Occurrence and Distribution of Entomopathogenic Nematodes in Termite Prone Areas of Eastern and Western Ethiopia

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Abstract

Entomopathogenic nematodes (EPNs) are simple roundworms that naturally kill insects and are abundantly present in the soil. A survey of EPNs was conducted in selected agroecologies of eastern and western Ethiopia with termite problems aiming to assess the species' diversity and distribution. From a total of 80 soil samples collected, 20% of them were positive for EPNs. They were found in all of the habitats where the study was conducted, but they were more abundant in soil samples collected from tree litters than in any others. Molecular analysis of the recovered EPNs of the internal transcribed spacer ITS region and D2\D3 expansion segments of the 28SrRNA gene were conducted. The results of the molecular analysis revealed the presence of three types of PNs viz., *Oscheius tipulae*, *Heterorhabditis indica and Caenorhabditis briggsae*. Among the recovered nematodes, *Oscheius* spp. are obtained from eight, *H. indica* from six, and *C. briggsae from two soil samples. Oscheius spp.* belong to the group of insectivores whereas *Heterorhabditis* belongs to the obligate parasite group of nematodes. All the three species recorded in the study were reported for the first time from Ethiopia. Further surveys should be conducted in the future to ensure the untouched sites of the country.

Keywords: *Caenorhabditis,* Ethiopia, *Heterorhabditis,* ITS and D2D3, Phylogenetic analysis, *Oscheius*

Introduction

Nematodes are roundworms that belong to the Phylum Nematoda (Kaya and Gaugler, 1993). They are the most abundant organisms on earth, with their numbers thought to exceed 1 million per m^2 (Floyd *et al.*, 2002). The term entomopathogenic usually refers to microorganisms and viruses capable of causing disease in insect's host (Onstad *et al.*, 2006). Entomopathogenic nematodes quickly kill their host with the help of their symbiotic bacteria and pass on the associated bacteria to the host (Dillman *et al.*, 2012). According to Grewal *et al.* (2005), around 30 nematode families were recognized as either parasite to insects or live in association with insects. Even though, Steinernematidae and Heterorhabditidae (in association with their symbiotic bacteria in the genera *Xenrorhabdus* and *Photorahabdus*) are considered the most effective in their biocontrol potential against soil insect pests (Lacey *et al.*, 2015).

The population of EPNs is influenced by the insect's age and habitat such as soil type,

pesticide use, agricultural practices, and location (Mietkiewski *et al.*, 1997; Chidawanyika *et al.*, 2012; Usta, 2013). Globally multiple surveys have been conducted in search of EPNs to obtain new resources for the biological control of insect pests (Bhat *et al.*, 2020). Several decades of applied research and hundreds of surveys have revealed that EPNs are ubiquitous, as they have been found in a wide range of ecologically diverse soil habitats, including cultivated fields, woodlands, grasslands, deserts, and even ocean beaches of every continent except Antarctica (Griffin *et al.*, 1990; Tarasco *et al.*, 2015). More than 100 valid species of EPNs in the genus *Steinernema* and 21 species in *the Heterorhabditis* genus have been reported from different countries (Bhat *et al.*, 2020). Ethiopia covers an area of over 1.1 million km^2 with a variety of agro-ecological conditions, ranging from arid and semiarid to rainforest; and from 110 meters below sea level to highlands with altitudes of over 4500 meters above sea level. It is estimated that 16 million ha of land is cultivated, and 20 million ha are permanent pastures. Its complex topography and wide altitudinal variations also ensure a variety of temperature and rainfall patterns (FAO, 2016).

Termites cause a significant yield loss in eastern and western Ethiopia (Abdurahman *et al.*, 2010) affecting mainly maize (Demissie *et al.*, 2019), teff, sorghum, barley, sugarcane, and pepper (Dako, 2004), groundnut (Chala *et al.*, 2014), and several species of tree seedlings (Debelo and Degaga, 2017). Different termite management practices are available including chemical (Gentz, 2009), cultural (Demissie *et al.*, 2019) and biological (Grace, 1997) This study would provide information regarding the diversity and abundance of EPNs in eastern and western Ethiopia. Knowing the species diversity will help to select virulent strains among these species and utilize them for biological control of soil insect pests including termites in the future. Moreover, the best performing EPN isolates can also be mass-produced and commercialized for farmers and growers who are suffering from soil insect pests mainly termites. Therefore, the main objective of the study was to assess the species diversity and distribution of entomopathogenic nematodes in selected agro-ecologies of termite problematic areas of Ethiopia.

