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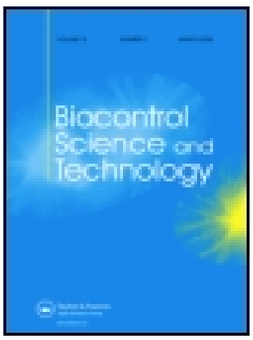

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RESEARCH ARTICLE

Occurrence and distribution of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in Morocco

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ABSTRACT

A survey of entomopathogenic nematodes (EPN) was conducted in Morocco during 2015 and 2016 in three regions with varying topography, climate and vegetation (Meknès-El Hajeb, Ifrane and Tafilalet). EPN were isolated from 14 of 169 soil samples (8%). Molecular and morphological techniques were used for species determination. Nine samples contained heterorhabditids and five samples contained steinernematids. Eight heterorhabditids were identified as *Heterorhabditis bacteriophora* Poinar 1976, two steinernematids as *Steinernema feltiae* (Filipjev, 1934), three as belonging to the *S. feltiae*-group, and one as an unidentified isolate (*Heterorhabditis* sp.) which gives dark burgundy colour for *G. mellonella* cadavers. These EPNs were collected mainly from agricultural soils rather than natural habitats, with potential adaptation to specific geography, climate and soil conditions (texture, pH and organic matter content), including six EPN isolates from heavy soils. These data demonstrate that EPN are present in Moroccan soils but at relatively low frequency, so opportunity exists to augment these populations to gain biological control benefits.

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Biodiversity; distribution; entomopathogenic nematodes; *Heterorhabditis*; *Steinernema*; Morocco

Introduction

Entomopathogenic nematodes (EPN) in the Heterorhabditidae and Steinernematidae are potentially lethal pathogens of hundreds of insect species (Georgis et al., 2006; Grewal et al., 2001). In these families, *Steinernema* and *Heterorhabditis* spp. are known to have symbiotic bacteria to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare et al., 1993), and are thus promising candidates for biocontrol of insect pests, given their ability to infest insects in cryptic habitats, their high reproductive potential, and safety to non-target organisms, humans and the environment (Koppenhöfer et al.,

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2012). EPN can be mass cultured and formulated as biopesticides for use against insect pests in agriculture and forestry (Campos-Herrera, 2015) including *Diaprepes abbreviates* (L., 1758), *Thrips* spp. and several other dipteran and lepidopteran larvae (Georgis et al., 2006; Kaya & Gaugler, 1993).

EPN are found worldwide, with over 95 species of *Steinernema* and 16 species of *Heterorhabditis* currently described (Hunt & Nguyen, 2016). The detection and identification of resident EPN is of great potential value, as these populations will have virulence and environmental adaptation enabling survival and reproduction under local conditions (Stock, 2009). Thus investigation of resident EPN is receiving increased attention, with surveys conducted in many areas around the world showing widespread distribution and local diversity (Hominick, 2002). Surveys conducted in Mediterranean countries, including in Israel (Glazer et al., 1991), Italy (Triggiani & Tarasco, 2000), Turkey (Hazir et al., 2003), Spain (Campos-Herrera et al., 2007) and Egypt (Shamseldean & Abd-Elgawad, 1994), have found species and genetic diversity.

