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Natural association of the entomopathogenic nematode *Heterorhabditis indica* (Rhabditida: Heterorhabditidae) from the Philippines with the non-symbiotic bacterium *Ochrobactrum anthropi* (Proteobacteria: Brucellaceae)

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Abstract

Background: Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are widely used as biocontrol agents against diverse insect pests. Their association with symbiont bacteria is found to be the primary cause of insect mortality. The present study reported a comprehensive characterization of a local Philippine EPN isolate using congruent morpho-taxometrical and molecular data and the associated bacterium in the infective dauer juveniles.

Results: Using an insect-baiting technique, a nematode isolate labeled F2H was recovered from the sandy soils in the coastal area of southern Philippines. Based on morphology and morpho-taxometrics, the F2H isolate collected from Iligan City was identified as *Heterorhabditis indica*. Analysis of the ITS region of rDNA revealed the highest sequence match to *H. indica*. The sequences of the bacterial 16S rDNA gene showed that the symbiont of *H. indica* presented 100% similitude with the sequences of *Ochrobactrum anthropi*. Further, pairwise alignment and phylogenetic analysis demonstrated that *O. anthropi*, *O. lupini* and *O. cytisi* shared 100% similarity and form a monophyletic clade. These strains have not presented any differences in the phylogenetic and 16S rDNA data, and *O. lupini* and *O. cytisi* should be considered a later heterotypic synonym of *O. anthropi*.

Conclusions: The description of *H. indica*-*O. anthropi* association was the first report in the Philippines. This provides additional account and collection of naturally isolated EPN-bacteria, contributing to limited knowledge on its diversity in the Philippines, which can be utilized in the biocontrol of pests after further comprehensive assessments.

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Background

The increasing need to reduce chemical pesticide use has driven the interest in utilizing entomopathogenic nematodes (EPNs) as biological control agents (BCA) against economic agricultural pests (Georgis et al. 2006). Unlike chemicals, EPNs are target-specific with a wide range, ecofriendly, and most notably, user safe (Ehlers 2003). Their effectiveness and success as BCAs depend on their adaptability to the environment. Local species or strains are of particular interest due to better adaptation to abiotic factors, local climate and other regulators (Campos-Herrera et al. 2012). The most successful EPN strains belong to the family of Heterorhabditidae. The genus *Heterorhabditis* (Nematoda: Rhabditida) has a worldwide natural occurrence with currently 19 valid species throughout the globe (Machado et al. 2021).

The two *Heterorhabditis* species, *H. bacteriophora* and *H. indica*, are suggested to have a global distribution, especially in the tropics and subtropics (Bhat et al. 2020). *Heterorhabditis indica* was first isolated from southern India as a type strain (Poinar et al. 1992) and later on in many other parts of the world with the most recent record in Fiji Islands (Kour et al. 2020) and in eastern Australia (Aryal et al. 2022). In the Philippines, new populations of *H. indica* from banana, rice (Navarez et al. 2021), peanut (Dichusa et al. 2021) and sugarcane fields (Ramos et al. 2022) were recently documented from Davao del Sur and Cotabato provinces in Mindanao island.

Morphological discrimination between EPN species starts with differences in cadaver coloration to morphometrics. However, *Heterorhabditis* have been shown to exhibit slight morphological differences between species (Dolinski et al. 2008), making species identification extremely difficult without the aid of a molecular technique (Darissa and Iraki 2014). Molecular characterization was done by the analysis of internal transcribed spacer (ITS) rDNA sequences, among many others (Nasmith et al. 1996). This gene is considered as informative marker for species identification, barcoding and phylogeographical studies of *Heterorhabditis* species (Dhakal et al. 2020). Even so, false assignment of sequences to a taxon can occur, creating risks in the genetic database. Thus, for an increased resolution and reliability, combining morphological, morpho-taxometrical, molecular and phylogenetic studies are suggested (Spiridonov 2017).

The EPNs form a mutualistic complex with specific symbiotic bacteria, of which the latter is the primary cause of insect mortality (Ciche 2007). They depend on the EPNs for penetration into insect hosts through natural openings while bacterial cells are carried in the infective juveniles' (IJs) gut. Once inside the host, they are released into the hemolymph, producing virulence factors like the toxin complex a (Tca) among many others produced by *Photorhabdus* (Proteobacteria: Morganellaceae) symbionts, killing the insect by septicemia within 48 h. (Waterfield et al. 2009). The bacteria then degrade host tissues, supplying nutrients for the growth and development of the nematode population (Hazir et al. 2003). It is known that symbiotic bacteria also produce compounds to antagonize competitors like other bacterial species, fungi, and scavengers that may disturb the nematode development (Waterfield et al. 2009). However, several non-symbiotic bacteria have also been isolated and reported in association with EPN species, for instance, *Ochrobactrum anthropi, O. intermedium,* and *Pseudomonas protegens* (Ogier et al. 2020). It was reported recently that the presence of non-symbiotic bacteria is likely to reduce the recycling ability of *H. indica* in other insect larvae (Upadhyay and Mohan 2021). Despite this, their functions, specific requirements, and relationships are by far still unclear.

