated with nematodes in the *Affine*-clade such as *Steinernema affine* and *Steinernema intermedium* on one hand and with nematodes in *Feltiae*-clade such as *Steinernema feltiae* on another hand.

Analysis of 16S rRNA, gyrB and recA genes sequences of *Photorhabdus* isolates, demonstrated that they belong to the *P. luminescens* cluster. They constitute two separate sub-clusters (A) and (B) within the *P. luminescens* group in the phylogenetic trees of 16S rRNA and concatenated gyrB and recA genes (Figures 4.2 and 4.3). Based on the concatenated recA and gyrB sequence analysis, the two sub-clusters (A) and (B) show similar levels of sequence similarity (approx. 94 to 97%) to several other *P. luminescens* subspecies. These relatively low similarity values

indicate that the two sub-clusters (A) and (B) probably represent two different new subspecies within *P. luminescens* group.

Isolates contained in sub-cluster (A) were isolated from Gm infected with nematodes identified, based on ITS region analysis (Supplementary Figure 4.1), as *H. sonorensis*, a senior synonym of *H. taysearae* (Hunt and Subbotin 2016). Bacterial isolates in sub-cluster (B) were isolated from Gm infected with nematode isolates grouping with *H. indica* (Supplementary Figure 4.1). *H. taysearae* was described as having *P. luminescens* subsp. *sonorensis* as bacterial symbiont (Orozco et al. 2013) while *H. indica* has been reported to live in association with *P. luminescens* subsp. *akhurstii* (Fischer-Le Saux et al. 1999). In our study, we found *H. taysearae* and *H. indica* to be in association with new sub-clusters (A) and (B) respectively. Both *Heterorhabditis* species belong to the *H. indica* clade with each of them belonging to one of two proposed subclades (Spiridonov and Subbotin 2016).

In this study, in addition to the conserved 16S rRNA genes, also the more variable housekeeping genes gyrB and recA were sequenced to assess for the first time the diversity of *Photorhabdus* and *Xenorhabdus* isolates from EPNs recovered from soil from Benin. Some or all three of these genes have been used in recent years to characterize EPN bacteria (Cimen et al. 2016b; Fukruksa et al. 2017; Muangpat et al. 2017; Thanwisai et al. 2012). However, for more comprehensive taxonomic characterization and definition of new groups, a multigene approach involving more housekeeping genes (recA, gyrB, gltX, dnaN and infB) has recently been proposed (Tailliez et al. 2010; Tailliez et al. 2012) to increase the robustness of the phylogeny of *Photorhabdus* and *Xenorhabdus* bacteria. A threshold of 97% nucleotide identity has been proposed (Tailliez et al. 2010) to distinguish species in *Xenorhabdus* and subspecies in *Photorhabdus* groups. In our study, in accordance with 16S rRNA gene analysis, concatenated recA and gyrB sequence phylogeny clearly supported the clustering of Beninese *Photorhabdus* isolates in separate sub-clusters (A) and (B). Nevertheless, further molecular information based on gltX, dnaN and infB genes and some phenotypic studies are needed to fully clarify the status of sub-clusters (A) and (B) isolates in the *P. luminescens* group as potential new subspecies of *Photorhabdus luminescens*.

Overall, this molecular characterization study of symbiotic bacteria of Beninese EPNs allowed us to find two new groups of *Photorhabdus luminescens* strains associated with *H. taysearae* and *H. indica*. In addition, *Xenorhabdus indica* was identified in association with EPNs that cluster with

Steinernema abbasi based on ITS region analysis. These bacteria will be used in large scale multiplication of the associated Beninese EPNs, as they constitute their major food source, for insect pest biological control purposes.

4.6 Acknowledgements

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4.7 References

Aguillera MM, Hodge NC, Stall RE, Smart GC (1993) Bacterial symbionts of *Steinernema scapterisci*. Journal of Invertebrate Pathology 62:68-72

Akhurst R (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. Journal of General Microbiology 121:303-309

Akhurst R (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061-3065

Akhurst R, Boemare N, Janssen P, Peel M, Alfredson D, Beard C (2004) Taxonomy of Australian clinical isolates of the genus *Photorhabdus* and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. *australis* subsp. nov. International Journal of Systematic and Evolutionary Microbiology 54:1301-1310

Babic I, Fischer-Le Saux M, Giraud E, Boemare N (2000) Occurrence of natural dixenic associations between the symbiont *Photorhabdus luminescens* and bacteria related to *Ochrobactrum* spp. in tropical entomopathogenic *Heterorhabditis* spp.(Nematoda, Rhabditida). Microbiology 146:709-718

Baimey H, Zadji L, Afouda L, Moens M, Decraemer W (2015) Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from Southern Benin and control of underground termite nest populations. Nematology 17:1057-1069

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Bird A, Akhurst R (1983) The nature of the intestinal vesicle in nematodes of the family Steinernematidae. International Journal for Parasitology 13:599-606

Boemare N (2002) Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler R (ed), Entomopathogenic Nematology CABI Wallingford, UK, pp 35-56

Boemare N, Akhurst R (1988) Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). Journal of General Microbiology 134:751-761

Boemare N, Akhurst R, Mourant R (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal

to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249-255

Bonifassi E, Fischer-Le Saux M, Boemare N, Lanois A, Laumond C, Smart G (1999) Gnotobiological study of infective juveniles and symbionts of *Steinernema scapterisci:* a model to clarify the concept of the natural occurrence of monoxenic associations in entomopathogenic nematodes. Journal of Invertebrate Pathology 74:164-172

Cimen H, Půža V, Nermuť J, Hatting J, Ramakuwela T, Faktorova L, Hazir S (2016) *Steinernema beitlechemi* n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from South Africa. Nematology 18:439-453

Cleenwerck I, Camu N, Engelbeen K, De Winter T, Vandemeulebroecke K, De Vos P, De Vuyst L (2007) *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. International Journal of Systematic and Evolutionary Microbiology 57:1647-1652

Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J (2002) Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. International Journal of Systematic and Evolutionary Microbiology 52:1551-1558

Coenye T, Falsen E, Vancanneyt M, Hoste B, Govan JR, Kersters K, Vandamme P (1999) Classification of Alcaligenes faecalis-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49:405-413

Dreyer J, Malan AP, Dicks LM (2017) Three Novel *Xenorhabdus–Steinernema* Associations and Evidence of Strains of *X. khoisanae* Switching Between Different Clades. Current Microbiology 74:938–942

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Elawad S, Robson R, Hague N (1999) Observations on the bacterial symbiont associated with the nematode, *Steinernema abbasi* (Steinernematidae: Nematoda). In: COST. pp 105-111

Emelianoff V, Sicard M, Le Brun N, Moulia C, Ferdy J-B (2007) Effect of bacterial symbionts *Xenorhabdus* on mortality of infective juveniles of two *Steinernema* species. Parasitology Research 100:657-659

Ferreira T, Van Reenen C, Tailliez P, Pagès S, Malan A, Dicks L (2016) First report of the symbiotic bacterium *Xenorhabdus indica* associated with the entomopathogenic nematode *Steinernema yirgalemense*. Journal of Helminthology 90:108-112

Fischer-Le Saux M, Viallard V, Brunel B, Normand P, Boemare NE (1999) Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49:1645-1656

Forst S, Dowds B, Boemare N, Stackebrandt E (1997) *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annual Reviews in Microbiology 51:47-72

Forst S, Nealson K (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiological Reviews 60:21-43

Fukruksa C, Yimthin T, Suwannaroj M, Muangpat P, Tandhavanant S, Thanwisai A, Vitta A (2017) Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against *Aedes aegypti*. Parasites & Vectors 10:440

Grewal PS, Selvan S, Gaugler R (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. Journal of Thermal Biology 19:245-253

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors), Advances in Taxonomy and Phylogeny of Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Kazimierczak W, Skrzypek H, Sajnaga E, Skowronek M, Waśko A, Kreft A (2017) Strains of *Photorhabdus* spp. associated with polish *Heterorhabditis* isolates: their molecular and phenotypic characterization and symbiont exchange. Archives of Microbiology 199:979–989

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120

Lee M-M, Stock SP (2010) A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp.(γ-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. Journal of Invertebrate Pathology 104:67-74

Liu J, Berry RE, Blouin MS (2001) Identification of symbiotic bacteria *(Photorhabdus* and *Xenorhabdus*) from the entomopathogenic nematodes *Heterorhabditis marelatus* and *Steinernema oregonense* based on 16S rDNA sequence. Journal of Invertebrate Pathology 77:87-91

Lysenko O, Weiser J (1974) Bacteria associated with the nematode *Neoaplectana carpocapsae* and the pathogenicity of this complex for *Galleria mellonella* larvae. Journal of Invertebrate Pathology 24:332-336

Martens EC, Goodrich-Blair H (2005) The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. Cellular Microbiology 7:1723-1735

Muangpat P, Yooyangket T, Fukruksa C, Suwannaroj M, Yimthin T, Sitthisak S, Chantratita N, Vitta A, Tobias NJ, Bode HB (2017) Screening of the Antimicrobial Activity against Drug Resistant Bacteria of *Photorhabdus* and *Xenorhabdus* Associated with Entomopathogenic Nematodes from Mae Wong National Park, Thailand. Frontiers in Microbiology 8:1142

Nguyen K, Hunt D (2007) Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Hunt DJ, Perry RN (series editors), vol 5. Nematology Monographs and Perspectives. Brill, Leiden. 816pp.

Orozco RA, Hill T, Stock SP (2013) Characterization and phylogenetic relationships of *Photorhabdus luminescens* subsp. *sonorensis* (γ-Proteobacteria: Enterobacteriaceae), the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae). Current Microbiology 66:30-39

Peat SM, Waterfield NR, Marokházi J, Fodor A, Adams BJ (2010) A robust phylogenetic framework for the bacterial genus *Photorhabdus* and its use in studying the evolution and maintenance of bioluminescence: a case for 16S, gyrB, and glnA. Molecular Phylogenetics and Evolution 57:728-740

Pitcher D, Saunders N, Owen R (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters in Applied Microbiology 8:151-156

Rainey F, Ehlers R-U, Stackebrandt E (1995) Inability of the polyphasic approach to systematics to determine the relatedness of the genera *Xenorhabdus* and *Photorhabdus*. International Journal of Systematic and Evolutionary Microbiology 45:379-381

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425

Sergeant M, Baxter L, Jarrett P, Shaw E, Ousley M, Winstanley C, Morgan JAW (2006) Identification, typing, and insecticidal activity of *Xenorhabdus* isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the xpt toxin loci. Applied and Environmental Microbiology 72:5895-5907

Smigielski AJ, Akhurst RJ, Boemare NE (1994) Phase variation in *Xenorhabdus nematophilus* and *Photorhabdus luminescens*: differences in respiratory activity and membrane energization. Applied and Environmental Microbiology 60:120-125

Somvanshi VS, Lang E, Ganguly S, Swiderski J, Saxena AK, Stackebrandt E (2006) A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. Systematic and Applied Microbiology 29:519-525

Spiridonov SE, Subbotin SA (2016) Phylogeny and phylogeography of *Heterorhabditis* and *Steinernema*. Advances in Entomopathogenic Nematode Taxonomy and Phylogeny 12:413-427

Stock SP (2015) Diversity, biology and evolutionary relationships. In: Campos - Herrera R (ed), Nematode Pathogenesis of Insects and Other Pests. Springer, pp 3-27

Strauch O, Ehlers R-U (1998) Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Applied Microbiology and Biotechnology 50:369-374

Szállás E, Koch C, Fodor A, Burghardt J, Buss O, Szentirmai A, Nealson KH, Stackebrandt E (1997) Phylogenetic evidence for the taxonomic heterogeneity of *Photorhabdus luminescens*. International Journal of Systematic and Evolutionary Microbiology 47:402-407

Tailliez P, Laroui C, Ginibre N, Paule A, Pagès S, Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X.*

vietnamensis sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60:1921-1937

Tailliez P, Pagès S, Edgington S, Tymo LM, Buddie AG (2012) Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. International Journal of Systematic and Evolutionary Microbiology 62:1761-1765

Tailliez P, Pages S, Ginibre N, Boemare N (2006) New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. International Journal of Systematic and Evolutionary Microbiology 56:2805-2818

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725-2729

Thanwisai A, Tandhavanant S, Saiprom N, Waterfield NR, Long PK, Bode HB, Peacock SJ, Chantratita N (2012) Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. PLoS One 7:e43835

Thomas GM, Poinar GO (1979) *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. International Journal of Systematic Bacteriology 29:352-360

Torres-Barragan A, Suazo A, Buhler WG, Cardoza YJ (2011) Studies on the entomopathogenicity and bacterial associates of the nematode *Oscheius carolinensis*. Biological Control 59:123-129

Tóth T, Lakatos T (2008) *Photorhabdus temperata* subsp. *cinerea* subsp. nov., isolated from *Heterorhabditis* nematodes. International Journal of Systematic and Evolutionary Microbiology 58:2579-2581

Vrain T, Wakarchuk D, Levesque A, Hamilton R (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15:563-573

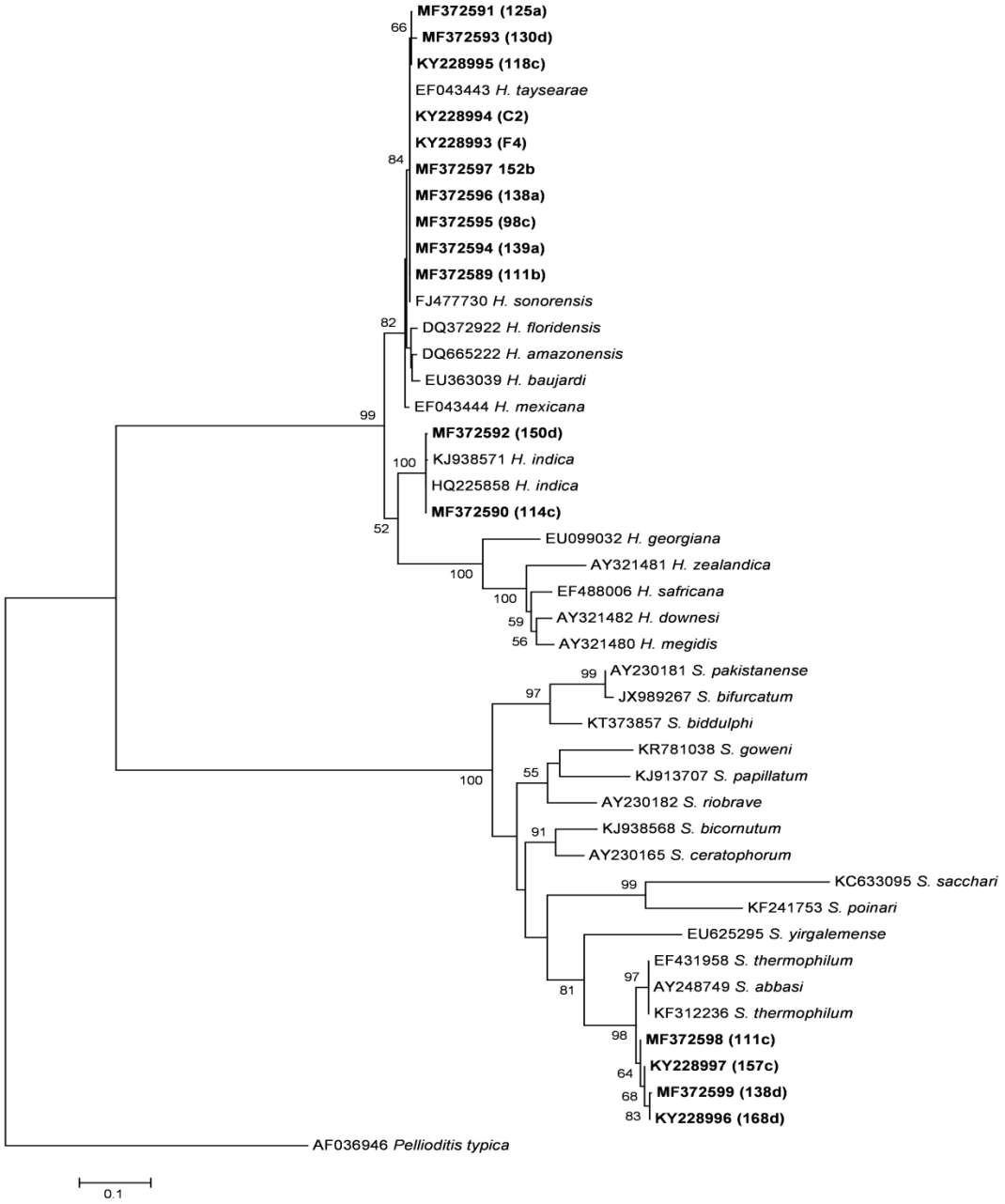
White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

Zadji L, Baimey H, Afouda L, Houssou FG, Waeyenberge L, de Sutter N, Moens M, Decraemer W (2013) First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Southern Benin. Russ J Nematol 21:117-130

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014) Comparative susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Isoptera: Termitidae) to entomopathogenic nematodes from Benin. Nematology 16:719-727

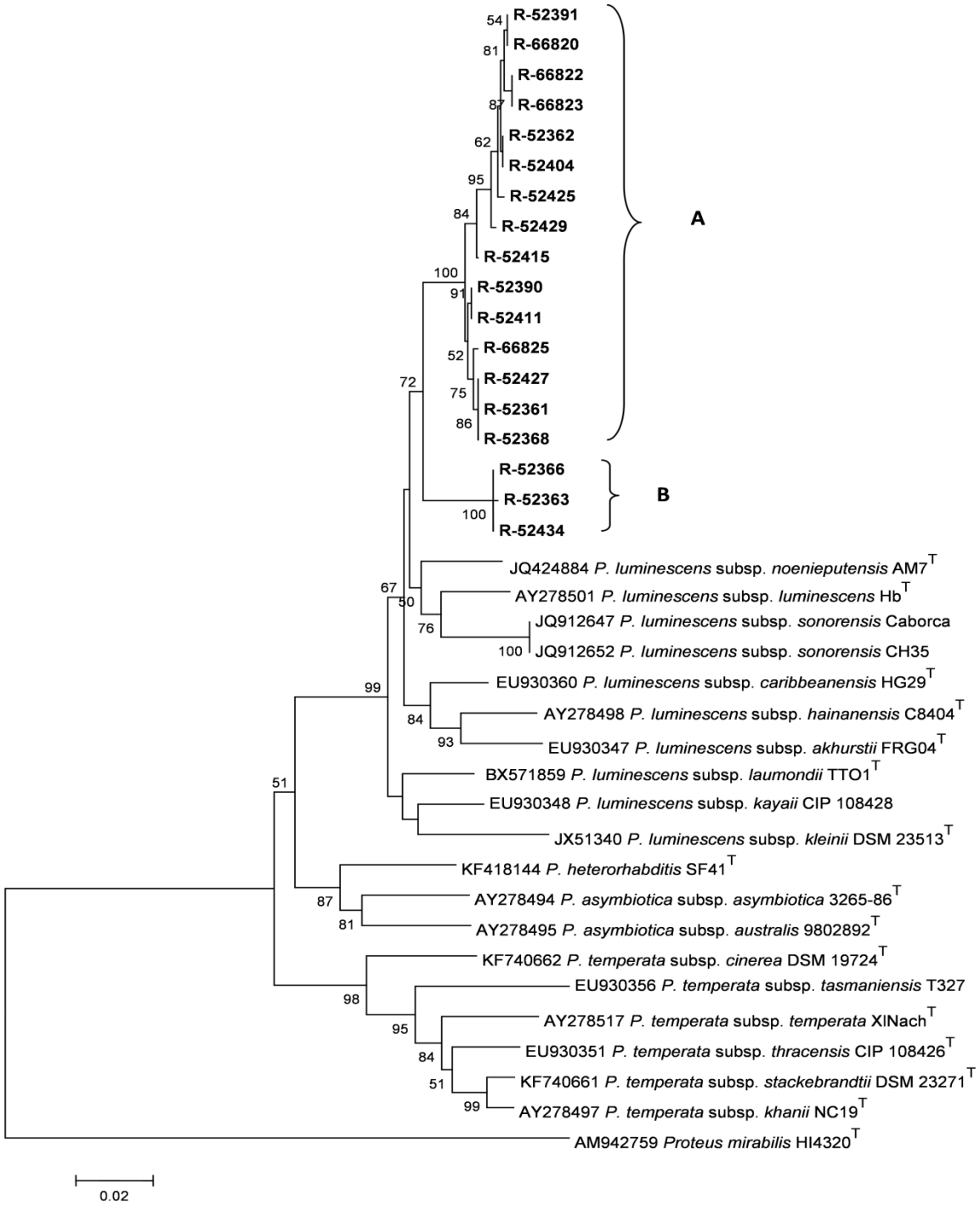
Zhang C, Liu J, Xu M, Sun J, Yang S, An X, Gao G, Lin M, Lai R, He Z (2008) *Heterorhabditidoides chongmingensis* gen. nov., sp. nov.(Rhabditida: Rhabditidae), a novel member of the entomopathogenic nematodes. Journal of Invertebrate Pathology 98:153-168

4.8 Supplementary materials

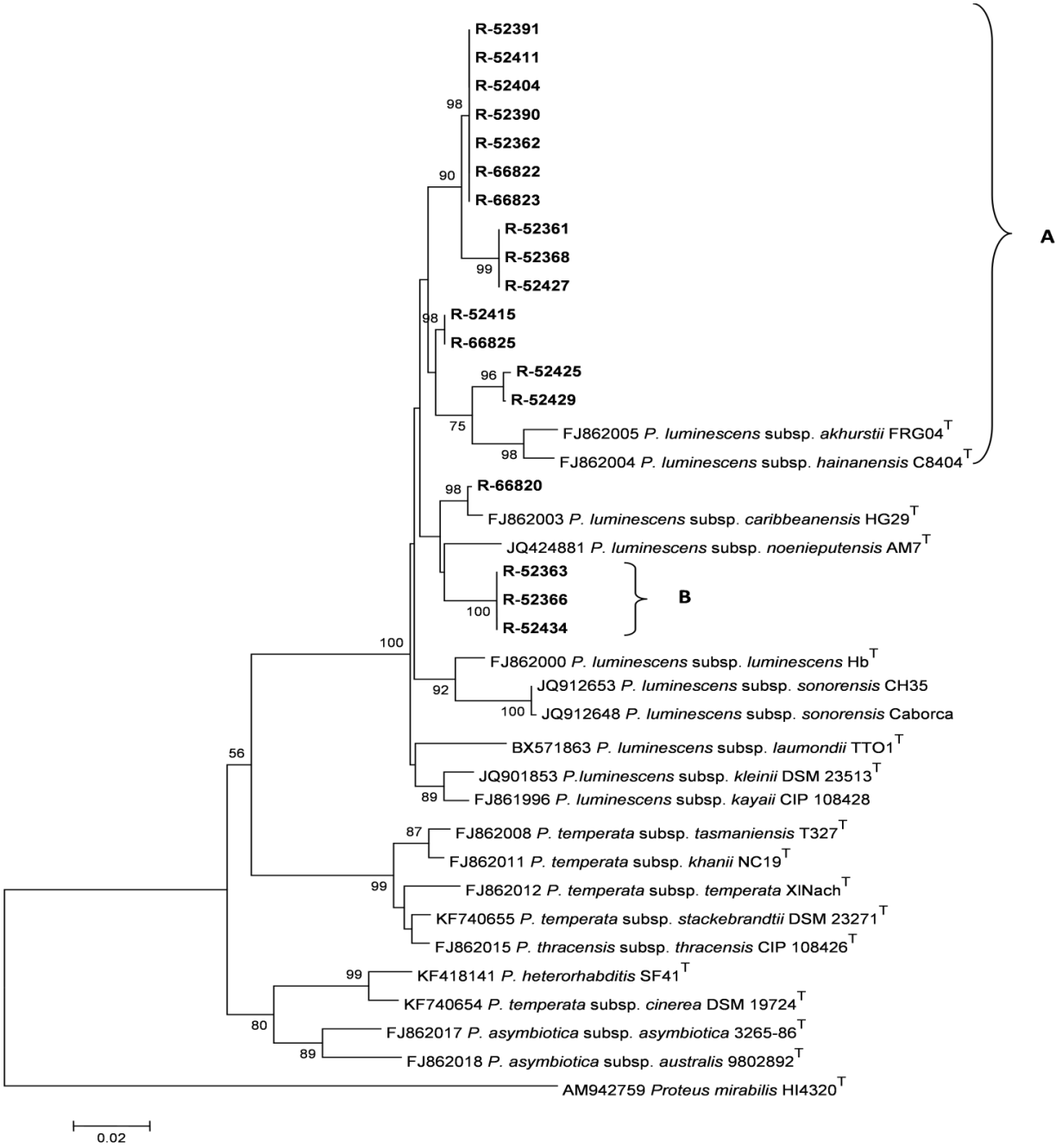


Supplementary Figure 4.1 Neighbor joining tree based on partial ITS sequences of entomopathogenic nematodes recovered from Beninese soil. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before strains name correspond to GenBank accession number. Beninese isolates are shown in bold. *Pellioditis typica* was used as outgroup.

