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ORIGINAL PAPER

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

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Abstract

The diversity of 43 bacterial strains isolated from Beninese entomopathogenic nematodes was investigated molecularly by analyzing the 16S rRNA, recA, and gyrB genes. Based on 16S rRNA sequence analysis, 15 bacterial strains were identified as *Xenorhabdus* sp., 27 strains as *Photorhabdus* sp., and one as *Serratia* sp. The *Xenorhabdus* strains were isolated from *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* strains, all isolated from *Heterorhabditis* nematodes, resulted in two separate sub-clusters (A) and (B) within the *Photorhabdus luminescens* group, distinct from the 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* $AM7^T$.

Keywords GyrB · RecA · Entomopathogenic nematodes · Symbiotic bacteria · *Photorhabdus luminescens* · *Xenorhabdus indica*

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, 2–3 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 the EPNs' 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

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(Akhurst 1982; Boemare 2002; 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 29 strains identified as *Heterorhabditis sonorensis* and three as *Heterorhabditis indica* (Zadji et al. 2013). Several EPNs' isolates 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, 2014). 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 this study of new isolates from EPN from Benin, gyrB and recA gene sequences were, therefore, used in addition to 16S rRNA genes.

Materials and methods

Isolation and identification of entomopathogenic nematodes and their phylogenetic position

EPNs considered in this study were isolated from soil samples collected in the North and Center of Benin (Table 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 100IJs/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).

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

Mature Gm larvae were infected with IJs of pure culture of each nematode isolate at the rate of 100 IJs per Gm larva. After 48 h, 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.

Morphological examination of bacterial colonies

Visual characteristics such as colony diameter, shape, and color after 48 h of incubation at 28 °C on NBTA plates were recorded. In addition, the capacity for bioluminescence of

Table 1 Morphological features of isolated *Photorhabdus* and *Xenorhabdus* strains