Vast natural genetic diversity in EPN populations across the country is yet to be explored and exploited. The collection of local EPNs may bring higher infectivity to target insect pests as a result of better adaptation. In this study, characterizing a local EPN strain collected from Iligan City, as part of the leading agricultural producer in the second largest island in the Philippines, Northern Mindanao was aimed. By employing complementary morphological, morpho-taxometrical, and molecular approaches, a more comprehensive characterization of our local *H. indica* isolate was conducted, providing an additional account to existing knowledge of EPNs in the country. Furthermore, while symbionts have biocontrol potential, some non-symbiotic bacteria such as *Ochrobactrum* (Proteobacteria: Brucellaceae) were reported to be opportunistic and pose high risks to humans' health (Kettaneh et al. 2003). Hence prior to virulence assays and mass production, the non-symbiotic bacteria associated with our EPN species was also identified using the 16S rDNA region (Kämpfer et al. 2003). The outcomes of this study will be pivotal for the future establishment of biocontrol programs in the country.

Methods

Nematode and bacterial isolates

Heterorhabditis indica F2H was isolated from a sandy soil sample collected from a coastal area in Iligan City, Mindanao, Philippines at 15 cm depth (N 8°12′ 5.10″) in January 2019. Soil samples were moistened and placed in plastic containers, with the subsequent addition of five super worm larvae, *Zophobas morio* (Coleoptera: Tenebrionidae) in each container (Bedding and Akhurst 1975). Containers with the soil and insect baits were incubated in the dark at 25 ± 2 °C. Regular checking of the samples to obtain infected cadavers was conducted every 2–3 days until required. A modified White trap method was used to isolate IJs from cadavers. Dextrose saline solutions (NSS, 0.9% NaCl) were poured into the Petri plate (5 cm diam.) and sealed with parafilm. The pure culture was obtained by re-inoculating EPNs thrice in sand assays and isolating fresh nematodes a new with modified white traps after every infection (Hoy et al. 2008). This monoculture was used for characterization. For the bacterial isolate, *Ochrobactrum anthropi* F2B was extracted directly from a total of five clean monocultured IJs of *H. indica* F2H, following the description of Aryal et al. (2022). Briefly, IJs were surface sterilized by soaking in 2% NaOCl for 5 min to avoid secondary contaminations.

Morphological characterization of the nematode

For morpho-taxometrical characterization, IJs were collected directly from the White trap, while adults were obtained through the dissection of cadavers 3–6 days after reinfection. For mounting and fixation of IJs and adults, a modified method originally described by Seinhorst (1959) was followed. A stepwise transfer of heat-killed IJs, hermaphrodite, male and female nematodes to anhydrous glycerol were done. Three following solutions were prepared: Solution 1 (containing 50:50 ratio of formalin (4%) and glycerin), Solution 2 (containing 50:50 ratio of 96% ethanol and 4% formalin) and lastly, Solution 3 (containing pure glycerin). Nematodes were then picked and mounted in clean glass slides with wax rings within a drop of pure glycerin. A maximum of five nematodes were placed on each permanent slide. A Carl Zeiss inverted routine microscope (ZEISS AxioVert. A1) equipped with a camera and AxioVision imaging software were used for studying morphology and measuring the key characters. The isolates were classified using taxonomic keys from Nguyen and Hunt (2007) and Machado et al. (2021).

Molecular characterization of the nematode and bacterium

DNA extraction was performed following the method described by Spiridonov et al. (2004) and using a tissue genomic DNA extraction kit (Dongsheng, Biotech). Briefly, the sterilized IJs were crushed by a sterile scalpel in a 1.5-ml microcentrifuge tube containing buffer, followed by adding 20 μl of Proteinase K. This was incubated at 55 °C until a homogenous solution was obtained. Different solutions provided within the kit were added following the manufacturer's protocol after each brief vortexing and centrifugation process. After elution, the obtained purified DNA was then kept at -20 °C in a deep freezer. A fragment of rDNA containing the ITS rDNA was amplified using the primers TW81 (5′-GTT TCCGTAGGTGAACCTGC-3′) as a forward primer and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) as a reverse primer (Joyce et al., 1994). The thermal cycler program for EPNs was as follows: 1 cycle of 94 °C for 7 min, followed by 35 cycles of amplification at 94 °C for 60 s, 54 °C for 60 s and 72 °C for 60 s and a final extension at 72 °C for 10 min.