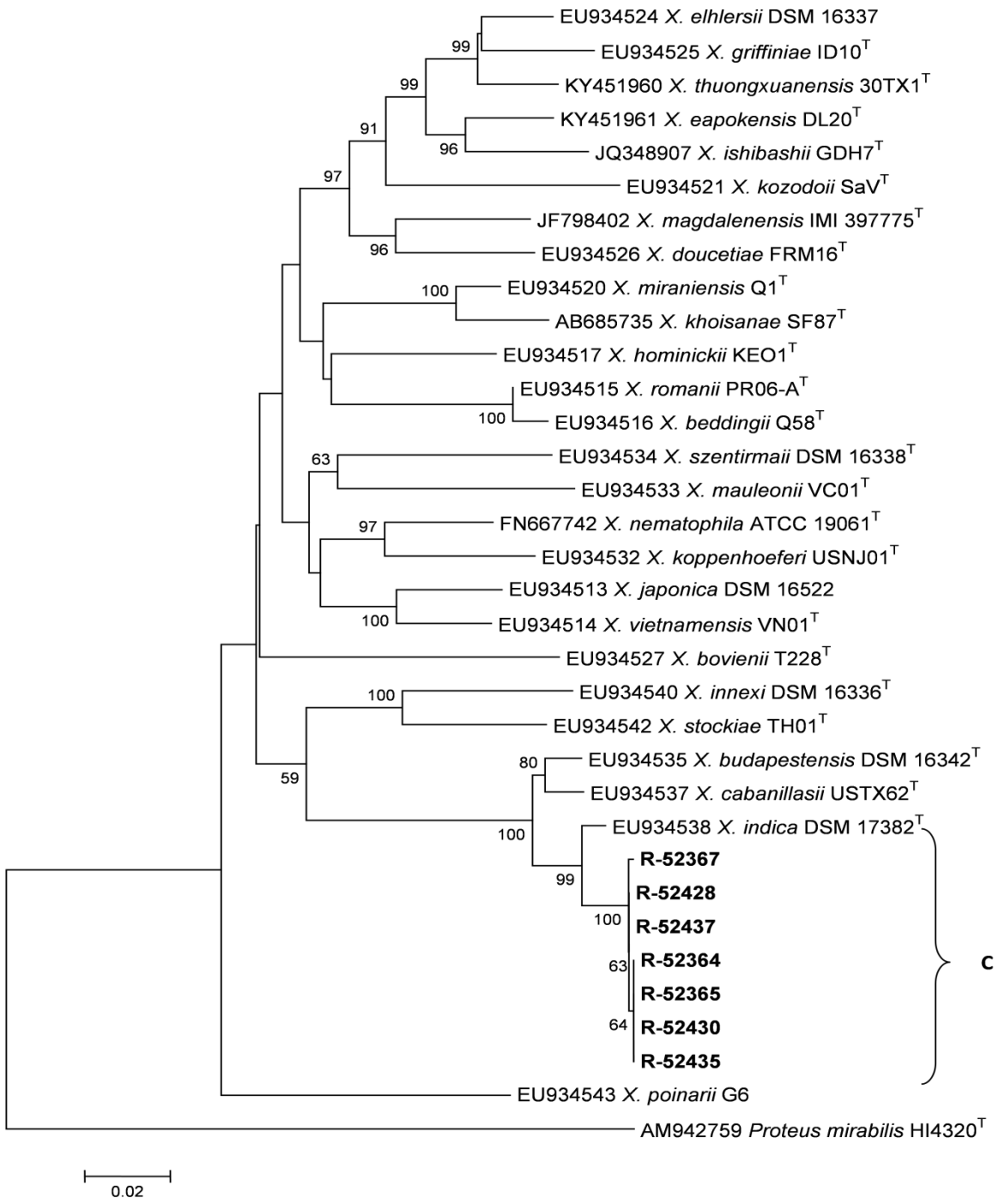
Supplementary Figure 4.2 Neighbor joining tree based on partial 16S rRNA gene sequences for 43 bacterial isolates (in bold) isolated from Gm infected with entomopathogenic nematodes recovered from Beninese soils. Bootstrap values above 30% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup.



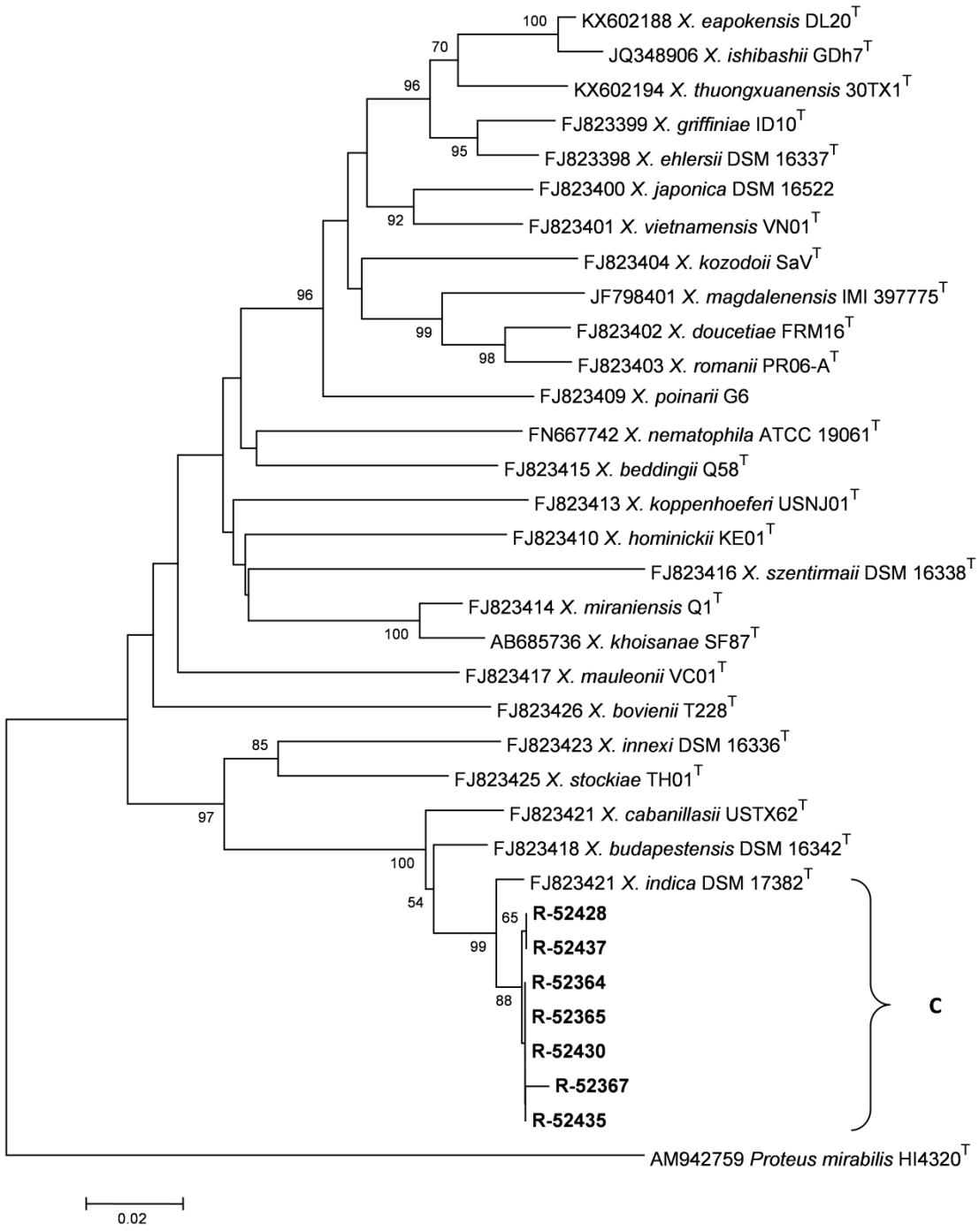
Supplementary Figure 4.3 Neighbor joining tree based on partial gyrB sequences for 18 *Photorhabdus* isolates (in bold). Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup.



Supplementary Figure 4.4 Neighbor joining tree based on partial recA sequences for 18 *Photorhabdus* isolates (in bold). Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup.



Supplementary Figure 4.5 Neighbor joining tree based on partial gyrB sequences for 7 X*enorhabdus* isolates (in bold). Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup.



Supplementary Figure 4.6 Neighbor joining tree based on partial recA sequences for 7 X*enorhabdus* isolates (in bold). Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. Numbers before reference strains name correspond to GenBank accession numbers. *Proteus mirabilis* was used as outgroup.

Supplementary Table 4.1 overview of Accession numbers of 16S rRNA, recA and gyrB gene sequences included in the phylogenies. Beninese isolates are indicated in Bold.

Bacterial isolates	**16S rRNA**	**recA**	**gyrB**	**Bacterial isolatess**	**16S rRNA**	**recA**	**gyrB**
R-66822	MF353507	MF356978	MF357004	*P. temperata* subsp. *tasmaniensis* T327^{T}	EU930339	FJ862008	EU930356
R-66823	MF353506	MF356980	MF357005	**R-52436**	MF353489	N/T	N/T
R-66825	MF353505	MF356977	MF357003	*Serratia nematodiphila* DZ 0503SBS1^{T}	EU036987	N/T	N/T
R-52429	MF353494	MF356975	MF357001	**R-52428**	MF353510	MF356983	MF357009
R-52373	MF353447	N/T	N/T	**R-52437**	MF353512	MF356987	MF357012
R-52403	MF353442	N/T	N/T	**R-52382**	MF353454	N/T	N/T
R-52416	MF353441	N/T	N/T	**R-52377**	MF353452	N/T	N/T
R-52415	MF353490	MF356970	MF356996	**R-52417**	MF353450	N/T	N/T
R-52402	MF353445	N/T	N/T	**R-52367**	MF353511	MF356982	MF357008
R-52380	MF353443	N/T	N/T	**R-52379**	MF353448	N/T	N/T
R-52425	MF353500	MF356972	MF356997	**R-52435**	MF353514	MF356985	MF357011
R-52362	MF353495	MF356968	MF356994	**R-52364**	MF353515	MF356979	MF357006
R-52404	MF353491	MF356964	MF356990	**R-52365**	MF353513	MF356981	MF357007
R-52412	MF353444	N/T	N/T	**R-52430**	MF353508	MF356984	MF357010
R-52410	MF353446	N/T	N/T	**R-52405**	MF353453	N/T	N/T
R-52390	MF353496	MF356967	MF356993	**R-52426**	MF353451	N/T	N/T
R-52411	MF353498	MF356963	MF356989	**R-52406**	MF353449	N/T	N/T
R-52383	MF353440	N/T	N/T	**R-52372**	MF353509	N/T	N/T
R-52368	MF353502	MF356971	MF356998	*Xenorhabdus beddingii* Q58^{T}	AY278675	FJ823415	EU934516
R-52361	MF353497	MF356965	MF356991	*Xenorhabdus cabanillasii* USTX62^{T}	AY521244	FJ823425	EU934537
R-52427	MF353493	MF356974	MF357000	*Xenorhabdus doucetiae* FRM16^{T}	DQ211709	FJ823402	EU934526
R-52389	MF353439	N/T	N/T	*Xenorhabdus magalenensis* IMI 397775^{T}	HQ877464	JF798401	JF798402
R-66820	MF353504	MF356976	MF357002	*Xenorhabdus nematophila* ATCC 19061^{T}	AY278674	FN667742	FN667742
R-52391	MF353499	MF356962	MF356988	*Xenorhabdus stokiae* TH01^{T}	DQ202309	FJ823425	EU934542
R-52434	MF353501	MF356973	MF356999	*Xenorhabdus bovienii* T228^{T}	AY278673	FJ823426	EU934527
R-52366	MF353503	MF356969	MF356995	*Xenorhabdus kozodoii* SaV^{T}	DQ211716	FJ823404	EU934521

Bacterial isolates	**16S rRNA**	**recA**	**gyrB**	**Bacterial isolatess**	**16S rRNA**	**recA**	**gyrB**
R-52363	MF353492	MF356966	MF356992	*Xenorhabdus innexi* DSM16336 ^{T}	AJ810292	FJ823423	EU934540
Photorhabdus luminescens subsp. *sosnorensis* CH35	JQ912649	JQ912653	JQ912652	*Xenorhabdus japonica* DSM22392	D78008	FJ823400	EU934513
Photorhabdus luminescens subsp. *sosnorensis* Caborca	JQ912644	JQ912648	JQ912647	*Xenorhabdus vietnamensis* VN01^{T}	DQ205447	FJ823401	EU934514
Photorhabdus luminescens subsp. *luminescens* Hb ^{T}	AY278640	FJ862000	AY278501	*Xenorhabdus hominickii* KEO1^{T}	DQ211719	FJ823410	EU934517
Photorhabdus luminescens subsp. *kayaii* DSM 15198	AJ560630	FJ861996	EU930348	*Xenorhabdus budapestensis* DSM16342 ^{T}	AJ810293	FJ823418	EU934535
Photorhabdus luminescens subsp. *kleinii* DSM23513 ^{T}	JX513408	JQ901853	JX51340	*Xenorhabdus koppenhoeferi* USNJ01^{T}	DQ205450	FJ823413	EU934532
Photorhabdus luminescens subsp. *laumondii* TT01 ^{T}	AJ007404	BX571863	BX571859	*Xenorhabdus szentirmaii* DSM16338 ^{T}	AJ810295	FJ823416	EU934534
Photorhabdus luminescens subsp. *noenieputensis* AM7 ^{T}	JQ424880	JQ424881	JQ424884	*Xenorhabdus miraniensis* Q1^{T}	DQ211713	FJ823414	EU934520
Photorhabdus luminescens subsp. *akhurstii* FRG04 ^{T}	AJ007359	FJ862005	EU930347	*Xenorhabdus mauleonii* VC01^{T}	DQ211715	FJ823417	EU934533
Photorhabdus luminescens subsp. *caribbeanensis* HG29 ^{T}	EU930345	FJ862003	EU930360	*Xenorhabdus romanii* PR06-A^{T}	DQ211717	FJ823403	EU934515
Photorhabdus luminescens subsp. *hainanensis* C8404 ^{T}	EU930342	FJ862004	AY278498	*Xenorhabdus griffiniae* ID10 ^{T}	DQ211710	FJ823399	EU934525
Photorhabdus temperata subsp. *khanii* NC19 ^{T}	AY278657	FJ862011	AY278497	*Xenorhabdus poinarii* G6	D78010	FJ823409	EU934543
Photorhabdus temperata subsp. *temperata* XINach ^{T}	AJ007405	FJ862012	AY278517	*Xenorhabdus eapokensis* DL20 ^{T}	KX602187	KX602188	KY451961
Photorhabdus temperata subsp. *thracensis* CIP 108426^{T}	AJ560634	FJ862015	EU930351	*Xenorhabdus thuongxuanensis* 30TX1 ^{T}	KX602193	KX602194	KY451960
Photorhabdus temperata subsp. *cinerea* DSM19724 ^{T}	EU136626	KF740654	KF740662	*Xenorhabdus ishibashii* GDH7 ^{T}	GQ149086	JQ348906	JQ348907
Photorhabdus temperata subsp. *stackebrandtii* DSM23271 ^{T}	EF467859	KF740655	KF740661	*Xenorhabdus ehlersii DSM16337* ^{T}	AJ810294	FJ823398	EU934524
Photorhabdus asymbiotica subsp. *australis* 9802892 ^{T}	AY280572	FJ862018	AY278495	*Xenorhabdus indica* DSM17382 ^{T}	AM040494	FJ823421	EU934538
Photorhabdus asymbiotica subsp. *asymbiotica* 3265-86 ^{T}	Z76755	FJ862017	AY278494	*Xenorhabdus khoisanae* SF87 ^{T}	HQ142625	AB685736	AB685735
Photorhabdus heterorhabditis SF41 ^{T}	HQ142626	KF418141	KF418144	*Proteus mirabilis* H14320 ^{T}	AM942759	AM942759	AM942759

5 Chapter 5:

Description of *Photorhabdus luminescens* subsp. *beninensis* subsp. nov., a novel symbiotic bacterium associated with *Heterohabditis taysearae* (Nematoda, Heterorhabditidae) nematodes isolated from Benin.

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Author's contribution:

AG and AW designed the experiments. AG performed the experiments. AG and AW analyzed the data. AG, WD, LA, HB and AW wrote the manuscript.

5.1 Abstract

Thirteen bacterial symbionts of *Heterorhabditis taysearae* nematodes collected in Benin were characterised phenotypically and genotypically to establish their taxonomic position. They share the main phenotypic features of *Photorhabdus* bacteria. Molecular analysis of the 16S rRNA gene sequences showed their close similarity to *Photorhabdus luminescens* subsp. *luminescens* Hb^T (98.9 – 99.4%) and *Photorhabdus luminescens* subsp. *sonorensis* Caborca^T (99 – 99.6% 16S rRNA). However, a multilocus analysis based on five housekeeping genes (recA, gyrB, dnaN, gltX and infB) indicated they represent a new subspecies of *Photorhabdus luminscens*, sharing at most 96.8%, 96.2% and 95.6% nucleotide similarity with *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *noenieputensis* and *P. luminescens* subsp. *luminescens*, respectively. The name *P. luminescens* subsp. *beninensis* is proposed for Beninese *Photorhabdus* strains. In contrast to *P. luminescens* subsp. *luminescens* Hb^T, *P. luminescens* subsp. *beninensis* subsp. nov. was able to ferment erythritol and ribose and did not produce hydrogen sulfide (H_2S). It can also be differentiated from *P. luminescens* subsp. *caribbeanensis* HG29^T by its positive reaction for esculin hydrolysis, negative amygdalin assimilation, and a weak positive fermentation of Methyl-α D-manopyranoside. Furthermore, *P. luminescens* subsp. *beninensis* subsp. nov. can be separated from *P. luminescens* subsp. *noenieputensis* AM7^T by its capacity to ferment mannitol and not arbutin. The type strain is LMG 30373^T (=DSM xxxxx^T). It was found to be highly pathogenic to *Galleria mellonella* mature larvae.

5.2 Introduction

Photorhabdus bacteria were initially described by Thomas and Poinar (1979) under the name *Xenorhabdus luminescens*, but later reclassified into a new genus *Photorhabdus* (Boemare et al. 1993) with type species *Photorhabdus luminescens*. Bacteria of the genus *Photorhabdus* belong to the family *Enterobacteriaceae* (Gammaproteobacteria) and are gram-negative. These bacteria live in symbiotic association with *Heterorhabditis* nematodes. Located in the intestine of the non-feeding infective juveniles of the nematodes, *Photorhabdus* bacteria are transported by the associated *Heterorhabditis* nematode and released into the hemolymph of an insect host when parasitized by the nematode. Here the bacteria multiply quickly and kill the insect host within 24h – 48 h by septicemia, allowing nematodes to resume development, feed on the bacteria (Koppenhöfer et al. 2007) and complete two to three generations inside the insect host (Kaya and Gaugler 1993). This pathogenic feature of the symbiotic partnership *Heterorhabditis* - *Photorhabdus*, as demonstrated on a wide range of insect pests in many studies, make them widely used in several biological control programs against insect pests around the world (Ehlers 2001; Ehlers 2005; Stock and Hunt 2005).

Up to the time of writing, 17 subspecies have been described within the genus *Photorhabdus* and grouped into four valid species: *Photorhabdus heterorhabditis* (Ferreira et al. 2014), *P. temperata*, *P. asymbiotica* (Fischer-Le Saux et al. 1999), and *P. luminescens* (Boemare et al. 1993; Thomas and Poinar 1979). Within the species *Photorhabdus luminescens*, 8 subspecies have been validly recognized: *Photorhabdus luminescens* subsp. *noenieputensis* (Ferreira et al. 2013a), *P. luminescens* subsp. *kleinii* (An and Grewal 2011), *P. luminescens* subsp. *caribbeanensis* and *P. luminescens* subsp. *hainanensis* (Tailliez et al. 2010), *P. luminescens* subsp. *kayaii* (Hazir et al. 2004), *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *laumondii* (Fischer-Le Saux et al. 1999) and *P. luminescens* subsp. *luminescens* (Boemare et al. 1993; Thomas and Poinar 1979). In addition, *P. luminescens* subsp. *sonorensis* (Orozco et al. 2013) has recently been described but not yet officially validated. *Photorhabdus temperata* comprises 6 subspecies which are: *Photorhabdus temperata* subsp. *temperata* (Fischer-Le Saux et al. 1999), *Photorhabdus temperata* subsp. *khanii* and *Photorhabdus temperata* subsp. *tasmaniensis* (Tailliez et al. 2010), *Photorhabdus temperata* subsp. *stackebrandtii* (An and Grewal 2010) and *Photorhabdus temperata* subsp. *thracensis* (Tailliez et al. 2010), initially

known as *Photorhabdus luminescens* subsp. *thracensis* (Hazir et al. 2004). Only two subspecies of *Photorhabdus asymbiotica* have been so far recognized: *Photorhabdus asymbiotica* subsp. *asymbiotica* (Akhurst et al. 2004; Fischer-Le Saux et al. 1999), and *Photorhabdus asymbiotica* subsp. *australis* (Akhurst et al. 2004). *Photorhabdus heterorhabditis* (Ferreira et al. 2014) is the most recent described species within the *Photorhabdus* genus and has no subspecies so far.

Previously, a proposal of a new subspecies within the *Photorhabdus* genus was mainly based on analysis of the 16S rRNA gene sequences in addition to some phenotypic features (Liu et al. 2001), but recently, a multigene-based analysis of protein coding genes like recA, gyrB, dnaN and gltX (Tailliez et al. 2010) and infB gene (Ferreira et al. 2013a; Tailliez et al. 2012) is also used. It has been demonstrated that within the genus *Photorhabdus*, strains that share less than 97% nucleotide identity of concatenated recA, gyrB, gltX, dnaN and infB gene sequences, could be delineated into new subspecies (Tailliez et al. 2010; Tailliez et al. 2012).

Several *Photorhabdus* isolates have been recovered from *Galleria mellonella* infected with entomopathogenic nematodes isolated from Benin (Godjo et al. 2018a). Based on the analysis of three genes (16S rRNA, recA, gyrB), these isolates were placed into two new sub-clades within the *Photorhabdus luminescens* group (3 isolates and 24 isolates) suggesting that they may represent two different new subspecies within *P. luminescens*. In this study, 13 strains from the largest of these groups are further characterized and described using Multi Locus Sequence Analysis (MLSA) based on 5 housekeeping genes complemented with phenotypic characterizations. The three bacterial isolates in the other group lost their viability for unkown reason and were therefore not included in the present work.

5.3 Material and methods

5.3.1 Bacterial isolates and culture conditions

Photorhabdus isolates identified in this study were obtained from the hemolymph of moribund *Galleria mellonella* (Gm) infected with *Heterorhabditis* nematode isolates recovered from Benin as described earlier (Godjo et al. 2018a) by plating them on Nutrient Bromothymol blue Agar (NBTA) plates (Akhurst 1980) and incubation in darkness for 48h at 28°C. *Photorhabdus luminescens* subsp. *luminescens* Hb^T (LMG 7797^T), *Photorhabdus luminescens* subsp.

caribbeanensis HG29^T (LMG 29448^T) and *Photorhabdus luminescens* subsp. *noenieputensis* AM7^T (LMG 29523^T) were used in this study as most closely related reference strains for comparison to the new *Photorhabdus* isolates.

5.3.2 Molecular identification

5.3.2.1 DNA extraction

Bacterial DNA was extracted as described earlier (Pitcher et al. 1989), using freshly harvested bacterial colonies grown on NBTA medium for 24h at 28°C.

5.3.2.2 Amplification and sequencing

The 16S rRNA gene and two housekeeping genes (recA and gyrB), were previously analyzed for partial identification of Beninese *Photorhabdus* strains (Godjo et al. 2018a). In addition to these genes, we amplified and sequenced parts of three more protein coding genes (dnaN, gltX and infB) using primers previously described (Tailliez et al. 2010; Tailliez et al. 2012).

Sequencing was performed using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an Applied Biosystems 3130xl DNA sequencer, using the protocol of the manufacturer (Applied Biosystems).

Sequences were assembled in Bionumerics 7 prior to an alignment performed using Clustal W incorporated in Mega 6.6 software (Tamura et al. 2013). Aligned sequences of each gene were visually inspected and trimmed to the common length of the *Photorhabdus* reference strains extracted from GenBank. Aligned nucleotide sequences of the protein coding genes were concatenated using an in-house Python script. Concatenated sequences and sequences of each gene were realigned and Neighbor joining trees were reconstructed using Kimura two parameter model in Mega 6.6.

Accession numbers of all bacterial sequences used for the concatenation of the 5 protein coding genes are provided in Supplementary Table 5.1.

5.3.3 Phenotypic characters

Four Beninese *Photorhabdus* isolates (Table 5.1) were randomly selected and phenotypically characterized together with type strains of three related *Photorhabdus luminescens* subsp. (Table 5.1). All bacterial isolates were grown on Tryptone Soya Agar (TSA) plates for 2 days in

darkness at 28°C before use. Each test was carried out in duplicate for each bacterial isolate if not specified otherwise.