Bacterial strain	Identification based on partial 16S rRNA	Bioluminescence feature	Colony shape	Colony size (mm)	Colony color on NBTA	Associated EPNs isolate	EPNs identification based on ITS region	EPNs isolation source (vegetation)	Origin of EPNs in Benin (GPS coordinates)
R-66822	*Photorhabdus* sp.	+	Irregular	< 1	Dark-green	F4	*Heterorhabditis* sp.	Mango	09°22.287′N 02°40.233′E
R-66823	*Photorhabdus* sp.	+	Irregular	2	Green-reddish	F4	*Heterorhabditis* sp.	Mango	09°22.287′N 02°40.233′E
R-66825	*Photorhabdus* sp.	w	Irregular	2	Green-reddish	C2	*Heterorhabditis* sp.	Mango	09°22.356′N 02°41.175′E
R-52429	*Photorhabdus* sp.	+	Irregular	2	Dark-green	138a	*Heterorhabditis* sp.	Cashew	08°59.467′N 02°35.347′E
R-52373	*Photorhabdus* sp.	+	Irregular	2	Green-reddish	138a	*Heterorhabditis* sp.	Cashew	08°59.467′N 02°35.347′E
R-52403	*Photorhabdus* sp.	+	Round	3	Green-reddish	138a	*Heterorhabditis* sp.	Cashew	08°59.467′N 02°35.347′E
R-52416	*Photorhabdus* sp.	+	Round	3	Green	138a	*Heterorhabditis* sp.	Cashew	08°59.467′N 02°35.347′E
R-52415	*Photorhabdus* sp.	+	Irregular	1	Green-reddish	139a	*Heterorhabditis* sp.	Grassland	09°04.251′N 02°33.538′E
R-52402	*Photorhabdus* sp.	+	Irregular	2	Red	139a	*Heterorhabditis* sp.	Grassland	09°04.251′N 02°33.538′E
R-52380	*Photorhabdus* sp.	NT	Irregular	1	Red	139a	*Heterorhabditis* sp.	Grassland	09°04.251′N 02°33.538′E
R-52425	*Photorhabdus* sp.	+	Irregular	3	Dark-green	152b	*Heterorhabditis* sp.	Shea	09°48.441′N 02°39.275′E
R-52362	*Photorhabdus* sp.	NT	Irregular	1	red	98c	*Heterorhabditis* sp.	Cashew	08°07.263′N 02°14.534′E
R-52404	*Photorhabdus* sp.	+	Irregular	1	Transparent	98c	*Heterorhabditis* sp.	Cashew	08°07.263′N 02°14.534′E
R-52412	*Photorhabdus* sp.	+	Irregular	1	Transparent	98c	*Heterorhabditis* sp.	Cashew	08°07.263′N 02°14.534′E
R-52410	*Photorhabdus* sp.	+	Irregular	1,5	Reddish	98c	*Heterorhabditis* sp.	Cashew	08°07.263′N 02°14.534′E
R-52390	*Photorhabdus* sp.	NT	Irregular	3	Blue	130d	*Heterorhabditis* sp.	Cashew	08°10.223′N 02°37.173′E
R-52411	*Photorhabdus* sp.	NT	Irregular	2	Red	130d	*Heterorhabditis* sp.	Cashew	08°10.223′N 02°37.173′E
R-52383	*Photorhabdus* sp.	NT	Irregular	2	Red	130d	*Heterorhabditis* sp.	Cashew	08°10.223′N 02°37.173′E
R-52368	*Photorhabdus* sp.	+	Irregular	3	Green-reddish	111b	*Heterorhabditis* sp.	kpai (Tchabe)	08°35.847′N 01°41.202′E
R-52361	*Photorhabdus* sp.	NT	Round	1	Red	111b	*Heterorhabditis* sp.	kpai (Tchabe)	08°35.847′N 01°41.202′E
R-52427	*Photorhabdus* sp.	+	Irregular	2	Red	111b	*Heterorhabditis* sp.	kpai (Tchabe)	08°35.847′N 01°41.202′E
R-52389	*Photorhabdus* sp.	NT	Irregular	1, 5	Red	111b	*Heterorhabditis* sp.	kpai (Tchabe)	08°35.847′N 01°41.202′E
R-66820	*Photorhabdus* sp.	w	Irregular	2	Green	118c	*Heterorhabditis* sp.	Cashew	08°07.520′N 01°57.912′E
R-52391	*Photorhabdus* sp.	NT	Irregular	2	Dark-green	125a	*Heterorhabditis* sp.	Grassland	07°59.256′N 02°16.428′E
R-52434	*Photorhabdus* sp.	+	Round	2	Green	150d	*Heterorhabditis* sp.	Forest	09°49.556′N 02°42.837′E
R-52366	*Photorhabdus* sp.	+	Irregular	3	Green	150d	*Heterorhabditis* sp.	Forest	09°49.556′N 02°42.837′E

Table 1 (continued)