For the bacterium, the universal primers used to amplify 16S rDNA genes were 27F (5′-AGAGTTTGA TCCTGGCTCAG-3′) as the forward primer and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as the reverse primer (Frank et al. 2008). The PCR was performed with the following conditions: 1 cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 60 s, 72 °C for 60 s and a final extension at 72 °C for 10 min. The amplified products were Sanger sequenced by Macrogen, Inc. (Seoul, South Korea). The sequencing results of ITS and 16S rDNA of nematodes and bacteria were submitted in the GenBank under accession numbers, MT023075 and MT012075, respectively.

Phylogenetic analyses of nematode and bacterium

The generated sequences were first edited using Chromas 2.6.6. A nucleotide basic local alignment search tool (BLASTn) was performed in order to compare to all sequences deposited in the National Centre for Biotechnology (NCBI) (Altschul et al. 1997). A cut of $\geq 97\%$ identity was considered for the same nematode species (Bhat et al. 2021) and $\geq 99\%$ identity for bacterial sequence (Wang et al. 2015). ITS and 16S rDNA sequences were aligned with their closest BLAST search matches, using ClustalW multiple alignment. Poorly aligned regions were removed from the alignments using MEGA-7. The base substitution model was evaluated using jModeltest 0.1.1 (Posada 2008). Phylogenetic trees were elaborated using the Bayesian inference method as implemented in the program MrBayes 3.2.7 (Ronquist et al. 2012). The HKY + (gamma distribution of rate variation with a proportion of invariable sites) model was selected. The selected model was initiated with a random starting tree and ran with the Markov chain Monte Carlo (MCMC) for 1×10^7 generations. The Bayesian tree was

ultimately visualized using the FigTree program 1.4.4 (Rambaut 2018). The out group taxons used for phylogenetic analysis were *Heterorhabditis zealandica* in case of *Heterorhabtidis*, and *Xenorhabdus nematophila* and *Photorhabdus akhurstii* in bacteria. In addition, genetic pairwise distances were estimated using MEGA-7 software (Kumar et al. 2016).

Results

Morphological characterization of the nematode

Key morphological characters were identified according to Nguyen and Hunt (Nguyen and Hunt 2007). Infective juveniles ($n=20$) and only few males ($n=5$) were obtained, fixed and measured in μm. F2H isolate closely matched the original description of *H. indica.* Infective juveniles were characterized by their body length of 488.5 (441–550) μm, tail length of 95.5 (79.9–105.6) μm, maximum body diameter of 24.2 (18–28) μm and ratio *E%* of 98.8 (85.9–112.5), and ratio *F* of 0.3 (0.2–0.4). Males are found to be slightly smaller (642, 585–697 μm) than the type strain (721, 573–788 μm) and the *H. indica* (872, 765–988 μm) documented from Cebu, Philippines; however, comparable size can be observed with *H. indica* H.TN48 populations in Vietnam with males size of 689 (596–818) μm. They are characterized by their spicule length (40.8, 36–46 μm), gubernaculum length (21.1, 20.9–24 μm), and ratio SW% (1.8, 1.6–1.9), closely matching other described strains. Females are observed to have protruding vulval lips with transverse slit, located

near mid-body and generally smaller compared to other *H. indica* female populations (data not shown). Comparative morphometrics of IJs and males of the present isolates with previously described *H. indica* populations are presented in Tables 1 and 2, respectively.

Molecular characterization of the nematode and bacterium

Nucleotide sequence analysis of the ITS rDNA indicated the F2H isolate as *H. indica.* There were 699 bp for the strain F2H. Upon BLASTn analysis of the ITS rDNA sequences, F2H showed 99.86% similarity with the *H. indica* strains reported from different geographical regions of the globe. When aligned with ITS rDNA sequences of already known *H. indica*, it displayed two nucleotide differences with the sequences of *H. indica* from India (MF973067). The distance matrix analyses based on ITS rDNA sequences showed that the present isolate of *H. indica* (F2H) is separated from other reported *Heterorhabditis* nematodes by 10–189 bp (Additional file 1: Table S1).

The sequencing of amplified product of 16S rDNA resulted in the production of a partial sequence of 1220 bp which is the size expected for 16S rDNAs. Using the BLASTn algorithm, the 16S rDNA gene of the present bacterial strain was identified as the most closely related to *Ochrobactrum* sequences available in the GenBank database. The highest levels of identity were obtained with *O. anthropi*: the 16S rDNA sequence of isolate F2B had 100% identity with *O. anthropi* reported

Table 1 Compendium of infective juveniles of *Heterorhabditis indica* F2H isolate with the type strain and other populations