In North Africa, surveys of EPN have been limited with few studies reported from Morocco. Akalach and Wright (1995) evaluated the entomopathogenic activity of two exogenous EPN (*Steinernema carpocapsae* and *S. feltiae*) (commercial products from USA) against larvae of the beet weevil *Conorhynchus mendicus* (Coleoptera: Curculionidae) in the Gharb area. Recently, Benseddik et al. (2018) reported the presence of five indigenous EPN isolates during a preliminary study carried out in 2008 in four sites, *i.e.* National School of Agriculture (ENAM) experimental farm, Aït Yazem, Haj Keddor, and Sidi Adi, which were identified based on coloration of *Galleria* dead larvae (red for *Heterorhabditis* and brown for *Steinernema*). Furthermore, a study conducted in Germany by Premachandra et al. (2003) investigated the efficacy of nine EPN strains, one of which is a Moroccan isolate (*Steinernema* sp.), against soil-dwelling stages of the western flower thrips *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae). Based on these previous preliminary works, the field of EPN seems to be very interesting, but, is not fully understood. For this reason, more deeper and enlarged studies are needed to fill this existing gap in this field and to allow the identification of more indigenous species from Morocco. The natural distribution of EPN depends on precipitation and temperature, and is closely related to natural habitats, vegetation and presence of insect hosts. Campos-Herrera et al. (2011) considered that soil type and texture are particularly important parameters that influence EPN distribution. In Morocco, the diversity of climates (Mediterranean, oceanic, continental, alpine, semiarid and arid) and landforms (fertile coastal plains, plateaus, mountains and deserts), gives the country great biodiversity in both flora and fauna (Verner et al., 2018). After Turkey, Morocco is the second most biodiverse country in the Mediterranean basin, and likewise has diverse agricultural activities (e.g. cereals, fruit farming, vegetable crops and oasis agriculture), and may therefore also have a diverse EPN fauna (Verner et al., 2018). To the best of our knowledge, Moroccan EPN have only been identified based on the coloration of *G. mollenela* hence, there is no reports available on the diversity of their genetics (Benseddik et al., 2018). For this reason, we conducted this extended survey to explore local EPN in Morocco for use against local insect pests as a viable eco-friendly alternative to chemical control, especially as the country envisages increasing ecologically based agricultural production.

The objectives of this study were (1) to survey EPN from the families Heterorhabditidae and Steinernematidae in Morocco and (2) to identify their species, habitat and soil type associations for possible use of EPN in biocontrol of local soil-dwelling insect pests.

Material and methods

Survey regions and soil sampling

Soil samples were collected in 2015 and 2016 in three regions of Morocco: Meknès-El Hajeb, Ifrane and Tafilalet (Figure 1). In total, 169 samples were collected from sites distributed over 35 communities (see Table S1). Natural habitats and agricultural lands in the surveyed regions are detailed in Table S2. Soil characteristics of the positive sampling site are given in Table S3. Each soil sample (about 1 kg) was a composite of five subsamples, taken to a depth of 20 cm over an area of 20 m^2. The subsamples were mixed, placed in polyethylene bags to prevent water loss and kept in coolers (about 15°C) as recommended by Kaya and Stock (1997) for transport to the laboratory. The sampling equipment was disinfected with 70% ethanol before leaving each sampling site to avoid cross contamination of samples. Details recorded for each sample included site name, geographic coordinates, habitats and vegetation.

Nematodes isolation

EPN were isolated from soil samples using an insect-baiting technique (Bedding & Akhurst, 1975). About 300 g of each sample was transferred to a plastic container

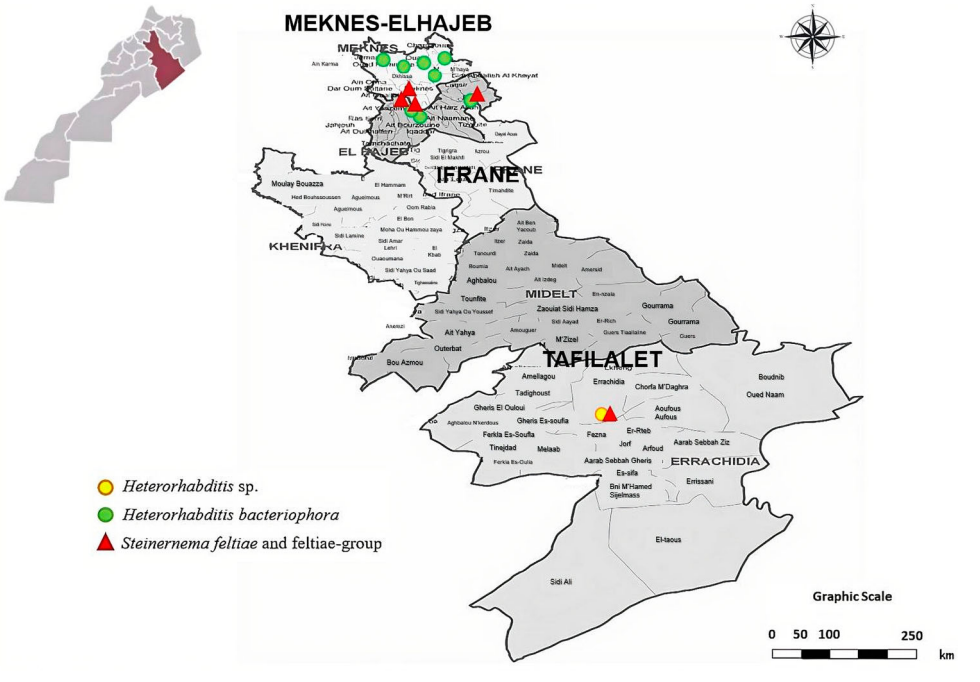

Figure 1. Map of three Moroccan regions (Meknès-El Hajeb, Ifrane and Tafilalet) showing geographical distribution of sampling locations where different isolates of EPN were found.

