First report of *Steinernema longicaudum* and its bacterial symbionts, *Xenorhabdus* species, in pummelo orchards of Davao region, Philippines

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ABSTRACT

Davao region is the largest pummelo-producing area in the Philippines. Like other tropical fruits, pummelo is infested with several insect pests which consequently lead to the application of synthetic chemicals as deterrent. One potential alternative control measure is the use of soil-dwelling, entomopathogenic nematodes (EPNs), each with its own suite of preferred host, which can be used to control a wide range of insect pests. A survey and soil samples were collected to isolate and identify EPNs in ten pummelo orchards in the Davao region. EPNs were extracted from soil samples using the insect-baiting technique, and were found in only two of the ten sampling areas. EPN isolates were subjected to molecular identification using the 28S ribosomal DNA (rDNA) while its bacterial symbionts were identified using 16S rDNA genes. Both EPN isolates were identified as *Steinernema longicaudum*. This is the first report of *S. longicaudum* isolate in the Philippines which can be used as biological control agent against insect pests of pummelo such as citrus rind borer and flower thrips.

Keywords: entomopathogenic nematodes, pummelo, *Steinernema longicaudum*, *Xenorhabdus ehlersii*, bacterial symbionts

INTRODUCTION

The Philippines is a tropical country with abundant fruits like pummelo (*Citrus maxima*), which is one of the economically important fruits mainly produced by Region XI (Pangan 2007) with a total of 1,748 ha planted producing a total volume of 15,824 metric tons (mt) (Bureau of Agricultural Statistics 2009). Pummelo also has a big potential for export, considering that only Malaysia and Thailand are actively selling the fruit to Singapore, Hong Kong, and other countries. More importantly, competition created by imported China pummelo threatens the small- and big-scale pummelo growers in Mindanao and intensifies the need to produce high-quality fruits. Pummelo production in the Philippines utilized synthetic chemicals to control pests since it is the easiest to apply, most efficient, and cheapest among control methods available. Most of the insect pest problems were linked to insecticide resistance, resurgence, and secondary pest outbreak that result from misuse and overuse of these synthetic chemicals. Increasing public awareness on the importance of food safety coupled with the initiative of the Philippine government through the Republic Act 10068, known as the Organic Act of 2010, have necessitated alternative control measures that are environment-friendly and pose lower risks to human and animals.

One potential alternative control measures is the use of entomo-pathogenic nematodes (EPNs), which are soil-dwelling organisms that attack a wide range of soil-borne insect pests as well as those that occur in cryptic habitats (Hazir *et al*. 2003). EPNs from the families Steinernematidae and Heterorhabditidae have proven to be the most effective biological control organisms (Kaya & Gaugler 1993). EPNs can be used to control wide range of insect pests, including a variety of lepidopteran larvae, scarab beetle larvae, craneflies, thrips, and fungus gnats (Miles *et al*. 2012). One of the common insect pests affecting pummel, for instance, is thrips which often drop to the soil to pupate. Use of EPNs was tested to be effective against western flower thrips (*Frankliniella occidentalis*) where the mortality ranged from 2.6% to 60% (Ebssa *et al*. 2003).

According to Kaya & Koppenhofer (1999) as cited by Miles *et al*. (2012), there are over 80 species of EPNs identified and 11 of them have been successfully commercialized worldwide. EPNs are cultured on a large scale in laboratories and are available from many commercial suppliers.

in North America and Europe making them expensive and inaccessible for the farmers in the Philippines. There are only few researchers in the Philippines to date who focus their research on EPNs and their potential in pest management. This study aims to create a preliminary list of EPNs found in pummelo orchards in Region XI, Philippines to serve as the basis for future studies and to identify collected EPN isolates using molecular techniques.

METHODOLOGY

Collection of soil

Pummelo orchards considered for soil sampling were identified in coordination with the Department of Agriculture offices in the different municipalities in Region XI. Using an auger, soil samples were randomly collected from the identified study areas. Only 200 g of composite soil samples were brought to the laboratory in a plastic bag for baiting. Plastic bags were labeled indicating the date and place of collection.