Materials and Methods

Site description and sampling procedures

The study was conducted in eastern and western Ethiopia during the 2019/20 cropping season. Survey sites were selected based on the presence of termites in the area and on their accessibility and proximity to the road. A total of 80 survey sites were selected and similar numbers of composite soil samples were collected. The survey sites were located in eastern Ethiopia (40 sites) and western Ethiopia (40 sites) (Figure 1). Samples were randomly collected at 3 to 5 km intervals (Table 1).

From each of the survey sites, five random sub samples at 10 meters intervals were taken at a depth of 15–20 cm (Abate *et al.*, 2018) and the sub samples are then mixed to make 1 kg of composite soil samples and were placed in polyethylene bags to prevent water loss. After each sampling, collecting materials such as a shovel, tubular soil sampler, and measuring tape were cleaned and disinfected with 70% ethanol and 0.5% bleach solution to prevent contamination of the samples. The collected samples were properly labeled and kept in an icebox (Abate *et al.*, 2018) and samples collected from western Ethiopia were transported to Ambo Agri. Research Center Plant

Protection Laboratory and those samples collected from eastern Ethiopia were transported to Haramaya University Plant Protection Laboratory. Insects found dead at the sampling sites were also collected during the survey to check the presence of nematodes. The dead insects were surface sterilized using 70% ethanol and rinsed with sterilized distil water. The insects then were place in the 90cm Petri dish on moist filter paper. Nematode emergence was checked for 10 days.

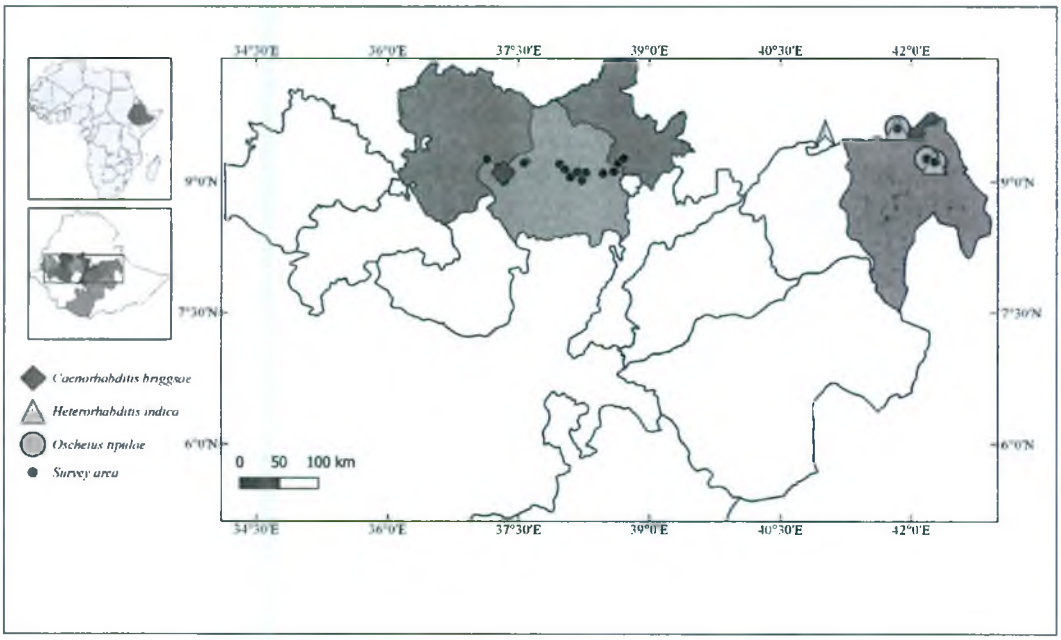

Figure 1. Map showing surveyed areas of entomopathogenic nematodes in eastern and western Ethiopia.

