Philipp Ent 36 (1): 25-43 ISSN 0048-3753 January-June 2022

CHARACTERIZATION OF THREE PHILIPPINE *HETERORHABDITIS INDICA* ISOLATES BASED ON MORPHOMETRIC, MOLECULAR, AND VIRULENCE DATA[1]

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ABSTRACT

Heterorhabditis indica **populations from Palawan (Hi MAP), Surigao Del Sur (Hi BSDS), and Batangas (Hi PBCB), Philippines were characterized based on their morphometrics, partial *cytochrome oxidase subunit 1* (*COI*) gene sequence pathogenicity and virulence against the Asian corn borer (*Ostrinia furnacalis)*, mealworm (*Tenebrio molitor*) and wax moth (*Galleria* sp.). Morphometric analyses of the three *H. indica* isolates based on some diagnostic characters observed, particularly in males, amphimictic females, and hermaphrodites showed little intraspecific differences. The approximately 686 bp partial *COI*-based barcode generated for each isolate revealed single nucleotide polymorphisms between Hi MAP and the other two isolates that have identical *COI* sequences. Phylogenetic analysis further revealed two putative groups of *H. indica* namely, *H. indica* group 1 (Hi MAP) and *H. indica* group 2 (Hi BSDS and Hi PBCB). On their virulence, the *H indica* isolates showed high level of infection towards the test insects used, but no significant differences at 24 hours post infection (HPI) was observed. The IJ penetration rate that caused the same level of mortality, however, was significantly lower in Hi BSDS-infected *Galleria* sp. than those exposed to Hi MAP and Hi PBCB. In terms of median lethal time (LT_{50}), no significant difference was observed among the three *H. indica* isolates when tested on *Galleria* sp. and *O. furnacalis*. However, in *T. molitor*, Hi BSDS killed significantly faster than Hi PBCB and Hi BSDS. This study revealed variation in the pathogenicity of the three *H. indica* isolates; it showed higher level of virulence of Hi BSDS isolate than the other *H. indica* isolates studied.**

Key words: *cytochrome C oxidase I* (*COI*), entomopathogenic nematodes, EPN, *Galleria* sp., LT_{50}, *Ostrinia furnacalis, Tenebrio molitor,* mortality

INTRODUCTION

The utilization of entomopathogenic nematodes (EPN) to control insect pest population has been practiced as early as the 17th century (Nickle, 1984). EPN are small, soft-bodied and non-segmented roundworms, which attack many insect orders. These nematodes belong to the genera *Heterorhabditis* and *Steinernema*, under the Heterorhabditidae and Steinernematidae family, respectively. *Steinernema* are known for their specific symbiotic association with the bacteria *Xenorhabdus* Thomas & Poinar, while *Heterorhabditis* with *Photorhabdus* Boemare, Akhurst & Mourant (Ehlers, 2007). These EPN occur naturally in the soil and locate their host in response to carbon dioxide, vibration and other chemical cues (Kaya & Gaugler, 1993). They are used either solely as biological control agents or as components of an integrated pest management program against economically-important insect pests (Grewal et al., 2005). These organisms are considered as safe and environment-friendly biopesticides, since they are non-toxic to humans and relatively specific to their target insect pest. Moreover, EPN can be applied using standard pesticide equipment (Shapiro-Ilan et al., 2006).

Entomopathogenic nematology is a budding field in the Philippines. Currently, there are few studies on entomopathogenic nematode (EPN) discovery and investigations of their biological control potential against several insect pests in the local setting. Some of the notable studies include the discovery of *Steinernema abassi* and *Heterorhabditis indica* isolates from Batangas with biological potential against corn lepidopteran pests (Caoili & Latina et al., 2018), *H. indica* isolates from banana and rice fields from Davao del Sur (Navarez et al., 2021), *H. indica*, *Metarhabditis rainai, Oscheius insectivora*, and *Oscheius* sp. from Cotabato and some *Steinernema* and *Heterorhabditis* spp. from sweet potato fields in the Visayas region (Gapasin et al., 2017).

Recently, two EPN populations previously identified as *H. indica* through ITS sequence analysis, was found along the shores of Bretania, Surigao del Sur (Latina and Caoili, 2017), and Magbabadil, Aborlan, Palawan (R. A. Latina, personal communication, 2019). These isolates, however, require characterization and bio-efficacy tests; hence, this study which was designed to comparatively analyze the *H. indica* populations from Palawan, Surigao Del Sur, and Batangas, Philippines. Specifically, this study aimed to 1) obtain morphometric data from the *H. indica* populations using infective or second stage juveniles (IJ/J2), adult males, and hermaphroditic and amphimictic females; 2) assess the genetic variation of the three *H. indica* populations based on DNA barcodes; and 3) compare their pathogenicity and virulence against three insect pest species of economic importance such as Asian corn borer (*Ostrinia furnacalis)*, wax moth (*Galleria* sp.), and mealworm (*Tenebrio molitor*).

MATERIALS AND METHODS

Source of Test Insects and Entomopathogenic Nematodes

Heterorhabditis indica isolates used in the study, which were originally extracted from soil samples using *O. furnacalis* from Magbabadil, Aborlan, Palawan (Hi MAP), Puting Bato, Calaca, Batangas (Hi PBCB), and Bretania, Surigao del Sur (Hi BSDS), are currently cultured and maintained at the Insect Pathology and Molecular Biology Laboratory (IPMBL) and Tropical Phytonematology Laboratory (TPL) of the Institute of Weed Science, Entomology and Plant Pathology (IWEP), UP Los Baños (**Table 1**). The larvae of *O. furnacalis* (Asian corn borer), *Galleria* sp. (greater wax moth), and *Tenebrio molitor* (mealworm), reared at IPMBL, were used as host insects for *in vivo* mass-culturing of the EPN.