Extraction of nematodes from soil

EPNs were extracted from the soil following the insect-baiting technique using *Achroia grisella* larvae since *Galleria mellonella*, the most common insect species used in baiting EPN, was not available in the area. Each 200 g soil sample was placed in a plastic box and baited with ten *A. grisella*. Boxes were stored at 25°C for five days, and then transferred to a White trap (Kaya & Stock 1997) to collect infective juviniles (IJs). Collected IJs were placed in a vial containing sterile distilled water ready for shipment to the Stock Laboratory at the University of Arizona, Tucson, AZ, USA where molecular and morphological identifications were conducted. Appropriately labelled vials were stored in a refrigerator until shipment.

Identification of EPNs

Infection was set-up in the Stock Laboratory to collect fresh specimen that were used for identification. This was done by infecting five *G. mellonella* larvae with 1 mL of IJs suspension and incubated for three days

at 25°C. Dead larvae were dissected using M9 buffer. Adult females were collected and stored in Tris EDTA (TE) buffer for molecular identification.

Genomic DNA was extracted from the digestion supernatant using phenol-chloroform enrichment and ethanol/ammonium acetate precipitation (Ausbel *et al*. 1989). The resulting pellet was washed with 70% ethanol, re-suspended in TE buffer (pH 8.0), treated with 50 µg of RNAse A (1 h, 37°C), and DNA was recovered following re-precipitations with ethanol. DNA from phenol-chloroform extracts was quantified by spectrophotometry (NanoDrop 1000 Spectrometer, Thermo Fisher Scientific, DE, USA) with 100–200 ng used per Polymerase Chain Reaction (PCR) reaction.

A region within the 5'-end of the nuclear large-subunit (LSU) ribosomal DNA that included the D2 and D3 domains was amplified using PCR (Stock *et al*. 2001a). PCR conditions, e.g., annealing temperature and $MgCl_2$ concentration, were adjusted empirically to optimize reaction specificity for individual species. Proofreading polymerase (ID Proof, ID Labs Biotechnology, or Finnzymes DyNAzyme EXT, MJ Research, Watertown, MA, USA) was used for PCR amplification (BioRad MyCycler, CA, USA). The PCR reactions include 1.5 µL primer (391: AGCGGAGGAAAAGAAACTAA and 501: TCGGAAGGAACCAGCTACTA), 1.0 µL sample DNA at 100 µg/µL, 13 µL Taq DNA polymerase, and 10 µL water for a total reaction volume of 27 µL. PCR cycling parameters followed the standard protocol of each primer.

Three µL of each PCR amplification was used for agarose gel electrophoresis (Thermo OWL EasyCast B1A, Thermo Fisher Scientific, DE, USA) to confirm product size and yield. PCR products were prepared for direct sequencing using spin-filtration or enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product Presequencing Kit, Amersham Corporation, Piscataway, NJ, USA). For spin-filtration, excess PCR primers and dNTPs were removed by washing the product three times with 0.1× TE buffer (pH 8.0) using a Millipore filter (Ultrafree-MC 30,000 NMWL, Millipore Corporation, Bedford, MA, USA). Sequencing reactions were performed using BigDye (Perkin-Elmer, Norwalk, CT, USA) terminator cycle sequencing chemistry and reaction products were separated and detected using an ABI automated DNA Sequencer. Both 28S and ITS sequences were deposited in GenBank.

Contig assembly and sequence ambiguity resolution were performed with the aid of Geneious v8.0. Sequences of new species/isolates were compared with a library of more than 30 EPN species (Stock *et al*. 2001b, Nadler *et al*. 2006) using Basic Local Alignment Search Tool (BLAST).

Identification of bacterial symbionts of EPNs

Bacterial symbionts of the EPNs were also isolated and identified. A two-day old dead infected larva was surface sterilized by dipping in 95% ethanol and blot-dried using laboratory tissue. The third segment from the mouth parts of dead *G. mellonella* larvae were opened using fine sterile forceps to obtain the hemolymph containing the bacterium. A loopful of hemolymph was transferred and mixed well into 1 mL Luria-Bertani (LB) broth, while another loopful was streaked on nutrient bromothymol blue-triphenyltetrazolium chloride agar (NBTA) plate. The plates and the LB broth were incubated at 29°C overnight. After 24 h, a single colony was transferred to NBTA plates to obtain a pure culture of the bacterium.