Nematode extraction

The collected soil samples were transferred to a 2-liter glass jar in the laboratory. Each jar was infested with 20 last instars larvae of *Galleria mellonella* (Lepidoptera, Galleriidae) and placed in a dark area to bait the nematodes. Dead *G. mellonella* larvae were collected daily starting from the fourth day and placed in a moist White trap at 24 °C in the dark (White, 1927). After the dead larvae placed in to the White trap, infective juveniles (IJs) baited from the soil samples were collected at the emergence day from the dead *G. mellonella* larvae.

Each dead insect collected from the field was surface sterilized with 70% ethanol and placed on a filter paper in a different Petri dish. The Petri dish which contains the dead insects then placed on a bigger Petri dish having tap water and the bigger Petri dish was covered with a shallow transparent lid, resembling a slightly wider version of the dish itself. After 10–25 days, the IJs were collected, and new cultures were obtained using *G. mellonella* larvae (White, 1927; Orozco *et al.*, 2014). Sample specimens of the recovered nematodes were sent to Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium for molecular analysis.

Table 1. Geographic coordinates of soil samples positive to EPNs.

Sample number	Site name	Admirative zone	Sampling zones	GPS coordinates	Altitude (m.a.s.l.)	Habitat
8	Bako	West Wollega	Western Ethiopia	09°05'66" N 037°08'70"E	1752	Farm soil
19	Bako	West Wollega	Western Ethiopia	08°59'89" N 037°19'97"E	1752	Farm soil (Maize)
41	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'57"N 41°50'19"E	1152	Tree litter
43	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'58"N 41°50'19"E	1152	Tree litter
50	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'43"N 41°50'28"E	1152	Tree litter
52	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'59"N 41°50'20"E	1152	Tree litter
58	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'59"N 41°50'17"E	1152	Tree litter
59	Dire Dawa	Dire Dawa	Eastern Ethiopia	9°36'43"N 41°50'27"E	1152	Tree litter
67	Babile	East Hararghe	Eastern Ethiopia	9°13'49"N 42°15'44"E	1328	Farm soil (Groundnut)
68	Babile	East Hararghe	Eastern Ethiopia	9°13'49"N 42°15'45"E	1328	Farm soil (Groundnut)
69	Babile	East Hararghe	Eastern Ethiopia	9°13'50"N 42°15'44"E	1328	Farm soil (Groundnut)
70	Babile	East Hararghe	Eastern Ethiopia	9°13'50"N 42°15'45"E	1328	Tree litter
71	Babile	East Hararghe	Eastern Ethiopia	9°13'50"N 42°15'43"E	1328	Tree litter
72	Babile	East Hararghe	Eastern Ethiopia	9°13'52"N 42°15'46"E	1328	Tree litter
73	Babile	East Hararghe	Eastern Ethiopia	9°13'50"N 42°15'50"E	1328	Tree litter
74	Babile	East Hararghe	Eastern Ethiopia	9°13'51"N 42°15'49"E	1328	Tree litter
76	Harar	Harar	Eastern Ethiopia	9°14'29"N 42°14'50"E	1319	Tree litter

Morphological characterizations of EPNs

Microscope observations were made on live and mounted specimens of IJs for morphological identification of the recovered isolates. For morphological analysis 25 IJs for each species were randomly selected from different *G. mellonella* cadavers for microscopic observation. Specimens were examined with a Leica DM2500 compound microscope with interference contrast of up to 1000× magnification. Characters such as presence of cheilorhabdions, pharynx, nerve ring and other parts were observed (Lam and Webster, 1971; Stock and Kaya, 1996; Stock *et al.*, 1999). Morphological identification was made at Ambo plant protection research laboratory.

Molecular characterization of EPN

DNA extraction, amplification, and sequencing

DNA was extracted according to the manufacturer's instructions of the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). An rDNA fragment containing the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA gene, was amplified by PCR using the forward primer TW81 5'-GTTTCCGTAGGTGAACCTGC-3' and the reverse primer AB28 5'-ATATGCTTAAGTTCAGCGGGT- 3' (Joyce *et al.*, 1994). A second rDNA fragment containing the D2D3 expansion segment of the 28S rRNA gene was amplified using the forward primer D2A 5'-ACAAGTACCGTGAGGGAAAGTTG-3' and the reverse primer D3B 5'-TCGGAAGGAACCAGCTACTA-3'(De Ley et al., 1999). The master mix contained 12.5 µL of the 2X KAPA HiFi HotStart ReadyMix (KAPA biosystems, Boston, MA, USA), 0.3 µM of each primer, 1 µL DNA extract, and MilliQ water up to a volume of 25 µL. The temperature profile was as follows: 95 °C for 3 min; 30 cycles of 98 °C for 20, 45 and 55 °C, respectively for 15 s, 72 °C for 30 s; and a final step of 72 °C for 2 min.