(500 ml) to which five final instar larvae of *Galleria mellonella* (L., 1758) were added as bait. Containers were covered with lids, inverted and kept at room temperature (22°C) in darkness. Every 2–3 days for 15 days, dead larvae of potential interest were removed and rinsed with distilled water until all *G. mellonella* were removed from the container. Dead larvae with signs of EPN infection, i.e. soft, flaccid, odourless larvae with a change in color (usually red/purple for heterorhabditids and brown to black for steinernematids), were removed and rinsed in sterile distilled water and individually placed in modified White traps (Kaya & Stock, 1997) for emergence of the infective-stage juveniles (IJs). The larvae in the White traps were checked for emergence initially after 36 h and then on a daily basis. Emerging nematodes were pooled for each sample and used to inoculate fresh *G. mellonella* larvae to verify their pathogenicity and to obtain nematodes for identification and to establish of cultures. Positive soil samples were assayed (texture, pH and organic matter content) by the Department of Soil Science, National School of Agriculture, Meknès, Morocco. In total, 14 EPN isolates were obtained.

Nematodes identification

A combined approach, including examination of morphological characters of first-generation males, IJs and DNA sequences were used for determination and characterisation of the EPN isolates.

A preliminary morphological determination to genera was performed using the following criteria: (1) colour of cadavers, *i.e.* red, brick-red or orange in *Heterorhabditis* and yellow-brown or black in *Steinernema* (Emelianoff et al., 2008); (2) presence of bursa in male *Heterorhabditis* and bursa absence in *Steinernema* (Kaya & Stock, 1988; Nguyen & Smart, 1996) and (3) position of excretory pore in IJs, distance anterior to nerve ring in *Steinernema* and distance posterior to nerve ring in *Heterorhabditis*. Morphology of 20 first-generation males and 20 IJs was examined for each isolate (Hominick et al., 1997; Stock & Hunt, 2005). The heat-killed (60°C Ringer's solution) specimens were placed in triethanolamine-formalin fixative (Kaya & Stock, 1997) and processed to anhydrous glycerin for mounting (Seinhorst, 1959). Observations were made from live and mounted specimens using an Olympus BX51 compound microscope equipped with an Olympus image-capture system.

Species identification based on morphological traits was confirmed by molecular analysis as recommended by Stock and Reid (2004). Two nuclear genes, the internal transcribed spacer region (ITS) and large subunit (28S) rDNA, were assayed for Heterorhabditidae and Steinernematidae, respectively. DNA extraction was based on Nguyen (2007). Polymerase chain reaction (PCR) and DNA sequencing were conducted to identify *Heterorhabditis* and *Steinernema* spp. The primers used for *Heterorhabditis* ITS rDNA were TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (Hominick et al., 1997) and for amplifying *Steinernema* 28S rDNA were 391-F (5'-AGCGGAGGAAAAGAAACTAA-3') and 501-R (5'-TCGGAAGGAACCAGCTACTA-3') (Stock et al., 2001). DNA amplification by PCR and sequencing followed the protocols described by Nguyen (2007) and Stock et al. (2001) for Heterorhabditidae and Steinernematidae, respectively. The sequences obtained were compared with sequences of *Heterorhabditis* and *Steinernema* using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (www.ncbi.com) and deposited on the GenBank.

An alignment of the ITS rDNA and 28S rDNA sequences was generated using Clustal W (Thompson et al., 1997). Phylogenetic analyses of ITS and 28S rDNA were performed by the maximum likelihood method and Kimura 2-parameter model (Kimura, 1980) of MEGA X software (Kumar et al., 2018). Phylogenetic trees were evaluated by bootstrap analysis based on 1000 replicates. *Caenorhabditis elegans* (Maupas, 1899) (X03680) was used as outgroups for ITS phylogenetic analysis and *Panagrellus redivivus* (Linnaeus, 1767) (AF331910) for the 28S analysis.