Bacterial strain	Identification based on partial 16S rRNA	Bioluminescence feature	Colony shape	Colony size (mm)	Colony color on NBTA	Associated EPNs isolate	EPNs identification based on ITS region	EPNs isolation source (vegetation)	Origin of EPNs in Benin (GPS coordinates)
R-52363	*Photorhabdus* sp.	NT	Irregular	2	Dark-green	114c	*Heterorhabditis* sp.	Cashew	08°25.904′N 01°51.900′E
R-52428	*Xenorhabdus* sp.	–	Irregular	3	Green	168d	*Steinernema* sp.	Eucalyptus	11°21.993′N 03°03.959′E
R-52437	*Xenorhabdus* sp.	–	Irregular	3	Red	168d	*Steinernema* sp.	Eucalyptus	11°21.993′N 03°03.959′E
R-52382	*Xenorhabdus* sp.	–	Irregular	2	Blue	168d	*Steinernema* sp.	Eucalyptus	11°21.993′N 03°03.959′E
R-52377	*Xenorhabdus* sp.	–	Irregular	3	Blue	168d	*Steinernema* sp.	Eucalyptus	11°21.993′N 03°03.959′E
R-52417	*Xenorhabdus* sp.	–	Irregular	2	Red	168d	*Steinernema* sp.	Eucalyptus	11°21.993′N 03°03.959′E
R-52367	*Xenorhabdus* sp.	–	Irregular	3	Green	157c	*Steinernema* sp.	Forest	10°11.472′N 02°39.266′E
R-52379	*Xenorhabdus* sp.	–	Round	3	Green	157c	*Steinernema* sp.	Forest	10°11.472′N 02°39.266′E
R-52435	*Xenorhabdus* sp.	–	Irregular	1	Green	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52364	*Xenorhabdus* sp.	–	Irregular	3	Green	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52365	*Xenorhabdus* sp.	–	Round	2	Red	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52430	*Xenorhabdus* sp.	–	Irregular	2	Green	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52405	*Xenorhabdus* sp.	–	Round	1	Green	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52426	*Xenorhabdus* sp.	–	Irregular	3	Red	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52406	*Xenorhabdus* sp.	–	Round	2	Green	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52372	*Xenorhabdus* sp.	–	Round	3	Green-reddish	111c	*Steinernema* sp.	Baobab	08°35.847′N 01°41.202′E
R-52436	*Serratia*. sp.	–	Irregular	2	Transparent	138d	*Steinernema* sp.	Cashew	08°59.467′N 02°35.347′E

Strains selected for full 16S rRNA, recA, and gyrB genes analyses (Genebank accession numbers provided in Supplementary Table 1) are shown in bold

+ means the bacterial strain produced light in the darkness/ − means the bacterial strain did not produce light in the darkness/w means the bacterial strain produced weak light in the darkness/NT means bioluminescence was not tested

some isolates was visually assessed in darkness (Kazimierczak et al. 2017).

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 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 isolated bacterial strains 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 strain from each nematode isolate were represented. Full sequencing of the 16S rRNA gene was performed on the selected strains using six sequencing primers described previously (Coenye et al. 1999). In addition, amplification and partial sequencing of two housekeeping genes, the recA and gyrB, were 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 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 neighbor-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 realigned 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.

Results

Phylogenetic assignment of nematodes

The phylogenetic analysis of the ITS fragments showed that collected nematode isolates (Table 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 Fig. 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.

Morphological examination of bacterial colonies

In total, we obtained 43 bacterial strains from the 16 nematode isolates considered in this study (Table 1). These bacterial strains were assigned an R number (accession number) and were stored in the research collection of the Laboratory of Microbiology at Ghent University (LM-UGent) at − 80 °C. All bacterial strains were able to grow after 48 h with maximum colony diameter of 3 mm. Color of the colonies varied. They appeared on NBTA plate as either green or blue and sometimes reddish. Most of the bacterial strains were able to absorb dye on NBTA plates and bioluminescence was observed for some *Photorhabdus* strains and not for *Xenorhabdus* strains after 48 h of incubation (Table 1).

Molecular characterization of symbiotic bacteria

16S rRNA gene

Comparison of partial 16S rRNA gene sequences (414 bp) of the 43 isolated bacterial strains with the type strains of references species showed that 27 and 15 strains were, respectively, distributed within *Photorhabdus* and *Xenorhabdus* groups. One bacterial strain, isolated from *Steinernema* sp., formed a distinct cluster (D) together with *Serratia nematodiphila* (Supplementary Fig. 2). All bacterial strains isolated from *Heterorhabditis* nematodes, grouped with *Photorhabdus* reference strains and bacteria isolated from *Steinernema* nematodes clustered within *Xenorhabdus* species. The 27 *Photorhabdus* strains were all distributed within the *Photorhabdus luminescens* group with 24 of them forming a separate sub-cluster (A) (Supplementary Fig. 2). The 3 other strains grouped in a sub-cluster (B) together with *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *noenieputensis*. All *Xenorhabdus* strains 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 strains from *Xenorhabdus* and *Photorhabdus* groups were selected for further molecular analysis.

