Journal of Helminthology

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Natural occurrence of entomopathogenic nematodes (Steinernema and Heterorhabditis) and *Pristionchus* nematodes in black truffle soils from Spain

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Research Paper

Cite this article: Julià I, Morton A and Garcia-del-Pino F (2023). Natural occurrence of entomopathogenic nematodes (Steinernema and Heterorhabditis) and *Pristionchus* nematodes in black truffle soils from Spain. *Journal of Helminthology*, **97**, e76, 1–10
https://doi.org/10.1017/S0022149X23000615

Received: 04 July 2023
Revised: 05 September 2023
Accepted: 25 September 2023

Keywords:
Biodiversity; distribution; *Steinernema feltiae*; *Heterorhabditis bacteriophora*; *Pristionchus*; necromenic; phoretic; *Tuber melanosporum*

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Abstract

The European truffle beetle *Leiodes cinnamomeus* is the most important pest in black truffle (*Tuber melanosporum*) plantations. Current control methods against it are inefficient, so entomopathogenic nematodes (EPNs) could play an important role in their population regulation due to their efficacy against many soil-dwelling insect pests. A survey of EPNs and *Pristionchus* nematodes was conducted in truffle soils of Spain, considering environmental and physical–chemical soil factors. A total of 164 soil samples were collected from forests, productive plantations and null-low productive plantations, representing three distinct black truffle-growing habitat types. EPNs were isolated from seven soil samples (4.3%); four nematodes were identified as *Steinernema feltiae* and three as *Heterorhabditis bacteriophora.* Both species were sampled in three types of soil texture (loam, sandy loam or sandy clay loam), characterized by alkaline pH (7.5 to 8.5) and high organic matter (2.1–11.04%). The presence of these EPNs was influenced by habitat type and organic matter content. *Pristionchus* nematodes were isolated from truffle soil, around truffle fruit bodies and under the elytra of *L. cinnamomeus,* with *Pristionchus maupasi* being the most commonly identified species. No significant associations were found between environmental and soil factors and the occurrence of *Pristionchus* nematodes. These nematodes were found in alkaline soils (pH 7.75 to 8.7), across all seven sampled soil textures, with variable organic matter content (0.73%–5.92%). The ecological trends and the presence of *Pristionchus* may affect the occurrence of EPNs and their prospective use as biological control agents against *L. cinnamomeus* in black truffle plantations.

Introduction

The black truffle, *Tuber melanosporum* Vittad. (Pezizales: Tuberaceae), is a hypogeal fungus that establishes a mycorrhiza relationship mainly with the *Quercus* genus (Bonito et al. 2010) that has been traditionally collected from wild forests and in the last decades cultivated due to its high gastronomic value and economic interest (Oliach et al. 2020). *T. melanosporum* usually starts to fructify when the host tree is about 6–7 years old and continues until it is 20–25 years old, when the production starts to drop (Martín-Santafé, 2020). During this period, the mycorrhiza develops a burnt area around the host tree where the herbaceous cover is scarce (Splivallo et al. 2011) due to the production of mainly two truffle volatiles (ethylene and indole-3-acetic acid) that act as potent herbicides at high concentrations (Hansen and Grossmann 2000; Grossmann 2003). Moreover, alkaline soils are required for its development, with pH that ranges from 7.0 to 8.9, with a median of 7.9 (Jaillard et al. 2016). This fungus is also benefited by high organic matter content and balanced textures, generating loamy soils that are well-structured, porous, aerated and without excess of water (Jaillard et al. 2016).

The economic importance of this fungus has led to a monoculture situation that along with certain agricultural practices like irrigation have favoured the presence of some insect species that then become pests (Martín-Santafé 2020). The European truffle beetle, *Leiodes cinnamomeus* (Panzer) (Coleoptera: Leiodidae), is one of the most serious pests in black truffle plantations (Arzone 1971; Martín-Santafé et al. 2014; Navarro-Llopis et al. 2021). Adults and larvae feed on *T. melanosporum* fruiting bodies, causing galleries which reduce quality and can generate up to 70% of economic losses in plantations (Barriuso et al. 2012). Cultural practices, such as frequent collections of truffles and the use of traps for mass capture of adults, are recommended (Martín-Santafé et al. 2014; Navarro-Llopis et al. 2021). However, these practices are not enough to reduce the population of *L. cinnamomeus* to acceptable levels. Thus, alternative biological control methods are needed.