Using the pure culture, a single colony was transferred to 1 mL of LB broth and incubated at 29°C overnight. The culture was then used for DNA extraction following the modified Rapid Extraction of Bacterial DNA procedure by Orozco (2009). DNA product was subjected to PCR using 16S Primer (16s Universal F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 16s Universal R: 5'-GGT TAC CTT GTT ACG ACTT-3'). To confirm product size and yield, PCR reactions were run in 1% agarose gel electrophoresis with 1× Tris/borate/EDTA (TBE). PCR products were prepared for direct sequencing using spin-filtration or enzymatic treatment with exonuclease I and shrimp alkaline phosphatase. For spin-filtration, excess PCR primers and dNTPs were removed by washing the product three times with 0.1× TE buffer (pH 8.0) using a Millipore filter. Sequences were analyzed using Geneious v8.0 and sequences were compared with a library of bacterial species using BLAST.

Morphological details

All isolates were propagated *in vivo* with last instar larvae of *G. mellonella* (L.) at 22 + 3°C (Woodring & Kaya 1988). Insect cadavers were dissected on days 3–4 to recover first-generation adults of steinernematids.

For each isolate, 30 specimens from first-generation adults were randomly collected from 10 *G. mellonella* cadavers. Nematodes were examined live or heat-killed in 60°C Ringer's solution. Samples were fixed in triethanolamine formalin (TAF) and processed in anhydrous glycerine for permanent mounting (as reviewed by Stock & Goodrich-Blair 2012). Specimens were mounted on glass slides supported with glass rods to avoid their flattening. Observations of live and mounted specimens were made using a Nikon Eclipse E600 microscope equipped with differential interference contrast optics. Specimen measurements were made using Scion Image software (Frederick, MD, USA) calibrated using a stage micrometer.

RESULTS AND DISCUSSION

A total of 10 pummelo orchards in Davao region, Philippines were included in soil collection (Table 1) based on their locations and history to establish the presence of EPNs. All pummelo orchards were established for more than five years and planted with 'Magallanes' pummelo. Except for orchard 1, which is utilized for academic research purposes, all orchards were operated for fruit production; consequently massive synthetic chemical application was practiced. The most common insect pest problem encountered by these orchards were citrus rind borer (*Prays endolemma*) and a species of flower thrips. Synthetic chemicals were applied on a weekly basis to manage these pests.

Locations were coded as indicated in Table 1. EPNs were found in only two of the ten sampling areas, namely *barangay* (i.e., the smallest political unit in the Philippines) Sta. Filomena, Asuncion, Davao del Norte (Xsf2) covering 1.5 ha of pummel-bearing trees and from a 1-ha orchard in *barangay* Del Pilar, New Corella, Davao del Norte (Xnc4). Between the two isolates, Xsf2 isolates yielded a better infection performance based on the number of cadavers retrieved; however, in terms of development, Xsf2 isolates developed slower than those from Xnc4. Nematodes from larva cadavers infected with Xsf2 isolates dissected after four days were still in juvenile stage 2 with more male than female, while those from Xnc4-infected cadavers after four days were already in juvenile stage 4 with more female than male. The brown coloration of the cadavers suggests that both isolates were *Steinernema* spp.

Table 1. Presence of entomopathogenic nematodes in ten pummelo orchards in Region XI.

Orchard* no.	Location	EPN present	Sample code	Length of deduced gene sequence	Species of the most significant BLAST hit	% Identity	NCBI Accession No.
1	Mabini, Compostela Valley**	–	Xmc1				
2	Sto. Tomas, Davao del Norte	–	Xst1				
3	Sto. Tomas, Davao del Norte	–	Xst2				
4	Sto. Tomas, Davao del Norte	–	Xst3				
5	New Corella, Davao del Norte	+	Xnc4	607	*Steinernema longicaudum*	100%	GU569054
6	Asuncion, Davao del Norte	+	Xsf2	607	*Steinernema longicaudum*	99%	GU569054
7	Asuncion, Davao del Norte	–	Xsf1				
8	Panabo, Davao del Norte	–	Xpd1				
9	Calinan, Davao City	–	Xcd1				
10	Calinan, Davao City	–	Xcd1				

*The orchards' identity remain confidential as requested by the owners.
**Pummelo orchard of the University of Southeastern Philippines

Morphological characteristics were typical of the *S. longicaudum* species (Figures 1 and 2), the body curved posteriorly forming a C- or J-shape when heat killed as previously described by Stock *et al.* (2001b). First generation females and males of *S. longicaudum* were measured (Tables 2 and 3, respectively), with an average female body length of 7,702.2 ± 1,548.7 µm while males measured an average of 1,915.1 ± 142.4 µm.