5.3.3.1 Maximum temperature for bacterial growth

Bacterial strains were cultured in triplicate on TSA plates at 25, 28, 37, 40 and 42°C. Growth was checked based on visual observations over 2 to 5 days incubation.

5.3.3.2 Catalase and oxidase test

Catalase test was performed by placing a drop of hydrogen peroxide (11% v/v) on a colony of each bacterial isolates, as previously described (Ferreira et al. 2013a). Oxidase reaction was performed on bacterial colonies using 1-4 phenyldiammoniumchloride (1% g/v) and positive reaction was recorded when a blue color was observed.

5.3.3.3 Antibiotic reaction

Resistance to Ampicillin (10 µg/disc), Gentamicin (10 µg/disc), Chloramphenicol (30 µg/disc), Tetracycline (30 µg/disc), Bacitracin (0.04 IU) and Vancomycin (30 µg/disc) were tested using disc diffusion test on Muller-Hinton agar plates (per one litre of distilled water, 2 g Beef extract, 17.5 g Casein Hydrolysate, 1.5 g starch and 17 g Agar).

5.3.3.4 Enzymatic reactions

DNase reaction was performed by plating bacterial colonies on DNase test agar (*BD Difco*™). Hemolysis and lipase reactions were tested by plating bacterial colonies onto, respectively, Columbia Agar plates supplemented with Sheep blood (Oxoid) and on bacteriological peptone Agar supplemented with 1% tween 20, 40, 60 and 80 as previously described (Sierra 1957). Lecithinase test was performed using Nutrient Agar supplemented with egg yolk (Oxoid) as previously proposed (Ferreira et al. 2013a).

5.3.3.5 Carbon sources utilization

API 20NE (bioMérieux) systems were used to evaluate the assimilation of some carbohydrates. Furthermore, carbon sources utilization (fermentation/Oxidation) by *Photorhabdus* isolates was tested with API 50 CH, API 20 and Biolog GEN III MicroPlate™ systems according to the

manufacturer's instructions. Results of the reactions were recorded after 72h (API 20E, 20NE and 50CH), or 24 and 48h for the GEN III MicroPlate^{TM} based on visual observations.

5.3.3.6 Dye absorption

The ability of bacterial isolates to absorb dye was previously confirmed on NBTA plates (Godjo et al. 2018a). In this study, we checked their ability to absorb the neutral red (0.075 g/L) contained in Mac Conkey Agar plates (Oxoid). Bacterial isolates were individually streaked onto the medium in duplicate and incubated at 28°C. Color of the colonies was observed after 48 hours.

5.3.4 Entomo-pathogenicity test

Beninese Photorhabdus strain LMG 30373^{T} was tested for its entomo-pathogenic capacity by injection into Gm larvae which were reared on artificial diet in laboratory as described previously (Givaudan and Lanois 2000). A modified protocol described by Ferreira et al. (2013a) was used. *Escherichia coli* FIRDI 675 (LMG 2092) was used as negative control. Bacterial isolates were grown overnight on Tryptic Soy Broth (TSB) medium at 28°C. Fifty microliters of the growing bacterial culture was transferred into a new flask containing 5 ml of Nutrient broth. The Optical Density was monitored and when the OD_{600} value reached 0.7, bacterial cells were harvested in 1.5 ml Eppendorf tubes and rinsed 3 times with 0.8% sterile NaCl and adjust to OD_{600} 0.1. Ten µl of these prepared LMG 30373^{T} (6 x10^{2} CFU) or *E. coli* LMG 2092 (2 x 10^{4} CFU) suspensions were individually injected using a sterile syringe with 0.5 x 25mm needle into the hemocoel of 20 Gm larvae through one of the hindmost prolegs pre-sterilized with 70% ethanol. Insect death was recorded 24 to 48 hours after incubation at 28°C.

5.4 Results and discussion

5.4.1 Molecular characterization

Based on 16S rRNA gene sequences, Beninese isolate appeared to belong to the *Photorhabdus* genus and grouped with type strains of *Photorhabdus luminescens* subspecies as previously demonstrated (Godjo et al. 2018a). They shared 98.9 – 99.4% and 99 – 99.6% 16S rRNA

sequence similarity with *Photorhabdus luminescens* subsp. *luminescens* Hb^T and *Photorhabdus luminescens* subsp. *sonorensis* Caborca^T, respectively.

RecA, gyrB, gltX, dnaN and infB genes phylogenies are presented in supplementary Figures 5.1 to 5.5. The phylogeny of the concatenated five protein coding genes revealed that the 13 studied isolates clustered separately with 100% bootstrap value and shared among them 98.8 - 100% nucleotide similarity (Figure 5.1). They showed close similarity to *P. luminescens* subsp. *caribbeanensis* (96 - 96.8%), *P. luminescens* subsp. *noenieputensis* (96.1 - 96.2%) and *P. luminescens* subsp. *luminescens* (95.1 – 95.6%). This separate clustering of the Beninese isolates is observed in the phylogenies of gyrB, dnaN and infB genes included in the concatenated sequence analysis. However, the recA and gltX genes phylogenies showed some variations (supplementary Figures 5.1 and 5.3). In the recA phylogeny, a sub-cluster containing *P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *hainanensis* and two Beninese isolates was obtained (76% bootstrap value) within the main distinct cluster of the new *Photorhabdus* isolates. GltX gene sequence analysis showed that the Beninese *Photorhabdus* isolates clustered with *P. luminescens* subsp. *caribbeanensis* with which they displayed 97.6 – 99.2% nucleotide similarity. These variations in the phylogenies of recA and gltX genes may suggest that some of the sequences included in the comparison were probably involved in lateral gene transfer as it was previously observed with *Photorhabdus luminescens* subsp. *kayai* (type strain = CIP 108428^T) (Tailliez et al. 2010). Furthermore, the phylogeny of the concatenated recA, gyrB, gltX and dnaN genes revealed that *Photorhabdus luminescens* subsp. *sonorensis* Caborca and CH35 (for which no information on infB gene is available) shared 94.5 – 95% nucleotide similarity with Beninese isolates (Supplementary Figure 5.6).

These similarity values between sequences of the concatenated protein coding genes recA, gyrB, gltX, dnaN and infB of the new bacterial isolates and their close bacterial neighbors, are low compared to the threshold (97%) earlier proposed (Tailliez et al. 2010; Tailliez et al. 2012) to split subspecies among *Photorhabdus* group. Therefore, we propose to classify the Beninese isolates as a new subspecies, *Photorhabdus luminescens* subsp. *beninensis* subsp. nov. within *P. luminescens*.

Nematode hosts of the new bacterial isolates were identified, based on ITS region analysis, as *Heterorhabditis taysearae* (Godjo et al. 2018b). *Photorhabdus luminescens* subsp. *sonorensis*

strains have been reported to live in association with *Heterorhabditis sonorensis*, a junior synonym of *Heterorhabditis taysearae* (Hunt and Subbotin 2016; Orozco et al. 2013). Therefore, our results show that *Heterorhabditis taysearae* may host more than one *P. luminescens* subspecies. The diversity in the association of *Heterorhabditis* nematodes and *Photorhabdus* bacteria have previously been reported in other studies (Kazimierczak et al. 2017; Maneesakorn et al. 2011) which revealed the association of the same *Heterorhabditis* species with more than one subspecies or species of *Photorhabdus* bacteria. For instance, the same authors reported the association of *Heterorhabditis bacteriophora*, *Heterorhabditis zealendica* and *Heterorhabditis georgiana* with both *P. luminescens* and *P. temperata* subspecies. These results represent a great challenge to the established concept of one-to-one species association between EPNs and their bacterial symbionts (Akhurst 1982; Thomas and Poinar 1979). In this study, *Heterorhabditis taysarae* is reported for the first time to be in association with *Photorhabdus luminescens* subsp. *beninensis* subsp. nov.

5.4.2 Phenotypic characters of *Photorhabdus luminescens* subsp. *beninensis* subsp. nov.

All seven isolates (Table 5.1) included in the phenotypic tests catalase positive and they were all negative for the oxidase reaction. Bacterial colonies absorbed the red dye from Mac Conkey agar plates. Phenotypic characters recorded using API 20E, 20NE, 50CH and Biolog GEN III MicroPlate^{TM} for the *Photorhabdus* isolates are presented in Table 5.1. The results showed that the *Photorhabdus* isolates from Benin were all able to hydrolyze esculin and gelatin. They were able to ferment or assimilate glucose, mannitol, inositol, glycerol, fucose, N-acetyl-glucosamine and citrate. Unlike *P. luminescens* subsp. *noenieputensis* AM7^{T}, the four Beninese isolates were unable to produce acid from arbutin, fermented mannitol and showed a weak or negative reaction for the urease test (Table 5.1). Furthermore, these new isolates can be differentiated from *P. luminescens* subsp. *caribbeanensis* HG29 by their positive reaction for esculin hydrolysis, their negative amygdalin assimilation, and their weak positive fermentation of methyl-α D-mannopyranoside. *P. luminescens* subsp. *luminescens* Hb weakly produced H_2S in contrast to the new isolates which showed a negative reaction (Table 5.1). Moreover, fermentation of erythritol and ribose were positive for strains LMG 30373^{T} and R-66823 and negative for *P. luminescens* subsp. *luminescens* Hb^{T}.

5.4.3 Entomo-pathogenicity test

The pathogenicity test conducted with *Photorhabdus luminescens* subsp. *beninensis* subsp. nov. LMG 30373^{T} and *E. coli* LMG 2092 as a control demonstrated that the tested bacterial strain is highly pathogenic to Gm as all the injected larvae turned brown and died within 24 hours while larvae which were injected with *E. coli* suspension remained white and survived. This result confirms the pathogenicity of Beninese *Heterorhabditis* EPNs previously reported (Godjo et al. 2018b) and their association with highly insect pathogenic bacteria (*Photorhabdus luminescens* subsp. *beninensis* subsp. nov.) might be of great interest in biological control of local insect pests.

Table 5.1 Phenotypic characteristics of bacterial symbionts based on conventional tests (API 20E, API 20NE, API 50CH, Biolog GEN III MicroPlates^{TM}). 1= *P. luminescens* subsp. *luminescens* Hb^{T}; 2= *P. luminescens* subsp. *caribbeanensis* HG29^{T}; 3= *P. luminescens* subsp. *noenieputensis* AM7^{T}; 4= isolate LMG 30373^{T}; 5= isolate R66823; 6= isolate R-66825; 7= isolate R-52404

[a] +, positive reaction; -, negative reaction; (+), weak positive reaction.

Characteristics[a,b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Characteristics**[a,b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Maximum temperature for growth (°C)	37	40	40	40	40	40	40	N-acetylglucosamine	(+)	+	+	(+)	+	(+)	-
Reduction of nitrates to nitrogen	-	-	(+)	-	+	-	+	Arbutin	-	-	+	-	-	-	-
Indole production (Tryptophane)	-	+	(+)	-	(+)	+	+	Esculin	+	-	(+)	+	+	+	+
Arginine dihydrolase	(+)	(+)	-	-	-	(+)	-	Maltose	-	+	+	-	-	+	(+)
Urease	-	-	+	(+)	(+)	-	-	Lactose	-	-	-	(+)	-	-	-
Hydrolysis of esculin	+	-	(+)	+	+	+	+	Trehalose	(+)	+	(+)	-	(+)	+	(+)
β-galactosidase (ONPG)	-	-	-	(+)	-	-	-	Xylitol	-	-	-	(+)	-	-	-
H2S production	(+)	-	-	-	-	-	-	Gentiobiose	-	-	-	(+)	-	-	-
Tryptophane deaminase	+	+	+	(+)	+	(+)	+	L-Fucose	+	(+)	+	+	+	+	+
Fermentation/ Oxydation of								Potassium 5-ketogluconate	(+)	-	-	-	(+)	-	-
Erythritol	-	(+)	-	+	(+)	-	-	Methyl-αD-mannopyranoside	-	(+)	-	-	-	-	-

Characteristics[a,b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Characteristics**[a,b]	**1**	**2**	**3**	**4**	**5**	**6**	**7**
D-Arabinose	-	(+)	-	(+)	-	-	-	Methyl-αD-glucopyranoside	-	-	-	-	-	-	-
L-Arabinose	-	-	(+)	-	(+)	-	-	Mannose	(+)	+	+	(+)	+	+	(+)
Ribose	-	+	(+)	+	+	-	-	**Assimilation of**							
D-Xylose	-	(+)	-	-	-	-	-	Mannose	+	(+)	(+)	(+)	(+)	+	(+)
L-Xylose	-	(+)	-	-	-	-	-	Mannitol	+	+	-	+	+	+	+
Adonitol	-	(+)	-	-	-	-	-	Maltose	-	(+)	(+)	(+)	-	+	(+)
Methyl-βD-xylopyranoside	-	(+)	-	(+)	(+)	-	-	Potassium gluconate	(+)	-	(+)	-	-	(+)	-
Galactose	-	(+)	-	(+)	-	-	-	Malate	+	-	-	(+)	-	(+)	(+)
Fructose	-	+	(+)	-	(+)	(+)	-	Trisodium citrate	+	+	+	(+)	(+)	+	+

[a] +, positive reaction; -, negative reaction; (+), weak positive reaction.

[b] All isolates were positive for: the hydrolysis of gelatin; fermentation/oxydation of inositol and glucose; and the assimilation of N-acetyl-glucosamine and citrate. They were all negative for: the reduction of nitrates to nitrites, β-galactosidase (PNPG), lysine decarboxylase, ornithine decarboxylase, acetoin production (VP); the fermentation/oxydation of inulin, melezitose, raffinose, starch, glycogen, turanose, lyxose, tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, L-sorbose, dulcitol, salicin, cellobiose; and the assimilation of arabinose, capric acid, adipic acid, and phenylacetic acid.

5.5 Description of *Photorhabdus luminescens* subsp. *beninensis* subsp. nov

Photorhabdus luminescens subsp. *beninensis* [be.ni.nen'sis N.L. fem. adj. *beninensis* pertaining to Benin country, the source of the nematode host, *Heterorhabditis taysearae* strain KorobororouF4, from which the type strain was isolated (Godjo et al. 2018b)].

They are bioluminescent and have a maximum temperature for growth of 40°C on TSA medium. DNase test was negative and hemolysis test positive for all tested isolates. Lecithinase test was negative. Positive for catalase and negative for oxidase. They utilize mannitol, N- acetyl glucosamine and citrate, and are positive for tryptophane deaminase, hydrolysis of gelatin and esculin. They produce acid from glucose, mannitol, inositol, glycerol, mannose, amygdalin (weak) esculin and L-fucose. They are resistant to Ampicillin, Bacitracin and Vancomycin. However they are susceptible to Chloramphenicol, Tetracyclin and Gentamicin with the latter giving a smaller inhibition zone compared to the first two.

The type strain is LMG 30373^{T} (= R-66822^{T}, DSM number to be assigned)

5.6 Acknowledgements

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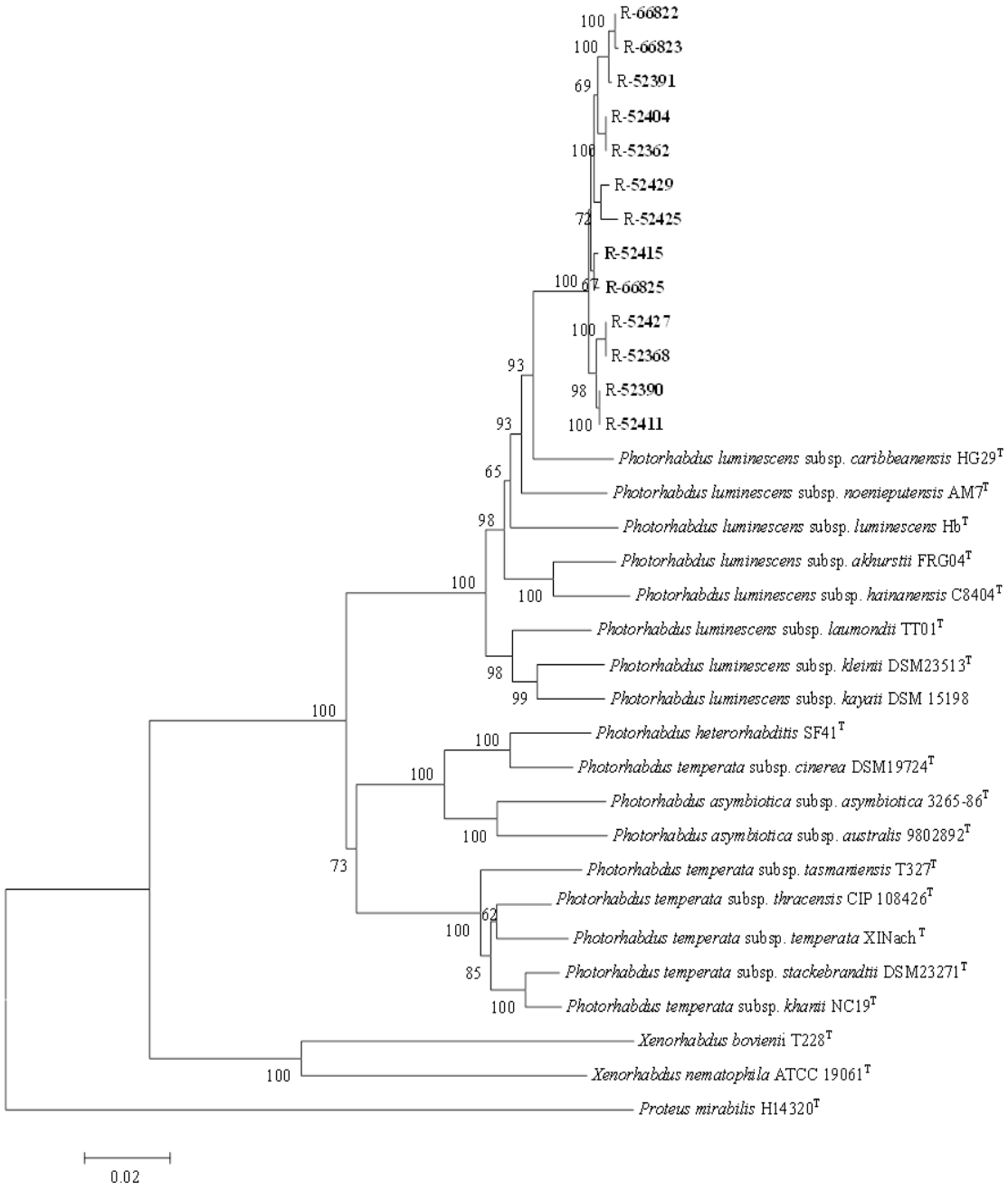


Figure 5.1 Neighbor joining tree based on concatenated recA, gyrB, gltX, dnaN and infB gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup

5.7 References

Akhurst R (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. Journal of General Microbiology 121:303-309

Akhurst R (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061-3065

Akhurst R, Boemare N, Janssen P, Peel M, Alfredson D, Beard C (2004) Taxonomy of Australian clinical isolates of the genus *Photorhabdus* and proposal of *Photorhabdus asymbiotica* subsp. *asymbiotica* subsp. nov. and *P. asymbiotica* subsp. *australis* subsp. nov. International Journal of Systematic and Evolutionary Microbiology 54:1301-1310

An R, Grewal PS (2010) *Photorhabdus temperata* subsp. *stackebrandtii* subsp. nov.(Enterobacteriales: Enterobacteriaceae). Current Microbiology 61:291-297

An R, Grewal PS (2011) *Photorhabdus luminescens* subsp. *kleinii* subsp. nov.(Enterobacteriales: Enterobacteriaceae). Current Microbiology 62:539-543

Boemare N, Akhurst R, Mourant R (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249-255

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Ehlers R (2005) Forum on safety and regulation. Nematodes as Biocontrol Agents(Grewal, PS, Ehlers, R-U and Shapiro-Ilan, DI eds), CABI Publishing, Wallingford:107-114

Ferreira T, Van Reenen C, Pagès S, Tailliez P, Malan AP, Dicks LM (2013) *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov., a symbiotic bacterium associated with a novel *Heterorhabditis* species related to *Heterorhabditis indica*. International Journal of Systematic and Evolutionary Microbiology 63:1853-1858

Ferreira T, van Reenen CA, Endo A, Tailliez P, Pagès S, Spröer C, Malan AP, Dicks LM (2014) *Photorhabdus heterorhabditis* sp. nov., a symbiont of the entomopathogenic nematode *Heterorhabditis zealandica*. International Journal of Systematic and Evolutionary Microbiology 64:1540-1545

Fischer-Le Saux M, Viallard V, Brunel B, Normand P, Boemare NE (1999) Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. International Journal of Systematic and Evolutionary Microbiology 49:1645-1656

Givaudan A, Lanois A (2000) flhDC, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. Journal of Bacteriology 182:107-115

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2018a) Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin. Archives of Microbiology doi:10.1007/s00203-017-1470-2

Godjo A, Zadji L, Decraemer W, Willems A, Afouda L (2018b) Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*) under laboratory conditions. Biological Control 117:68-77 doi:10.1016/j. biocontrol.2017.10.009

Hazir S, Stackebrandt E, Lang E, Schumann P, Ehlers R-U, Keskin N (2004) Two new Subspecies of *Photorhabdus luminescens*, Isolated from *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae): *Photorhabdus luminescens* subsp. *kayaii* subsp. nov. and *Photorhabdus luminescens* subsp. *thracensis* subsp. nov. Systematic and Applied Microbiology 27:36-42

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors), Advances in Taxonomy and Phylogeny of Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Kaya HK, Gaugler R (1993) Entomopathogenic nematodes. Annual Review of Entomology 38:181-206

Kazimierczak W, Skrzypek H, Sajnaga E, Skowronek M, Waśko A, Kreft A (2017) Strains of *Photorhabdus* spp. associated with polish *Heterorhabditis* isolates: their molecular and

phenotypic characterization and symbiont exchange. Archives of Microbiology 199:979–989

Koppenhöfer AM, Grewal PS, Fuzy EM (2007) Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. Journal of Invertebrate Pathology 94:184-195

Liu J, Berry RE, Blouin MS (2001) Identification of Symbiotic Bacteria (*Photorhabdus* and *Xenorhabdus*) from the Entomopathogenic Nematodes *Heterorhabditis marelatus* and *Steinernema oregonense* Based on 16S rDNA Sequence. Journal of Invertebrate Pathology 77:87-91

Maneesakorn P, An R, Daneshvar H, Taylor K, Bai X, Adams BJ, Grewal PS, Chandrapatya A (2011) Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (*Heterorhabditis*: Rhabditida) and their symbiotic bacteria (*Photorhabdus*: Enterobacteriaceae). Molecular Phylogenetics and Evolution 59:271-280

Orozco RA, Hill T, Stock SP (2013) Characterization and phylogenetic relationships of *Photorhabdus luminescens* subsp. *sonorensis* (γ-Proteobacteria: Enterobacteriaceae), the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae). Current Microbiology 66:30-39

Pitcher D, Saunders N, Owen R (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters in Applied Microbiology 8:151-156

Sierra G (1957) A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek 23:15-22

Stock SP, Hunt DJ (2005) Morphology and systematics of nematodes used in biocontrol. Nematodes as Biocontrol Agents(Grewal, PS, Ehlers, R-U and Shapiro-Ilan, DI eds), CABI Publishing, Wallingford:3-43

Tailliez P, Laroui C, Ginibre N, Paule A, Pagès S, Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp.