Table 1. Collection data for *Heterorhabditis indica* from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB)

Isolate	Collection Site	Geography	Climate	Soil Type	Associated Vegetation
Hi MAP	Magbabadil, Aborlan, Palawan	Sandy shore	Hot and humid	Sandy	Coconut
Hi BSDS	Bretania, Surigao Del Sur	Sandy shore	Hot and humid	Sandy	Coconut
Hi PBCB	Puting Bato, Calaca, Batangas	Coastal Plain	Hot and humid	Sandy Loam	Mango

Morphological Characterization and Preparation of Permanent Mounts of *Heterorhabditis indica*

Twenty (20) larvae of *Galleria* sp., and *O. furnacalis* were placed in a 6-cm Petri dish lined with filter paper (Whatman No.1) saturated with nematode suspension containing 200 infective juveniles (IJ) from each of the *H. indica* isolates. After 3 to 5 days post-infection (dpi), 10 insect cadavers were dissected to get hermaphroditic females, amphimictic females and males. The collected first- and second-generation adults and IJs were heat-killed and temporarily fixed with formalin-glycerol 2x (10ml 40% formaldehyde, 1ml glycerol, 45ml distilled water). Permanent fixing was done using ethanol-glycerol technique. Permanent mounts were prepared by picking out fixed nematodes in pure glycerin and putting them in small drop of glycerol as mounting medium on clear glass slide lined with parafilm wax (ring). For IJ, live mounts were used.

Images were documented, observed and recorded using Dino Eye Capture 2.0 software (AnMo Electronics Corporation, New Taipei City, Taiwan). The characters measured were total body length (L), greatest body with (W), distance from anterior end to excretory pore (EP), distance of anterior end to nerve ring (NR), distance from anterior end to end of pharynx (ES), distance of vulva from anterior end/L x 100 (V%), tail length without sheath (TL) and anal body width (ABW) for hermaphroditic and amphimictic females, and total body length (L), greatest body width (W), distance from anterior end to excretory pore (EP), distance of anterior end to nerve ring (NR), distance from anterior end to end of pharynx (ES), testis reflexion (TR), tail length without sheath (TL), anal body width (ABW), spicule length (SL),gubernaculum length (GL), and ratios of EP/ES x 100 (%D) and SP/ABW× 100 (%SW) for males. For IJs, similar characters as with the males were obtained with the exception of TR, SL and GL.

DNA Extraction and PCR Amplification

Total genomic DNA of the entomopathogenic nematodes species was extracted using the Animal and Fungi DNA Isolation Kit (Jena Bioscience, Jena, Germany) following the manufacturer`s standard protocol with slight modifications. After the DNA extraction, PCR amplification was done immediately using on an S-96 Thermal Cycler (Quanta Biotech, Ltd., Surrey, UK). The ITS region of Hi MAP was amplified using the general primers, TW81: 5'- GTT TCC GTA GGT GAA CCT GC-3' (forward) and AB28: 5' –ATA TGC TTA AGT TCA GCG GGT-3' (reverse) (Joyce et al., 1994) following the PCR profile of Nguyen et al. (2004); initial denaturation at 95 °C for 7 min followed by 35 cycles of 94 °C for 60 sec, 45 °C for 60 sec, and 72 °C for 1 min, and final extension at 72 °C for 10 min. The ITS nucleotide sequences of Hi MAP was compared with those of Hi BSDS (Latina & Caoili, 2017) and Hi PBCB (Caoili & Latina et al., 2018). Meanwhile, amplification of mitochondrial *cytochrome C oxidase I* (*COI*) region for the three isolates was carried out using the primer pairs, 507: 5'-AGT TCT AAT CAT AAR GAT ATY GG-3'(forward) and 588: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (reverse) with the following thermal profile: initial denaturation at 94 ºC for 3 min followed by 36 cycles of 94 ºC for 60 sec, 40 ºC for 60 sec and 72 ºC for 60 sec, and final extension at 72 ºC for 7 min (Nadler et al., 2006).

The amplicons were resolved in 1.2% agarose gel in 0.5% TBE buffer using MupidX (Mupid-One, Nippon Genetics) run at 100 volts for 35 minutes. The gel was stained with GelRed® (Biotium, Fremont, California, USA) and viewed using AlphaImager® transilluminator (ProteinSimple, California USA).

Pathogenicity and Virulence Testing

The test insects used for this experiment were 10-day-old *T. molitor* and 9-day old *O. furnacalis* and *Galleria* sp. Each of the *H. indica* isolates was applied onto sterile Whatman filter paper in UV-sterilized 4-cm Petri plates at a 200 IJs per plate. The number of dead insects was monitored every 4 hours interval after the infection process and was expressed as percentage mortality. Three trials of three replicates, with 50 insects per replicate per trial were performed. The control set-up had sterile distilled water-saturated filter papers instead of EPN suspensions.

Lethal Time Assay and Percentage Penetration

The mortality of the test insects (*T. molitor, O. furnacalis* and *Galleria* sp.) was monitored every 4 hours after the application of the three strains of *H. indica*, as indicated in the pathogenicity and virulence assays described above. Lethal time was estimated using PoloPlus (LeOra Software LLC; http://leora-software.com). Three trials were conducted with three replicates. Each replicate contained fifty insects per trial.

The percentage penetration of the different *H. indica* isolates in each insect species was estimated using a single larva representing a biological replicate. Ten biological replicates were used for each insect species. The bioassays were done in a Petri dish with a larva infected with 200 IJs. After 24 hours post inoculation (HPI), dead larvae were individually collected, washed with distilled water, and dissected. The number of IJs that entered in each larva that caused death of the test insect was counted and recorded. Statistical analysis was carried out using the GraphPad Prism software.

RESULTS

Morphometric Analyses

Tables 2-5 show the morphometric data obtained from the three *H. indica* isolates namely, Hi PBCB, Hi MAP and Hi BSDS, based on selected male, female (amphimictic and hermaphroditic) and infective juvenile (J3) characters, together with the measurements from the type specimen and a locally reported *H. indica* Hagonoy isolate.

In terms of the male characters, most of the measurements obtained from the three *H. indica* isolates are similar and/or within the size ranges, and are in agreement with those from the type strain, specifically the values for L, W,

EP, ES, SP and GL. Meanwhile, these characters, along with the %D and %SW from the three Hi isolates, are bigger than the Hagonoy isolate. Only the NR and TR measurements from the three isolates are similar to the Hagonoy isolate. TL, on the other hand, appears to be longer in the Hagonoy isolate as compared to the three Hi isolates. Nevertheless, most of the measurements of the Hagonoy isolate are within the size ranges (**Table 2**).

Table 2. Morphometric data obtained from males of *Heterorhabditis indica* from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB) along with the type strain and locally reported *H. indica* from Hagonoy Davao del Sur.