After electrophoresis of 5 µl PCR product in a 1.5% TAE buffered agarose gel (1 h, 100 V) containing Midori Green Advance DNA stain (0.008%; Nippon Genetics Europe GmbH, Düren, Germany), the gel was photographed under UV light (Figure 2). In case of a positive result, the remainder of the PCR product was purified after electrophoresis in a 1% TAE buffered agarose gel (1 h, 100 V) following the instructions included in the Smartpure Gel DNA Purification Kit (Eurogentec, Seraing, Belgium).

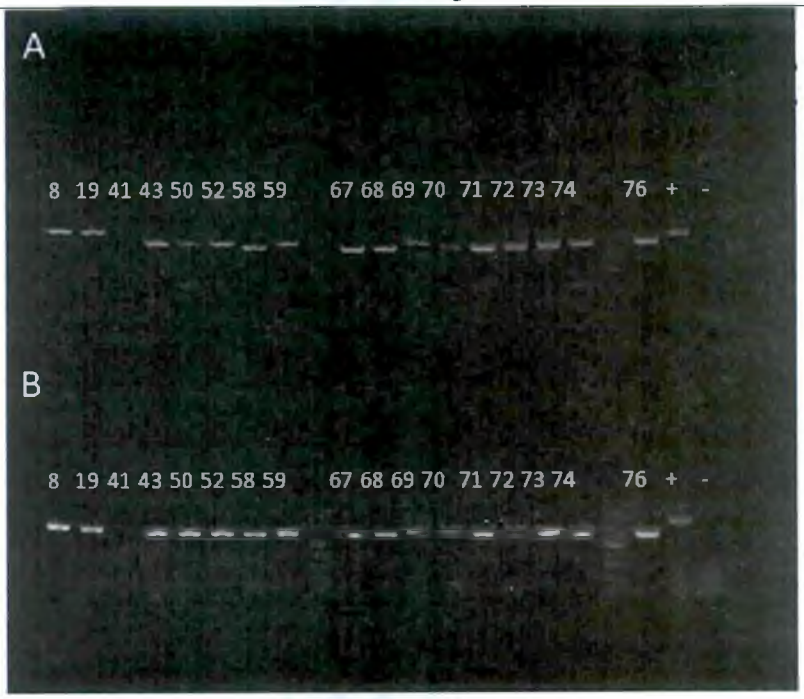

Figure 2. Amplicons visualized on agarose gel. ITS-region (A), D2D3-region (B). 100bp ladder included in duplicate. + = Globodera pallida, - = NTC.

Subsequently, concentrations of the purified PCR products were measured using a UV spectrophotometer (Nanodrop ND-1000, Isogen Life Sciences, Sint-Pieters-Leeuw, Belgium). Approximately 50ng/µl of the purified amplicons were used to sequence the amplicons in both directions to obtain overlapping sequences of the forward and reverse DNA strand (Macrogen Inc, Seoul, Korea). Finally, the sequences were visualized, edited, and analyzed using BioNumerics 7.6 (http://www.applied-maths.com/). Two DNA regions were sequenced: ITS using TW81/AB28 primers and D2D3 of the 28SrRNA gene using D2a/D3b primers. *Globodera pallida* were used as a positive control. NTC = No Template Control (negative control, no DNA). Sequences were analyzed by BioNumerics 7.6. The obtained sequences were blasted against the Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), to compare the similarity of the obtained sequence with a sequence in Genbank.

Phylogenetic analysis

The evolutionary history of the isolates was inferred using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The NJ analyses were carried out using MEGA 7 (Tamiru *et al.*, 2012). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) was used (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and were in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter

= 1). This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 1130 positions in the final dataset.