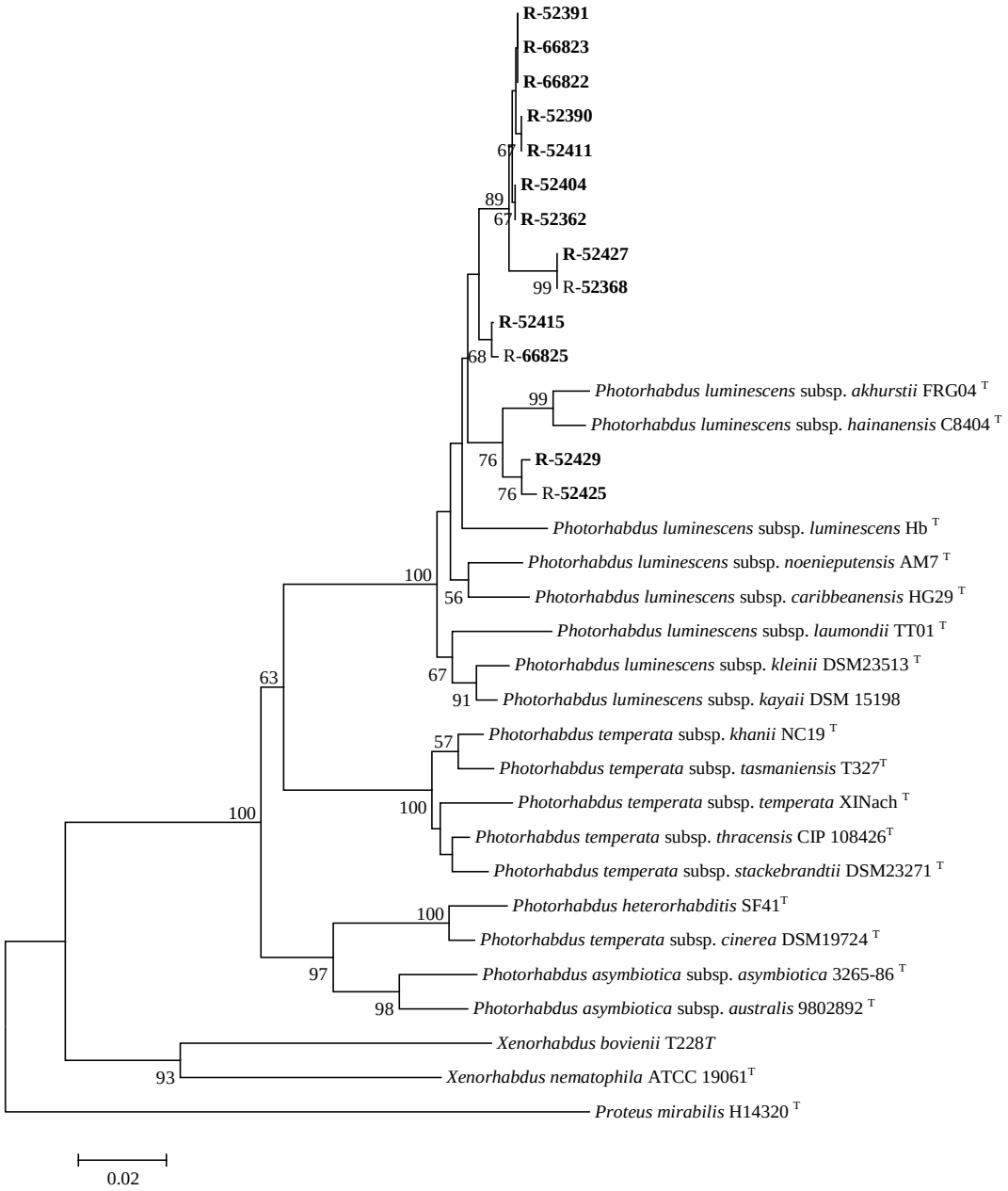
thracensis as *P. temperata* subsp. *thracensis* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60:1921-1937

Tailliez P, Pagès S, Edgington S, Tymo LM, Buddie AG (2012) Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. International Journal of Systematic and Evolutionary Microbiology 62:1761-1765

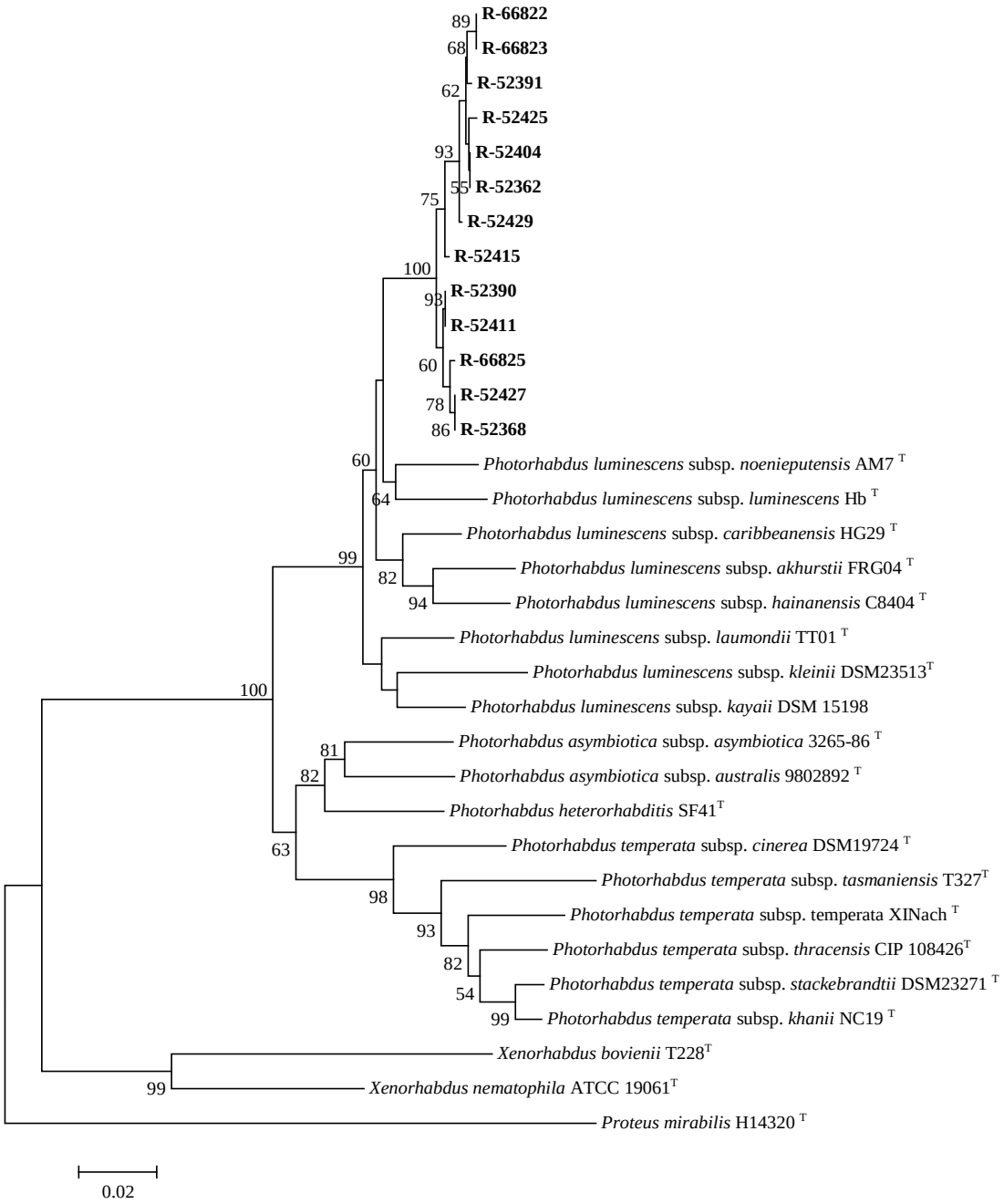
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725-2729

Thomas GM, Poinar GO (1979) *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. International Journal of Systematic Bacteriology 29:352-360

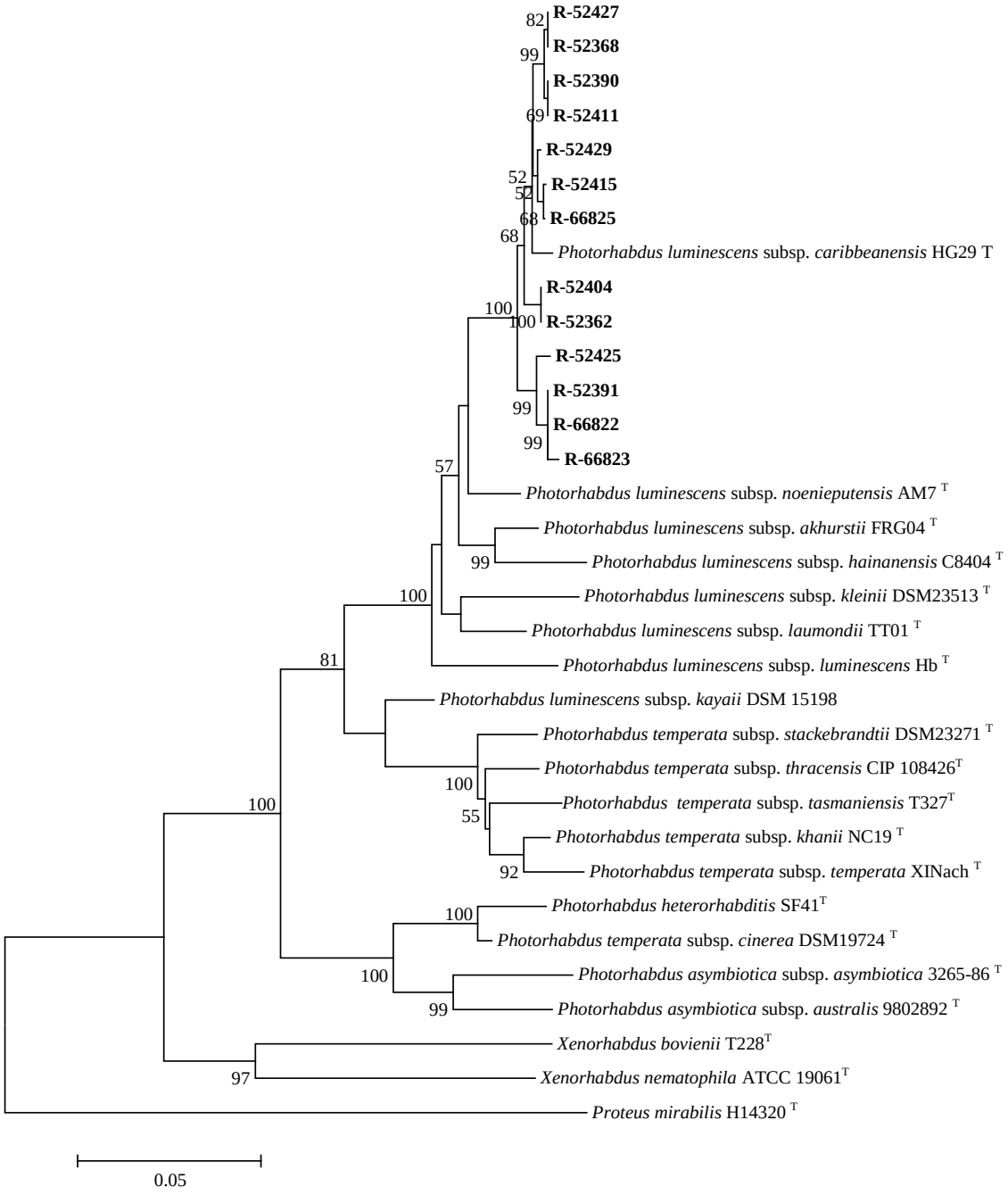
5.8 Supplementary materials



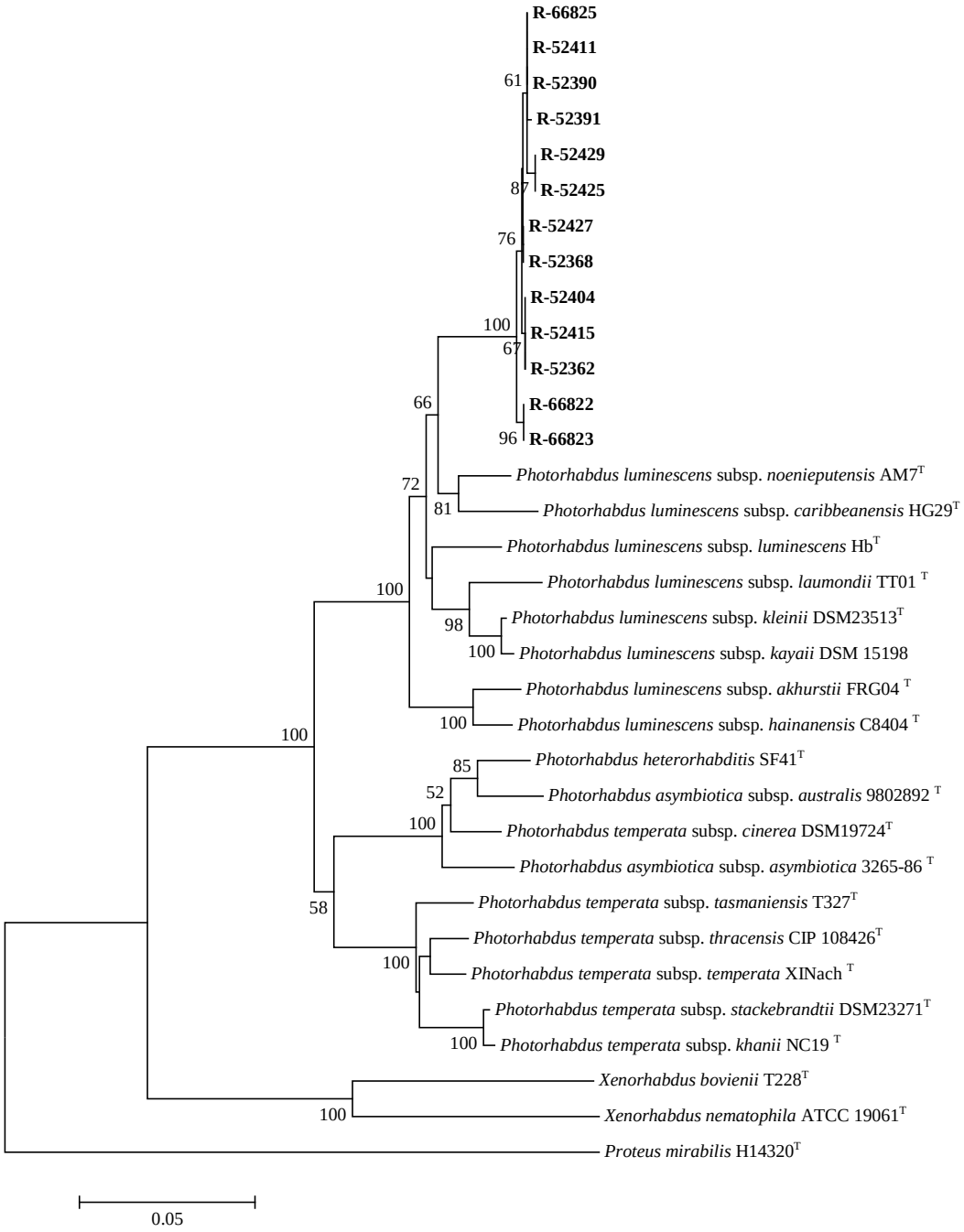
Supplementary Figure 5.1 Neighbor joining tree based on recA gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.



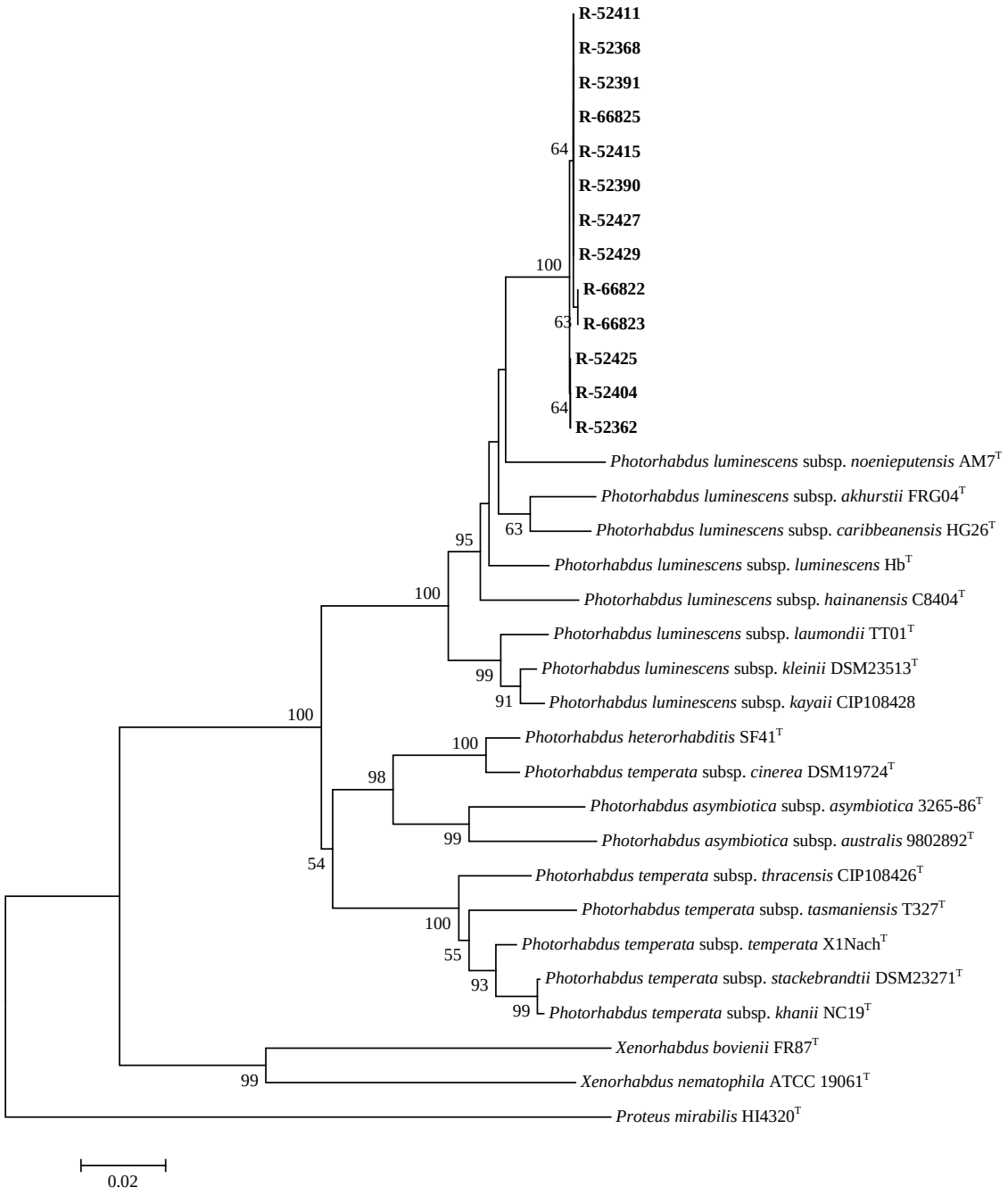
Supplementary Figure 5.2 Neighbor joining tree based on gyrB gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.



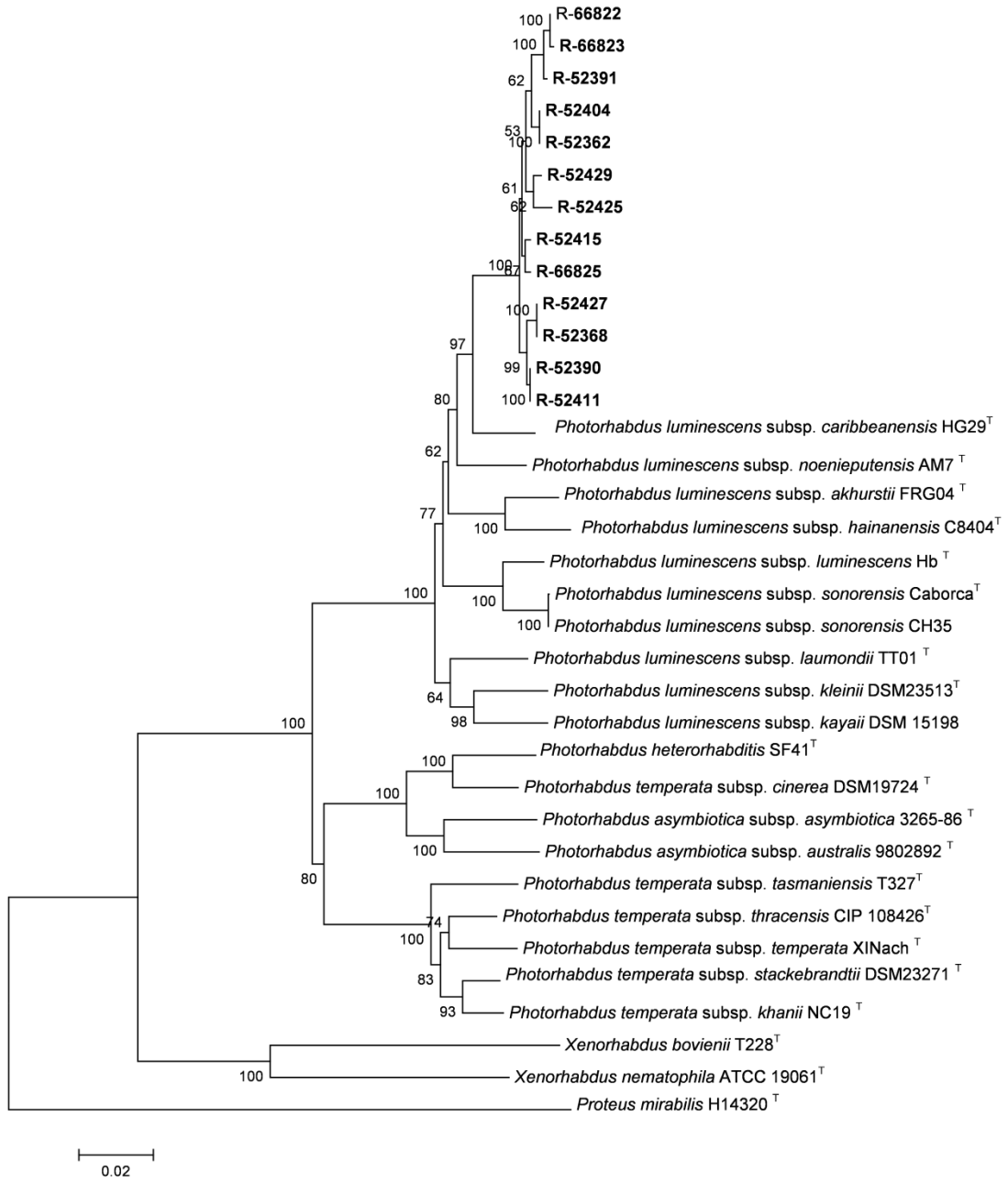
Supplementary Figure 5.3 Neighbor joining tree based on gltX gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.



Supplementary Figure 5.4 Neighbor joining tree based dnaN gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.



Supplementary Figure 5.5 Neighbor joining tree based on infB gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.



Supplementary Figure 5.6 Neighbor joining tree based on concatenated recA, gyrB, gltX,and dnaN gene sequences showing the phylogenetic position of Beninese *Photorhabdus* isolates (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* and *Xenorhabdus* sp. were used as outgroup.

Supplementary Table 5.1 Overview of accession numbers of sequences used for MLSA and 16S rDNA analyses.

Bacterial isolates	16S rDNA	recA	gyrB	dnaN	gltX	infB
R-66822 = LMG 30373^{T}	MF353507	MF356978	MF357004	MF353536	MF353552	MF353518
R-52429	MF353494	MF356975	MF357001	MF353537	MF353553	MF353519
R-52425	MF353500	MF356972	MF356997	MF353538	MF353554	MF353520
R-52404	MF353491	MF356964	MF356990	MF353567	MF353556	MF353522
R-52427	MF353493	MF356974	MF357000	MF353540	MF353557	MF353523
R-52390	MF353496	MF356967	MF356993	MF353541	MF353558	MF353524
R-52415	MF353490	MF356970	MF356996	MF353542	MF353559	MF353525
R-52362	MF353495	MF356968	MF356994	MF353543	MF353560	MF353526
R-66825	MF353505	MF356977	MF357003	MF353544	MF353561	MF353527
R-52391	MF353499	MF356962	MF356988	MF353545	MF353562	MF353528
R-52368	MF353502	MF356971	MF356998	MF353566	MF353563	MF353530
R-52411	MF353498	MF356963	MF356989	MF353547	MF353564	MF353531
R-66823	MF353506	MF356980	MF357005	MF353549	MF353565	MF353533
Photorhabdus luminescens subsp. *sosnorensis* CH35	JQ912649	JQ912653	JQ912652	JQ912650	JQ912651	N/A
Photorhabdus luminescens subsp. *sosnorensis* caborca	JQ912644	JQ912648	JQ912647	JQ912645	JQ912646	N/A
Photorhabdus luminescens subsp. *luminescens* Hb	AY278640	FJ862000	AY278501	FJ831500	FJ844911	JQ901851
Photorhabdus luminescens subsp. *kayaii* CIP108428	AJ560630	FJ861996	EU930348	FJ831494	FJ844917	JQ901845
Photorhabdus luminescens subsp. *kleinii* DSM23513	JX513408	JQ901853	JX51340	JQ901854	JQ901855	JQ901849
Photorhabdus luminescens subsp. *laumondii* TT01	AJ007404	BX571863	BX571859	BX571859	BX571863	BX571874
Photorhabdus luminescens subsp. *noenieputensis* AM7	JQ424880	JQ424881	JQ424884	JQ424882	JQ424883	JQ424885
Photorhabdus luminescens subsp. *akhurstii* FRG04	AJ007359	FJ862005	EU930347	FJ831503	FJ844913	JQ901840
Photorhabdus luminescens subsp. *caribbeanensis* HG26	EU930345	FJ862003	EU930360	FJ831499	FJ844916	JQ901842
Photorhabdus luminescens subsp. *hainanensis* C8404	EU930342	FJ862004	AY278498	FJ831502	FJ844914	JQ901843
Photorhabdus temperata subsp. *khanii* NC19	AY278657	FJ862011	AY278497	FJ831486	FJ844921	KF740642
Photorhabdus temperata subsp. *temperata* X1Nach	AJ007405	FJ862012	AY278517	FJ831485	FJ844918	JQ723516
Photorhabdus temperata subsp. *thracensis* CIP108426	AJ560634	FJ862015	EU930351	FJ831481	FJ844927	KF740640
Photorhabdus temperata subsp. *cinerea* DSM19724	EU136626	KF740654	KF740662	KF740659	KF740651	KF740645
Photorhabdus temperata subsp. *stackebrandtii* DSM23271	EF467859	KF740655	KF740661	KF740658	KF740650	KF740646
Photorhabdus temperata subsp. *tasmaniensis* T327	EU930339	FJ862008	EU930356	FJ831478	FJ844926	KF740641
Photorhabdus asymbiotica subsp. *australis* 9802892	AY280572	FJ862018	AY278495	FJ831489	FJ844930	JQ723517
Photorhabdus asymbiotica subsp. *asymbiotica* 3265-86	Z76755	FJ862017	AY278494	FJ831491	FJ844929	KF740643
Photorhabdus heterorhabditis SF41	HQ142626	KF418141	KF418144	KF418142	KF418143	KF418145
Xenorhabdus nematophila ATCC19061	AY278674	FN667742	FN667742	FN667742	FN667742	FN667742
Xenorhabdus bovienii FR87	AY278673	FJ823426	EU934530	FJ831466	FJ840514	KF946019
Proteus mirabilis H14320	AM942759	AM942759	AM942759	AM942759	AM942759	AM942759

6 Chapter 6:

Evaluation of the ability of indigenous isolates of *Heterorhabditis taysearae* and *Steinernema* n. sp. to control mango fruit fly *Bactrocera dorsalis* under laboratory, semi-field and field conditions in Northern Benin

Redrafted from:

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Author's contribution:

AG, LZ, HB and LA designed the experiments. AG, NC, PD and OB performed the experiments. AG analyzed the data and wrote the manuscript. BH, SAAC, BAH, LZ, WD, AW and LA revised the manuscript.