Analysis of full 16S rRNA gene sequences (1427 bp) revealed that the *Xenorhabdus* strains were highly similar (100% sequence similarity) and confirmed their highest sequences similarity (99.7%) with *X. indica* DSM 17382^T (Fig. 1). In addition, as noted with the partial 16S rRNA gene analysis above, *Photorhabdus* strains formed two separate sub-clusters (A) and (B) (Fig. 2) based on the analysis of full sequences of 16S rRNA gene. Strains of sub-cluster (A) showed 7–14 nucleotide differences of the 16S rDNA (1349 positions compared) sequences with strains 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) (Fig. 2). *P. luminescens* subsp. *luminescens* Hb shared 98.7–99.3 and 98.7–98.8% sequence similarities with strains 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 strains in sub-cluster (A) and (B),

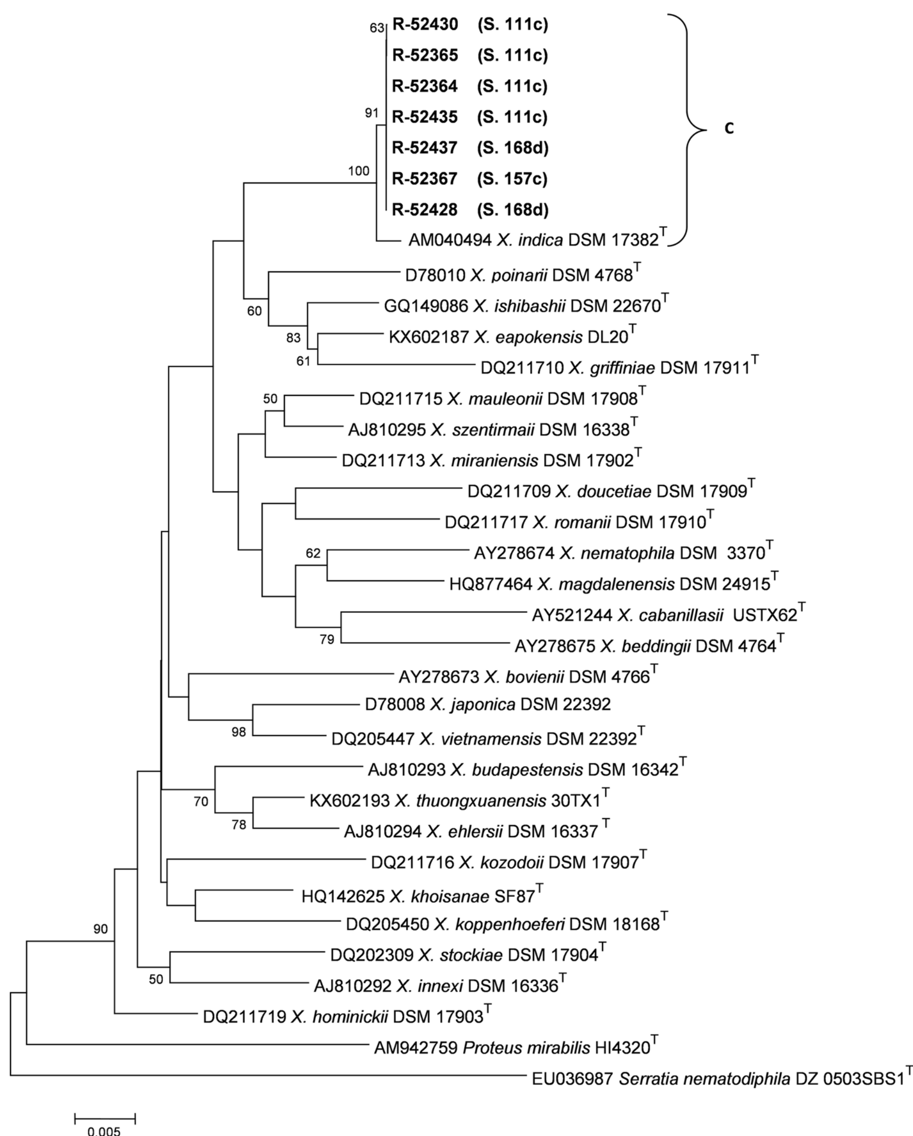

Fig. 1 Neighbor-joining tree based on 1,427 kb 16S rRNA sequences for 7 *Xenorhabdus* strains (indicated in bold), from entomopathogenic nematodes (EPN strains in brackets next to the bacterial strain) recovered from Beninese soils and reference strains. Bootstrap values above 50% based on 1000 replicates 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*, *Steinernema* strain

respectively. The *Serratia* strain R-52436 shared 99.7% 16S rRNA nucleotide identity with *S. nematodiphila* with high bootstrap value (100%).

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 Figs. 3 and 4. Sequences of individual genes were also used to build neighbor-joining phylogenetic trees which are presented in Supplementary Fig. 3 to 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 (Fig. 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 sub-cluster (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)