Results

EPN were isolated from 14 of 169 samples (8%; Figure 1) and from 9 of 35 sampling locations (26%; Table S1). The Meknès-El Hajeb region had the most positive samples (10 samples from 6 locations). However, only two EPN isolates each were found in Ifrane and Tafilalet (see Table S1). For habitat associations (see Table S2), the highest number of EPN isolates was found in association with olives (29% of isolates), followed by stone fruit (21%) and about 14% each with date palms and oak and vegetables. Only one isolate (7%) was found in association with fig trees. EPN were found in both natural habitats (14% in oak forests) and agricultural lands (86%). Heterorhabditid isolates (64%) were found in nine samples and steinernematid isolates (36%) in five soil samples. Eight isolates were identified as *Heterorhabditis bacteriophora* Poinar, 1976, two as *Steinernema feltiae* (Filipjev, 1934). Species determination of three isolates is undergoing and they were determined as belonging to the *S. feltiae*-group, which has IJ with overall body length of 800–1000 µm (Stock & Hunt, 2005). *Heterorhabditis bacteriophora* was the most prevalent, with eight isolates found in agricultural lands in the Meknès-EL Hajeb region (melons, olives, plums and potatoes) and in natural habitats in Ifrane (oak forests) (see Table S3). *Steinernema feltiae* was found in Ifrane and Tafilalet (date palms and oak forests). The three isolates in the *S. feltiae*-group were all found in Meknès-El Hajeb (figs and olives). A single isolate identified as *Heterorhabditis sp.* (HJo-MOR14) was concomitant with *S. feltiae* in El Jorf-Fezna (Tafilalet), in association with date palms. EPN were extracted from all soil types sampled, ranging from sand to clay, and neutral to alkaline, with pH ranging from 7.2 to 8.6 and organic matter from 1.2 to 5.9% (see Table S3).

Based on the PCR analysis, sequencing and BLASN search, eight isolates of *Heterorhabditis* (GenBank accession nos MN420270, MN420405, MN420697, MN420689, MN420696, MN420695, MN420687 and MN420691) were identified as *H. bacteriophora* with 99% identity (Figure 2) and two as *S feltiae* (GenBank accession nos MN749619 and MN752176) with similarity greater than 95%. The three steinernematids and one heterorhabditis did not match any described species, or submitted 28S rDNA or ITS sequence, and are likely to represent new species. The results of the phylogenetic analysis of the isolates are shown in Figures 3 and 4 with eight isolates associated with *H. bacteriophora* and two with *S. feltiae*.

Discussion

The present study was the most systematic survey aimed to isolate and characterise native EPN in Morocco to the species level. During this study, 14 isolates were found: 8 strains of

H. bacteriophora, 2 strains of *S. feltiae* and 4 non-identified isolates (3 isolates of *S. feltiae*-group and *Heterorhabditis* sp.). Although the prevalence was low (14 of 169 samples; 8%), this was consistent with reports from other countries. Globally, EPN prevalence in soil samples has been reported as ranging from 0.7% to 50% (Hominick, 2002; Pillay et al., 2009). However, the prevalence in Morocco is similar to other Mediterranean and African countries: 5–14% in Italy (Ehlers et al., 1991; Tarasco & Triggiani, 1997; Tarasco et al., 2015), 10% in Egypt (Shamseldean & Abd-Elgawad, 1994), 7% in Ethiopia (Mekete et al., 2005), 5% in Spain (Campos-Herrera et al., 2007) and 10% in Cameroon (Kanga et al., 2012). The measured prevalence of EPN is influenced by sampling method, insect bait used and soil type (Adams & Nguyen, 2002; Cheruiyot et al., 2013; Garcia del Pino & Palomo, 1996). Also, EPN prevalence may vary with sampling time and edaphic factors such as soil texture, soil moisture, temperature, pH and biotic factor (Cheruiyot et al., 2013). In the present study, soil samples collected from agricultural lands had more EPN (12 isolates) than those from natural habitats, which is consistent with the findings of other studies (Campos-Herrera et al., 2007; Garcia del Pino & Palomo, 1996; Mracek & Webster, 1993). Although, the opposite has also been found, with EPN more prevalent in natural habitats than agricultural contexts (Stock et al., 2008; Valadas et al., 2013).