Entomopathogenic nematodes (EPNs) are a group of species that have been studied and used as biological control agents for decades (Lacey and Georgis 2012; Shapiro-Illan et al. 2017). These

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nematodes, species of the genus *Steinernema* and *Heterorhabditis,* which are the most common, are widely distributed throughout the world and have been reported from a wide variety of soils (Hominick 2002). The presence and survival of EPNs are influenced by multiple factors, including geographical location, climatic conditions, habitat type and soil properties, such as pH, organic matter content and texture (Stuart et al. 2015). EPNs are believed to be adapted to the soil-specific conditions where they were isolated (Kung et al. 1991). Julià et al. (2023) have already observed the susceptibility of *L. cinnamomeus* adults and larvae to EPNs under laboratory conditions, suggesting that the presence of these nematodes in truffle plantations could naturally regulate the population of this beetle.

Free-living bacterivorous nematodes (FLBNs) species can interfere and compete with EPNs for host resources (Duncan et al. 2003; Campos-Herrera et al. 2012). Some species of the genus *Pristionchus* exhibit facultative insect parasitic, necromenic and nematophagous behaviour (Félix et al. 2018). For example, *Pristionchus pacificus* Sommer, Carta, Kim and Sternberg (Rhabditida: Diplogastridae) can display dimorphic mouth structures, differing in the number and shape of teeth and in the complexity of other mouth armature. This dimorphism enables it to adopt a predator behaviour towards other nematodes when bacterial food is scarce (Meyer et al. 2017). Moreover, previous studies have also observed that species such as *P. pacificus* and *Pristionchus maupasi* (Potts) (Rhabditida: Diplogastridae) form necromenic or phoretic associations with various species of beetles (Herrmann et al. 2006, Hong et al. 2008; Félix et al. 2018).

The discovery of new strains and species of EPNs has been important in their commercial success as biocontrol agents against pests (Shapiro-Ilan et al. 2002; Lacey and Georgis 2012) due to the importance of being adapted to the environmental conditions of the site of application (Bedding 1990). There is currently a lack of studies examining the presence of EPNs in truffle soils. Therefore, the main objectives of this research were: (1) to isolate EPNs and study their ecological requirements in truffle soils from the regions of Teruel and Catalonia (Spain); (2) assess the presence of species of *Pristionchus* nematodes that could interfere with the presence of EPNs.

Material and methods

Field sampling and soil characterization

A total of 164 soil samples in 112 and 52 locations of Teruel and Catalonia (Spain), respectively, were collected from different black truffle-growing areas (Figure 1) from autumn (October 2020) to spring (March 2021). Each soil sample weighed about 1 kg, resulted from the mixture of four subsamples of about 200 cm^3 dug from 0 to 20 cm deep in soil around the host tree (Campbell et al. 1998).

In 21 productive plantations in Teruel, both nonburnt areas (without the presence of *T. melanosporum*) and burnt areas were sampled to assess whether the volatiles of *T. melanosporum*, which act as herbicides, influenced the occurrence of nematodes. The sampling methodology employed was the same as explained above.

The locations and altitudes of the sampled soils were recorded using global positioning system equipment. Data of annual average air temperature and rainfall were recorded from maps of the Government of Aragon and Government of Catalonia. The study area in Teruel lies between 888 and 1760 m above sea level, with mean annual temperatures of 9–13 ºC and mean annual rainfall of 400–600 mm. In the case of Catalonia, the study area lies between 514 and 1484 m above sea level, with mean annual temperatures of 8–12 ºC and mean annual rainfall of 600–800 mm. The samples were categorized based on habitat type, with 20.1% obtained from forests known to naturally produce *T. melanosporum*, 67.1% from productive truffle plantations and 12.8% from low-productive or nonproductive plantations. This last category includes plantations less than 6 years old or more than 25 years old.