Diagnostic Character	Hi MAP n=20	Hi BSDS n=20	Hi PBCB n=20	*H. indica* Type Specimen (Poinar et al., 1992) n=12	*H. indica* Hagonoy isolate (Navarez et al., 2021) n=8
L	705.84 ± 59.45 (597.2 - 796.92)	698.62 ± 40.93 (610.18-745.26)	697.15 ± 65.20 (578.03-787.07)	721 (573-788)	557 (516-629)
W	40.67 ± 2.82 (35.09-45.90)	38.49 ± 3.12 (33.14-45.20)	39.14 ± 2.94 (34.39-45.86)	42 (35-46)	30 (26–36)
EP	121.67 ± 7.06 (109.09-133.3)	125.00 ± 8.70 (109.67-137.35)	119.74 ± 11.73 (90.18-137.75)	123 (109-138)	78 (42–99)
NR	77.25 ± 3.99 (72.07-84.88)	85.98 ± 13.65 72.09-122.12)	76.58 ± 4.66 (65.10-84.33)	-	75 (48–103)
ES	98.87 ± 4.67 (92.63-108.68)	101.99 ± 4.57 (91.44-108.91)	100.46 ± 4.62 (93.63-108.68)	101 (93-109)	113 (77–133)
TR	107.65 ± 17.10 (81.12-143.02)	106.91 ± 20.32 (62.25-144.85)	112.30 ± 19.87 (17.69-76.31)	91 (35-144)	106 (93–116)
TL	27.83 ± 2.17 (24.15-31.83)	28.22 ± 2.39 (25.10-34.34)	27.28 ± 2.95 (20.07-31.94)	28 (24-32)	42 (29–50)
ABW	21.32 ± 1.89 (19.85-23.55)	23.56 ± 2.21 (20.02-29.49)	21.80 ± 1.38 (19.00-23.95)	23 (19-24)	21 (20–22)
SP	41.98 ± 4.02 (35.84-47.87)	38.16 ± 3.57 (29.50-44.69)	38.63 ± 3.53 (32.52-46.81)	43 (35-48)	22 (19–24)
GL	20.45 ± 1.65 (18.15-22.51)	21.14 ± 2.21 (18.10-26.85)	19.46 1.56 (15.66-22.72)	21 (18-22)	11.0 (11–12)
%D	123.32 ± 9.26 (104.80-138.38)	122.77 ± 10.05 (107.60-149.65)	119.45 ± 13.08 (90.05-139.57)	-	70 (50–70)
%SW	196.82 ± 13.97 (173.62-219.73)	162.90 ± 18.77 (138.18-208.99)	177.95 ± 20.89 (149.01-227.69)	-	102 (86–114)

Abbreviations: L = total body length; BW = greatest body width; EP = distance from anterior end to excretory pore; NR = distance of nerve ring from anterior end; ES = distance from the anterior end to end of pharynx; TR = testis reflexion; TL = tail length; ABW = anal body width; SP = spicule length; GL = gubernaculum length; %D = EP/ES x 100; %SW = SP/ABW× 100. The measurements are in µm and presented as mean ± standard deviation and range (n=20). Unavailable data are designated by -.

As for the amphimictic females, L, EP, NR of the three Hi isolates show similar measurements as with the type strain. The Hagonoy isolate seems to be the smallest, with measurements outside the size ranges. Although the value of its NR, TL, %V are also bigger than the rest, the values for NR are not within the size range. On the other hand, all isolates share similar values for W, ES and ABW. The values for %D and %SW of the three Hi and Hagonoy isolates are comparable with each other (**Table 3**).

Table 3. Morphometric data obtained from amphimictic females of *Heterorabditis indica* from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB) along with the type strain and locally reported *H. indica* from Hagonoy Davao del Sur.

Diagnostic Character	Hi MAP n=20	Hi BSDS n=20	Hi PBCB n=20	*H. indica* Type Specimen (Poinar et al., 1992) n=12	*H. indica* Hagonoy isolate (Navarez et al., 2021) n=10
L	1687.45 ± 184.73 (1236.74-1975.61)	1596.42 ± 119.54 (1376.76-1786.90)	1647.73 ± 132.86 (1398.31-1882.24)	1600 (1200–1800)	1148 (1127–1185)
W	117.49 ± 17.61 (89.85-159.94)	88.45 ± 6.28 (78.22-99.22)	100.46 ±7.29 (84.76-109.74)	95 (76–113)	98 (86–125)
EP	145.48 ± 17.05 (120.16-171.39)	129.48 ± 4.36 (124.68-137.75)	131.32 ± 3.39 (123.67-136.82)	127 (118–138)	-
NR	95.72 ± 7.76 (86.14-113.22)	91.18 ± 3.11 (85.24-95.77)	91.79 ± 2.03 (89.47-95.72)	92 (88–96)	156 (147–176)
ES	138.29 ± 9.99 (122.59-163.72)	129.30 ± 3.92 (123.50-138.06)	129.91 ± 4.04 (120.74-136.85)	131 (120–139)	123 (105–147)
V%	48.58 ± 2.29 (43.72-51.73)	46.72 ± 3.21 (41.38-52.53)	45.00 ± 2.51 (41.12-48.43)	-	57 (51–75)
TL	72.78 ± 6.31 (58.69-84.36)	80.51 ± 5.35 (70.14-87.87)	74.61 ± 4.25 (70.03-86.27)	-	95 (73–104)
ABW	33.32 ± 7.78 (22.97-47.75)	29.51 ± 6.77 (23.36-56.42)	28.78 ± 2.12 (24.68-31.44)	26 (22–32)	34 (24–40)

Abbreviations: L = total body length; W = greatest body width; EP = distance from anterior end to excretory pore; NR = distance of nerve ring from anterior end; V% = distance of vulva from anterior end/L x 100; ES = distance from the anterior end to end of pharynx; TL = tail length; ABW = anal body width. The measurements are in µm and presented as mean ± standard deviation and range (n=20). Unavailable data are designated by -.

Measurements for the hermaphroditic females of the three Hi isolates were only compared with the type strain due to unavailability of data for the Hagonoy isolate. All measured characters (L, W, EP, NR, ES, V%, TL and ABW) agree to the measurements indicated for the type specimen (**Table 4**).