The diversity of the EPN found in Morocco was relatively low, with only four taxa detected: *H. bacteriophora*, *Heterorhabditis* sp., *S. feltiae* and *S. feltiae*-group with *H. bacteriophora* the most common (57%). This finding is consistent with many studies in other Mediterranean countries (Campos-Herrera et al., 2007; de Doucet & Gabarra, 1994; Glazer et al., 1993; Hazir et al., 2003; Noujeim et al., 2011; Rosa et al., 2000; Stock et al., 2008; Tarasco et al., 2009, 2015; Valadas et al., 2013). *H. bacteriophora* was also isolated from a wide range of contexts, both agricultural (melons, olives, plums and potatoes) and natural (oak forests). Some studies have reported that *H. bacteriophora* is frequently found in maritime contexts, such as beaches (Emelianoff et al., 2008; Stock et al., 1999) and

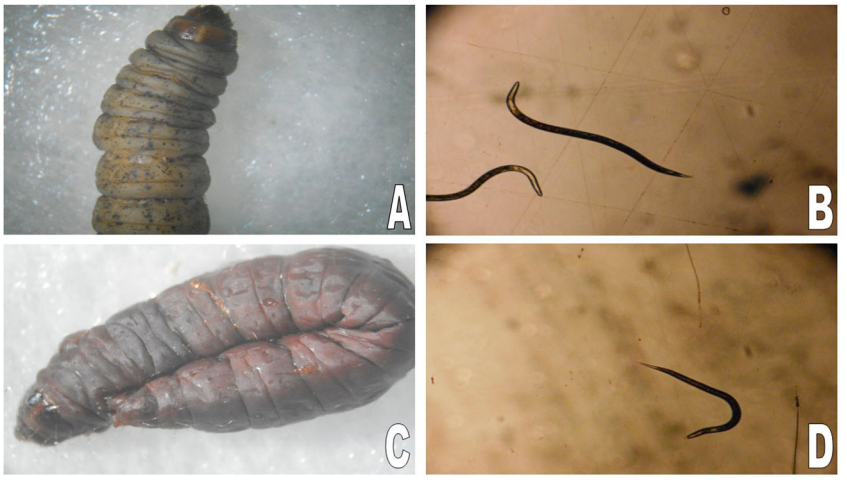

Figure 2. *Galleria mellonella* cadavers and infective juveniles of two Moroccan EPN isolates. A: *G. mellonella* larvae killed by HB-MOR3; B: Infective juvenile of HB-MOR3; C: *G. mellonella* larvae killed by the isolate *Heterorhabditis sp.*; D: IJ of *Heterorhabditis* sp.