6.1 Abstract

Looking for a sustainable way to prevent damage caused by *Bactrocera dorsalis* to mango fruits in Benin as in the whole West Africa region, we investigated the use of entomopathogenic nematodes (EPNs) for the biological control of this insect pest. One *Steinernema* n. sp. and two *Heterorhabditis taysearae* were investigated for their invasion time and virulence to third instar larvae in laboratory and semi field tests, respectively. In addition, the persistence of the same nematode isolates in soil under field conditions was tested. Results showed that all three EPN isolates were highly pathogenic to *B. dorsalis* under semi field conditions with *H. taysearae* Hessa1 being the most virulent (70.84% ± 10.46 insect mortality). This result confirmed our previous laboratory test which reported high susceptibility of larvae and pupae to the same nematode isolate. In addition, all three tested nematode isolates could penetrate insect larvae after 2 hours of exposure time suggesting that EPNs require a short time to invade larvae under laboratory conditions and therefore may represent good candidates to reduce larvae population, which spend relatively short time in soil before pupating. Furthermore, insect mortality was higher when EPNs were applied 3 days before insect inoculation than when they were applied at the same moment. *Steinernema* n. sp. Thui persisted in soil up to 32 weeks and its infective juveniles (IJs) were more often trapped in the upper layer of soil (0-10 cm), whereas for *H. taysearae* Hessa 1 and Koroborororou, IJ density was higher in the soil layer of 10-20 cm and they persisted up to 30 weeks post application in the mango orchard. In general, the density of IJs two weeks upon nematode application decreased considerably and was lower than expected. We therefore suggest 2 to 3 nematode applications to enhance nematode presence and establishment in the soil. Furthermore, the application time should also be considered with care to ensure EPN efficiency in mango orchards as our results confirmed the presence of third instars larvae from March onward during the fructification stage of mango trees.

6.2 Introduction

Fruit flies (Diptera: Tephritidae) constitute the most economically important insect pest for tropical horticulture including mango production (Vayssieres et al. 2009a; White and Elson-Harris 1992). Worldwide, mango yield and, more importantly, fruit quality are severely affected by infestations of many tephritid species. These insects are difficult to eradicate once they are established in an environment (Minas et al. 2016). Furthermore, due to their severe damage, most tephritids appear on the quarantine list of the USA and several countries in Europe which offer to African mango growers most of the international export markets.

In Benin, mango is grown, as in all the tropical areas around the world, for its juicy fruits and shade. Due to its exceptional nutritional value, mango represents a source of essential nutrients for rural populations living in relatively poor areas (Vayssieres et al. 2008). The production of mango is constantly growing and the need of supplying undamaged mangos becomes important to reduce poverty in Benin as in the whole Western Africa region where mango has a great economical value. However, the Oriental fruit fly, *Bactrocera dorsalis* Hendel (Tephritidae), represents the most invasive and damaging species to the mango crop as well as other fruit crops (Clarke et al. 2005; Drew 1989; Gnanvossou et al. 2017; Goergen et al. 2011; Vayssières et al. 2009b; Vayssieres et al. 2008). It was initially described as *Bactrocera invadens* Drew, Tsuruta & White, but later regarded as a junior synonym of *B. dorsalis* (Schutze et al. 2015). Adult female flies lay eggs under the skin of fruits and the eggs develop into larvae that fully complete their development inside the infested fruit. Mature larvae exit the fruit and fall on the ground where they pupate in the top 4 cm of the soil layer after a short dispersal period (Alyokhin et al. 2001; Hou et al. 2006). More than 75% of yield loss has previously been attributed to these insect pests in Benin (Vayssieres et al. 2008). To reduce damage caused by *B. dorsalis*, mango growers lack appropriate methods for efficient control and traditionally rely on chemical treatments (Sinzogan et al. 2008). Given the extent of the losses, human health, wildlife and environmental risks associated with the use of insecticides, alternative integrated control methods to achieve more sustainable mango production are being investigated. Therefore, a combination of most economical and environmentally friendly methods is required for an Integrated Pest Management (IPM) approach. In this respect, the use of insecticidal bacterial (*Saccharpolyspora spinosa*) product, Spinosad (GF-120) fruit fly bait, Dow groSciences, was recently explored (Vayssieres et

al. 2009a). Although the Spinosad product was classified as a reduced-risk compound, a sustainable reduction of *B. dorsalis* population may alternatively be achieved by developing a range of several natural enemies which may attack most of its developmental stages. Efforts have been made in this sense during the past few years to investigate natural enemies of tephritid flies. For example, predators such as weaver ants (*Oecophylla longinoda*) were investigated for conservation biological control of tephritids (Anato et al. 2015b; Van Mele 2008; Van Mele et al. 2007; Vayssières et al. 2015b). Despite their potential ability to reduce tephritid populations, these ants were reported to harm mango growers during harvest which is done manually on small scale plantations (Sinzogan et al. 2008). In addition to weaver ants, the use of parasitoids such as *Fopius arisanus* has equally been explored (Gnanvossou et al. 2016). In the same context, entomopathogenic nematode (EPN) species have all qualifications to be inserted in the IPM guidelines for the control of soil stages of fruit flies, especially *B. dorsalis*.

Indeed, EPN species of the genera *Heterorhabditis* and *Steinernema* living in soil in association with their symbiotic bacteria *Photorhabdus* and *Xenorhabdus*, respectively, are commonly used to control insect pests at reasonable cost (Ehlers 2001). Once inside the insect host, the infective juveniles (IJs) of the nematode release the bacterial symbiont which produces toxins that kill the host within 24 to 48 hours (Dowds and Peters 2002). Many advantages qualify EPNs as commercially valuable biocontrol agents. They are safe for both user and the environment; they are persistent and have the potential to recycle inside the host insect causing a long term and sustainable effect on the targeted pest (Peters 1996). EPNs are highly effective and can be produced at large scale in liquid culture (Ehlers 2001) at reasonable costs (Ehlers 1996; Ehlers et al. 1998). Several tephritid species such as *Bactrocera oleae, Bactrocera dorsalis, Ceratitis capitata* and *Ceratitis rosa* have earlier been reported to be susceptible to EPNs (Langford et al. 2014; Lin et al. 2005; Lindegren and Vail 1986; Malan and Manrakhan 2009; Sirjani et al. 2009) under laboratory conditions. More specifically, we recently confirmed the susceptibility of *B. dorsalis* third instar larvae and 1 to 3 day-old pupae to Beninese EPN isolates under laboratory conditions (Godjo et al. 2018b). However, field experimentations to investigate such ability of EPNs to control fruit fly population in natural environment are poorly documented. In China, up to 86.3% mortality of *B. dorsalis* larvae and pupae was achieved with 300 IJs/cm² density of *Steinernema carpocapsae* under field conditions (Lin et al. 2005). In addition, a recent study

revealed that *Heterorhabditis baujardi* could be well adapted for *Ceratitis capitata* control under field conditions in guava orchards (Minas et al. 2016).

In this study, a first attempt at field implementation of the use of Beninese EPNs to reduce fly populations in mango orchards is investigated. Our objectives were to i) evaluate the minimum time needed by EPNs to invade *B. dorsalis* larvae, ii) evaluate the appropriate period for nematode application in mango orchards, iii) investigate the virulence of two native nematode species (two isolates of *Heterorhabditis taysearae* and one of *Steinernema* n. sp.) on *B. dorsalis* under semi-field conditions, and iv) evaluate the persistence of the three EPN isolates in mango orchards throughout the mango season 2016 in Northern Benin.

6.3 Material and methods

6.3.1 Insects

B. dorsalis late instar (L3) larvae were obtained from naturally-infested mango fruits directly collected from mango orchards in Northern Benin. Infested fruits were incubated at room temperature in plastic containers containing 10% (V/V) moist sand as earlier detailed (Vayssières et al. 2015a). Fresh L3 larvae, newly exited from infested fruits to pupate in sand, were used in our experiments. *Galleria mellonella* used for EPN multiplication were reared on artificial diet in the laboratory as previously described (Birah et al. 2008).

6.3.2 Nematodes

Three Beninese EPN isolates including two *Heterorhabditis taysearae* isolates (KoroborororouF4 and Hessa1) and one *Steinernema* n. sp. isolate (Thui) were included in all laboratory and field experiments performed in this study. The nematode isolates were selected to represent one isolate native to mango orchard (isolate KoroborororouF4), one isolate with good performance in laboratory experiments (isolate Hessa1) and one isolate from different genus (isolate Thui, which induced higher insect mortality in laboratory assays compared to the *Steinernema* isolate Bembereke). These EPN isolates were obtained from the Laboratoire de Phytotechnie, d'Amélioration et de Protection des Plantes (LaPAPP), Benin. Mass reproduction of nematode isolates was performed using methods of *G. mellonella* baiting (Bedding and Akhurst 1975) and

white trap (White 1927). Newly harvested IJs were used for experiments and the remaining suspensions were stored at 13°c for further use.

6.3.3 Field experimental site

Field experiments were conducted in Northern Benin, Borgou department which is characterized by a Sudanian agro climate with a unimodal rainfall (1000-1100 mm yearly generally from end of April till end of October) (Vayssieres et al. 2008). Two mango cultivars (IFAC and ELDON) were considered. Experiments were performed at the same time in a mango orchard at 09°22'13''N, 02°40'16''E planted with the cultivar IFAC and in a mango orchard at 09°22'32''N, 02°40'59''E planted with the cultivar ELDON. The two mango orchards cover at least an area of 1 ha each and were located approximately 1.5 km apart. The abiotic data related to both experimental sites are presented in Table 6.1.

No insecticide treatments against the fruit fly had been previously applied in the two mango orchards nor in the nearby plots where cashew trees were the main crop.

Table 6.1 Agro meteorological data of Parakou in 2016.

Abiotic factors		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Mean temperature (°C)	Min	19.40	22.36	24.64	23.79	23.09	22.14	21.94	21.89	22.25	22.37	21.36	21.17
	Max	34.12	36.78	37.42	34.69	33.11	30.74	28.87	28.67	32.05	35.10	34.78	34.69
Mean Relative Humidity (%)	Min	11.67	15.76	32.51	49.77	52.16	59.51	68.09	68.70	54.25	29.30	21.93	21.22
	Max	38.16	50.06	85.90	91.40	92.48	94.44	95.96	96.38	94.19	85.50	61.03	58.29
Precipitations (mm)		0	13.40	32.70	168.6	133.7	91.20	200.5	99.80	82.20	70.60	0	0.30

Source, ASECNA Aérodrome station, Parakou

6.3.4 Laboratory test: Invasion time

In order to evaluate the time needed by EPNs to parasitize *B. dorsalis* L3 larvae, the three Beninese isolates described above, were used to set an exposure-time-assay under laboratory conditions. The experimental arena consisted of 3 cm-diameter plastic dishes (2 cm height) which were individually filled with 10 ml sterile sand. Hundred IJs of each EPN isolate, suspended in 1 ml of distilled water, were scattered on top of the sand. Controls received 1 ml of distilled water without nematodes. This addition of 1 ml of distilled water resulted in a sand humidity level of 10% (V/V). After the water had been absorbed and thoroughly dispersed in the dishes, we

assumed that nematodes had equally been distributed in the sand and one L3 larva was placed on top of the sand and the dish was closed with its lid and incubated in the dark at 28°C for 2, 4, 6, 8 and 24 hours. After each exposure time, the L3 larva was retrieved from the sand, rinsed with distilled water to remove all attached IJs on their cuticle, and incubated at 28°C in a Petri dish lined with a moist Whatman N°1 paper. After 48 hours of incubation, larvae were individually dissected in Ringer's solution under a stereomicroscope to count the number of penetrated IJs. For each combination (nematode isolate x exposure time), 10 repetitions were performed. The whole experiment was repeated four times with different batches of nematodes and *B. dorsalis* larvae.

6.3.5 Field and semi-field tests

6.3.5.1 Larval drop monitoring

An appropriate forecasting of the presence of tephritid larvae and pupae in mango orchards is a major challenge for EPN success in fly control. In order to evaluate the amount of larval drop and to determine the possible period of nematode application, the two aforementioned mango orchards were monitored from January to July 2016. In each orchard, 5 trees (repetitions) were selected at 20 m distance from each other. A 1 m^2 wood-framed experimental plot (5 cm high) sealed at the bottom with fine-mesh gauze (1.5 mm), was placed under the canopy of each tree (Figure 6.0). The bottom of each plot was filled with about 1.5 cm layer of sand collected locally from the orchard in order to provide suitable substrate for the larvae for eventual pupation. The sand was initially sieved to remove all residual insects. Plots were monitored weekly by individually sieving the sand in each plot to count any larvae/pupae that would have dropped from infested fruits. After each evaluation, the insect-free sand was placed back in the plots. Fruits on the evaluated mango trees were not harvested until larvae were no longer recorded in the plots.

6.3.5.2 Nematode persistence and vertical distribution in mango orchards

Only the mango orchard with IFAC cultivar was considered to evaluate the persistence of the three local nematode isolates under field conditions. Four rows of mango trees (repetitions), with regular spacing between trees (10m x 10m), were selected. Each mango row consisted of four trees. A plot of $1m^2$ was delimited on the ground under the canopy of each tree to serve as evaluation area for the experiment. Prior to EPN application, soil samples were taken from each plot (as described below) to ascertain the initial absence of nematodes. Thereafter, *G. mellonella*

larvae of approximatively the same size (0.23 ± 0.021 g) were infested with one of the nematode isolates at the dose of 100 IJs/ larva with 10% (V/V) substrate humidity.

Figure 6.0 Fruit fly larval drop monitoring in mango orchard in Northern Benin

A week later, these EPN-infested *G. mellonella* cadavers were randomly applied on each plot (50 cadavers/ plot), approximately 3 cm deep in the soil and covered with a layer of sand. Control plots received no nematode treatment. Afterwards, each plot received 5 liters of water (manually applied with a watering can) to enhance the emergence of nematodes into the soil as the experiment was set at the end of the dry season (March 2016) and no rain was forecast for the following days.

Data collection: A modified method of Ferguson et al. (1995) was used to collect the data. On each plot including the control plots, soil samples were randomly taken at different depth (0 -5 cm, 5 - 10 cm, 10 – 15 cm and 15-20 cm) every two weeks using PVC tubings (4 cm diameter) of 20 cm long marked with 5cm intervals. On each plot, the marked tubing was introduced in the ground, at the same point to subsequently collect the top 5, 10, 15 and 20 cm layer of the soil. This was repeated randomly at three different points of each plot and soil sub-samples of the same layer were mixed to constitute a soil sample. The *Galleria* baiting method (Bedding and Akhurst 1975) was used to trap IJs in each soil sample. After 2 days of incubation in the darkness (to allow penetrated IJs to develop and increase somewhat in size for easy recognition under stereomicroscope without giving any progeny), dead *G. mellonella* were removed daily and dissected in Ringer's solution under a stereomicroscope to count the number of penetrated IJs. Dead *G. mellonella* larvae were replaced with living ones in the incubation pots until no new dead larvae were observed. The total number of IJs was estimated by adding all nematodes found in every dead *G. mellonella* from the same soil sample over the baiting time.

6.3.5.3 Nematode virulence under semi field tests

The virulence of the three above mentioned nematode isolates under semi-field conditions was investigated. Plastic pots (height: 25 cm, diameter: 28 cm) were half-buried under the canopy of mango trees in the IFAC orchard described above. Pots were placed in the shady area of the trees precluding excessive rain water penetration during the experiment. Each pot was filled with approximately 5 dm^3 of local soil taken in the orchard. The soil was sieved beforehand to remove all initially present fly larvae or pupae and adjusted to 10% (V/V) humidity. Nematodes were applied to soil as two week-old EPN infested *G. mellonella* larvae, buried at 3cm in soil and 10 EPN-infested *G. mellonella* larvae were applied per pot. These larvae were obtained by infesting

G. mellonella larvae at the dose of 100 IJs/ larva with 10% (V/V) substrate humidity as describe above. Thereafter, 50 third instar *B. dorsalis* larvae were introduced in each container including the control pots which received no nematode treatment. Two different moments of EPN application were tested: three days before *B. dorsalis* larvae introduction in the pots (BI) and at the same time as *B. dorsalis* larvae introduction in the pots (ST). Plastic pots were individually covered with fine-mesh gauze fixed with an elastic band to prevent adult flies from flying away upon emergence. For each treatment (isolate x moment of EPN application), three replicates were assigned and pots were arranged in a complete randomized bloc (repetition) design. Ten days later, when adult flies had completely emerged in the control pots, dead larvae/ pupae were evaluated in each pot by sieving the soil under a mosquito net to prevent any adult from escaping. The number of unemerged pupae was added to that of dead larvae as described in our previous studies (Godjo et al. 2018b) to obtain the total number of dead flies in each pot. The whole experiment was repeated two times with different batches of nematodes and insects.

6.3.6 Data analysis

Experimental data were analyzed using SAS (version 9.3). To stabilize the variance of means, nematode densities were arcsine transformed and subjected to a General Linear Model analysis. Insect mortality registered in the control pots of the semi-field assay was used to correct insect mortality in the other pots using Abbott's formula (Abbott 1925). One way Analysis of Variance (ANOVA) was used to evaluate the effect of exposure times on nematode penetration in *B. dorsalis* larvae, and also to evaluate if the moment of EPN application was crucial for EPN efficacy to control *B. dorsalis* larvae. Student-Newman Keul's (SNK) test when $P<0.05$ was carried out to assess efficacy differences among nematode isolates. To evaluate the persistence of IJs and their vertical distribution at different soil depths in mango orchard, the Repeated Measures Analysis of Variance (RMANOVA) was conducted to compare the density of EPNs in soil at different sampling dates. Using this test, the potential correlations existing in between observations at successive dates were considered. In addition, the Dunnett's test was employed to compare nematode densities on treated plots with the control plots. Adjusted means of IJ density were extracted to construct the graphs illustrating nematode population dynamic and their vertical distribution in the orchard soil during the mango season.

6.4 Results

6.4.1 Invasion time under laboratory conditions

At each exposure time, IJs of the three EPN isolates penetrated inside *B. dorsalis* larvae with significant different percentages (F= 17.41; df= 2; P<0.0001). In addition, significant differences of IJ penetration percentages were recorded among larval exposure times for each nematode isolate (F= 92.82; df = 4; P< 0.0001). IJs of all tested EPN isolates were able to penetrate *B. dorsalis* larvae after 2h exposure time even though the penetration percentages remained low. The highest IJ penetration percentage inside a single *B. dorsalis* larva was recorded with *H. taysearae* Hessa1 with 24.42% after 24 h exposure time (Figure 6.1). For the same nematode isolate, lower IJ penetration percentages were recorded at 2 h (0.4%) and 4h (0.8%) exposure times.

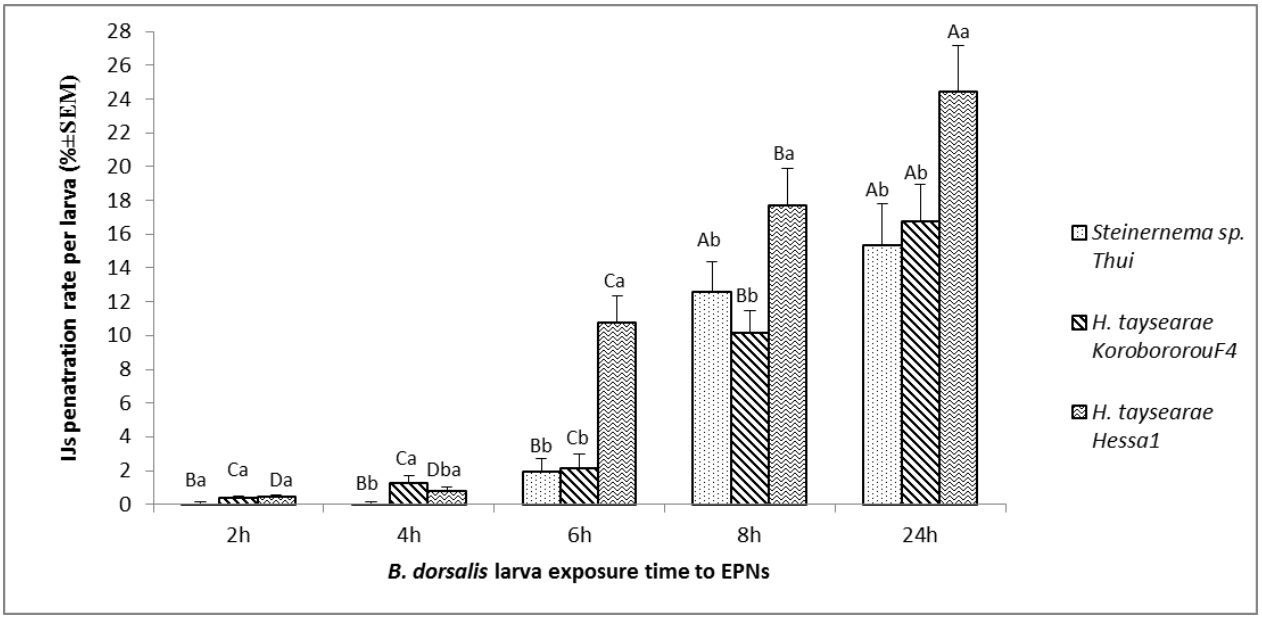


Figure 6.1 Penetration percentages (% IJs of 100 inoculated IJs ±SEM) of IJs of three different EPN isolates (*H. taysearae* Hessa1, *H. taysearae* KoroborourouF4 and *Steinernema* n. sp. Thui) inside a *Bactrocera dorsalis* third instar larva after 2, 4, 6, 8 and 24h exposure times. Vertical bars are standard error of the means. Bars with the same uppercase letters stand for

Figure 6.1 (legend continued) non-significant differences of the same nematode isolate among different exposure times. Bars with the same lowercase letters stand for non-significant differences among nematode isolates at the same exposure time (SNK's test at $P<0.05$)

6.4.2 Larval drop Monitoring

Fruit fly larvae were present in the orchards from fructification stage (mid-March 2016) of the trees (Figure 6.2). The highest populations were observed during the whole evaluation period in the ELDON orchard. The population of larvae reached its peak in May in the ELDON orchard (148.4 larvae/m² of soil surface) and the IFAC orchard (21.4 larvae/m^2 of soil surface) as well (Figure 6.2).