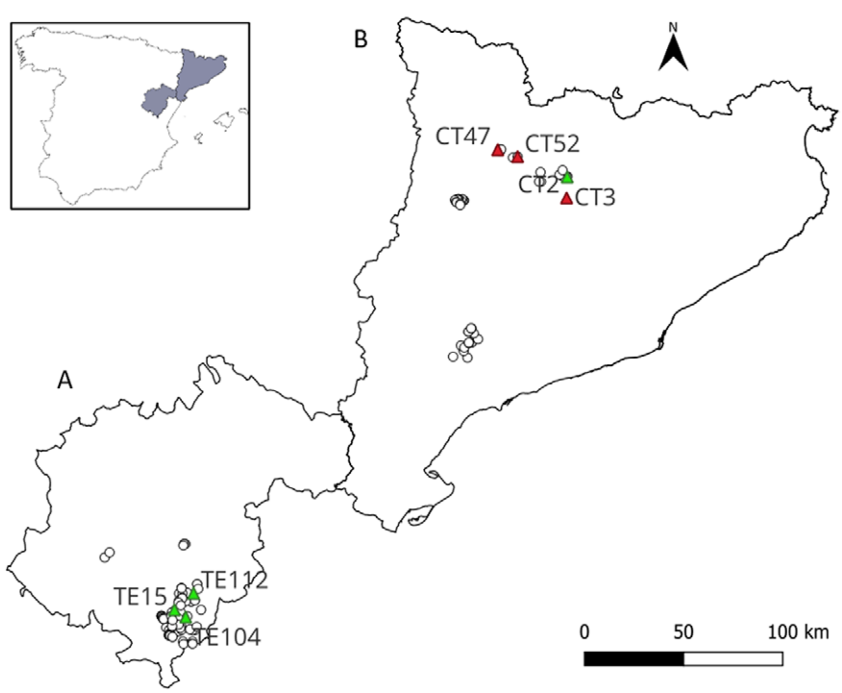

Figure 1. Geographical distribution of EPN sampling locations in the regions of A. Teruel and B. Catalonia (Spain). Green triangles: sites with *S. feltiae.* Red triangles: sites with *H. bacteriophora.* White circles: sites without nematodes.

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For each collected sample, pH, soil organic content and soil particle size were measured. The soil pH was measured from a 1:2.5 soil/mQ-water suspension and the total organic matter was determined by wet oxidation (MAPA 1975). To determine the soil texture, particle size analysis was performed to calculate the percentage of silt, sand and clay using the Bouyoucos method (MAPA 1975).

Isolation of nematodes

Isolation of EPNs

The insect bait method (Galleria trap) described by Bedding and Akhurst (1975) was used to isolate EPNs. For each soil sample, five Petri dishes containing five last-stage larvae of *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) were covered with soil. After incubating for one week at 24 ºC, dead insects that showed symptoms of infection were rinsed in sterile Ringer solution and placed individually into modified White traps (White 1927) to collect the emerged infective juveniles (IJs). The assay was conducted twice. To confirm their pathogenicity, harvested nematode strains were reared at 24 °C in last instar larvae of *G. mellonella*, according to the method of Woodring and Kaya (1988). They were maintained at 9 °C until their molecular identification.

Isolation of Pristionchus nematodes

The presence of *Pristionchus* and other FLBNs was determined using a similar methodology. For each soil sample, one Petri dish containing three frozen dead last-stage larvae of *G. mellonella* was covered with soil. After incubating for one week at 24 ºC, nematodes detected around the *G. mellonella* cadavers were collected in Eppendorf tubes with ethanol 70%. The assay was conducted twice.

The presence of *Pristionchus* nematodes was also determined directly from the surface of black truffle fruit bodies to confirm their presence on this fungus. Eight fruit bodies of *T. melanosporum* were sampled in Teruel. The nematodes around them were rinsed and collected in Eppendorfs with ethanol 100%. Their presence was also determined under the elytra of *L. cinnamomeus* to confirm the phoretic relationship between nematode and insect. Twenty Petri dishes with nutrient agar were used, with two elytra per dish. After 7–10 days of incubation, the percentage of Petri dishes with *Pristionchus* nematodes was calculated.