Table 2. Morphometrics of the first generation females of *Steinernema longicaudum* Davao
strain, *n* = 25

Character	Range (µm)	Average (µm)
Body length	10,115.1–4,188.4	7,702.2 ± 1,548.7
Greatest body width	408.2–200.8	287.6 ± 53.9
Anterior end to excretory pore	237.9–94.5	156.6 ± 29.2
Anterior end to nerve ring	282.2–108.1	156.6 ± 29.2
Anterior end to oesophagus	414.3–220.3	269.3 ± 48.0
Body width to anus	189.1–112.4	146.3 ± 18.0
Tail length	136.2–69.8	94.6 ± 17.5
Anterior end to vulva	5,136.0–2,063.3	3,982.4 ± 855.9
V%	56.1–48.0	51.6 ± 2.6
D%	79.3–38.4	58.6 ± 9.3
E%	233.2–111.4	166.7 ± 30.0

V%, is the distance from the anterior end to vulva divided by body length × 100%.
D%, is the distance from anterior end to excretory pore divided by oesophagus length ×
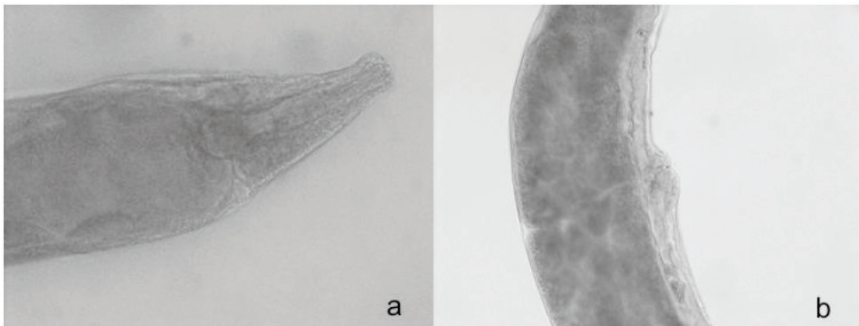100%.

Figure 1. *Steinernema longicaudum* Davao strain female under microscope; (a) anterior part showing the esophagus, nerve ring and excretory pore opening, (b) vulva (500×).

Molecular identification of EPN isolates

Despite differences between the two isolates based on the number of days to infection and the stage of nematode recovered upon dissection four days after infection, molecular identification revealed that the two isolates were both *S. longicaudum*. After analysis of the LSU of rDNA of the Xnc4 and Xsf2 isolates, the length of the sequence of the consensus obtained

Table 3. Morphometrics of the first generation males of *Steinernema longicaudum* Davao strain, *n* = 20