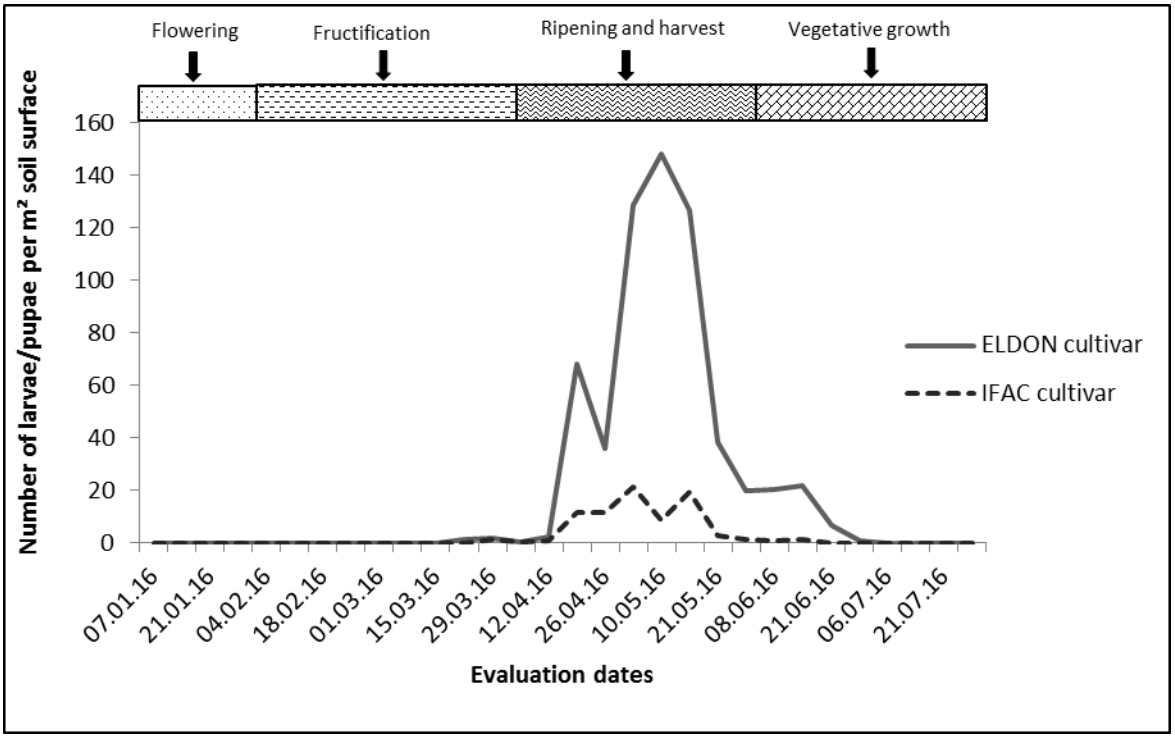


Figure 6.2 Number of fruit flies larvae/pupae dropped per square meter of surface soil in two different mango orchards (Eldon and IFAC) cultivars in Northern Benin during the period of January to July 2016.

6.4.3 Nematode persistence and vertical distribution in mango orchards

6.4.3.1 Nematode persistence

Based on the Repeated Measures Analysis of Variance, density of IJs in the mango orchard soil was significantly different at each sampling date after nematode treatment (F= 56.48, df= 18, P< 0.0001). In addition, the density of IJs at each sampling date (up to 20 cm depth) was significantly different among nematode isolates (F= 8.83, df= 54, P < 0.0001). Figure 6.3 shows that IJ density in the soil was relatively high the first two weeks for all tested EPN isolates with *Steinernema* n. sp. Thui displaying the highest IJ density (1023.05 IJs/dm^3). IJ density decreased dramatically four weeks after nematode application and afterwards remained variable depending on the isolate considered. No *Heterorhabditis* isolates persisted in the soil more than thirty weeks after nematode application whereas *Steinernema* isolate Thui persisted until thirty-two weeks after nematode application. The comparison of IJ densities on treated plots and control plots (Table 6.2) showed significant differences up to 4 weeks after *Steinernema* n. sp. Thui application, 8 weeks after *H. taysearae* Hessa1 application and 14 weeks after *H. taysearae* KorobororouF4 application.

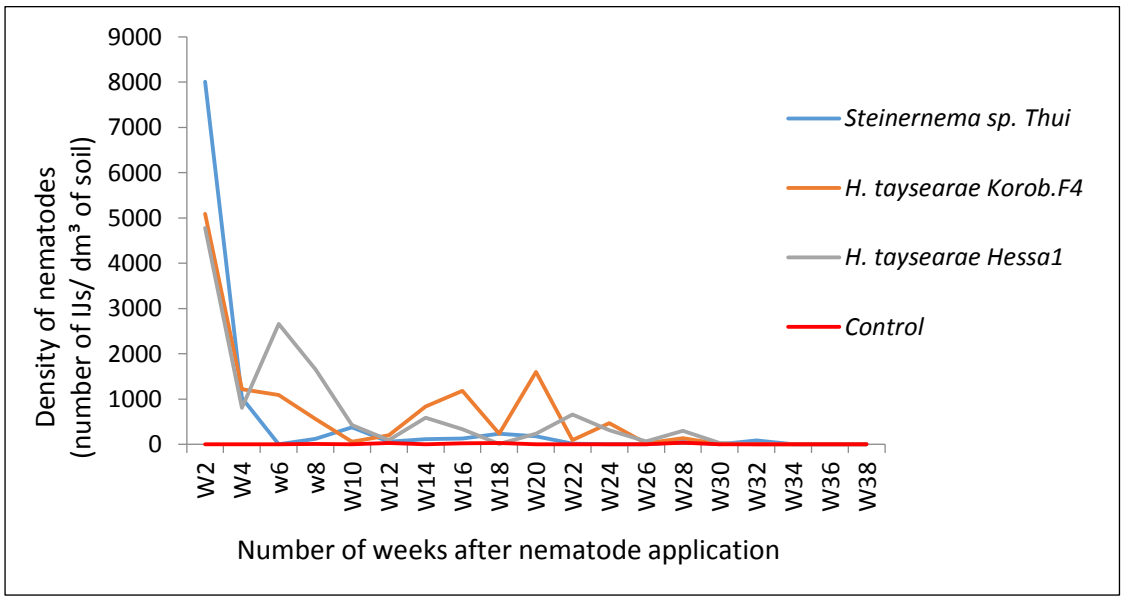


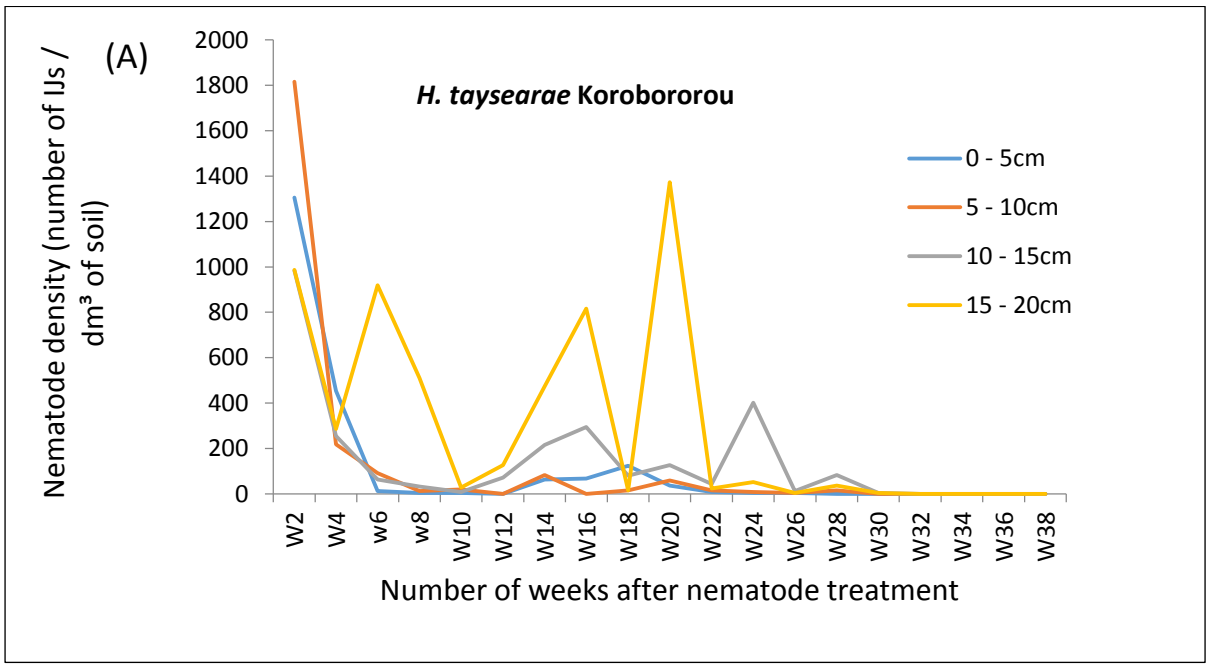
Figure 6.3 Persistence in the soil (up to 20 cm depth) of *Steinernema* n. sp. Thui, *H. taysearae* KorobororouF4 and *H. taysearae* Hessa1 in mango orchard after application of 50 nematode-infested *Galleria mellonella* cadavers (100IJs/insect) per plot.

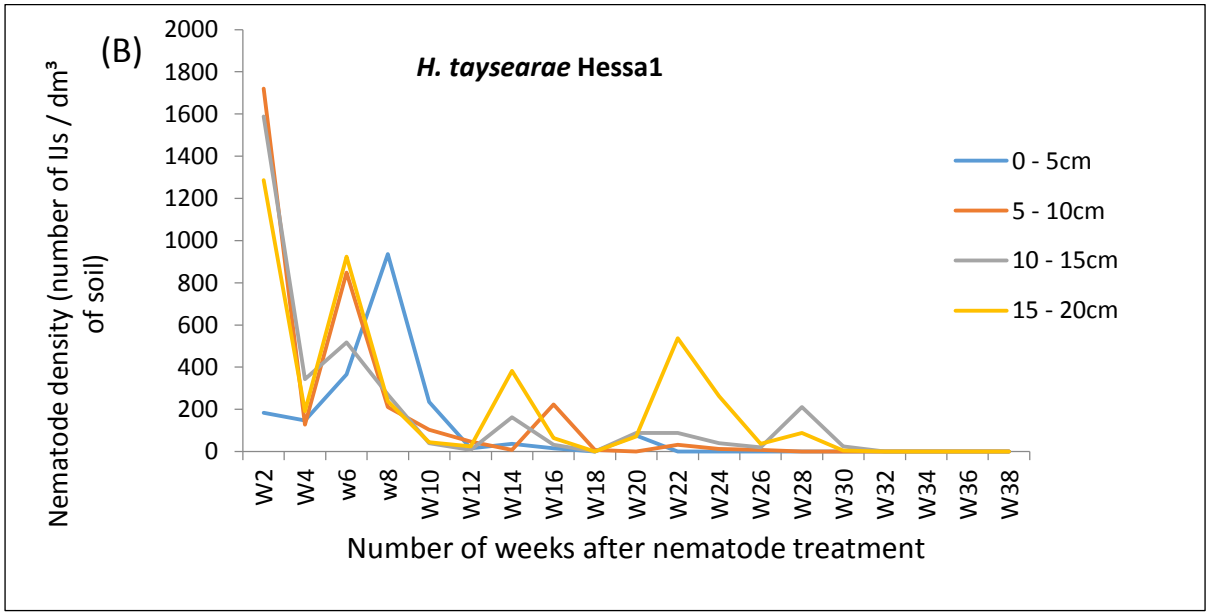
6.4.3.2 Nematode vertical distribution in soil

At each sampling date, IJ density was significantly different among different soil depth (F= 2.23, df= 54, P= 0.0140). The highest densities were observed, two weeks after nematode application, with *Heterorhabditis* isolates as well as with the *Steinernema* isolate, at all evaluated depths (Figure 6.4). In addition, *Heterorhabditis* isolates were trapped more at 5-10 cm, KorobororouF4 (1815 IJs/dm^3) (Figure 6.4A) and Hessa1 (1719 IJs/dm^3) (Figure 6.4B), whereas the *Steinernema* isolate was trapped more at 0-5 cm, Thui (3387 IJs/dm^3) (Figure 6.4C). During the remaining evaluation period, *Heterorhabditis* isolates were frequently trapped highly at 10-15 cm and 15-20 cm, whereas the *Steinernema* isolate was frequently trapped highly at 0-5 cm.

6.4.4 Nematode virulence under semi field tests

Fly mortality was significantly different among nematode isolates (F= 19.11; df= 2; P< 0.0001). In addition, insect mortality was significantly different depending on the moment of nematode application (F= 11.82; df = 1; P= 0.0017). The greatest insect mortality was recorded with *H. taysearae* Hessa1 (70.84% ± 10.46) when nematodes where applied 3 days before larval introduction in the pots (Figure 6.5). *Steinernema* n. sp. Thui induced low insect mortality with 7, 72% ± 3.25 and 6.61% ±3.63 when EPNs were applied 3 days before and at the same time as *B. dorsalis* larvae introduction to the pots, respectively (Figure 6.5).


(A)
H. taysearae Koroborororou
Nematode density (number of IJs / dm³ of soil)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 - 5cm
5 - 10cm
10 - 15cm
15 - 20cm
W2
W4
w6
w8
W10
W12
W14
W16
W18
W20
W22
W24
W26
W28
W30
W32
W34
W36
W38
Number of weeks after nematode treatment


(B)
H. taysearae Hessa1
Nematode density (number of IJs / dm³ of soil)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 - 5cm
5 - 10cm
10 - 15cm
15 - 20cm
W2
W4
w6
w8
W10
W12
W14
W16
W18
W20
W22
W24
W26
W28
W30
W32
W34
W36
W38
Number of weeks after nematode treatment

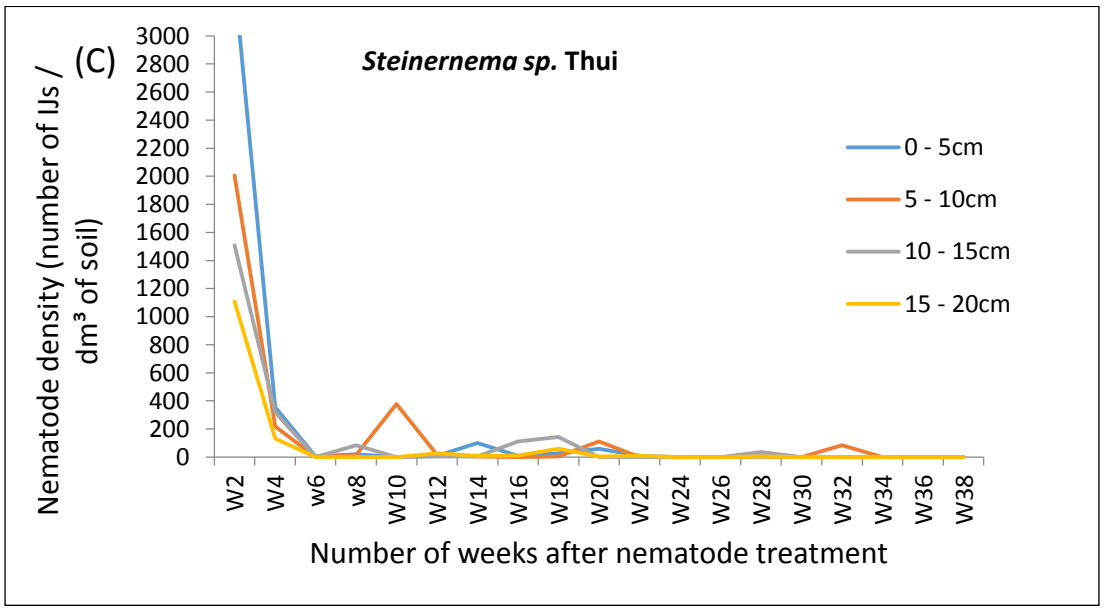


Figure 6.4 Vertical distribution of *H. taysearae* KorobororouF4 (A), *H. taysearae* Hessa1 (B) and *Steinernema* n. sp. Thui (C), infective juveniles in the top 20 cm of soil in mango orchard during the evaluation period (March to December 2016)

Table 6.2 Differences between IJ density (number/ dm^3) on treated plots and control plots during the observation period in the mango orchard based on Dunnett's test.

Nematode treatment	W2	W4	W6	W8	W10	W12	W14	W16	W18	W20	W22	W24	W26	W28	W30	W32	W34	W36	W38
Steinernema* sp. *Thui	2002^{***}	256^{***}	1^{ns}	26^{ns}	94^{ns}	6^{ns}	29^{ns}	28^{ns}	51^{ns}	44^{ns}	4^{ns}	0	0	3^{ns}	0	21^{ns}	0	0	0
***H. taysearae* Hessa1**	1609^{***}	202^{ns}	664^{***}	411^{***}	105^{ns}	16^{ns}	147^{ns}	79^{ns}	6^{ns}	59^{ns}	164^{ns}	79^{ns}	16^{ns}	66^{ns}	7^{ns}	0	0	0	0
***H. taysearae* Korobo-rorou F4**	1273^{***}	303^{***}	272^{ns}	136^{ns}	15^{ns}	42^{***}	209^{***}	290^{ns}	51^{ns}	40^{ns}	23^{ns}	116^{ns}	6^{ns}	24^{ns}	2^{ns}	0	0	0	0

Difference significant at the 0.05 level are indicated by ***; ^{ns} =means non-significant, W(x) = number of weeks after nematode application

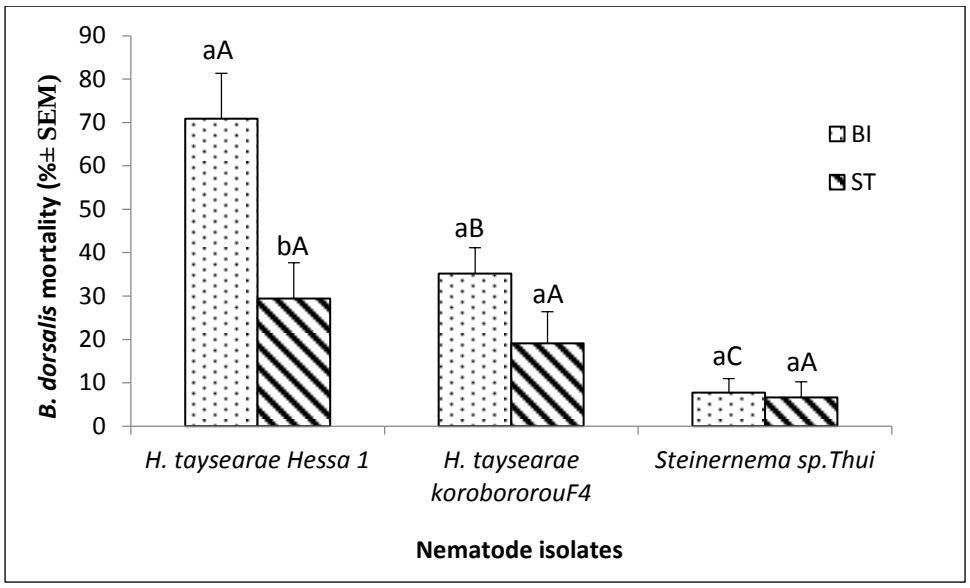


Figure 6.5 Mortality (% of introduced larvae in pots ±SEM) of *B. dorsalis* insect induced by three nematode isolates (*H. taysearae* Hessa1, *H. taysearae* KoroborororouF4 and *Steinernema* n. sp. Thui) inoculated 3 days before (BI) and at the same time as insect introduction into experimental pots under semi field conditions. Vertical bars are standard error of the means. Bars with the same uppercase letters stand for non-significant differences of insect mortality for the same time of EPN inoculation among nematode isolates. Bars with the same lowercase letters stand for non-significant differences of insect mortality of the same nematode isolate depending on the time of EPN application (SNK's test at $P<0.05$).

6.5 Discussion

6.5.1 Invasion time under laboratory conditions

The penetration percentages of *H. taysearae* Hessa1, *H. taysearae* KoroborororouF4 and *Steinernema* n. sp. Thui IJs inside *B. dorsalis* larvae increased with time of insect exposure to IJs. *H. taysearae* Hessa1 displays the highest percentage (24.42%) after 24 h exposure time. Hominick and Reid (1990) suggested the consideration of the penetration ability of a nematode as an indication of their virulence. These results are therefore in agreement with our previous laboratory results (Godjo et al. 2018b) which revealed the virulence of *H. taysearae* Hessa1 on larvae and pupae of *B. dorsalis* at relatively low IJ concentration. In this respect, based on its high penetration percentage, the isolate *H. taysearae* Hessa1 may be a good candidate to control *B. dorsalis* in mango orchards.

After 2 hours of exposure, all three tested EPN isolates were able to penetrate the mature insect larvae even though the percentage remained low. This short invasion time needed by EPNs may indicate that late instar larvae would be exposed to EPN infestation in soil before they shift to pupae stage. Indeed, these larvae are believed to spend a short time in soil before they pupate in the top 4 cm (Hou et al. 2006). This dispersal period of the larvae in soil is highly variable depending on the environmental conditions and therefore not clearly specified in the literature. In our preliminarily experiments, first pupae were harvested in room conditions 24 hours after larvae had exited the infested fruits for pupation in the soil (Godjo et al. 2018b). In other unspecified conditions, mature larvae could spend 2 days before shifting to pupae stage (Hou et al. 2017). Furthermore, it has earlier been reported that pupae are less susceptible to EPNs compared to third instar larvae (Godjo et al. 2018b) suggesting that larvae represent the best developmental stage to target. Based on the invasion time results, we can therefore assume that relatively short time is needed for EPNs to penetrate *B. dorsalis* larvae, confirming their efficacy on the insect pest.

6.5.2 Larval drop monitoring

Fruit flies third instar larvae were collected in the two orchards from mid-March (early stage of mango fructification) until July with highest population recorded in May when mangos started maturing. These results are in agreement with Vayssières et al. (2015a) who reported the period of abundance of *B. dorsalis* and *C. cosyra* to be in May-June. The early appearance of larvae in soil in the orchards, suggests that EPNs need to be applied early in mango orchards to allow their establishment in the soil before larvae start dropping on the ground for pupation. We propose the application of EPNs before the fructification stage, that begins generally at end January for IFAC and ELDON cultivars (Vayssieres et al. 2008), to increase their chance to establish and efficiently control the flies. The abundance of larvae in the ELDON orchard compared to the IFAC orchard could be explained by the high susceptibility of ELDON cultivar to tephritids (Vayssieres et al. 2008).

6.5.3 Nematode persistence and vertical distribution in mango orchards

The significant differences in IJ density recorded with the three nematode isolates could be explained by the fact that they adapt differently to soil conditions and nematode movement in soil is believed to vary among species (Shapiro-Ilan et al. 2012b). *H. taysearae* Hessa1 was earlier

demonstrated to have a Cruiser type foraging strategy (Zadji et al. 2014c) which may differ in the other two isolates. The high density of IJs in soil the first two weeks after nematode application could be explained by mass emergence of IJs from *G. mellonella* cadavers applied in the experimental plots.

EPN activity in soil 4 weeks after IJ application was less than expected as the density of the three nematode isolates dramatically dropped. Similar results were found by Herz et al. (2006) when *Steinernema feltiae* was applied against *Rhagoletis cerasi*, the cherry fruit fly. Several environmental factors such as temperature, soil texture, soil moisture, UV radiation and pH could influence the survival and persistence of EPNs in soil (Grewal et al. 2005; Grewal et al. 1994; Kaya 1990). Among those factors, the soil temperature and moisture are susceptible to rapid change. In the present experiment, water was provided on each plot after EPN application, but this could not exclude the possibility of soil dryness some weeks later since the experiment was installed at the end of the dry season (March). As indicated by the agro-meteorological data of the experimental area (Table 6.1), the relative humidity (32.51- 85.9%) was low and there was little rain (32.7 mm) in March suggesting poor water supply in the soil. In addition, soil temperature was relatively high (Maximum of 37.42°C) in the period where IJ density dropped considerably, thus leading to the observed reduction of EPN density in the orchard. We suggest in the future two to three EPN applications at different dates to enhance their establishment and persistence in mango orchards.

Nematode distribution among different soil depths was significantly different between species suggesting that they had preferences to establish at different soil layers. Similar results were found when vertical distribution of nematodes in soil was previously studied (Ferguson et al. 1995; Georgis and Poinar Jr 1983; Moyle and Kaya 1981). The higher IJ density of the two *Heterorhabditis* isolates in the lower layers of soil (10-20 cm) indicated their preference to establish in the inner layer of soil unlike the *Steinernema* species that resided in the top layers. This result corroborated field study by Ferguson et al. (1995) and laboratory tests by Georgis and Poinar Jr (1983) who reported the preference of *S. carpocapsae* to stay near the soil surface and the *Heterorhabditis* species in deeper layers of soil probably due to their different host-search strategy (Kaya et al. 1993).

6.5.4 Nematode virulence under semi field tests

The high virulence of *H. taysearae* Hessa1 under semi field condition is in agreement with our previous laboratory experiments where the same isolate demonstrated high virulence on *B. dorsalis* larvae and pupae (Godjo et al. 2018b). *H. taysearae* Hessa1 represents, therefore, a good candidate for biological control of *B. dorsalis* in mango orchards. This result confirms the effectiveness of EPNs on *B. dorsalis* larvae and pupae as earlier reported by Lin et al. (2005). The significant differences in insect mortality recorded among EPN isolates in function of the application time confirms the importance of this parameter for optimizing EPN efficacy in field as previously reported (Pérez and Lewis 2002, 2004).