Identification of nematodes

EPNs and *Pristionchus* nematodes isolated were identified using molecular techniques. For each sample, a PCR reaction was performed to amplify an internal transcribed spacer (ITS) region from genomic DNA extracted from a single female. PCR amplification conditions followed procedures described by Hominick et al. (1997) using the primers 18s (5-AAAGATTAAGCCATGCATG-3) and 26s (5-CATTCTTGGCAAATGCTTTCG-3) (Vrain et al. 1992). Amplified samples were purified and sequenced before being compared with GenBank database sequences of *Steinernema*, *Heterorhabditis* and *Pristionchus* using Blastn (NCBI; http://www.ncbi.nlm.nih.gov), searching for sequence similarity matches at ≥ 98%.

An alignment of the ITS rDNA sequences was generated using Clustal W (Thompson et al. 1997) for *Steinernema* and *Heterorhabditis* species separately. Phylogenetic analyses were performed using the maximum parsimony (MP) method with MEGA X software (Kumar et al. 2018). The calculated phylogenetic trees were evaluated by bootstrap analysis based on 1000 replicates. *Caenorhabditis elegans* (Maupas) (X03680) was used as outgroup during calculation of the trees based on ITS sequences.

Data analysis

The data for EPNs and *Pristionchus* were assessed in relation to environmental and soil factors, using the recovery frequency (number positive samples/number total samples), and the number and percentage of positive samples per variable category. The data corresponding with EPNs and *Pristionchus* were analysed separately. Generalized linear model (GLM), with negative binomial distribution and a log link function, was used to examine the relationships between the nematodes and the environmental/soil variables. The variables were categorized into groups, as shown in Tables 1 and 2. The recovery frequency of nematodes obtained in the regions of Catalonia and Teruel and in the burnt and nonburnt areas were also compared using GLM with negative binomial distribution. All data were analysed with the R software (version 4.2.2) (R Core Team 2022). Any comparison was considered significant if the p-value was less than 0.05.

Table 1. Distribution of EPNs at different environmental variables

		Positive samples	
Categories (total samples)	Recovery frequency (%)	*S. feltiae* No (%)	*H. bacteriophora* No (%)
Habitat type			
Productive plantation (110)	0	0	0
Null-low productive plantation (21)	14.3	2 (50%)	1 (33.3%)
Truffle forest (33)	12.1	2 (50%)	2 (66.6%)
Altitude (masl)			
500–800 (22)	9.1	0	2 (66.6%)
800–1100 (90)	3.3	2 (50%)	1 (33.3%)
1100–1400 (41)	2.5	1 (25%)	0
>1400 (5)	20	1 (25%)	0

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Table 2. Distribution of EPNs at different physical–chemical variables

Categories (total samples)	Recovery frequency (%)	Positive samples	
		S. feltiae No (%)	*H. bacteriophora* No (%)
Soil pH			
7–7.5 (1)	0	0	0
7.5–8 (11)	18.2	2 (50%)	0
8–8.5 (129)	3.9	2 (50%)	3 (100%)
≥8.5 (23)	0	0	0
Organic matter			
≤1 (7)	0	0	0
1–2 (48)	0	0	0
2–3 (31)	9.7	2 (50%)	1 (33.3%)
3–4 (38)	0	0	0
4–5 (21)	4.8	1 (25%)	0
≥5 (19)	15.8	1 (25%)	2 (66.6%)
Texture			
Sandy loam (24)	16.7	3 (75%)	1 (33.3%)
Loam (59)	3.4	0	2 (66.6%)
Sandy clay loam (37)	2.7	1 (25%)	0
Silt loam (4)	0	0	0
Clay loam (36)	0	0	0
Sandy clay (1)	0	0	0
Clay (3)	0	0	0

Results

Isolation of entomopathogenic nematode species

Entomopathogenic nematodes were isolated from 7 of 164 soil samples (4.3%) distributed across plantations and wild truffle-producing forests. EPNs were recovered from 3 of 112 samples from Teruel (2.6%) and 4 of 52 samples from Catalonia (7.7%), with no significant differences between both regions ($\chi^2 = 1.92$, df = 1, $p = 0.17$).

Morphologic and molecular examinations revealed the presence of four isolates of *Steinernema* and three isolates of *Heterorhabditis*. BLASTn analysis of the ITS region showed that the three steinernematid recovered from Teruel and one from Catalonia shared sequence similarity of >99% with *Steinernema feltiae* (Filipev) (Panagrolaimida: Steinernematidea) (Figure 2). The second species isolated from three samples in Catalonia shared sequence similarity of >99% with *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae) (Figure 3).