Character	H. indica F2H	H. indica type strain	H. indica F05	H. indica H.CP6	H. indica H.TN48
Country	Philippines	India	Philippines	Vietnam	Vietnam
Reference	This study	Poinar et al. (1992)	Pascual et al. (2017)	Phan et al. (2003)	Phan et al. (2003)
n	20	25	20	25	25
Body length (L)	488 (441–550)	528 (479–573)	481 (432–560)	558 (490–614)	563 (348–622)
Max. body diam. (MBD)	24.2 (18–28)	20 (19–22)	19 (18–20)	19 (18–22)	20 (18–21)
a (L/MBD)	21 (17–24.9)	26 (25–27)	25 (23–27)	29 (27–31)	29 (27–33)
b (L/ES)	4.5 (4–5.6)	4.5 (4.3–4.8)	3.6 (3.5–3.9)	5 (5.3–6.3)	4.8 (2.8–6.3)
c (L/T)	5.4 (4.5–6)	5.3 (4.5–5.6)	6.3 (5.9–6.7)	5.8 (5.3–6.3)	5.7 (3.1–6.6)
Tail without sheath (T)	95.5 (79.9–105.6)	101 (93–109)	–	96 (86–105)	99 (88–112)
Anal body diam. (ABD)	10.6 (7.1–13.8)	–	11 (10–12)	13 (11–14)	13 (11–14)
Nerve ring-ant. end (NR)	76.7 (60.7–88.6)	82 (72–85)	85 (77–88)	82 (75–91)	83 (75–89)
Excretory pore-ant. end (EP)	94.3 (77.3–106.5)	98 (88–107)	105 (100–108)	95 (90–105)	96 (88–103)
Pharynx length (ES)	113 (79.6–129.4)	117 (109–123)	133 (125–138)	112 (108–117)	117 (108–24)
D (EP/ES)	0.8 (0.73–0.87)	0.8 (0.79–0.9)	79 (75–84)	0.8 (0.8–0.9)	82 (79–85)
E (EP/T)	0.9 (0.85–1.1)	0.9 (0.83–1.03)	137 (127–144)	0.9 (0.8–1.15)	0.9 (0.8–1.1)
F (MBD/T)	0.2 (0.18–0.35)	0.2 (0.18–0.22)	–	0.2 (0.1–0.22)	0.2 (0.1–0.2)

All morphometric measurements are in μm (except n, ratio and percentage) and expressed as means (range)

Table 2 Compendium of males of *Heterorhabditis indica* F2H with the type strain and other populations

Character	H. indica F2H	H. indica type strain	H. indica F05	H. indica H.CP6	H. indica H.TN48
Country	Philippines	India	Philippines	Vietnam	Vietnam
Reference	This study	Poinar et al. (1992)	Pascual et al. (2017)	Phan et al. (2003)	Phan et al. (2003)
n	5	12	–	15	15
Body length (L)	642.7 (585.4–69)	721 (573–788)	872 (765–988)	753 (708–847)	689 (506–818)
Max. body diam. (MBD)	41.6 (36.8–45.7)	42 (35–46)	35 (26–44)	46 (43–50)	49 (38–50)
a (L/MBD)	15.6 (13.4–18.2)	26 (25–27)	25 (22–29)	–	–
b (L/ES)	6.2 (5.9–6.7)	–	4.2 (4.0–4.4)	–	–
c (L/T)	23.4 (21.6–26.3)	–	55 (46–65)	–	–
Tail with sheath (T)	27.6 (25.5–30.9)	28 (24–32)	16 (13–18)	31 (26–34)	34 (27–41)
Anal body diam. (ABD)	23.2 (22.5–25.5)	23 (19–24)	8 (7–9)	20 (18–22)	24 (21–29)
Nerve ring-ant. end (NR)	75.9 (70.61–81.1)	–	141 (119–169)	–	–
Excretory pore-ant. end (EP)	120.7(106.7–131.2)	123 (109–138)	174 (158–191)	90 (83–97)	71 (63–78)
Pharynx length (ES)	104.2 (95.1–116.7)	101 (93–109)	210 (176–242)	98 (93–101)	95 (85–103)
D (EP/ES)	1.16 (1.1–1.19)	–	84 (73–98)	–	–
E (EP/T)	4.4 (3.93–5.14)	–	–	–	–
Testis reflexion	–	91 (35–144)	–	70 (60–80)	97 (77–117)
Spicule length (SL)	40.8 (35.9–45.7)	43 (35–48)	38 (35–44)	36 (27–39)	39 (34–44)
Gubernaculum length (GL)	21.1 (19.9–23.9)	21 (18–22)	18 (15–22)	19 (17–21)	20 (17–24)
GS (GL/SL)	51.9 (50.3–56.5)	0.5 (0.4–0.6)	47 (40–50)	0.5 (0.4–0.6)	0.52 (0.4–0.6)
SW (SL/ABD)	1.8 (1.6–1.9)	–	4.7 (4.2–5.5)	1.8 (1.3–2.1)	162 (132–197)

All morphometric measurements are in µm (except n, ratio and percentage) and expressed as means (range)

from different geographical regions of the world. When aligned with 16 rDNA sequences of already described *O. anthropi*, it showed zero nucleotide differences and 100 percentage similarity with the sequences of *O. anthropi* from France (AJ867290), India (EU826071), and other valid descriptions of *O. anthropi*. Further, the pairwise comparisons between the present *O. anthropi* from the Philippines and *O. lupini* from Spain showed a zero total character difference and 100% similarity, while with *O. cytisi* showed one total character difference and 100 percentage similarity. Additionally, *O. anthropi*, *O. lupini* and *O. cytisi* genomes presented a similar G + C (55.53, 56.52 and 55.56%, respectively), and A + C (44.47, 44.48 and 44.44, respectively) content demonstrating that each of them indeed represents single species. However, with other described species of *Ochrobactrum*, the present isolate F2B was separated by 18–68 bp (Additional file 1: Table S2).