Soil texture classes analysis

After nematode isolation was completed, soil texture classes of the soil samples were analyzed. Any stone, plant root and dead insect cadavers were removed from the soil samples. The soil was then transferred to a one liter jar of which three quarter of the jar was filled with the soil sample and water was then added to fill the jar leaving some air space for mixing. The jar was then well shacked by hand until all the soil particles were separated from each other. The jar was then set for one minute to allow the sand to settle, one hour for the clay and 24 hours for the silt to settle. In each time, a line was made using a marker on the jar on the top of each layer. The distance between each layer is measured and calculated as percentage. To determine the type of soil texture, the calculated values of percent sand, percent silt, and percent clay were compared against the standard soil texture triangle (Shirazi and Boersma, 1984).

Results

The species of *O. tipulae* was morphologically characterized with the following combination of characters: females were observed and characters examined include: Vulva slightly protruding, located posterior to middle part of body. Gymnostom longer than cheilostom, having well cuticularized lumen. Lip region continuous with body contour, having six rounded lips, bearing small papillae. Basal bulb ovoid, with valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Body length slightly curved ventrad after fixation The Morphological characters of *O. tipulae* species was further cross-checked with the descriptions provided by Lam and Webster (1971) (Lam and Webster, 1971) (Figure 3).

Figure 3. Female *O. tipulae* (Lam and Webster, 1971).

The morphological observation was also done for *H. indica* samples. Third-stage IJs of these *H. indica* isolates obtained within seven days of emergence had cross-checked with the morphological characters listed by Poinar *et al.* (1992) (Figure 4) which include: adults head truncate to slightly rounded; six distinct protruding lips surround mouth opening: head with six inner lip papillae and ten outer labial and submedial papillae. Cheilorhabdions present as a refractile ring just below the lips. The remaining meso-meta and telorhabdions are fused and enclosed by the anterior portion of the pharynx. The pharynx is cylindroid, lacks a distinct metacorpus but contains an isthmus and pronounced basal bulb containing some fine striations in the valve area. Nerve ring distinct, located in the middle of the isthmus in females but near the basal bulb in the males. The infective juveniles' lips contain a large dorsal tooth and the hemizonid is quite distinct and located just anterior to the excretory pore.

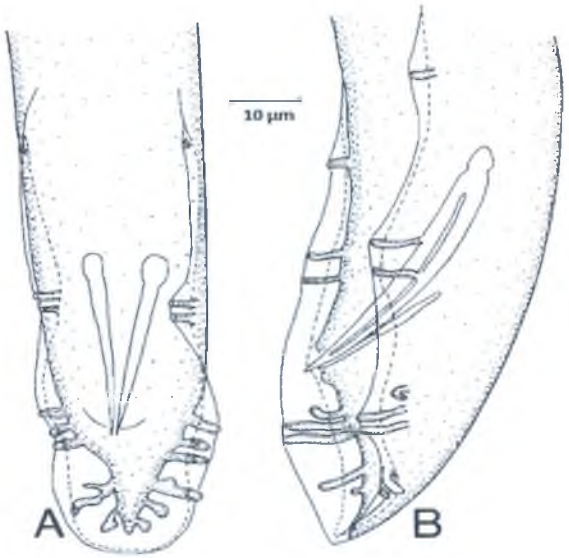

Figure 4. *H. indica*. Ventral view of male tail (A), lateral view of male tail (B) (Poinar *et al*., 1992)

When the sequences were compared with those in the GenBank through a Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI), sample 76 matches 100% with *Oscheius tipulae the remaining samples* (sample 43, 50, 52, 59, 69, 73, 74, and 76) *were further morphologically identified and found O. tipulae*. The second species isolated were *H. indica* observed in six samples (sample 58, 67, 68, 70, 71, and 72) were positive. The remaining two positive samples (8 and 9) belong in the genus *Caenorhabditis* and were 100% similar to *Caenorhabditis briggsae* with the sequence in the GenBank and because of this reason morphological identification was not made for *C. briggsae*.