| Character	Range (µm)	Average (µm)
Body length	2,135.0–1,663.5	1,915.1 ± 142.4
Greatest body width	102.6–68.2	81.7 ± 10.5
Anterior end to excretory pore	157.0–101.1	129.7 ± 21.3
Anterior end to nerve ring	137.4–91.1	117.3 ± 14.6
Anterior end to oesophagus	184.6–128.7	165.2 ±16.6
Testes reflexion	455.7–203.6	311.9 ± 67.6
Tail length	40.4–30.7	34.8 ± 3.0
Body width at cloaca	50.5–33.9	43.9 ± 4.8
Spicule length	98.0–69.4	43.9 ± 4.8
Gubernaculum length	69.4–47.4	60.7 ± 6.7
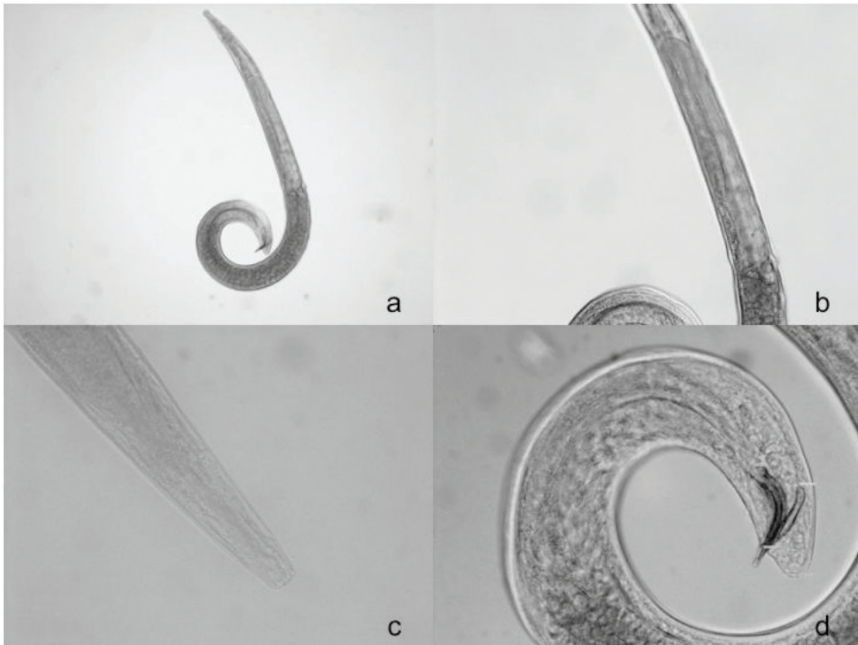
Figure 2. *Steinernema longicaudum* Davao strain male under microscope; (a) Male nematode showing the total body length (100×); (b) restes reflex TREF (200×); (c) anterior part (500×); (d) posterior part showing the tail, spicule and gubernaculum (500×)

were both 607 nt. Sequences were submitted to the National Center for Biotechnology Information (NBCI) database to be deposited with NBCI submission codes SUB2580156 and SUB2580164, for the *S. longicaudum* Xsf2 Davao strain and *S. longicaudum* Xnc4 Davao strain, respectively. Dissimilarity between the two isolates in terms of days to infection and the stage of nematode recovered could be attributed to the number of bacterial symbiont cells carried by the nematodes which affect their virulence. The number of bacterial cells carried by the nematodes differed dramatically affecting the type of interaction between symbiotic *Xenorhabdus* and *Steinernema* (Sicard *et al*. 2003). It appears that Xsf2 isolate carried more of this bacterium compared to the Xnc4 isolate.

S. longicaudum has been isolated and described first in China, and subsequently isolated in Australia (Hominick *et al*. 1996), California, USA (Stock *et al*. 1999) and South Korea (Stock *et al*. 2001a), with the Californian populations eventually being not conspecific with *S. longicaudum* (Stock *et al*. 2001b). This is the first record of *S. longicaudum* in the Philippines and first EPN isolates collected from pummelo orchards.

Molecular identification of bacterial symbionts

Similarly, bacterial symbionts of the two EPN isolates were identified. BLAST results suggest 95% and 94% similarities as *Xenorhabdus ehlersii* for both Xnc4 and Xsf2 bacterial symbiont isolates, respectively, thus the identification is inconclusive and further investigation is suggested. Interestingly, a different bacterial symbiont was isolated from *S. longicaudum* collected in Korea identified as *X. beddingii*, while *X. ehlersii* from Southern China was isolated from *Steinernema serratum* (Lengyel et al. 2005). These reports suggest the diversity of EPNs and bacterial symbionts association within Asia. Bacteria under the genus *Xenorhabdus* has been mutualistically associated with families of EPNs Steinernematidae and Heterorhabditidae. In this symbiotic relationship, the bacteria are pathogenic to the insect host when released into its haemolymph by the nematodes while also supporting nematode reproduction by producing nutrients and antimicrobial agents that inhibit the growth of a wide range of organisms (Akhurst & Boemare 1988).

CONCLUSION

S. longicaudum was isolated in two commercial orchards where synthetic chemicals were heavily applied for more than five years to control insect pests such as citrus rind borer (*Prays endolemma*) and flower thrips. This illustrates that despite long exposure to insecticide, *S. longicaudum* can survive and thrive in the soil. The potential of its use as a biological control agent can be explored as an alternative management of common insect pests of pummelo. Studies on its pathogenicity to citrus rind borer, flower thrips, and other insect pests and survivability in the environment must be conducted.

ACKNOWLEDGEMENT

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