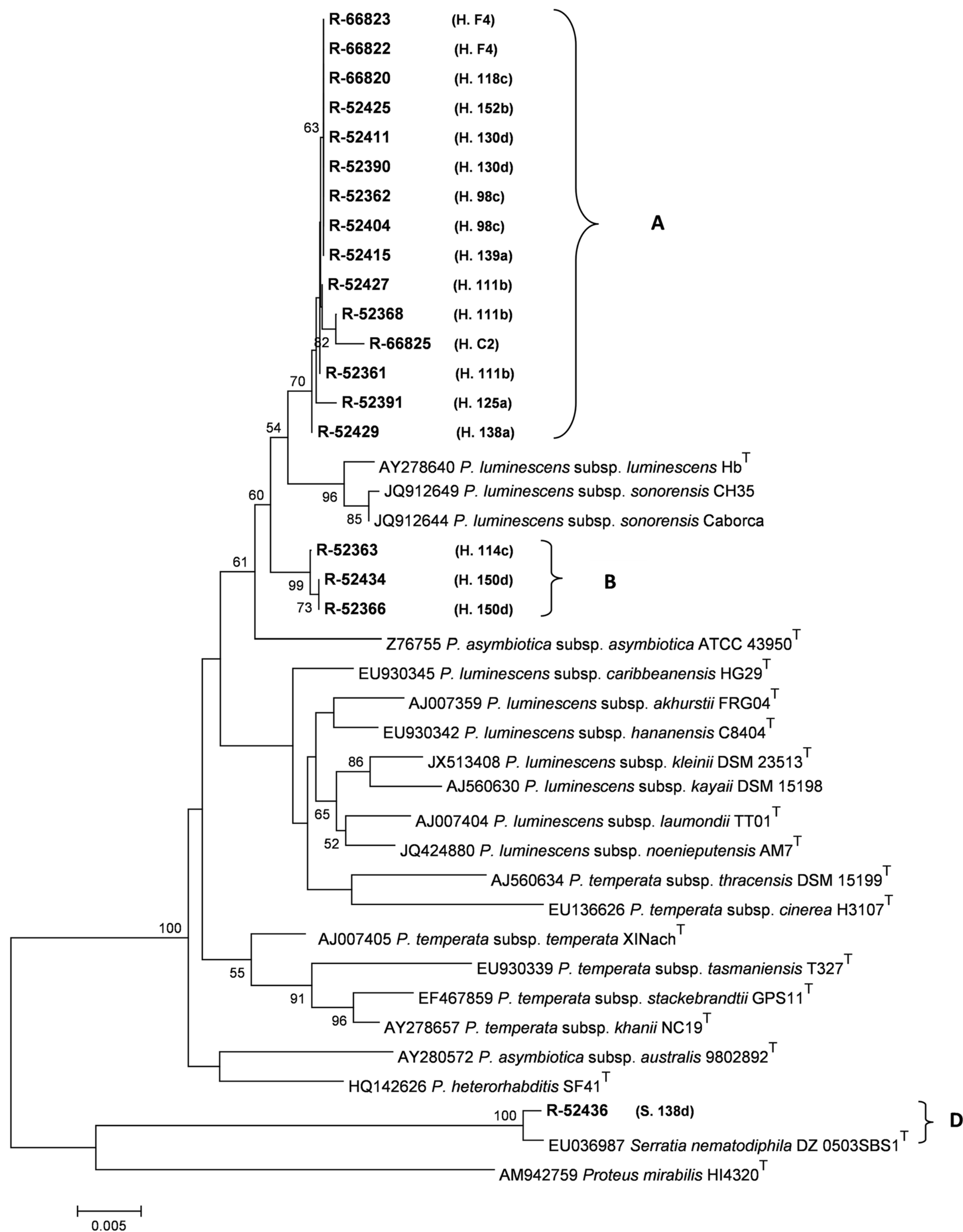

Fig. 2 Neighbor-joining tree based on 1,349 kb 16S rRNA sequences for 18 *Photorhabdus* and 1 *Serratia* strain (indicated in bold), from entomopathogenic nematodes (EPN strain in brackets next to the bacterial strain) recovered in Beninese soils and reference strains. Bootstrap values above 50% based 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, Heterorhabditis* strain, *S, Steinernema* strain

and (B) and showed the closest similarity (95.9%) to *P. luminescens* subsp. *noenieputensis* (Supplementary Fig. 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 Fig. 4).

For the *Xenorhabdus* strains, comparison of concatenated sequences of recA and gyrB (Fig. 4) with the reference type