6.6 Acknowledgements

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6.7 References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18:265-267

Alyokhin AV, Mille C, Messing RH, Duan JJ (2001) Selection of pupation habitats by oriental fruit fly larvae in the laboratory. Journal of Insect Behavior 14:57-67

Anato FM, Wargui RB, Sinzogan AA, Offenberg J, Adandonon A, Vayssières JF, Kossou DK (2015) Reducing losses inflicted by insect pests on cashew, using weaver ants as a biological control agent. Agricultural and Forest Entomology 17:285-291

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Birah A, Chilana P, Shukla UK, Gupta G (2008) Mass rearing of greater wax moth (*Galleria mellonella* L.) on artificial diet. Indian Journal of Entomology 70:389-392

Clarke AR, Armstrong KF, Carmichael AE, Milne JR, Raghu S, Roderick GK, Yeates DK (2005) Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. Annual Review of Entomology 50:293-319

Dowds BC, Peters A (2002) Virulence mechanisms. In: Gaugler R (ed), Entomopathogenic Nematology. CABI, Wallingford, UK, pp 79-98

Drew RA (1989) The tropical fruit flies (Diptera: Tephritidae: Dacinae) of the Australasian and Oceanian regions. Memoirs of the Queensland Museum 26:1-521

Ehlers R-U (1996) Current and future use of nematodes in biocontrol: practice and commercial aspects with regard to regulatory policy issues. Biocontrol Science and Technology 6:303-316

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Applied Microbiology and Biotechnology 56:623-633

Ehlers R-U, Lunau S, Krasomil-Osterfeld K, Osterfeld KH (1998) Liquid culture of the entomopathogenic nematode-bacterium-complex *Heterorhabditis megidis*/*Photorhabdus luminescens*. BioControl 43:77-86

Ferguson CS, Schroeder PC, Shields EJ (1995) Vertical distribution, persistence, and activity of entomopathogenic nematodes (Nematoda: Heterorhabditidae and Steinernematidae) in alfalfa snout beetle (Coleoptera: Curculionidae) infested fields. Environmental Entomology 24:149-158

Georgis R, Poinar Jr GO (1983) Effect of soil texture on the distribution and infectivity of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae). Journal of Nematology 15:308

Gnanvossou D, Hanna R, Bokonon-Ganta AH, Ekesi S, Mohamed SA (2016) Release, establishment and spread of the natural enemy *Fopius arisanus* (Hymenoptera: Braconidae) for control of the invasive oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) in Benin, West Africa. In: Ekesi S, Mohamed SA, Meyer MD (eds), Fruit Fly Research and Development in Africa-Towards a Sustainable Management Strategy to Improve Horticulture. Springer, pp 575-600

Gnanvossou D, Hanna R, Goergen G, Salifu D, Tanga C, Mohamed S, Ekesi S (2017) Diversity and seasonal abundance of tephritid fruit flies in three agro-ecosystems in Benin, West Africa. Journal of Applied Entomology 141:798-809

Godjo A, Zadji L, Decraemer W, Willems A, Afouda L (2018) Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*) under laboratory conditions. Biological Control 117:68-77 doi:10.1016/j. biocontrol.2017.10.009

Goergen G, Vayssières J-F, Gnanvossou D, Tindo M (2011) *Bactrocera invadens* (Diptera: Tephritidae), a new invasive fruit fly pest for the Afrotropical region: host plant range and distribution in West and Central Africa. Environmental Entomology 40:844-854

Grewal PS, Ehlers R-U, Shapiro-Ilan DI (2005) Nematodes as Biocontrol Agents. Grewal PS, Ehlers R-U, Shapiro-Ilan DI (series editors). CABI Publishing, Wallingford, UK

Grewal PS, Selvan S, Gaugler R (1994) Thermal adaptation of entomopathogenic nematodes: niche breadth for infection, establishment, and reproduction. Journal of Thermal Biology 19:245-253

Herz A, Köppler K, Vogt H, Elias E, Katz P, Peters A (2006) Biological control of the cherry fruit fly, *Rhagoletis cerasi* L.(Diptera, Tephriti-dae) by use of entomopathogenic nematodes: first experiences towards practi-cal implementation. In: Proceedings to the Conference Ecofruit-12th international conference on cultivation technique and phytopathological problems in organic fruit-growing, Weinsberg/Germany. Fördergemeinschaft Ökologischer Obstbau eV (FÖKO), Traubenplatz 5, D-74189 Weinsberg, pp 67-72

Hominick W, Reid A (1990) Perspectives on entomopathogenic nematology. In: Gaugler R, Kaya HK (eds), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, Florida, pp 327-345

Hou B, Xie Q, Zhang R (2006) Depth of pupation and survival of the Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) pupae at selected soil moistures. Applied Entomology and Zoology 41:515-520

Hou Q-L, Jiang H-B, Gui S-H, Chen E-H, Wei D-D, Li H-M, Wang J-J, Smagghe G (2017) A role of corazonin receptor in larval-pupal transition and pupariation in the oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae). Frontiers in Physiology 8:77 doi:10.3389/fphys.2017.00077

Kaya HK (1990) Soil ecology. In: Gaugler R, Kaya HK (eds), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FLorida, pp 93-115

Kaya HK, Burlando T, Thurston GS (1993) Two entomopathogenic nematode species with different search strategies for insect suppression. Environmental Entomology 22:859-864

Langford EA, Nielsen UN, Johnson SN, Riegler M (2014) Susceptibility of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), to entomopathogenic nematodes. Biological Control 69:34-39

Lin J, Zeng L, Liang G, Lu Y, Bin S (2005) Effects of entomopathogenic nematodes on the oriental fruit fly, *Bactrocera* (Bactrocera) *dorsalis* (Hendel). Acta Entomologica Sinica 48:736-741

Lindegren JE, Vail PV (1986) Susceptibility of Mediterranean fruit fly, melon fly, and oriental fruit fly (Diptera: Tephritidae) to the entomogenous nematode *Steinernema feltiae* in laboratory tests. Environmental Entomology 15:465-468

Malan AP, Manrakhan A (2009) Susceptibility of the Mediterranean fruit fly (*Ceratitis capitata*) and the Natal fruit fly (*Ceratitis rosa*) to entomopathogenic nematodes. Journal of Invertebrate Pathology 100:47-49

Minas RdS, Souza RM, Dolinski C, Carvalho RdS, Burla RdS (2016) Potential of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) to control Mediterranean fruit fly (Diptera: Tephritidae) soil stages. Nematoda 3:e02016 doi:10.4322

Moyle PL, Kaya HK (1981) Dispersal and infectivity of the entomogenous nematode, *Neoaplectana carpocapsae* Weiser (Rhabditida: Steinernematidae), in sand. Journal of Nematology 13:295

Pérez EE, Lewis EE (2002) Use of entomopathogenic nematodes to suppress *Meloidogyne incognita* on greenhouse tomatoes. Journal of Nematology 34:171

Pérez EE, Lewis EE (2004) Suppression of *Meloidogyne incognita* and *Meloidogyne hapla* with entomopathogenic nematodes on greenhouse peanuts and tomatoes. Biological Control 30:336-341

Peters A (1996) The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations. Biocontrol Science and Technology 6:389-402

Schutze MK, Mahmood K, Pavasovic A, Bo W, Newman J, Clarke AR, Krosch MN, Cameron SL (2015) One and the same: integrative taxonomic evidence that *Bactrocera invadens* (Diptera: Tephritidae) is the same species as the Oriental fruit fly *Bactrocera dorsalis*. Systematic Entomology 40:472-486

Shapiro-Ilan DI, Lewis EE, Campbell JF, Kim-Shapiro DB (2012) Directional movement of entomopathogenic nematodes in response to electrical field: effects of species, magnitude of voltage, and infective juvenile age. Journal of Invertebrate Pathology 109:34-40

Sinzogan AAC, Van Mele P, Vayssières J-F (2008) Implications of on-farm research for local knowledge related to fruit flies and the weaver ant *Oecophylla longinoda* in mango production. International Journal of Pest Management 54:241-246

Sirjani FO, Lewis EE, Kaya HK (2009) Evaluation of entomopathogenic nematodes against the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). Biological Control 48:274-280

Van Mele P (2008) A historical review of research on the weaver ant *Oecophylla* in biological control. Agricultural and Forest Entomology 10:13-22

Van Mele P, Vayssières J-F, Van Tellingen E, Vrolijks J (2007) Effects of an African weaver ant, *Oecophylla longinoda*, in controlling mango fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 100:695-701

Vayssières J-F, De Meyer M, Ouagoussounon I, Sinzogan A, Adandonon A, Korie S, Wargui R, Anato F, Houngbo H, Didier C (2015a) Seasonal abundance of mango fruit flies (Diptera: Tephritidae) and ecological implications for their management in mango and cashew orchards in Benin (Centre & North). Journal of Economic Entomology 108:2213-2230

Vayssières J-F, Korie S, Ayegnon D (2009b) Correlation of fruit fly (Diptera Tephritidae) infestation of major mango cultivars in Borgou (Benin) with abiotic and biotic factors and assessment of damage. Crop Protection 28:477-488

Vayssieres J-F, Korie S, Coulibaly O, Temple L, Boueyi SP (2008) The mango tree in Central and Northern Benin: cultivar inventory, yield assessment, infested stages and loss due to fruit flies (Diptera Tephritidae). Fruits 63:335-348

Vayssières J-F, Ouagoussounon I, Adandonon A, Sinzogan A, Korie S, Todjihoundé R, Alassane S, Wargui R, Anato F, Goergen G (2015b) Seasonal pattern in food gathering of the weaver ant *Oecophylla longinoda* (Hymenoptera: Formicidae) in mango orchards in Benin. Biocontrol Science and Technology 25:1359-1387

Vayssieres J-F, Sinzogan A, Korie S, Ouagoussounon I, Thomas-Odjo A (2009a) Effectiveness of spinosad bait sprays (GF-120) in controlling mango-infesting fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 102:515-521

White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

White IM, Elson-Harris MM (1992) Fruit flies of Economic Significance: their Identification and Bionomics. White IM, Elson-Harris MM (series editors). CAB International, Wallingford, UK

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014) Effectiveness of different *Heterorhabditis* isolates from Southern Benin for biocontrol of the subterranean termite, *Macrotermes bellicosus* (Isoptera: Macrotermitinae), in laboratory trials. Nematology 16:109-120

7 Chapter 7:

General discussion, conclusions and future prospects

The increasing need for reducing chemical use as a traditional solution for insect pest damage in agriculture, has led to the development of alternatives that promote the use of natural enemies to control these pests. Biocontrol is perceived as more respectful to the environment and more sustainable than chemical applications which in most cases eventually lead to pest resistance to insecticide (Sandhu et al. 2012). Tephritid fruit flies represent a serious threat for many important crops such as mango which contributes to food security of millions of Africans and particularly in Benin. In addition, the immature stages (egg and larvae) occur inside the fruit suggesting that only systemic insecticides may be used for efficient control of the insect pests (Reynolds et al. 2017). Considering the side effects of chemical measures on people and environment, a sustainable control of these insect pests mainly based on Integrated Pest Management (IPM) methods has been investigated by many researchers during the past few years (Vayssières et al. 2015a). Such considerations have motivated my effort in investigating the use of entomopathogenic nematode as an alternative way of reducing losses caused by fruit flies, especially *Bactrocera dorsalis* which represents the most invasive species in mango production (Vayssières et al. 2015a; Vayssieres et al. 2008).

7.1 Occurrence of entomopathogenic nematodes in mango orchards and their taxonomic identification

More than 90% of insect pests spend part of their life cycle in soil (Klein 1990; Stark and Lacey 1999). In soil, a large range of beneficial organisms are present and they can be used for the biological control of insect pests. Considering fruit flies, their last instar larval and pupal stages occur in soil (Hou et al. 2006; Stark and Lacey 1999). *Heterorhabditis* and *Steinernema* nematodes have been recovered from Beninese soil during several sampling campaigns. In my PhD study, 14 mango orchards located in Northern Benin, a region where more than 75% of mangos are produced, have been explored and only two *Heterorhabditis* isolates were recovered. These two EPN isolates (KoroborororouF4 and KoroborororouC2) have been identified as *Heterorhabditis taysearae* based on molecular and morphological/ morphometric data (chapter 2). The low percentage of nematode-positive soil samples (2.86%) could be explained by the fact that soil sampling was conducted in the dry season where most nematodes in the absence of humidity probably became less active or were more hidden in deeper layers of the soil than the sampling depth limit used (approximately 15cm depth). It has been documented that

Heterorhabditis species can survive up to 35 cm deep in loamy sand (Ferguson et al. 1995). However, the main research question related to this soil sampling exclusively in mango orchards was to investigate the natural occurrence of EPNs in mango orchards. Although a limited number of orchards were sampled, my results could give insights into the natural presence of EPNs in mango production environments. These two new *H. taysearae* isolates recovered from Beninese mango orchards were included in laboratory experiments (chapter 2) and one isolate (KorobororouF4) was included in the field trials (chapter 6) to investigate its efficiency to biologically reduce *B. dorsalis* population in mango orchard.

One of the major tasks in investigating a biological control alternative for an insect pest is the accurate identification of both the pest and its natural enemies. In my PhD study, I focused on the identification of newly collected EPN isolates from mango orchards (chapter 2) and the two undescribed native *Steinernema* isolates (chapter 3) previously included in pathogenicity tests against termite (Baimey et al. 2015) and here against *B. dorsalis* (chapters 2 and 6). The two *Heterorhabditis* isolates collected from mango orchards were identified as *H. taysearae* because they share all morphological characters with the described *H. taysearae* species (Shamseldean et al. 1996; Stock et al. 2009) and possessed the same ITS fragment as *H. sonorensis* strain Caborca (FJ477730) recently reclassified by Hunt and Subbotin (2016) as a junior synonym of *H. taysearae*. This result is a confirmation of the occurrence of *H. taysearae* in Benin as it was earlier reported as *H. sonorensis* by Zadji et al. (2013) and Houssou et al. (2014). The same species was recovered from soil in other locations around the world. It was originally described from Egypt (Shamseldean et al. 1996), and afterwards reported in Mexico in the Sonoran desert (Stock et al. 2009). ITS sequences of some additional isolates, SW1 (KP325085) and SW3 (KY092432) recently recovered from South Africa; EGAZ7-El-Kasasein_Ismaila (KY088208) and EGAZ8-El-Kasasein_Ismaila (KY088209) from Egypt and another isolate (KC633186) from Gaza strip have also been deposited in GenBank without being officially published.

The identification of the two Beninese *Steinernema* isolates (Bembereke157C and Thui168d) revealed that they belong to a separate species within *Steinernema* clade. For systematic studies of EPNs, molecular analysis of the ITS and the D2D3 regions have been used in many taxonomic studies to identify new nematode isolates (Liu et al. 1997). The analysis of these genes placed the two *Steinernema* isolates in a sister cluster to *S. abbasi* (97.3-97.6% ITS nucleotide similarity)

and *S. bifurcatum* (98.3-98.4% D2-D3 similarity) which were the most closely related species within the *Bicornutum* species group. For steinernematids, morphometrics and morphological data usually confirm identification inferred from molecular results (Spiridonov et al. 2004). According to Hominick et al. (1997), Nguyen and Hunt (2007) and Stock and Kaya (1996), morphometrics of males and IJs give the essential information for EPN identification. More specifically, Phan et al. (2003) suggested the consideration of characters such as IJs body diameter, spicule and gubernaculum length of males for species differentiation. However, some variations in the IJs measurements have been registered depending of their harvest time (Nguyen and Smart Jr 1995). Morphometrics of isolates Bembereke157C and Thui168d showed some intra-specific variations for some of the main characters such as the IJ body length, the spicule length of males and some other characters. Such variations have previously been observed with other species within the *Bicornutum* species group such as *Steinernema papillatum* (San-Blas et al. 2015). However, variation in IJs measurement could be related to the availability of food inside the insect host as freshly harvested IJs for both isolates were used for measurements. In addition to the IJs morphology, the number of the male's genital papillae has also been reported to be conserved within species (Nguyen et al. 2004). This criterion and the cross- hybridization support the classification of the two Beninese isolates as a new species.

7.2 Potential of Beninese EPN isolates to control *B. dorsalis* under laboratory conditions

During my PhD study, one of the main constraints I had to constantly deal with was the loss of some EPN isolates included in my experiments. These isolates for unknown reason failed to recycle inside *G. mellonella* and therefore lost their viability. The majority of nematode isolates included in my work were collected in 2012 during the Ecological Sustainable Citrus Production (ESCIP) project in Benin. These isolates were periodically (every two or three months) recycled in *G. mellonella* using the baiting (Bedding and Akhurst 1975) and white trap (White 1927) methods. However, some isolates unexpectedly lost their virulence and either failed to kill the *G. mellonella* larvae, or managed to kill the larvae but did not reproduce inside. This issue could be explained by the fact that i) the IJs lost their bacterial symbionts with repeated reproduction in *G. mellonella*, or ii) the bacterial cells once released into the insect host failed to reproduce thus compromising IJ development and reproduction. Variation of optimal temperature required by

each nematode isolate could also be a contributing factor as earlier pointed out by Zadji (2014) in his PhD dissertation. An alternative solution for this problem could be the preservation using liquid nitrogen as proposed by Popiel and Vasquez (1991) and at the same time this could solve the problem of regular checking which requires a lot of time and work.

The biocontrol potential of an organism is determined by its performance under challenging environmental conditions that the insect pest may encounter in nature. It is common to see researchers considering laboratory investigations as a startup point for developing a new biological control strategy of an insect pest. These laboratory investigations usually serve to select better candidates to be used in field assays and therefore avoiding huge costs associated with field experiments. In my case, 12 native EPN isolates were screened for their pathogenicity on *B. dorsalis* and three isolates were later selected for field investigations.
In Northern Benin where more than 75% of the national mango production comes from, the climate is characterized by a unimodal rainfall (approximately 1000-1100 mm yearly) with highest record in September (Vayssières et al. 2015a). Environmental conditions such as temperature, humidity, soil type, are controlling factors for EPN movement and reproduction in soil and therefore affecting their potential as Biocontrol agent.

In chapter 2, the reproduction potential of local *H. taysearae* and *Steinernema* n. sp. isolates inside the late instar larvae of *B. dorsalis* was studied. Results showed that all tested EPN isolates reproduced well inside the insect larvae with the *Heterorhabditis* isolates yielding higher numbers of IJs upon emergence from the *B. dorsalis* cadaver. This represents a key factor for EPN success in biological control of *B. dorsalis*. Their successful reproduction supports an eventual persistence of EPNs in mango field. EPNs may increase in numbers upon multiplication in insect hosts, thus favoring their established in the field.

In literature, EPNs have been tested on diverse tephritid species for their efficiency in reducing fly population. In most experiments, authors reported the virulence of EPNs on insect larvae and pupae developmental stages. However, the common question raised by researchers is the application cost-effectiveness. Some authors reported the high concentration of IJs needed to kill larvae/pupae of tephritid pest, constituting a limit for their implementation in field conditions (Rousse and Quilici 2009; Toledo et al. 2006a). In my study, as a first step, I found it relevant to determine the optimal concentration of Beninese EPN isolates needed to efficiently reduce *B.*

dorsalis population. Results from the laboratory assays showed that 32 IJs of *H. taysearae*/cm² was optimal to successively induce more than 90% insect mortality. In contrast, 237 IJs of *H. baujardi* (Minas et al. 2016) and 100 IJs of *S. riobrave* (Gazit et al. 2000) were needed per cm² of soil surface to achieve less than 88% mortality of *C. capitata* larvae in previous studies. Depending on the nematode species (Bedding and Molyneux 1982) and IJ size (Westerman 1998), the penetration percentage of IJs into the insect host may differ. In addition, it has been reported that the number of IJs that penetrate the insect host may influence the mortality of the latter (Pervez et al. 2012). However, that assumption was not confirmed with Beninese EPNs against termite pests (Zadji et al. 2014b) which resulted in the observation of low IJ penetration percentage with high virulent EPNs (*H. indica* and *H. taysearae*). Therefore, despite the small size (length) of *H. taysearae* IJs (418 µm) compared to *H. baujardi* (551 µm) and *S. riobrave* (622 µm), the efficient concentrations of these EPNs reported in the literature remain relatively high compared to my findings. It can be assumed that Beninese isolates express high performance in killing *B. dorsalis* insects as a low concentration of IJs (32 IJs/cm²) is required to induce a high mortality percentage (>90%) to larvae.

7.3 Characterization and identification of bacterial symbionts of Beninese EPN isolates

EPNs are known to live in symbiosis with bacteria that they use to prey on and cause the death of the insect host. A specific relationship exists between nematodes and their symbionts associating one nematode species to one bacterial species (Akhurst 1982; Thomas and Poinar 1979). However, evidence of the association of a bacterial species to more than one nematode species has been reported in many studies (Burnell and Stock 2000; Dreyer et al. 2017; Forst and Nealson 1996; Lacey et al. 2001; Lee and Stock 2010; Stock 2015). These bacterial symbionts play a key role in the infection process of the insect host. In chapter 4, the biological diversity of all bacterial symbionts associated with Beninese EPNs that were previously isolated from soil sampled in Central and Northern Benin, was investigated. Fifteen *Xenorhabdus* isolates were recovered and identified as *Xenorhabdus indica* based on a MLSA analysis including two housekeeping genes (recA and gyrB) and the 16S rRNA gene. Analysis of the same genes classified the 27 *Photorhabdus* isolates into two different new subspecies of *Photorhabdus luminescens*. While studying the diversity of these bacteria, initially, both *Photorhabdus* and

Xenorhabdus isolates were identified from the same EPNs belonging to two *Steinernema* isolates (Bembereke157c and Thui168d) described in chapter 3. These results contradicted the well-known associations *Heterorhabditis-Photorhabdus* and *Steinernema-Xenorhabdus*. Therefore, to confirm my observations, a clone library was prepared on the 16S rRNA gene products amplified from DNA directly extracted from infested hemolymph of *G. mellonella* with each of the two nematode isolates. Three repetitions (hemolymph of three different *G. mellonella* larvae) were used per nematode isolate and from each repetition, 100 colonies were picked and analysed. Sanger sequencing of partial 16S rRNA gene of the 600 clones revealed that all symbiotic bacteria shared identical partial 16S rRNA gene fragment and clustered with *X. indica*. These results did not support my initial observations which I therefore attributed to experimental error.

In prokaryotic taxonomy, different methods are used for bacterial characterization and identification. The 16S rRNA gene is commonly used for bacterial identification because it is present in all bacteria and well documented for most bacteria in public data bases such as NCBI although sequence quality may sometimes be poor. In addition, the gene possess 9 hypervariable regions that demonstrate considerable sequence diversity among different bacterial strains and therefore appear to be useful for species identification (Chakravorty et al. 2007; Van de Peer et al. 1996). However, in some cases, this gene can confound species delineation of bacteria as different species can possess highly similar 16S rRNA fragment (Clarridge 2004). For the identification of Beninese nematode bacterial symbionts, a MLSA method was used based on 2 to 5 housekeeping genes (recA, gyrB, gltX, dnaN and infB) in addition to the 16S rRNA gene to increase the robustness of the phylogenies (Tailliez et al. 2010; Tailliez et al. 2012). The same method, in addition to some phenotypic tests, was used in chapter 5 to fully identify one of the two new subspecies of *P. luminescens* reported while studying the biological diversity of the symbionts. I was able to proceed with the identification of only one sub-group in *P. luminescens* clade because the three bacterial isolates that formed the second sub-group unexpectedly lost their viability and could not be recovered. An attempt to re-isolate the bacterial isolates from the same nematodes failed because the latter could also not be recovered. A novel bacterial subspecies is therefore being proposed, *Photorhabdus luminescens* subsp. *beninensis* subsp. nov. It could be used in large scale multiplication of the associated nematode isolate for the control of *B. dorsalis* in mango orchards.

Nowadays, complete genome sequencing is being adopted in several molecular studies of *Xenorhabdus* and *Photorhabdus* strains (Dreyer et al. 2017; Kämpfer et al. 2017). Indeed genome sequencing has become an increasingly important tool to address various questions in microbiology. This method is considered more accurate as it is not restricted to specific genes and covers the whole genome. Furthermore, the cost for entire genome sequencing is decreasing considerably, suggesting that it will replace older methods in the near future for accurate bacterial identification. Therefore, this will resolve the problem of development of universal or genus specific primers for relevant housekeeping genes for species-delineation.

Next to a DNA-based approach for bacteria identification, MALDI-TOF MS might also have been used for dereplication of isolates and their rapid and reliable identification (Ghyselinck et al. 2011). For a first taxonomic grouping, this method could replace the partial 16S rRNA gene sequencing performed on all bacterial isolates in chapter 4, which is rather time and money consuming especially when dealing with a high number of isolates. However, identification would rely on the presence of protein profiles for reference strains of *Xenorhabdus* and *Photorhabdus* in the database.