EPN distribution in relation to environmental and soil characteristics

The habitat type significantly affected the recovery frequency of EPNs ($\chi^2 = 16.25$, df = 2, $p < 0.05$). Both species were isolated in truffle forest (12.1% of this habitat) and null-low productive plantations (14.3% of this habitat), but no EPNs were detected in productive plantations (Table 1). Altitude did not affect the occurrence of EPNs ($\chi^2 = 2.99$, df = 3, $p = 0.39$). However, *H. bacteriophora* strains were isolated at lower altitudes (694, 767 and 804 masl) than *S. feltiae* (899, 942, 1336 and 1417 masl) ($\chi^2 = 6.41$, df = 1, $p < 0.05$).

EPN distribution was also examined according to soil pH, organic matter and texture. Both EPN species isolated were found in alkaline soil samples with pH ranging from 7.58 to 8.47 (Table 2). However, there was no significant effect of soil pH to EPNs occurrence ($\chi^2 = 4.83$, df = 3, $p = 0.18$). Instead, the recovery frequency of *S. feltiae* and *H. bacteriophora* was significantly influenced by the content of organic matter ($\chi^2 = 12.85$, df = 5, $p < 0.05$), as these nematodes were predominantly isolated from soils with higher organic matter content (2.1–11.04%). The occurrence of EPNs was not significantly influenced by soil texture ($\chi^2 = 9.06$, df = 6, $p = 0.17$), although they were detected in sandy loam (16.7%), loam (3.4%) and sandy clay loam (2.7%) soils but not in silt loam, clay loam, sandy clay and clay soils (Table 2).

Presence of Pristionchus species

Species of genus *Pristionchus* were detected in 46 of 164 soil samples (28%) distributed across plantations and truffle forests surveyed. Individuals were recovered from 35 of 112 samples in Teruel (31.3%) and 11 of 52 samples in Catalonia (21.2%), with no significant differences between both regions ($\chi^2 = 1.85$, df = 1, $p = 0.17$).

BLASTn analysis of the ITS region showed that most of these isolates (39 of 46) shared sequence similarity of >98% with *P. maupasi*. The other isolates (7 of 46) were not similar to published ITS sequences of any specific species of this genus. Hence, they were considered as *Pristionchus* sp. Other free-living