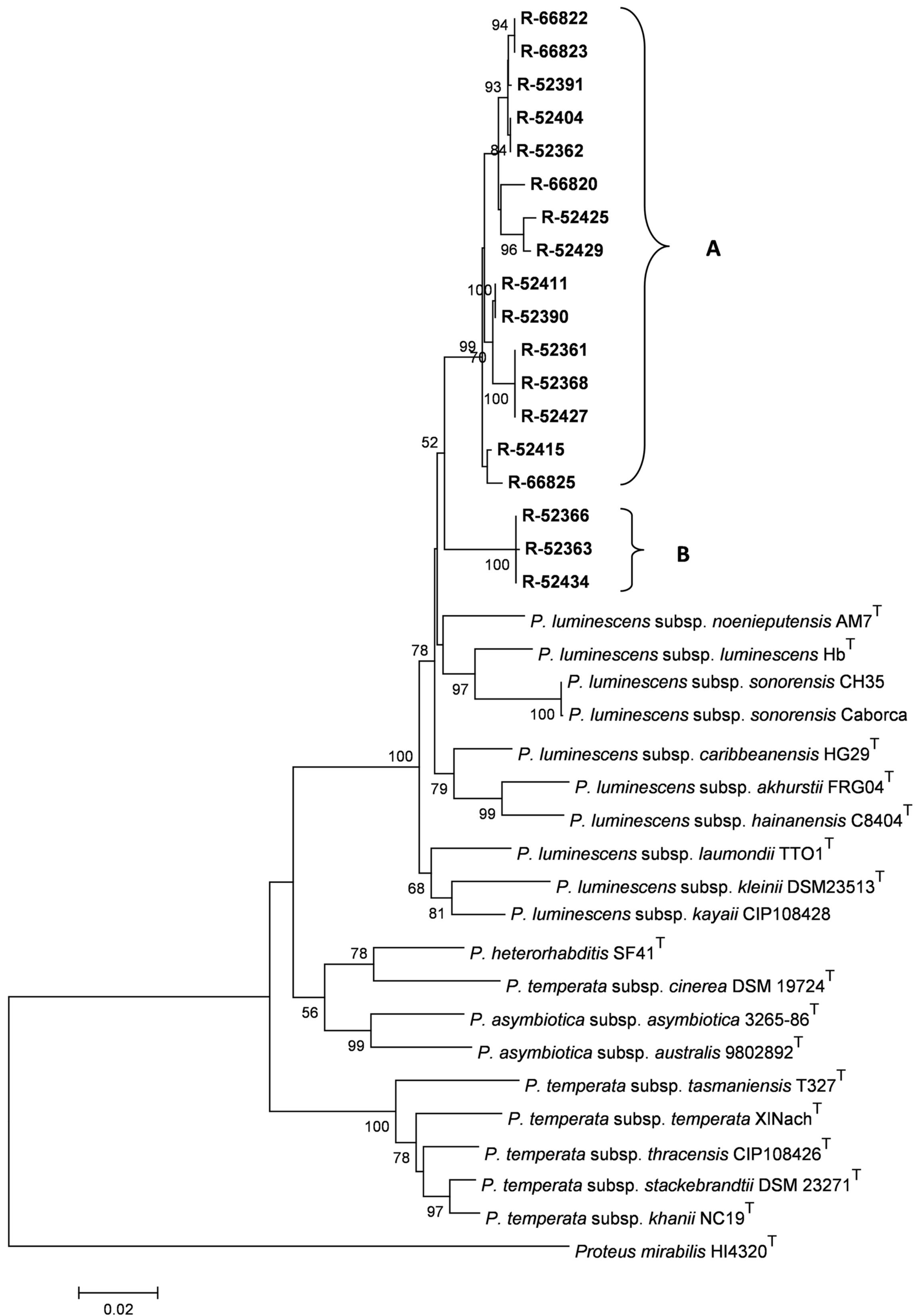

Fig. 3 Neighbor-joining tree based on concatenated recA and gyrB gene sequences showing the phylogenetic position of Beninese *Photorhabdus* strains (in bold) among type strains of described *Photorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* was used as outgroup