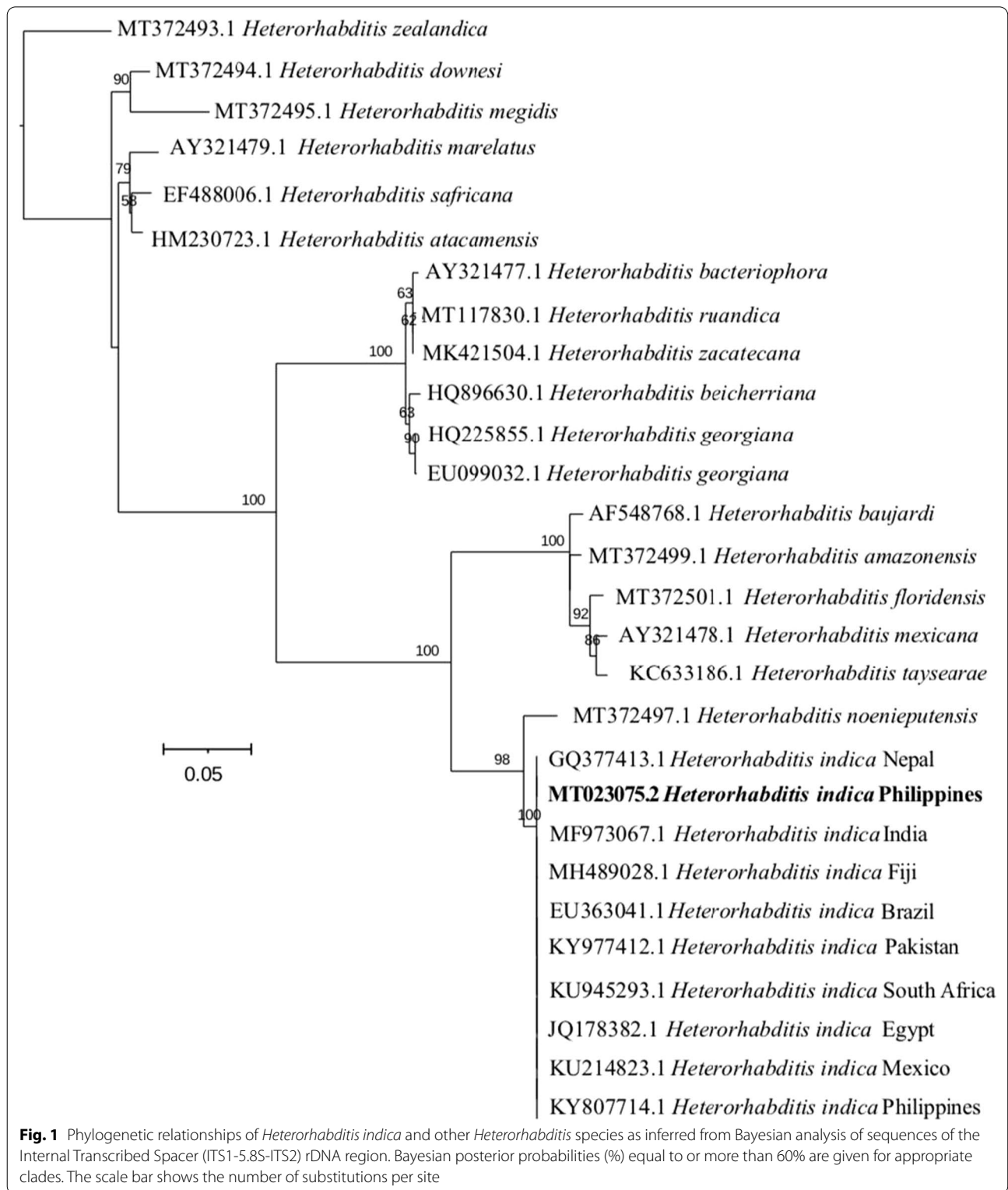### Phylogenetic analysis of the nematode and bacterium
Phylogenetic analyses performed based on ITS1-5.8S-ITS2 rDNA showed that the present *Heterorhabditis* isolate (F2H) formed a monophyletic clade with *Heterorhabditis indica* reported from different countries of the world, thus confirming its identification. This

monophyletic clade formed a group with closely related species, *H. noenieputensis* (Fig. 1) and together formed a group with other known members of *Indica*-clade. Members of *Indica*-clade formed a sister clade with described species of *Bacteriophora*- and *Megidis*-clade (Fig. 1). Thus, based on the phylogeny of ITS rDNA sequences, the F2H *Heterorhabditis* isolate from the Philippines was conspecific to *H. indica*.