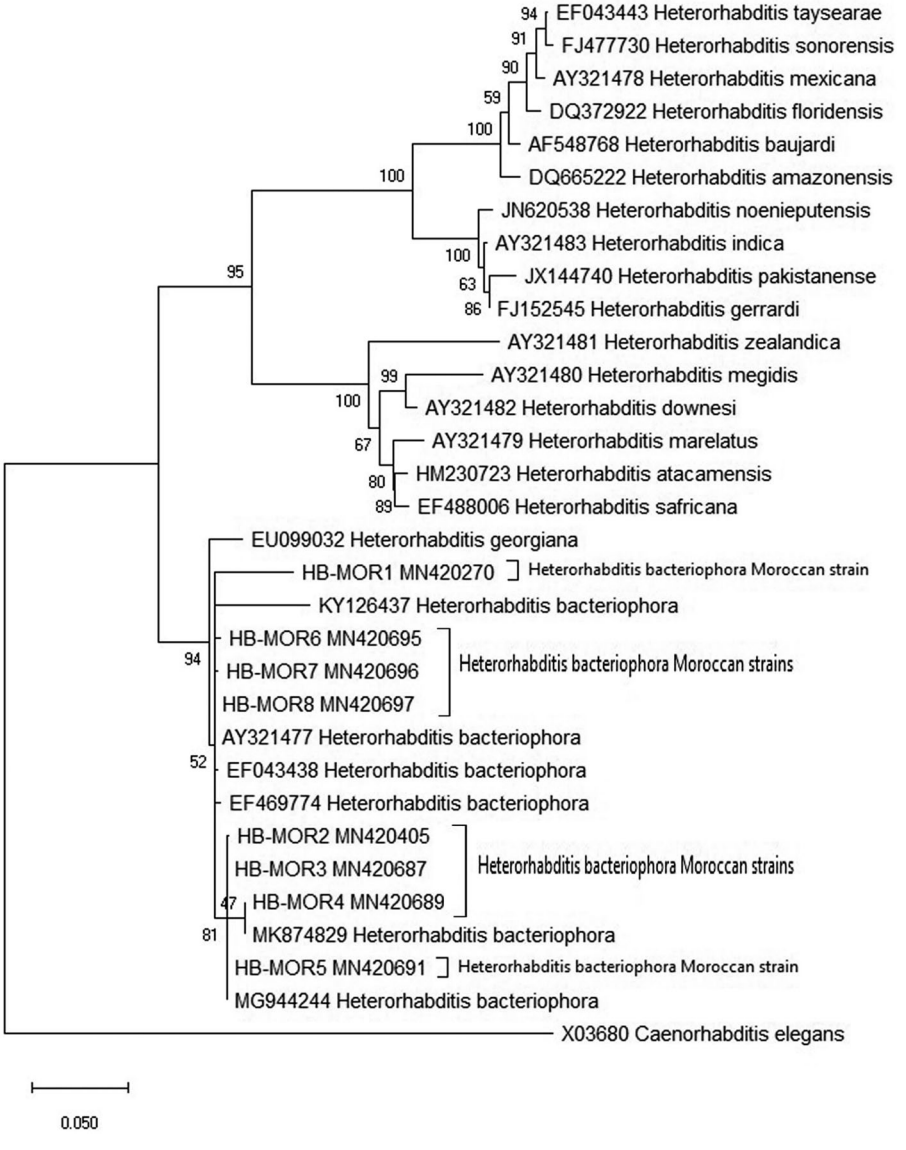

Figure 3. Phylogenetic relationship of Moroccan *Heterorhabditis bacteriophora* isolates and published sequences of selected *Heterorhabditis* based on the analysis of ITS region using maximum likelihood tree. *Caenorhabditis elegans* was used as outgroups. Numbers before species names correspond to GenBank accession numbers. For the list with the abbreviations of the isolate codes, see Table S3.

islands (Hara et al., 1991; Rosa et al., 2000). However, in Morocco, the sampled regions were well inland.

Steinernema feltiae was found at only two sites: near date palms and in oak forests. However, *S. feltiae* was found as the most common EPN species in some Mediterranean countries similar to Morocco in terms of topology and climate: 41–70% in Spain (Campos-Herrera et al., 2007; Garcia del Pino & Palomo, 1996), 87% in Algeria (Tarasco et al., 2009), 75% in continental Portugal (Valadas et al., 2013) and 38% in Italy (Tarasco et al., 2015).