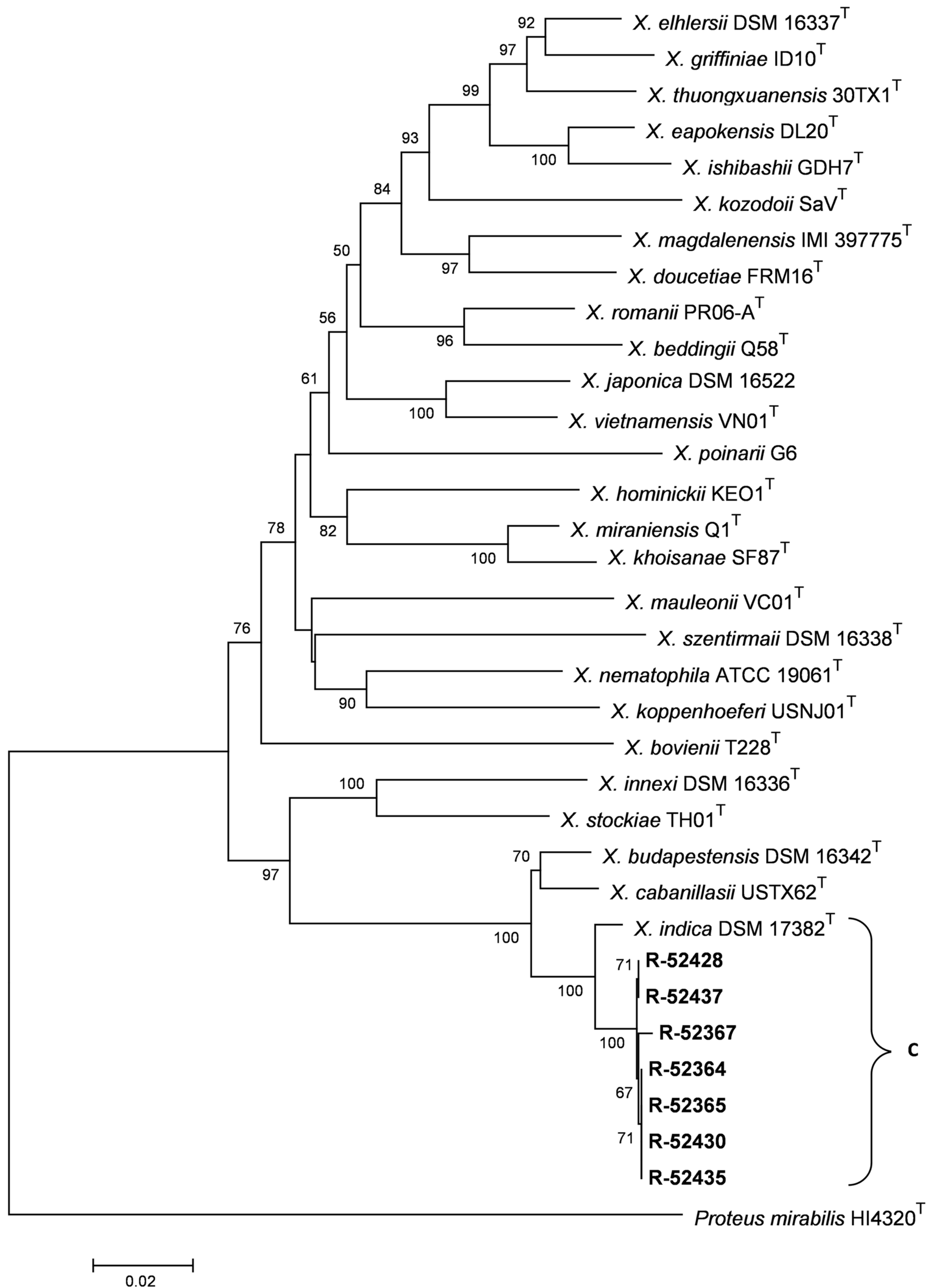

Fig. 4 Neighbor-joining tree based on concatenated recA and gyrB gene sequences showing the phylogenetic position of Beninese *Xenorhabdus* strains (in bold) among type strains of described *Xenorhabdus* species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. *Proteus mirabilis* was used as outgroup

strains in GenBank revealed that they shared 98.5% nucleotide identity with *X. indica*. Furthermore, analysis of gyrB (Supplementary Fig. 5) and recA (Supplementary Fig. 6) sequences confirmed their closest similarity with *X. indica* (98.3–98.4 and 98.8–98.9%, respectively).

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 Beninese *Steinernema* and *Heterorhabditis* nematodes, turned blue, dark-green, or red on NBTA plates after 48 h of incubation. This indicates that 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 Beninese EPNs indicated that most bacterial symbionts isolated from *Steinernema* nematodes were identified as *Xenorhabdus* species, and *Photorhabdus* species were isolated from *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 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 EPNs group. The present study is the first report of a *Serratia* strain isolated from *G. mellonella* 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 the previous studies, *Steinernema* have occasionally been found to be associated with bacteria other 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). In addition, for EPN 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* strains 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 for the first time by Somvanshi et al. (2006) and found to be associated with *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 nematodes clustering with *S. abbasi* based on ITS regions sequences analysis (Supplementary Fig. 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 with a single nematode species, although *S. yirgalemense* forms a sister clade to *S. abbasi*/*S. thermophilum* based on the ITS phylogeny (Supplementary Fig. 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* strains 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 (Figs. 2, 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.

Strains contained in sub-cluster (A) were isolated from nematodes identified, based on ITS region analysis

(Supplementary Fig. 1), as *H. sonorensis*, a junior synonym of *H. taysearae* (Hunt and Subbotin 2016). Bacterial strains in sub-cluster (B) were isolated from nematode isolates grouping with *H. indica* (Supplementary Fig. 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 sub-clades (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 EPN 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. 2016; 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, 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* strains 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) strains 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.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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