Based on the 16S rDNA gene sequences, the bacterial isolate F2B was closely related to *Ochrobactrum anthropi* and shares 100% sequence similarity. Phylogenetic relationship reconstructions confirmed this observation and suggested that the bacterial isolate F2B belongs to the *O. anthropi* species (Fig. 2). In the phylogenetic tree of the 16S rDNA genes computed with sequences from all type strains of *Ochrobactrum* species that known, *O. anthropi* strains clustered with *O. lupini* and *O. cytisi* forming a monophyletic clade. Phylogenetic 16S marker gene sequence extracted from *O. anthropi* genome present similarity of 99% with *O. lupini* and 100% with *O. cytisi*.
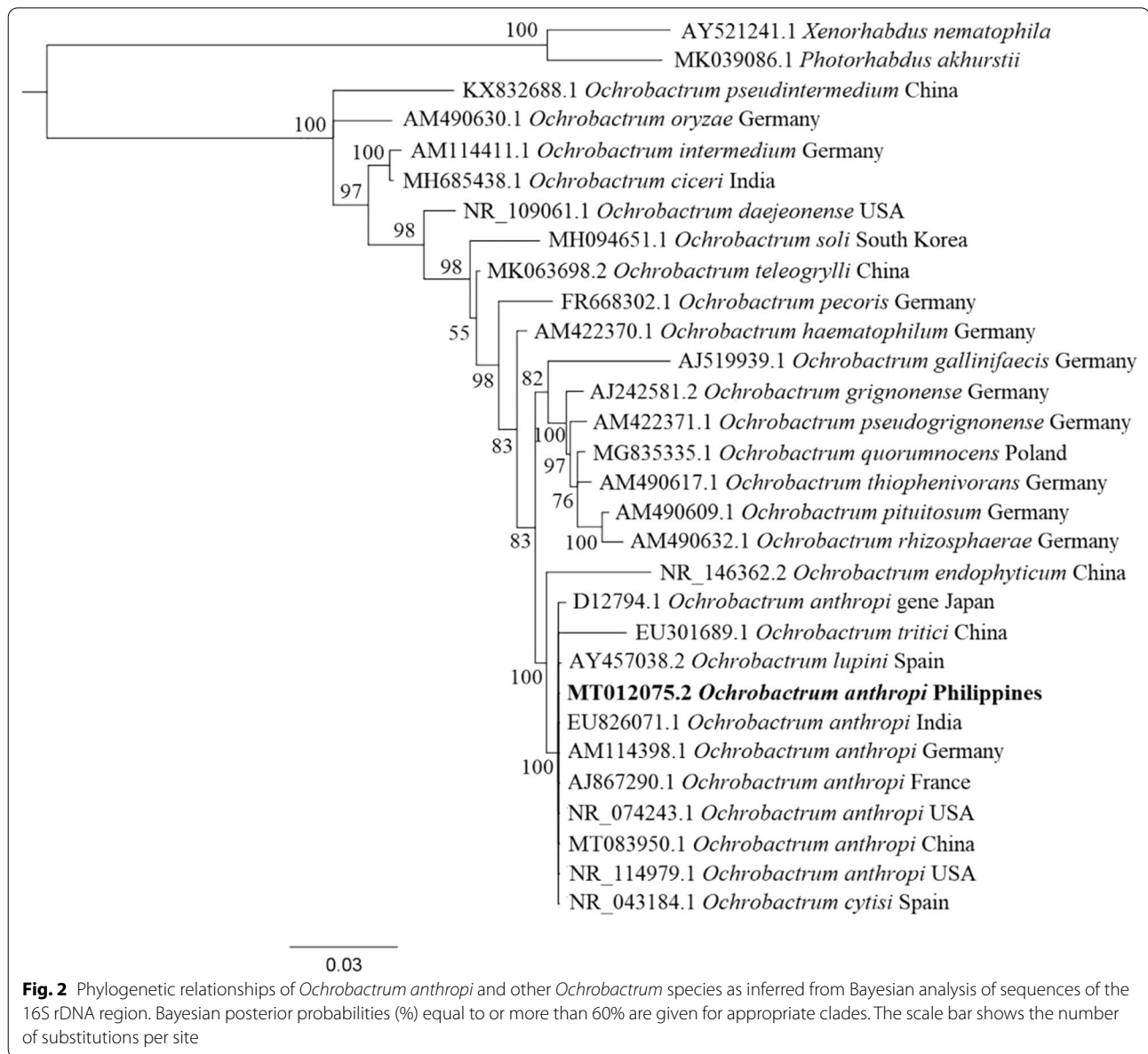
Discussion
Identification of these microscopic nematodes is often described to be rather difficult and requires meticulous efforts. At low taxonomic levels (i.e., order, family,