ITS analysis from the PCR fragments of nematodes baited using *G. mellonella* from the soil samples (58, 67, 68, and 71) showed that the sequences of the nematodes were almost identical, which suggests that they all belong to the same species, *H. indica also form the same clade on phylogenetic tree (Figure 5);* but sequence analysis of the D2D3-region of theses nematodes showed that they are similar with *Rhabditis rainia*. From the total of 80 soil samples collected during this study, 16 (20%) were positive for EPNs. Most of the positive soil samples were classified as sandy loam (35%), sandy clay loam (24%), and loamy sand (29%) (Table 2). The EPNs recovered belong to three genera of nematodes including *Oscheius*, *Heterorhabditis and Caenorhabditis*. Among this, the genus *Oscheius* was the most abundant (50%). Nematodes were not recovered from those dead insect samples.

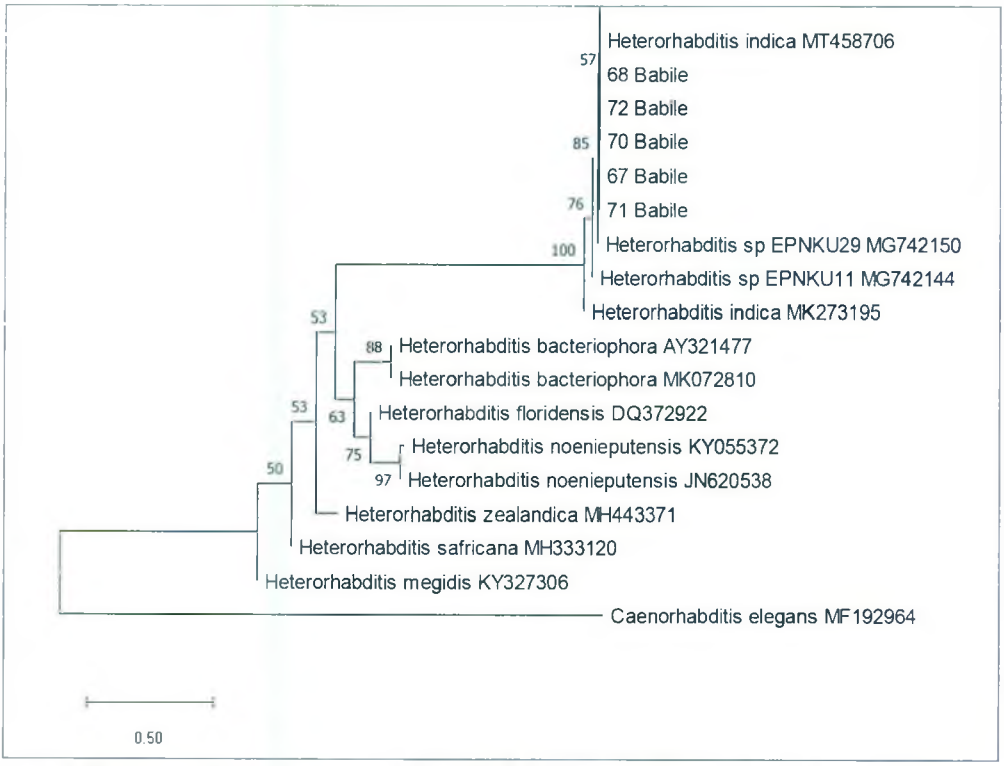

Figure 5. Phylogenetic identification of *Hetrorhabditis* indica isolates based on their ITS locus.

Table 2. Location and soil texture classes of entomopathogenic nematode positive soil samples assessed in different regions of Ethiopia.

Isolate number	Site name	Soil proportion			Soil texture class
		Clay	Silt	Sand	
8	Bako	5	20	75	Sandy loam
19	Bako	6	14	80	Loamy sand
41	Dire Dawa	10	36	54	Sandy loam
43	Dire Dawa	29	14	57	Sandy clay loam
50	Dire Dawa	33	15	51	Sandy clay loam
52	Dire Dawa	27	16	57	Sandy clay loam
58	Dire Dawa	9	7	84	Loamy sand
59	Dire Dawa	30	10	60	Sandy clay loam
67	Babile	11	8	81	Sandy loam
68	Babile	15	10	75	Sandy loam
69	Babile	8	5	87	Loamy sand
70	Babile	8	3	89	Loamy sand
71	Babile	11	2	87	Loamy sand
72	Babile	8	3	90	Sandy
73	Babile	11	9	80	Sandy loam
74	Babile	33	33	33	Clay loam
76	Harar	18	22	60	Sandy loam