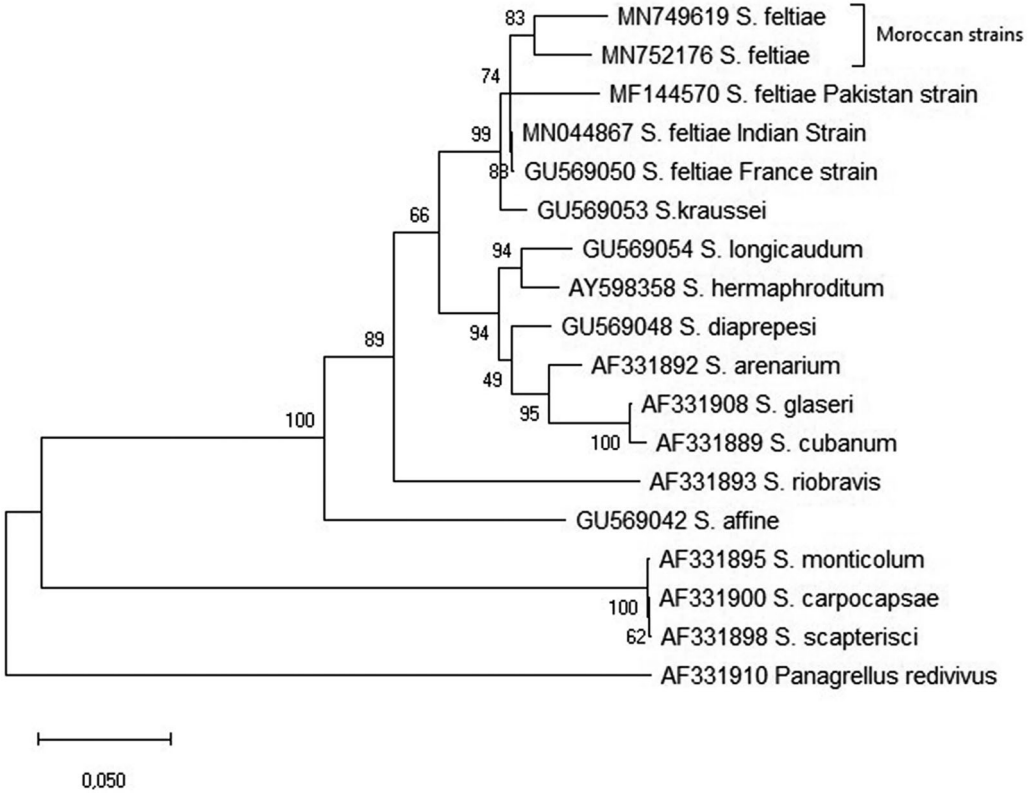

Figure 4. Phylogenetic relationship of Moroccan *Steinernema feltiae* isolates and published sequences of selected *Steinernema* based on the analysis of 28S rDNA region using maximum likelihood tree. *Panagrellus redivivus* was used as outgroups. Numbers before species names correspond to GenBank accession numbers.

Soil parameters, such as texture, moisture level, pH and organic matter, can affect EPN occurrence and infectivity (Koppenhöfer & Fuzy, 2006; Stuart et al., 2015). In some studies, EPN were commonly found in soils with high sand content (Khatri-Chhetri et al., 2010; Stock et al., 1999; Tarasco et al., 2015; Valadas et al., 2013). Light textured soils improve the mobility and survival of EPN compared to heavy textured soils (Stock et al., 1999). However, in the current survey, EPN were isolated from a wide range of soil types, from sand to clay. Notably, six isolates (HB-MOR3, HB-MOR4, HB-MOR6, HB-MOR8, SF-MOR12 and SF-MOR13) were obtained from heavy soils, so these could have potential for biological control of insects in these types of soil. Campos-Herrera et al. (2007) recommended using two Riojan (Spain) strains of *Steinernema carpocapssae* (Weiser, 1955) found in soils with high clay content as a commercial biocontrol agent in similar soils. Also, it is noteworthy that one *H. bacteriophora* isolate (HB-MOR3) was found at high altitude (1491 m) in a heavy soil in Aït Hammad, Ifrane. This site has low temperatures and snowfall in winter. It is possible that this is the first time *H. bacteriophora* has been found under such conditions. This isolate was also distinctive, causing a light grey colour and minute black spots in *G. mellonella* cadavers. Machado et al. (2018) have also reported isolates of *Heterorhabditis* that give grey rather than red cadavers.

In Morocco, this study found EPN in soil types ranging from neutral to alkaline. Khatri-Chhetri et al. (2010) found 90% of EPN in acidic soils and six isolates occurring in soils with pH <4. However, Campos-Herrera et al. (2007) found some EPN in alkaline soils of pH 8.3. Also, some studies have isolated EPN from desert habitats, which would be adapted to high temperature and low moisture (Ganguly & Singh, 2000; Shamseldean et al., 1996; Stock & Gress, 2006; Stock et al., 2008). In the present study, two isolates (SF-MOR9 and HJo-MOR14; S3) were found in Tafilalet (an arid area). In recent years, drought has become more frequent in Morocco, with extended periods (April to September) being dry and warm in many regions of the country. These conditions could make the isolation of an arid-zone EPN of potential importance for controlling insect pests in such contexts.

This survey has demonstrated that EPN, both *Heterorhabditis* and *Steinernema*, occur naturally in a range of ecosystems in Morocco, indicating that these EPN might have particular adaptations to local conditions which could give them potential to be developed as novel, non-chemical pest control agents. However, this potential needs to be assessed through research on the virulence of these EPN in local insect pest populations. Furthermore, the integration of other bait insects (coleopteran and dipteran) in the future studies may increase the recovery of EPN species and consequently improve our knowledge on the diversity and distribution of these agents in the country.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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