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Fig. 1 Phylogenetic relationships of *Heterorhabditis indica* and other *Heterorhabditis* species as inferred from Bayesian analysis of sequences of the Internal Transcribed Spacer (ITS1-5.8S-ITS2) rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given for appropriate clades. The scale bar shows the number of substitutions per site

and genus level), systematics was reported to be unstable. This is mainly due to their scantiness of observable and informative characters as well as overlapping

morphometrics (Holterman 2008). *H. indica* IJs of this study were separated from other *Heterorhabditis* species by their smaller body length (<600 μm). The rest of the

Fig. 2 Phylogenetic relationships of *Ochrobactrum anthropi* and other *Ochrobactrum* species as inferred from Bayesian analysis of sequences of the 16S rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given for appropriate clades. The scale bar shows the number of substitutions per site

characters are within the range of the original descriptions of Poinar et al. (1992). Males were slightly small at the type strain but comparable to the *H. indica* H.TN48 populations in Vietnam (Phan et al. 2003). However, *Heterorhabditis* exhibited slight morphological differences between species (Dolinski et al. 2008), making species identification extremely difficult (Darissa and Iraki 2014). With the advent of molecular diagnostic tools, morphological data can be complemented with modern techniques (Spiridonov 2017). In the present study, ITS rDNA region was used to identify and confirm the collected *Heterorhabditis* species.

Of particular highlight in this study was the bacterium detected in the IJs gut. These IJs initiate the parasitic-pathogenic life cycle of any EPNs as they carry bacteria in their guts or vesicle compartments while seeking their target insect hosts (Hazir et al., 2003). Thus, the natural bacteria present were determined in the IJs itself as it was rarely investigated in EPNs. Any phenotypic characterization, physiological, or biochemical profiling were not conducted to augment molecular analysis. Nonetheless, the conventionally used 16S rDNA gene analysis, which is the most used universal marker for identification of prokaryotes (Kämpfer et al. 2003) was used. For the first time, *Ochrobactrum anthropi* was reported as a

non-symbiotic bacterium naturally associated with the IJs of *H. indica* F2H from the Philippines. Previous reports relating to existence of non-EPN bacterial symbionts are commonly recognized either as laboratory contaminants (Poinar 1966), symbiont's inability to antagonize other species (Jackson et al. 1995), symbiont's phase variations (Razia et al. 2011), or ephemeral associations (Gouge and Snyder 2006). Some findings on the natural occurrence of dixenic associations between the *Ochrobactrum* and *Photorhabdus* in *H. indica* were reported specifically inside the infected insect cadaver (Razia et al. 2011). For this reason, the previous idea of general contamination was dismissed as the identified *Ochrobactrum* bacteria were fresh harvests without laboratory transfer (Babić et al. 2000) and the method of bacterial isolation entailed surface disinfection on the nematodes (Fischer-Le Saux et al. 1998). Furthermore, the pairwise alignment and phylogenetic analysis of the 16S rDNA genes revealed that *O. anthropi*, *O. lupini* and *O. cytisi* shared 100% similarity and form a monophyletic clade. Thus, it may be concluded that *O. cytisi* status as a separate species needs to be validated with a whole-genome sequencing project for this species-type strain as was clarified for *O. lupini* by Volpiano et al. (2019)

Associated bacteria are usually isolated and identified from the insect host hemolymph and seldom from the IJ. With the traditional culture-dependent approach, several other bacterial associates from nematodes remain cryptic (Gouge and Snyder 2006). To investigate its community dynamics, a heterorhabditid-infected insect cadaver was profiled for bacterial communities using a culture-independent next-generation sequencing tool. Some studies have been also carried out in the model organism, *C. elegans* (Dirksen et al. 2016). This recent approach for EPN-microbe interactions revealed a gradual shift from *Photorhabdus*-dominant community toward one shared with non-symbiotic bacteria in the insect environment (Wollenberg et al. 2016). In order to elucidate findings, Ogier et al. (2020) employed a modern and systematic approach to directly examine for the first time the IJ microbiota of the *Steinernema carpocapsae* by metabarcoding. The authors found that instead of the standard monoxenic model of the EPN-bacteria complex, it proposes the pathobiome view. *Ochrobactrum* sp. was among many other bacteria described from *Steinernema* species. They further reported *O. anthropi* from *H. bacteriophora* microbiota, which validates our recent findings on the *O. anthropi* association with the IJs of *H. indica*. Although the traditional method was used, nevertheless it was complemented. As a future prospect, the IJ microbiota of the tested *H. indica* tropical isolates will be investigated.