Discussion

The current study recorded three species of nematodes. These species were reported for the first time from Ethiopia. Previous surveys undertaken in Ethiopia on entomopathogenic nematodes revealed the presence of two nematode genera: *Steinernema* and *Heterorhabditis* (Nguyen *et al.*, 2004; Mekete *et al.*, 2005; Tamiru *et al.*, 2012). Ethiopia is rich in faunal, floral, and microbial diversity within its extraordinary number of the world's broad ecological zones and altitude range. The survey covered midland to highland regions. Accordingly, positive soil samples were analyzed with additional information collected, namely altitude, and soil type.

Entomopathogenic nematodes were detected in 20% of the samples collected. This rate is a better recovering rate as compared to Mekete *et al.* (2005), in which only 6.9% out of the total collected samples (288) were positive with EPNs in the genera *Steinernema* and *Heterorhabditis*. In the current study, three species of nematodes were isolated: *Oscheius tipulae* (50%), *H. indica* (37.5%), and *C. briggsae* (12.5%) out of the positive soil samples. Entomopathogenic nematodes were well recovered from sandy loam soil at a good rate (35%) followed by loamy sandy (29%) and sandy clay loam (24%). However, small proportions (6%) of EPNs were recovered from each sandy and clay loam soils. The result of this finding is similar to Choo *et al.* (1995) who reported more positive samples from sandy loam soils. Although dead insects were collected and investigated, nematode-infected insect were not found during the survey. Our result is similar to those by Griffin *et al.* (1991) and Choo *et al.* (1995) who reported the difficulty of finding nematode infected insects in the field.

Sequences from the D2D3 segment of the 28S rRNA showed that samples 58, 67, 68, 70, 71, and 72 are *Rhabditis rainai;* however, the result of the morphological study didn't support this. Even if, *R.rainai* is a parasitic nematode which lives in association with termite gut in the family Rhinotermitidae (Carta and Osbrink, 2005) the morphological characterization categorizes the species as *H. indica.* The result of the morphological study was supported by the known association of each nematode with its host or termite species. During the collection of the soil samples, termites were also collected from the same place and molecular identification of termite species was made in a separate study. Consequently, all termite species collected from these areas belong to the family Termitidae and none from Rhinotermitidae (unpublished data). This indicates that the nematodes are not *R. rainai* rather they are *H. indica. Moreover, G. mellonella* larvae infected with these nematode samples have shown a red color which is a typical characteristic of the genus *Heterorhabditis* due to the associated bacteria *Photorhabdus* (Kaya and Gaugler, 1993). The ITS regions are best regions for some nematode species than D2D3 regions. In a study conducted on *Pratylenchus* species, the ITS regions were the better regions for selecting nucleotide base sequences (Andrew *et al.*, 2004).

This study revealed that all the isolated nematodes, *O. tipulae, H. indica,* and *C. briggsae* are reported for the first time in Ethiopia. Previous surveys conducted in the country have reported three nematode spp: *Heterorhabditis bacteriophora* (Nguyen *et al.*, 2004; Mekete *et al.*, 2005), *Steinernema ethiopiense* (Tamiru *et al.*, 2012) from western Ethiopia and *Steinernema yirgalemense* (Nguyen *et al.*, 2004) from southern Ethiopia. A new form of EPN are being reported from the family Rhabditidae

including *Caenorhabditis briggsae* (Abebe *et al.*, 2010; Dillman *et al.*, 2012), *Oscheius chongmingensis* (Dillman *et al.*, 2012), *O. carolinensi* (Weimin *et al.*, 2010; Dillman *et al.*, 2012), *O. microvilli* (Zhou *et al.*, 2017) and *O. onirici* (Torrini *et al.*, 2015). *Oscheius* spp. carry symbiotic bacterial strains of *Serratia* in both the intestine and the cuticle (Kaya and Gaugler, 1993; Zhou *et al.*, 2017). Soil texture is one of the most important factors which can affect the nematodes movement inside the subterranean environment (Georgis and Poinar, 1982; Kaya and Entomol, 1991; Alekseev *et al.*, 2006). Studies revealed that coarser-textured soils are much preferred by the nematodes (Fourie *et al.*, 2017).

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