Table 4. Morphometric data obtained from hermaphroditic females of *Heterorabditis indica* from Palawan (Hi MAP), Surigao del Sur (Hi BSDS) and Batangas (Hi PBCB), along with the type strain

Diagnostic Character	Hi MAP n=20	Hi BSDS n=20	Hi PBCB n=20	*H. indica* Type Specimen (Poinar et al., 1992)
L	2843.05 ± 252.42 (2056.72-3094.82)	2931.84 ± 162.15 (2461.06-3086.04)	2902.05 ± 217.60 (2076.32-3098.02)	2700 (2300-3100)
W	131.51 ± 9.08 (110.54-143.19)	127.63 ± 14.93 (109.11-145.63)	130.37 ± 9.87 (110.08-144.34)	132 (107-145)
EP	176.31 ± 5.32 (165.87-186.02)	176.40 ± 7.59 (165.11-186.67)	175.43 ± 6.40 (165.83-185.93)	173 (163-187)
NR	114.34 ± 4.80 (106.45-121.17)	117.40 ± 3.92 (110.22-122.86)	116.37 ± 5.11 (107.41-122.68)	115 (104-123)
ES	172.48 ± 4.28 165.28-178.23()	169.84 ±9.07 (135.11-177.94)	161.35 ± 23.23 (105.00-178.32)	173 (163-187)
V%	47.89 ± 1.33 (45.01-49.77)	47.51 ± 1.54 (45.14-49.99)	46.16 ± 6.35 (45.70-49.58)	47 (45-50)
TL	88.32 ± 10.32 (74.55-106.33)	98.71 ± 7.21 (82.32-108.74)	99.11 ± 7.53 (83.11-109.72)	92 (72-110)
ABW	45.80 ± 3.80 (39.02-50.70)	44.46 ± 4.13 (39.13-50.37)	44.44 ± 5.17 (39.01-56.22)	44 (38-51)

Abbreviations: L = total body length; V = vulva; BW = greatest body width; EP = distance from anterior end to excretory pore; NR = distance of nerve ring from anterior end; ES = distance from the anterior end to end of pharynx; V% = distance of vulva from anterior end/L x 100; TL = tail length; ABW = anal body width. The measurements are in µm and presented as mean ± standard deviation and range (n=20). Unavailable data are designated by -.

Concerning the IJ character measurements, the three Hi isolates and the type specimen share the same values for L and TL, and appear to be slightly longer than the Hagonoy isolate. In terms of W and %E, however, all of the five specimen share similar values. The EP of the three Hi isolates are the biggest but within the size range of the type strain. EP of the Hagonoy isolate is the smallest and is outside the size range. For NR, only the values of the three Hi isolates and the type specimen agree with each other and appear to be smaller than the Hagonoy isolate. Similar to L, %D values of the three Hi isolates and the type specimen are concordant, while that from Hagonoy isolate is smaller and outside the size range. For ES, the type specimen has smaller values as compared to the three Hi isolates and Hagonoy isolate which share similar values (**Table 5**).

Table 5. Morphometric analyses of infective juvenile (IJ) of *Heterorabditis indica* from Palawan (Hi MAP), Surigao del Sur (Hi BSDS) and Batangas (Hi PBCB), along with the type strain and locally reported *H. indica* from Hagonoy Davao del Sur.

Diagnostic Character	Hi MAP	Hi BSDS	Hi PBCB	*H. indica* Type Specimen (Poinar et al., 1992) n=25	*H. indica* Hagonoy isolate (Navarez et al., 2021) n=15
L	558.87± 18.72 (513.10-574.35)	563.05 ± 18.66 (491.34-571.94)	556.13 ± 17.25 (505.64-571.79)	528 (479–573)	482 (403–529)
W	20.87 ± 0.70 (19.12-21.74)	21.24 ± 0.95 (19.63-22.75)	20.80 ± 1.02 (19.03-22.54)	20 (19–22)	21 (19–23)
EP	101.05 ± 3.43 (92.12-21.74)	100.51 ± 3.15 (92.32-105.65)	101.06 ± 2.59 (96.25-105.91)	85 (88–107)	61 (40–77)
NR	81.73 ± 2.00 (78.34-84.81)	80.17 ± 2.02 (75.43-83.84)	81.41 ± 2.42 (76.63-84.95)	82 (72–85)	95 (91–100)
ES	117.02 ± 5.29 (100.17-121.53)	117.77 ± 3.10 (110.36-121.38)	118.56 ± 3.03 (110.63-121.65)	98 (88–107)	124 (114–131)
TL	101.82 ± 3.16 (97.47-108.01)	102.42 ± 3.33 (97.51-108.43)	101.26 ± 2.34 (97.21-105.76)	101 (93–109)	-
%D	86.54 ± 5.43 (80.27-105.05)	85.41 ± 3.64 (79.70-95.46)	85.29 ± 3.10 (79.70-91.64)	84 (79–90)	49 (30–50)
%E	99.31 ± 4.13 (90.89-107.66)	98.23 ± 4.19 (87.49-104.47)	99.86 ± 3.82 (93.02-106.51)	94 (83–103)	110 (70–150)

Abbreviations: L = total body length; W = greatest body width; EP = distance from anterior end to excretory pore; NR = distance of nerve ring from anterior end; ES = distance from the anterior end to end of pharynx; TL = tail length without sheath; ABW = anal body width; %D = EP/ES x 100; %SW = SP/ABW× 100. The measurements are in µm and presented as mean ± standard deviation and range (n=20). Unavailable data are designated by -.

Molecular Analyses

The three *H. indica* isolates were further characterized using the mitochondrial *cytochrome C oxidase subunit 1* (*COI*) since the ITS marker used for initial species identification did not reveal any nucleotide variation among the three isolates (**Figure 1**). This is similar to the case of the three Venezuelan *H. amazonensis* strains which were identical to those of the original type species from Brazilian Amazonian Forest based on ITS (Morales et al., 2016). In another study, Kang et al., (2005) reported two *H. megidis* populations with

highly homologous total ITS rDNA sequence sharing only seven nucleotide differences. The molecular size of the partial *COI* gene amplified from the three