Interestingly, only *Ochrobactrum* was identified in this study and neither symbiotic bacterium nor dixenic associations was detected inside the IJs gut. Directly, the bacterial DNA inside the IJ gut was extracted and then identified using the 16S rDNA. The lone detection could be attributed to the methods used or for some other reasons. For example, the possibility of the insensitivity and tolerance of this *Ochrobactrum* strain against antimicrobials produced by the symbiont (Jackson et al. 1995) or that the antimicrobial activity of the non-symbiotic species may have affected the primary symbiont and contributed to interspecies competition (Ogier et al. 2020). For this, it was assumed that this *O. anthropi* F2B nonsymbiont may have dominated and decimated the symbiotic bacteria, or that they persisted well inside the IJ gut. This kind of sustainable association of bacteria other than the symbiont was demonstrated in the *Steinernema-Xenorhabdus* model, validating the persistence of non-symbionts like *Ochrobactrum* (Ogier et al. 2020). In a similar study, *Ochrobactrum* sp. was found persisting in the nematode gut of an EPN, *Oscheius chongmingensis* where it was also the most abundant bacterial genus at 59.82%, followed by *Bacillus* at 7.13% and others (Fu and Liu 2019). *Ochrobactrum* was further reported to be associated with the microbiome of other nematode species like the free-living cephalob nematode, *Acrobeloides maximus* (Baquiran et al. 2013), and in the model nematode *C. elegans* under stressful conditions (Dirksen et al. 2016), even indicated to coexist with several bacteria in nature. In general, *Ochrobactrum* is abundant in the soil, in the insect cadaver and in the IJ microbiota. As EPNs belong to the group of Rhabditida that feeds on microorganisms, they likely have retained this microbivorous behavior (Boemare et al. 1996), allowing non-symbionts to multiply in the nematode gut. Whether this bacterium is likewise maternally transmitted similar to a previously known elaborate pathway of *Heterorhabditis* symbionts (Ciche et al. 2008) is subject to further investigations.

The concern further is raised to health hazards. Although recently found not to be virulent on insects, the genus was earlier documented to be opportunistic pathogens to immune-compromised humans such as *O. anthropi* (Kettaneh et al. 2003). Nematodes then can be a reservoir of this opportunistic bacterium. As for the non-symbionts like *Ochrobactrum*, which can occur in laboratory culture, Boemare et al. (1996) emphasized that their association was not considered a risk because they cannot support long-term mass rearing of nematode populations. However, based on the documented persistence presented, risk cannot be ruled out. Thus, caution must always be practiced in the future outlook of applying EPN technology to the local agricultural industries for the farmers' benefit. When applied in the field, several

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natural associations may occur, but in handling EPN cultures in the laboratory and mass producing EPN-based products, in vitro monoxenic culture in bioreactors is highly recommended. Symbiont bacterial cultures must be isolated and later introduced with axenic nematodes. This enables more control of undesired non-symbionts (Peters et al. 2017) described by two methods: first by surface sterilizing the IJs, and second by sterilizing the eggs to produce aposymbiotic nematodes. Proper axenization and sterile techniques should be employed. Thus, careful practice and preparation of EPNs are needed prior to virulence tests or mass production as a biocontrol agent.

Conclusions

The EPN *Heterorhabditis indica* F2H, isolated from coastal sandy soils in Iligan, was the first report of occurrence in northern Mindanao, Philippines. This isolate showed a high homology to *H. indica* isolates reported from different geographical areas of the world, extending the documentation and geographic distribution of this particular species, which may be used as a biocontrol agent against insect pests in the country. The discovery of the natural association to *Ochrobactrum anthropi* F2B, a non-symbiont bacterium has provided additional evidence that this is present and abundant not only in the soils, but also in nematode microbiota, persisting in the IJs gut. This is the first record in the Philippine archipelago. Since the species is reported to be opportunistic to humans, this study will provide insights into practicing caution during EPN development as biocontrol agents in the country. Handling EPN cultures in the laboratory and their mass production must be done through in vitro monoxenic cultures, an existing approach where eggs are surface sterilized before introduction to its particular symbiotic bacterial culture. Further, full genome sequences are required to confirm the status of some controversial bacterial species.

Abbreviations

BCA: Biological control agent; EPN: Entomopathogenic nematodes; *H. indica*: *Heterorhabditis indica*; IJs: Infective juveniles; NCBI: National Center of Biotechnology Information; *O. anthropi*: *Ochrobactrum anthropic*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-022-00576-w.

Additional file 1: Table S1. Pairwise distances of the ITS region of the rDNA among species of the "*Heterorhabditis*". **Table S2.** Pairwise distances of the 16S region of the rDNA among species of the "*Ochrobactrum*".

Availability of data and materials

All sequence data have been deposited with GenBank (NCBI). Other data and material of this manuscript are available upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors of this manuscript do not have any kind of conflict of interest that needed disclosure to this journal.

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