7.4 Field adaptation of EPNs in mango orchards and their virulence on *Bactrocera dorsalis* under semi-field conditions

Environmental adaptation of EPNs is critical for their success in biological control of insect pests. An EPN isolate can show different virulence levels under laboratory conditions compared to field conditions where several uncontrolled parameters are present. Furthermore, the EPNs have to search for a host which was made readily available in laboratory investigations. One of the most important criteria for EPN field efficacy is their persistence in environmental conditions where the pest occurs (Jansson et al. 1993). In chapter 6, field performance of three EPN isolates from Benin was investigated. The three isolates persisted in the mango orchards for several months after EPN application. However, the dramatical drop of nematode densities for all the three isolates observed 4 weeks after nematode application could be due to a negative reaction to unfavorable environmental conditions. This assumption was supported by the high temperatures and low soil humidity (less rain water) recorded during that period on the experimental area. However, the method used to collect abiotic data of the experimental site was limited. The use of appropriate devices to record regular soil temperature and humidity on the experimental plots

would have allowed more precise information on environmental factor variation in the soil instead of merely collecting data of the general field area. Such data would allow better understanding of the vertical distribution of the tested EPN isolates which demonstrated some preferences to establish in upper or inner layer of soil (chapter 6). An alternative to counter the dramatic decrease in IJs density in the field would be the increase of the number of nematode applications at relevant dates as was earlier performed in a similar study (Herz et al. 2006) to enhance nematode establishment in the orchard. Obviously, it would be reasonable to study the cost effectiveness of the optimal number of nematode applications before presenting a practical protocol to mango growers.

Virulence of Beninese nematode isolates under semi-field conditions (chapter 6) confirmed the high pathogenicity of *H. taysearae* Hessa1to *B. dorsalis* as demonstrated in laboratory assays in chapter 2. This isolate would therefore represent a good candidate in *B. dorsalis* control. I also found that third instar larvae were more susceptible than pupae. This confirmed the observations by Yee and Lacey (2003) when *S. carpocapsae* and other EPN isolates were applied on *Rhagoletis indifferens*. The success of EPNs in the control of *B. dorsalis* would then depend on how efficient the insect larvae population is reduced, suggesting that time of EPN application is rather crucial as larvae spend relatively limited time in soil before pupation. It can be assumed that an early application would favor the establishment of nematodes before the abundance of larvae in mango orchards. Results of the invasion-time assay (chapter 6) give some hope for the success of EPNs to control *B. dorsalis* as a relatively short time (2 hours) is enough for the IJs to penetrate the insect larvae. Larvae that would escape parasitism by nematodes would still be exposed to them as pupae even though the chances for EPN invasion will be less.

In addition, local EPN treatments in a mango orchard would mainly decrease the fly population emerging from the same orchard. However, a possibility of infestation by flies from neighboring untreated mango orchards or other host crops cannot be avoided. Therefore, EPN establishment in orchard's soil at national or regional level should be encouraged. For example, the recent release of *Fopius arisanus* (parasitoid) in diverse agro-ecological zones in Benin led to its good establishment, persistence and spread in the targeted area (Gnanvossou et al. 2016). In the similar way, an alternative of inter-orchard infestations regarding EPN application could be the insertion of EPN treatments in national or regional programs aiming for fruit fly control based on IPM

methods such as PRIA (Plan Régional d'Investissement Agricole) or PNIA (Plans Nationaux d'Investissement Agricole). In addition, *B. dorsalis* can also be controled in alternative crop hosts such as *Irvingia gabonensis* (bush mango) which produces fruits like mangos throughout the whole year, or *Sarcocephalus latifolius* which produces fruits during the dry season where there are no mangos.

One of the main constraints encountered during field trials, was the presence of weaver ants in the two experimental orchards. These ants were reported to prey on larvae and pupae of the insect present in the orchard soil (Anato et al. 2015b; Van Mele 2008; Van Mele et al. 2007; Vayssières et al. 2015b) suggesting that densities of these developmental stages reported in chapter 6 were most likely underestimated.

7.5 Conclusion and future perspectives

In the present PhD study, local Beninese EPNs have been surveyed in several mango orchards (Chapter 2) to assess their natural occurrence in Northern Benin where mango is mostly produced. The collected EPN isolates together with the available isolates previously sampled from other vegetation in the whole country, were used to assess their potential in killing *B. dorsalis* under diverse abiotic factors in laboratory (chapter 2) and semi field conditions (Chapter 6) for the first time. Data in chapters 2 and 6 shed some light on the persistence and the virulence of local EPNs on soil developmental stages (larvae and pupae) of *B. dorsalis*, the most damaging mango pest in Benin and the whole West-Africa region. In addition, the isolation and identification of bacterial symbionts of Beninese EPNs included in this study have been performed (Chapter 4) and among the bacterial strains recovered, a new subspecies of *Photorhabdus luminescens* has been found and fully described (chapter 5). In the same respect, the EPN isolates newly recovered from mango orchards (Chapter 2) and two *Steinernema* isolates (chapter 3) previously isolated from the same geographical area (North Benin), were fully identified with the *Steinernema* isolates representing a new species. However, some aspects remain unclear or should be investigated further before the recommendation of Beninese EPNs for biological control of *B. dorsalis* or other tephritid insects.

1) The experiments conducted in Chapter 6 to assess the efficacy of nematodes in field conditions remain preliminary, and investigations should be furthered to assess, for example, the persistence of EPNs in different soil types and associated water regime, as

soil structure and its moisture content are important variables for EPN movement and therefore their virulence towards insect pests. In addition, for better extrapolation of field test results, it would be recommended to repeat the field assays in different mango orchards during two to three mango seasons. Moreover, these field tests in the future could consider more than one mango cultivar to have an idea of the performance of EPNs in the presence of resistant or more sensitive cultivars since EPN reproduction and recycling potential in the orchards will definitely depend on the presence of insect host.

2) Next to the application of EPNs on soil stages of *B. dorsalis*, further studies can investigate their efficacy on adult insects in soil. Application of EPNs in soil can therefore be ensured before the emergence of adults from pupae in such a way that, while exiting the pupae, the young insect adults could get infected by IJs before flying away. In this case, the EPN application time is very critical and should be as precise as possible to induce good control results.

3) In the context of IPM, further investigations should focus on the impact of EPN applications on beneficial organisms present in mango orchards such as the natural predators of flies (eg. weaver ants) or even the parasitoid wasps (eg. *Fopius arisanus*) being investigated for the same purpose of sustainable control of the insect pests.

4) The success of biological control of an insect pest can also be influenced by chemicals or fertilizers. Therefore, the effect of some commonly used insecticides or even the bacterial toxin-based insecticide (Spinosad GF-120) on EPN performance in mango orchards should be investigated to avoid any interference in case of both applications in the same area.

5) As a routine investigation for any biological control program, a cost effectiveness study of the use of EPNs to control *B. dorsalis* should be performed to evaluate the profitability of EPN applications compared to the existing insect control methods. In addition, awareness of agronomic researchers and mango growers regarding the use of EPNs, should be raised

through different information sessions and the grower's willingness to adopt EPNs as a new method to control the invasive *B. dorsalis* should be studied.

7.6 References

Akhurst R (1982) Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061-3065

Anato FM, Wargui RB, Sinzogan AA, Offenberg J, Adandonon A, Vayssières JF, Kossou DK (2015) Reducing losses inflicted by insect pests on cashew, using weaver ants as a biological control agent. Agricultural and Forest Entomology 17:285-291

Baimey H, Zadji L, Afouda L, Moens M, Decraemer W (2015) Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from Southern Benin and control of underground termite nest populations. Nematology 17:1057-1069

Bedding R, Akhurst R (1975) A simple technique for the detection of insect paristic rhabditid nematodes in soil. Nematologica 21:109-110

Bedding R, Molyneux A (1982) Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp. (Heterorhabditidae: Nematoda). Nematologica 28:354-359

Burnell AM, Stock SP (2000) *Heterorhabditis, Steinernema* and their bacterial symbionts—lethal pathogens of insects. Nematology 2:31-42

Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods 69:330-339

Clarridge JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical microbiology reviews 17:840-862

Dreyer J, Malan AP, Dicks LM (2017) Three Novel *Xenorhabdus–Steinernema* Associations and Evidence of Strains of *X. khoisanae* Switching Between Different Clades. Current Microbiology 74:938–942

Ferguson CS, Schroeder PC, Shields EJ (1995) Vertical distribution, persistence, and activity of entomopathogenic nematodes (Nematoda: Heterorhabditidae and Steinernematidae) in alfalfa snout beetle (Coleoptera: Curculionidae) infested fields. Environmental Entomology 24:149-158

Forst S, Nealson K (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. Microbiological Reviews 60:21-43

Gazit Y, Rossler Y, Glazer I (2000) Evaluation of entomopathogenic nematodes for the control of Mediterranean fruit fly (Diptera: Tephritidae). Biocontrol Science and Technology 10:157-164

Ghyselinck J, Van Hoorde K, Hoste B, Heylen K, De Vos P (2011) Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. Journal of Microbiological Methods 86:327-336

Gnanvossou D, Hanna R, Bokonon-Ganta AH, Ekesi S, Mohamed SA (2016) Release, establishment and spread of the natural enemy *Fopius arisanus* (Hymenoptera: Braconidae) for control of the invasive oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) in Benin, West Africa. In: Ekesi S, Mohamed SA, Meyer MD (eds), Fruit Fly Research and Development in Africa-Towards a Sustainable Management Strategy to Improve Horticulture. Springer, pp 575-600

Herz A, Köppler K, Vogt H, Elias E, Katz P, Peters A (2006) Biological control of the cherry fruit fly, *Rhagoletis cerasi* L.(Diptera, Tephriti-dae) by use of entomopathogenic nematodes: first experiences towards practi-cal implementation. In: Proceedings to the Conference Ecofruit-12th international conference on cultivation technique and phytopathological problems in organic fruit-growing, Weinsberg/Germany. Fördergemeinschaft Ökologischer Obstbau eV (FÖKO), Traubenplatz 5, D-74189 Weinsberg, pp 67-72

Hominick W, Briscoe B, del Pino FG, Heng J, Hunt D, Kozodoy E, Mracek Z, Nguyen K, Reid A, Spiridonov S (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. Journal of Helminthology 71:271-298

Hou B, Xie Q, Zhang R (2006) Depth of pupation and survival of the Oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) pupae at selected soil moistures. Applied Entomology and Zoology 41:515-520

Houssou G, Baimey H, Zadji L, De Sutter N, Waeyenberge L, Affouda L, Viaene N, Moens M, Decraemer W (2014) Taxonomic study of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) from Benin. Agronomie Africaine 26:231-245

Hunt DJ, Subbotin SA (2016) Taxonomy and systematics. In: Hunt DJ, Nguyen KB (eds), Hunt DJ, Perry RN (series editors), Advances in Taxonomy and Phylogeny of

Entomopathogenic Nematodes of the Steinernematidae and Heterorhabditidae, vol 12. Koninklijke Brill NV, Leiden, pp 13-58

Jansson RK, Lecrone SH, Gaugler R (1993) Field efficacy and persistence of entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae) for control of sweetpotato weevil (Coleoptera: Apionidae) in Southern Florida. Journal of Economic Entomology 86:1055-1063

Kämpfer P, Tobias NJ, Ke LP, Bode HB, Glaeser SP (2017) *Xenorhabdus thuongxuanensis* sp. nov. and *Xenorhabdus eapokensis* sp. nov., isolated from *Steinernema species*. International Journal of Systematic and Evolutionary Microbiology 67:1107-1114

Klein MG (1990) Efficacy against soil-inhabiting insect pests. Entomopathogenic Nematodes in Biological Control:195-214

Lacey LA, Frutos R, Kaya H, Vail P (2001) Insect pathogens as biological control agents: do they have a future? Biological Control 21:230-248

Lee M-M, Stock SP (2010) A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp.(γ-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. Journal of Invertebrate Pathology 104:67-74

Liu J, Berry RE, Moldenke AF (1997) Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. Journal of Invertebrate Pathology 69:246-252

Minas RdS, Souza RM, Dolinski C, Carvalho RdS, Burla RdS (2016) Potential of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) to control Mediterranean fruit fly (Diptera: Tephritidae) soil stages. Nematoda 3:e02016 doi:10.4322

Nguyen K, Hunt D (2007) Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Hunt DJ, Perry RN (series editors), vol 5. Nematology Monographs and Perspectives. Brill, Leiden. 816pp.

Nguyen KB, Smart Jr GC (1995) Morphometrics of infective juveniles of *Steinernema* spp. and *Heterorhabditis bacteriophora* (Nemata: Rhabditida). Journal of Nematology 27:206-2012

Nguyen KB, Tesfamariam M, Gozel U, Gaugler R, Adams BJ (2004) *Steinernema yirgalemense* n. sp. (Rhabditida: Steinernematidae) from Ethiopia. Nematology 6:839-856

Pervez R, Eapen S, Devasahayam S, Jacob T (2012) Efficacy of some entomopathogenic nematodes against insect pests of ginger and their multiplication. Nematologia Mediterranea 40

Phan KL, Subbotin SA, Nguyen NC, Moens M (2003) *Heterorhabditis baujardi* sp. n. (Rhabditida: Heterorhabditidae) from Vietnam and morphometric data for *H. indica* populations. Nematology 5:367-382

Popiel I, Vasquez EM (1991) Cryopreservation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. Journal of Nematology 23:432-437

Reynolds OL, Osborne TJ, Barchia I (2017) Efficacy of Chemicals for the Potential Management of the Queensland Fruit Fly *Bactrocera tryoni* (Froggatt)(Diptera: Tephritidae). Insects 8:49

Rousse P, Quilici S (2009) Recent advances in biological control of fruit fly pests. In: Bandeira RR (ed), Current Trends in Fruit Fly Control on Perennial Crops and Research Prospects. . Transworld Research Network, Kerala, India, pp 91-115

San-Blas E, Portillo E, Nermuť J, Půža V, Morales-Montero P (2015) *Steinernema papillatum n.* sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from Venezuela. Nematology 17:1081-1097

Sandhu SS, Sharma AK, Beniwal V, Goel G, Batra P, Kumar A, Jaglan S, Sharma A, Malhotra S (2012) Myco-biocontrol of insect pests: factors involved, mechanism, and regulation. Journal of Pathogens 2012:126819-126829 doi:10.1155/2012/126819

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

Spiridonov S, Krasomil-Osterfeld K, Moens M (2004) *Steinernema jollieti* sp n.(Rhabditida: Steinernematidae), a new entomopathogenic nematode from the American midwest. Russ J Nematol 12:85-95

Stark JEP, Lacey LA (1999) Susceptibility of western cherry fruit fly (Diptera: Tephritidae) to five species of entomopathogenic nematodes in laboratory studies. Journal of Invertebrate Pathology 74:206-208

Stock SP (2015) Diversity, biology and evolutionary relationships. In: Campos - Herrera R (ed), Nematode Pathogenesis of Insects and Other Pests. Springer, pp 3-27

Stock SP, Kaya HK (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. The Journal of Parasitology 82:806-813

Stock SP, Rivera-Orduño B, Flores-Lara Y (2009) *Heterorhabditis sonorensis* n. sp. (Nematoda: Heterorhabditidae), a natural pathogen of the seasonal cicada *Diceroprocta ornea* (Walker) (Homoptera: Cicadidae) in the Sonoran desert. Journal of Invertebrate Pathology 100:175-184

Tailliez P, Laroui C, Ginibre N, Paule A, Pagès S, Boemare N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., *P. luminescens* subsp. *hainanensis* subsp. nov., *P. temperata* subsp. *khanii* subsp. nov., *P. temperata* subsp. *tasmaniensis* subsp. nov., and the reclassification of *P. luminescens* subsp. *thracensis* as *P. temperata* subsp. *thracensis* comb. nov. International Journal of Systematic and Evolutionary Microbiology 60:1921-1937

Tailliez P, Pagès S, Edgington S, Tymo LM, Buddie AG (2012) Description of *Xenorhabdus magdalenensis* sp. nov., the symbiotic bacterium associated with *Steinernema australe*. International Journal of Systematic and Evolutionary Microbiology 62:1761-1765

Thomas GM, Poinar GO (1979) *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. International Journal of Systematic Bacteriology 29:352-360

Toledo J, Rasgado MA, Ibarra JE, Gómez A, Liedo P, Williams T (2006a) Infection of *Anastrepha ludens* following soil applications of *Heterorhabditis bacteriophora* in a mango orchard. Entomologia Experimentalis et Applicata 119:155-162

Van de Peer Y, Chapelle S, De Wachter R (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Research 24:3381-3391

Van Mele P (2008) A historical review of research on the weaver ant *Oecophylla* in biological control. Agricultural and Forest Entomology 10:13-22

Van Mele P, Vayssières J-F, Van Tellingen E, Vrolijks J (2007) Effects of an African weaver ant, *Oecophylla longinoda*, in controlling mango fruit flies (Diptera: Tephritidae) in Benin. Journal of Economic Entomology 100:695-701

Vayssières J-F, De Meyer M, Ouagoussounon I, Sinzogan A, Adandonon A, Korie S, Wargui R, Anato F, Houngbo H, Didier C (2015a) Seasonal abundance of mango fruit flies (Diptera: Tephritidae) and ecological implications for their management in mango and cashew orchards in Benin (Centre & North). Journal of Economic Entomology 108:2213-2230

Vayssieres J-F, Korie S, Coulibaly O, Temple L, Boueyi SP (2008) The mango tree in Central and Northern Benin: cultivar inventory, yield assessment, infested stages and loss due to fruit flies (Diptera Tephritidae). Fruits 63:335-348

Vayssières J-F, Ouagoussounon I, Adandonon A, Sinzogan A, Korie S, Todjihoundé R, Alassane S, Wargui R, Anato F, Goergen G (2015b) Seasonal pattern in food gathering of the weaver ant *Oecophylla longinoda* (Hymenoptera: Formicidae) in mango orchards in Benin. Biocontrol Science and Technology 25:1359-1387

Westerman P (1998) Penetration of the Entomopathogenic Nematode *Heterorhabditis* spp. into Host Insects at 9 and 20° C. Journal of Invertebrate Pathology 72:197-205

White G (1927) A method for obtaining infective nematode larvae from cultures. Science 66:302-303

Yee WL, Lacey LA (2003) Stage-specific mortality of *Rhagoletis indifferens* (Diptera: Tephritidae) exposed to three species of *Steinernema* nematodes. Biological Control 27:349-356

Zadji L (2014) Entomopathogenic nematodes as potential control agent of termite in citrus in Benin. Ghent University

Zadji L, Baimey H, Afouda L, Houssou FG, Waeyenberge L, de Sutter N, Moens M, Decraemer W (2013) First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Southern Benin. Russ J Nematol 21:117-130

Zadji L, Baimey H, Afouda L, Moens M, Decraemer W (2014) Comparative susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Isoptera: Termitidae) to entomopathogenic nematodes from Benin. Nematology 16:719-727

8 Curriculum vitae

Personal information

Name: Anique Tognisse Godjo

Date and Place of Birth: 19th March 1984, Abomey (Benin Republic)

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Work experience

2013-2018: PhD, Biology
Laboratory of Microbiology and Laboratory of Nematology, Faculty of Sciences, Ghent University, Belgium.
Research Assistant, Faculty of Agronomy, University of Parakou, Benin Republic.

2009-2011: Local coordinator of the cotton production management project (AIC-PARFCB), Savalou, Benin.

2008-2009: Crop production advisor at the international Non-Governmental Organization BorneFonden, Benin.

Education

2013-2018: **Doctor of Philosophy in Biology**, Laboratory of Microbiology and Laboratory of Nematology, Faculty of Sciences, Ghent University, Belgium.
Dissertation: Entomopathogenic nematodes and their symbiotic bacteria to control fruit flies (*Bactrocera dorsalis*) in mango cultivation in Benin
Promotors: Prof. Dr. Anne Willems, Prof. Dr. Wilfrida Decraemer, Prof. Dr. Leonard Afouda.

2011-2013: **Master of Science in Nematology**, Faculty of Sciences, Ghent University, Belgium.
Dissertation: Isolation, identification and characterization of symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*) from entomopathogenic nematodes (*Heterorhabditis* and *Steinernema*) from Benin.

Promotors: Prof. Dr. Anne Willems, Prof. Dr. Wilfrida Decraemer, Prof. Dr. Leonard Afouda.

2002-2008: Degree of Agricultural Engineer, Faculty of Agronomy, University of Parakou, Benin Republic.

Dissertation: Importance of seeds in the transmission of rice blast and brown spots to rice seedling in Gogounou, Benin.

Promotor: Prof. Dr. Leonard Afouda.

Skills

Scientific skills

- Nematode sampling, bacterial symbionts isolation and cultivation.
- Molecular techniques (eg. DNA extraction, Primer design, PCR, amplicon purification and sequencing and phylogenies reconstruction) related to the identification and description of bacteria and nematodes as well as their morphology or morphometric and phenotypic characterization.
- Entomopathogenic nematode cultivation, fixation and slides making
- Tephritid fruit fly (eg. *Bactrocera dorsalis*, *Ceratitis capitata*, *Ceratitis cosyra*) and *Galleria mellonella* rearing in laboratory conditions
- Laboratory and field experimental design
- Supervision of students for Master 1 project in Biochemistry and Biotechnology at LM-UGent and Master Thesis projects in Techniques and Crop Production at Parakou University.

Software

- MS Words, MS PowerPoint, MS Excel
- MEGA 6, SAS, SPSS, Bionumerics

Other abilities

- Perseverance and strong love for learning new things; motivated for team-work
- Driving Licenses B, A1 and A2

Poster and Oral presentations

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2017) Molecular diversity of *Photorhabdus sp.* and *Xenorhabdus sp.* bacteria symbiotically associated with entomopathogenic nematodes retrieved from soil in Benin. **Poster presentation** at the Belgium Society for Microbiology Meeting (BSM) "Current Highlights in Microbiology", October 20th, *2017,* Academy Palace, Brussels, Belgium.

Godjo A, Decraemer W, Willems A, Afouda L (2017) Infectivity and reproduction potential of *Heterorhabditis sonorensis* and *Steinernema* sp. isolates from Benin in late instar larvae of *Bactrocera dorsalis.* **Oral presentation** at the 69th International Symposium on Crop Protection. 23 May 2017. Ghent, Belgium.

Godjo A, Decraemer W, Willems A, Afouda L (2016) Infectivity and reproduction potential of *Heterorhabditis sonorensis* and *Steinernema* sp. isolates from Benin in late instar larvae of *Bactrocera dorsalis.* **Poster presented** at the 10th Symposium of the Ghent Africa Platform (GAPSYM10), December 8-9th 2016, Ghent, Belgium.

Godjo A, Afouda L, Baimey H, Zadji L, Decraemer W, Willems A (2016) Evaluation of Entomopathogenic nematodes to control mango fruit fly (*Bactrocera dorsalis*) in Benin. **Oral presentation** at the XVIIe édition des Journées Scientifiques Internationales de Lomé (JSIL 2016), 03-08 Octobre 2016, Lomé, Togo.

Godjo A, Decraemer W, Willems A, Afouda L (2015). Entomopathogenic nematodes isolated from Benin and their bacterial symbionts, **oral presentation** at the lab seminar, 12th November 2015, Laboratory of Microbiology (LM-UGent).

Scientific publications

A1 publications

Godjo A, Zadji L, Decraemer W, Willems A, Afouda L (2018) Pathogenicity of indigenous entomopathogenic nematodes from Benin against mango fruit fly (*Bactrocera dorsalis*) under laboratory conditions. Biological Control 117:68-77. doi: 10.1016/j.biocontrol.2017.10.009

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2018) Molecular diversity of *Photorhabdus* and *Xenorhabdus* bacteria, symbionts of *Heterorhabditis* and *Steinernema* nematodes retrieved from soil in Benin. Archives of Microbiology, in press. doi: 10.1007/s00203-017-1470-2

Godjo A, Afouda L, Baimey H, Couvreur M, Zadji L, Houssou G, Bert W, Willems A, Decraemer W (2018) *Steinernema* n. sp., a new *Steinernema* species (Rhabditida: Steinernematidae) from Northern Benin. Nematology, under review.

Godjo A, Chabi N, Dossou P, Zadji L, Batcho O**,** Baimey H, Decraemer W, Willems A, Afouda L (2018) Evaluation of the ability of indigenous isolates of *Heterorhabditis taysearae* and *Steinernema* sp. to control mango fruit fly *Bactrocera dorsalis* under laboratory, semi-field and field conditions in Northern Benin. In preparation

Godjo A, Afouda L, Baimey H, Decraemer W, Willems A (2018). Description of *Photorhabdus luminescens* subsp. *beninensis* subsp. nov., a novel symbiotic bacterium associated with *Heterohabditis taysarae* nematodes isolated from Benin. In preparation

Other publications

Godjo A, Decraemer W, Willems A, Afouda L (2017) Impact of temperature on the infectivity of *Heterorhabditis taysarae* and *Steinernema* sp. isolates from Benin to late instar larvae of *Bactrocera dorsalis.* Proceedings 69th International Symposium on Crop Protection (ISCP), Communications in Agricultural and Applied Biological Sciences, 82:245-247.

Zadji L, Baimey H, Afouda L, Houssou G, **Godjo A**, Waeyenberge L, De Sutter N, Moens M, Decraemer W (2013). Entomopathogenic nematodes as an alternative bio-control tool to pesticides for the management of *Macrotermes bellicosus*, a termite pest on citrus in Benin. In: Actes du 4ème Colloque des sciences, cultures et technologies de l'UAC-Benin.

Afouda L, **Godjo A,** Sere Y, Nouatin G, Akossou A (2010) Transmission de *Pyricularia grisea* (herbert) barr par les semences aux plantules de riz au Benin. Annales des Sciences Agronomiques (12) 2 spécial: 11-22, 2009 ISSN 1659-5009