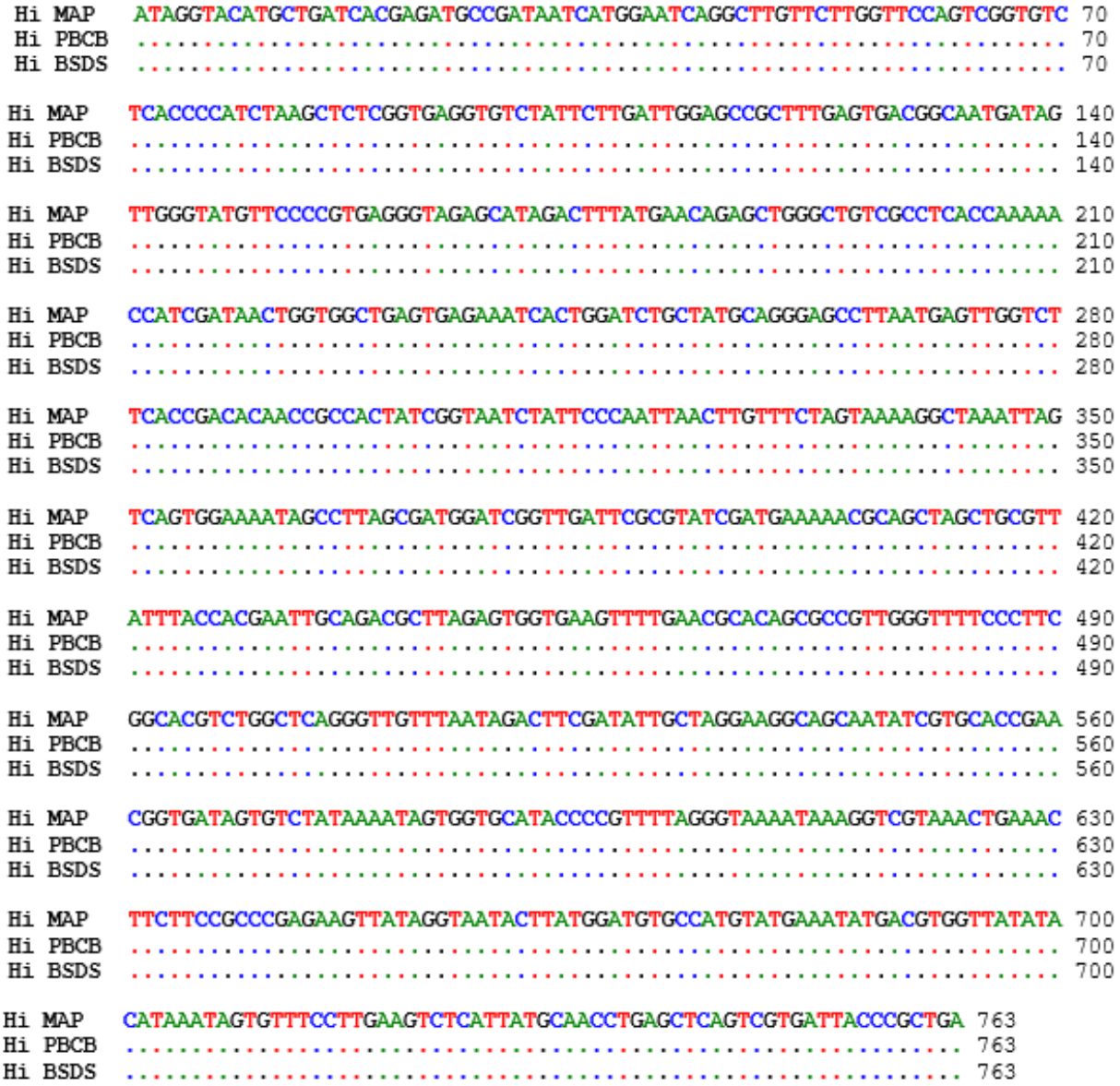


Figure 1. Multiple nucleotide sequence alignments of the partial ITS-rDNA consensus sequences of the three Philippine *Heterorhabditis indica* isolates from Palawan (Hi MAP), Surigao del sur (Hi BSDS; Latina & Caoili, 2017), and Batangas (Hi PBCB; Caoili & Latina et al. 2018).

H. indica isolates was approximately 700 bp, which is in agreement to the results of Nadler et al. (2006). After trimming the *COI* raw nucleotide sequences, a 686-nucleotide sequence for each population was generated that was subjected to BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analyses. The results

revealed a 98.16% (Hi MAP) and 98.77% (Hi BSDS and Hi PBCB) similarity to *H. indica* reported by Vanlalhlimpuia & Hrang Chal Lalramnghaki (2018) with GenBank Accession Number MF621247.1 (**Table 5**).

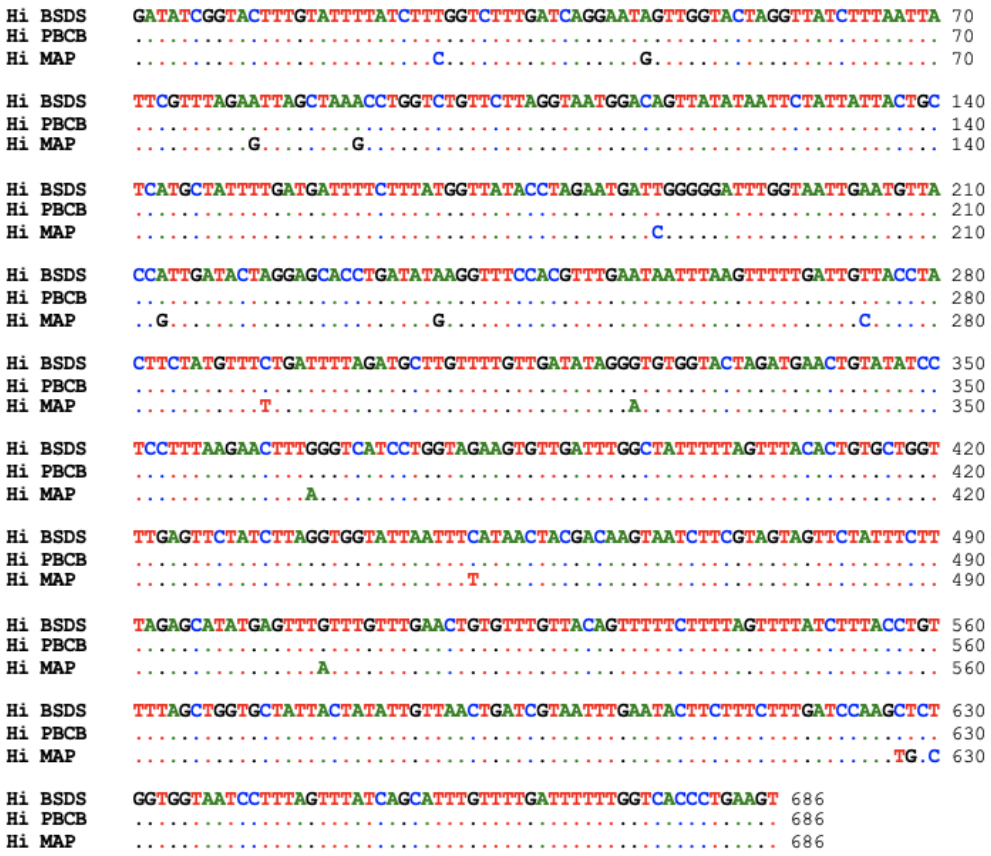
The multiple alignment of the partial nucleotide sequences of *COI* of the three isolates of *H. indica* revealed the presence of polymorphic sites as shown in **Figure 2A**. However, no nucleotide base difference between Hi BSDS and Hi PBCB was observed despite of their wide geographical distance. Whereas, a 15-nucleotide difference between Hi MAP and both Hi BSDS and Hi PBCB was noted. Further sequence analyses showed that out of the 15 polymorphic sites observed, 14 of which are transitions and only one is transversion. Nonetheless, the alignment of the amino acid sequences deduced from the partial nucleotide sequence of *COI* of *H. indica* isolates showed only a sole difference in Hi MAP at position 210 (**Figure 2B**). The partial *COI* protein of Hi MAP has alanine residue at this position while a serine residue was noted for both Hi BSDS and Hi PBCB.

The *COI* gene marker-based phylogenetic tree constructed showed that the three isolates formed a monophyletic group with *COI* nucleotide sequences of the reference *H. indica* from India (**Figure 3**), thus further confirming their species identity. Interestingly, the tree also revealed two putative groups of *H. indica* namely *H. indica* group 1 (Hi MAP) from Palawan and *H. indica* group 2 (Hi BSDS and Hi PBCB) from Surigao del Sur and Batangas. The grouping was due to the observed 15-base polymorphism in the nucleotide sequence of *COI.* Nonetheless, all the three isolates are still closely related with each other, since they are on the same clade. This further suggests that the location where the isolates came from has no effect on their molecular identities.

Pathogenicity and Virulence Test

The pathogenicity of the three *H. indica* isolates to *O. furnacalis* (Asian corn borer), *Galleria* sp. (greater wax moth), and *T. mollitor* (mealworm) were compared. **Figure 4** shows the percentage mortality of the test insects at 24 HPI exposed to Hi BSDS, Hi MAP and Hi PBCB. A significantly high mortality in *O. furnacalis*, *Galleria* sp., and *T. molitor* was observed in the insect groups exposed to Hi BSDS, Hi MAP and Hi PBCB as compared with the untreated counterpart. Based on the mortality data at 24 HPI for each of the test insects, statistical analysis revealed no significant differences among the *H. indica* isolates. The degree of variation was more apparent to the test insects used. Bioassay data revealed that *Galleria* sp. was the most susceptible among the test insects with 100% mortality at 24 HPI to all *H. indica* populations. In *O. furnacalis*, the mortality rate of insects exposed to Hi BSDS, Hi MAP and Hi PBCB ranged from 77 to 88%. On the other hand, there were only 50-70% mortality in *T. molitor* test insects. A 100% mortality was achieved in *O. furnacalis* and *T. molitor* at 48 HPI in all the *H. indica* treatments.

A. Nucleotide sequence



B. Amino acid sequence

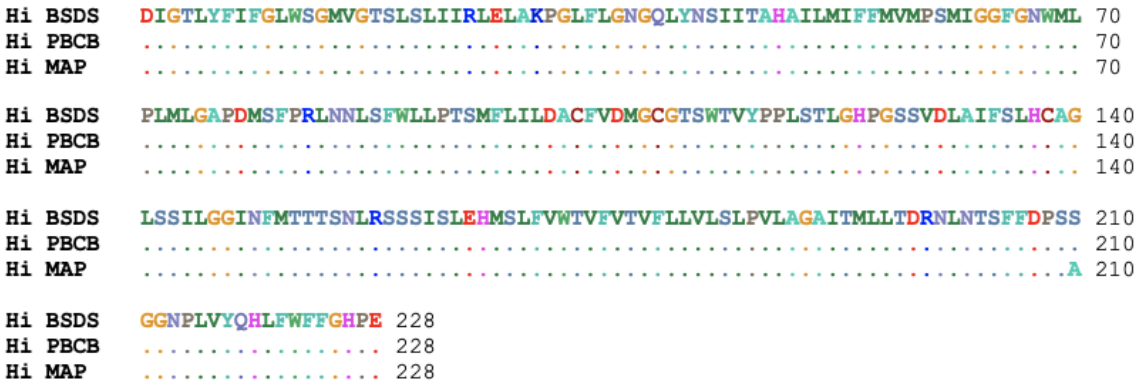


Figure 2. Multiple nucleotide sequence (A) and amino acid sequence (B) alignments of the partial *cytochrome C oxidase I* consensus sequences of the three Philippine *Heterorhabditis indica* isolates from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB). The difference in only one amino acid residue is shaded in yellow.

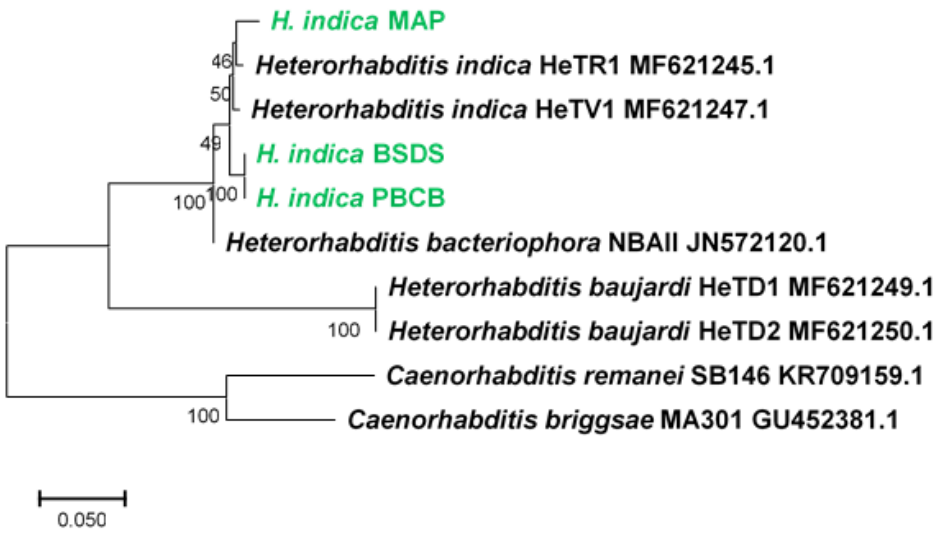


Figure 3. Phylogenetic tree of *cytochrome C oxidase I* (*COI*) partial nucleotide sequences of Philippine *Heterorabditis indica* isolates from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB) using Maximum Likelihood method based on Tamura-Nei model with invariable sites and 1000 bootstrap replications. *H. indica* sequences from India (GenBank Acc. No. MF621245.1 and MF621247.1) were included as the references. *Caenorhabditis remanei* (GenBank Acc. No. KR709159.1) and *C. briggsae* (GU452381.1) were used as outgroups. Phylogenetic analysis was constructed using MegaX software (Kumar et al., 2018).

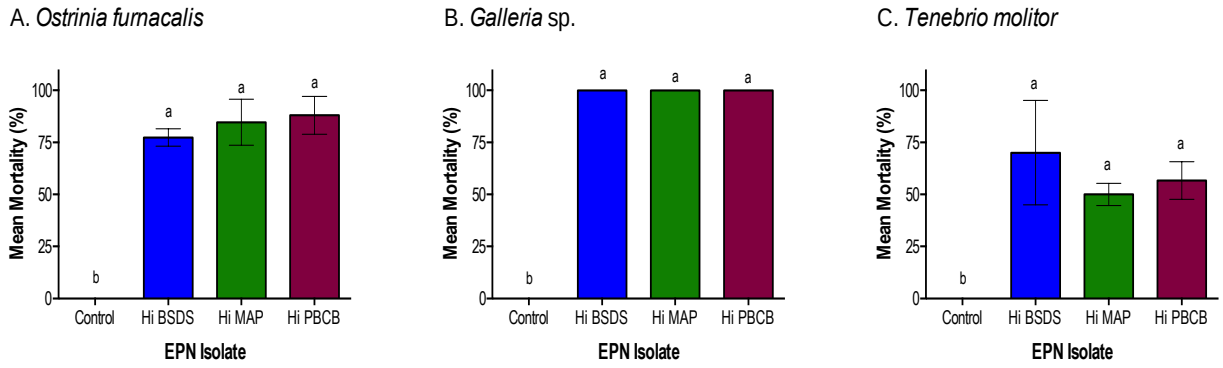


Figure 4. Pathogenicity of the Philippine *Heterorhabiditis indica* isolates from Surigao del sur (Hi BSDS), Palawan (Hi MAP), and *H. indica* Batangas (Hi PBCB) at 24 hours post infection to (A) *Ostrinia furnacalis* Guenee, (B) *Galleria* sp., and (C) *Tenebrio molitor.* The percentage mortality was computed from three independent trials with fifty insects per replicate per treatment per trial. Mean mortality was compared using ANOVA with Holm Sidak's as post-hoc test.

Virulence was measured through mean percentage penetration rate in dead larvae of *O. furnacalis*, *Galleria* sp., and *T. molitor* of the different *H. indica* isolates within 48 HPI. The EPN penetration among the insect species tested was compared to clearly identify to which EPN isolate the test insect was more permissive to infection (**Figure 5**). In general, the penetration rate of the *H. indica* isolates was highest in *Galleria* sp., which is ~50-60 % greater than in *O. furnacalis* and *T. molitor*. Among the isolates, a significantly higher number of IJs of Hi MAP and Hi PBCB penetrated in *Galleria* sp. than Hi BSDS at similar level of mortality at 24 HPI. The three *H. indica* isolates were equally virulent to both *O. furnacalis* and *T. molitor* as indicated by the insignificant difference in the mean percentage penetration.

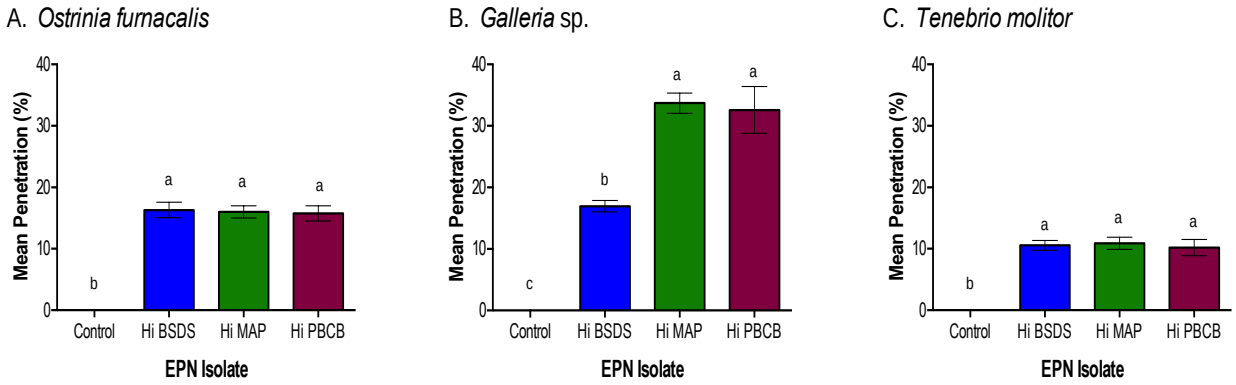


Figure 5. Mean percentage penetration in (A) *Ostrinia furncalis,* (B) *Galleria* sp., and (C) *Tenebrio molitor* of the Philippine *Heterorhabditis indica* isolates from Palawan (Hi MAP), *H. indica* Surigao del sur (Hi BSDS), and *H. indica* Batangas (Hi PBCB).

The virulence of the *H. indica* isolates was also evaluated using median lethal time (LT_{50}) estimates. The LT_{50} of *H. indica* isolates from Palawan (Hi MAP), Surigao del Sur (Hi BSDS), and Batangas (Hi PBCB) in *O. furnacalis* is presented in Table 6. Results indicated that the LT_{50} values observed in *O. furnacalis* infected with Hi BSDS (14.508 H), Hi MAP (16.263 H) and Hi PBCB (13.803 H) had no significant difference due to overlapping fiducial limits. In *Galleria* sp., the LT_{50} values observed in Hi BSDS (11.627 H), Hi MAP (12.197 H) and Hi PBCB (12.104 H) showed a similar trend. For *T. molitor,* LT_{50} estimate of Hi BSDS was the shortest (15.658 H) followed by Hi PBCB (18.611 H) and lastly by Hi MAP (19.755 H). LT_{50} estimates of Hi PBCB and Hi MAP were not significantly different, but both had significantly longer LT_{50} than Hi BSDS.

DISCUSSION

Based on the results of the morphometric analyses of the three *H. indica* isolates, intraspecific differences based on some diagnostic characters can be observed. Relative to the type specimen reported by Poinar et al. (1992), the mean measurements and the reported ranges of the three isolates based on males, amphimictic females and infective juveniles are comparable. Morphometrics of hermaphroditic females were also provided and were in agreement to the type strain. Surprisingly, a lot of striking differences of the Hagonoy isolate (Navarez et al., 2021) from the three *H. indica* isolates and the type specimen can be seen. In general, the Hagonoy isolate appears to be smaller than the type specimen and the three *H. indica* isolates under study. On the other hand, there were also some measurements that appear to be bigger in the Hagonoy isolate. Although some of the values of the Hagonoy isolate were within the size ranges of the type specimen, this isolate shows a higher intraspecific variability as compared to the three Hi isolates under study. Another interesting fact here is the phenotypic divergence of Hi BSDS and the Hagonoy isolate despite their closest geographic distance. The similarities of the three Hi isolates can be attributed to the similar environment they were isolated from. The three Hi isolates were all collected from sandy shores and coastal plains, while the Hagonoy isolate was isolated from silty clay and silty loam soils planted with banana and rice (Navarez et al., 2021). In a similar study done by Morales et al. (2016), all stages of three *H. amazonensis* isolates compared had morphometric differences. In hermaphroditic *H. amazonensis* studied, all measurements were similar except for NR, while in females L, NR, ES, and V % were variable. As for the males, EP, NR, ES, %D and %SW had significant differences. With the IJs, most extensive differences among the three *H. amazonensis* populations were found in the IJ's L, EP, NR, ES, T, the de Mann ratios a, b and c, and the value of %D, %E, and %H.

The mitochondrial gene *COI* has been widely used in many metazoan DNA barcoding studies including *Heterorhabditis* nematodes (Vanlalhlimpuia et al., 2018) because of its easy amplification due to the high copy numbers per cell, its haploid character and its relatively faster evolution than the coding regions of nuclear genes (Lin & Danforth, 2004). In this study, aside from being a barcode marker, the differences on the *COI* sequences among the two groups, although low, somehow demonstrated the utility of *COI* in studying intraspecific variations as compared to ITS.

Despite wide geographical isolation, the similarity in *COI* sequences of Hi BSDS and Hi PBCB possibly reflects low intraspecific variation among these *H. indica* isolates. This is probably a result of restricted gene flow since their only means of transportation are via infected insects, phoresis, or by wind or water, because of their patchy or clumped occurrence due to slow active movement, and their hermaphroditic lifestyle. In *H. marelatus*, this hypothesis was proven by looking at the diversity of its 58 populations based on the *ND4* gene found on

the mitochondrial DNA that codes for a membrane spanning polypeptide of the hydrophobic subunit of *NADH dehydrogenase* gene complex I. It was found out that *H. marelatus* populations from Oregon and California have a very low genetic diversity and very few haplotypes were present. The similarity of haplotypes of *H. marelatus* from these two states are somehow attributed to occasional long-distance migration via ocean currents as this nematode is a coastal species (Blouin et al., 1999). There is a strong possibility that the similarity between Hi BSDS and Hi PBCB is caused by the same phenomenon since both isolates were extracted along the sandy shores. In addition, they probably share the same maternal lineage, while MAP came from a divergent one. In a genetic variation study of different *Heterorhabdtis* species using isozyme profiling, Jagdale et al. (2006) found out that the genetic divergence among the populations of *H. bacteriophora* is relatively independent of geographic distance.

Bioassay results revealed that all the *H. indica* isolates were pathogenic to *O. furnacalis*, *Galleria* sp. and *T. molitor.* The percentage mortality of the test insects, although with different EPN sensitivity levels, did not vary significantly across the three *H. indica* isolates. In terms of rate of penetration, measured through the number of penetrated IJs recovered from dissected insects, only the Hi BSDS-infected *Galleria* showed significantly lower IJ numbers needed as compared to Hi MAP and Hi PBCB. The LT_{50} estimates also showed no significant differences among the isolates when exposed to both *O. furnacalis* and *Galleria* sp. In *T. mollitor*, however, Hi BSDS had significantly faster killing action than the other two isolates.

Caoili & Latina et al. (2018) conducted a study on *S. abbasi*, *S. minutum*, *S. tami*, and *H. indica* against lepidopterous pests of corn namely: *O. furnacalis*, *Spodoptera litura*, and *Helicoverpa armigera* larvae under laboratory conditions. Their results based on LC_{50} showed that *H. indica* PBCB was the most virulent to *S. litura.* The LT_{50} values also showed that *O. furnacalis* was the most susceptible to *H. indica* PBCB and *S. abbasi* MBLB. In the present study, the results of the penetration rate and biological assays indicated that all the three *H. indica* isolates are good candidate the biological control of O. *furnacalis*. However, Hi BSDS isolate, which required the least number of IJs to cause the same level of mortality to *Galleria* sp., and took shorter time to cause 50% mortality in *T. mollitor* appears to be the most virulent among the three.

Intraspecific variability based on pathogenicity and virulence of EPNs have been observed in many researches. In a study done by Zadji et al. (2014), both *H. sonorensis* and *H. indica* isolates were found to exhibit a cruiser type of search strategy and were capable of migrating, infecting and killing workers of *Macrotermes bellicosus* in 20-cm sand columns over a period of 3 days in varying degrees under laboratory conditions. In the same study, only three isolates of *H. sonorensis* caused 100% mortality to *M. bellicosus* at the greatest depth, while only the indigenous *H. indica* showed strong finding ability. In terms

of virulence, interspecific and intraspecific differences on their ability to invade workers of *M. bellicosus* was also observed. After 12 h post exposure, *H. sonorensis* Ze2 and *H. sonorensis* Azohoue2 exhibited the lowest invasion time with IT_{50} = 3.35 and 3.67 h, respectively, and a higher penetration rate (11.4% and 10%, respectively) compared with the other isolates. Another study using *G. mellonella* demonstrated intraspecific variation among *S. feltiae* isolates in terms of application concentration, mortality rate and exposure time. Out of the identified 17 *S. feltiae* isolates, only one population had outstanding potential (Yuksel & Canhilal, 2019). Differences among conspecific *S. feltiae* in terms of virulence were also evident on stored-product pests such as *Sitophilus oryzae* (Laznik et al., 2010), *Tribolium confusum* and *Ephestia kuehniella* (Athanassiou et al., 2008) in laboratory assay studies.

ACKNOWLEDGEMENT

EDR would like to express his sincerest thanks to the Accelerated Science and Technology Human Resource Development Program of the Philippine Department of Science and Technology – Science of Education Institute for the financial support they provided for the conduct of this research.

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