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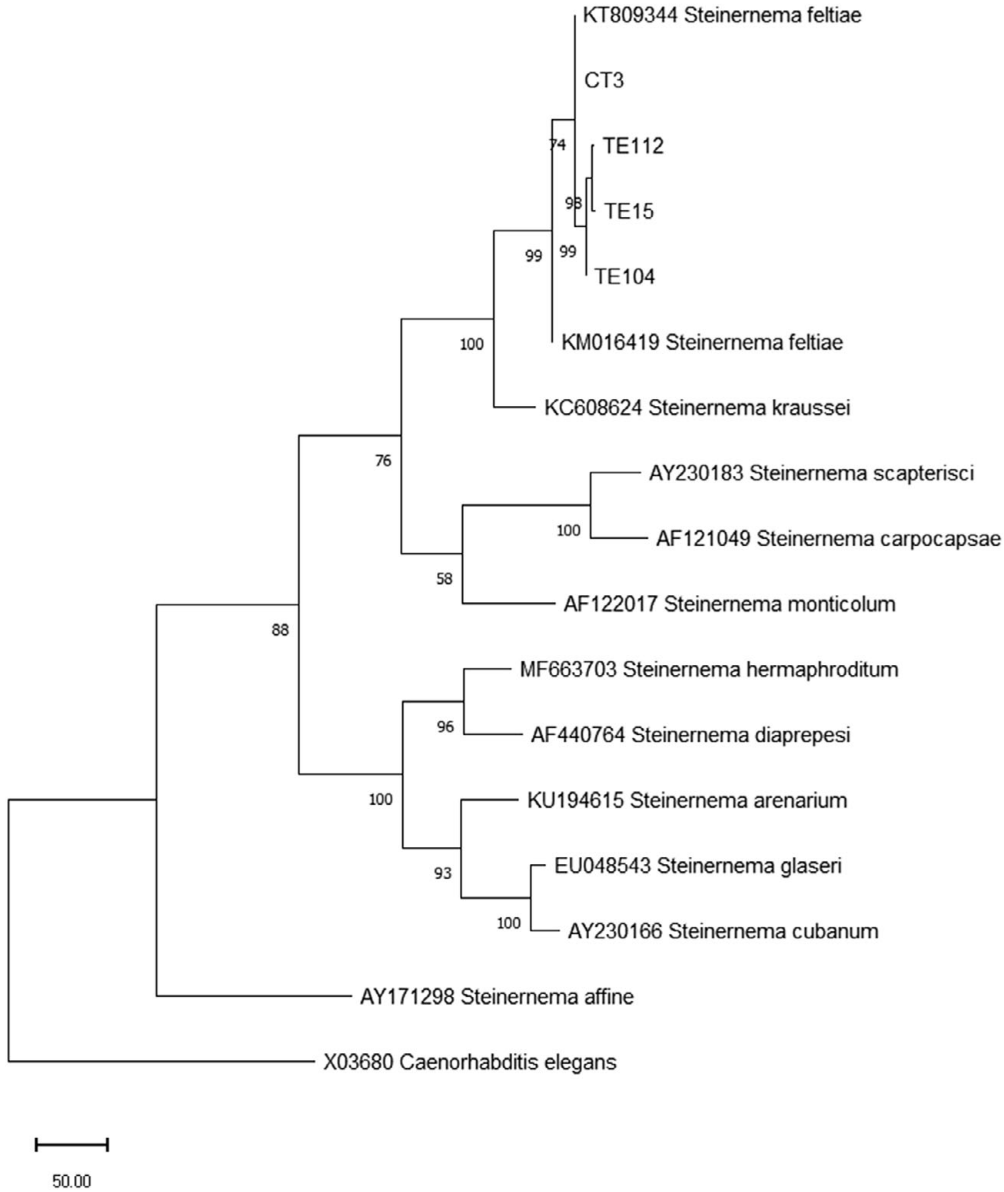

Figure 2. Phylogenetic relationship of isolated *S. feltiae* strains from Teruel (TE15, TE104 and TE112) and Catalonia (CT3) and published ITS sequences of *Steinernema* species using maximum parsimony tree. *C. elegans* was used as the outgroup. Numbers before species names correspond to GenBank accession numbers.

bacteriophage nematode species were also recovered and identified from nine samples in Teruel (8%) and six samples in Catalonia (11.5%) (Table 3).

BLASTn analysis also identified *P. maupasi* species around the eight fruit bodies of *T. melanosporum* sampled, sharing sequence similarities of >99% with this species. Moreover, *Pristionchus* nematodes were identified under the elytra of 45% of *L. cinnamomeus* adults. Some other nonidentified nematodes were also detected.

Pristionchus distribution in relation to environmental and soil characteristics

Pristionchus nematodes were isolated in all three sampled habitat types ($\chi^2 = 1.15$, df = 2, $p = 0.56$), including productive plantations (28.2%), null-low productive plantations (33.3%) and truffle forests (21.2%) (Table 4). Furthermore, these nematodes were found across all altitudes sampled ($\chi^2 = 1.77$, df = 3, $p = 0.62$).

Pristionchus distribution was also examined according to soil pH, organic matter and texture. None of these variables significantly influenced the presence of *Pristionchus* ($\chi^2 = 3.55$, df = 3, $p = 0.31$; $\chi^2 = 6.47$, df = 5, $p = 0.26$ and $\chi^2 = 4.1$, df = 6, $p = 0.66$, respectively). All nematodes were found in alkaline soil samples (7.75 to 8.7 pH), in all seven types of texture sampled with variable organic matter content (0.73–5.92%) (Table 5).

Presence of EPNs and *Pristionchus* in burnt and nonburnt areas

The number of isolated EPNs was not statistically analysed because no nematodes were recovered from the 42 samples (21 from burnt areas and 21 from nonburnt areas). In the case of *Pristionchus*, its recovery frequency was found to be significantly higher in burnt areas (38.1%) compared to nonburnt areas (4.8%) ($\chi^2 = 7.69$, df = 1, $p < 0.05$) (Table 6).

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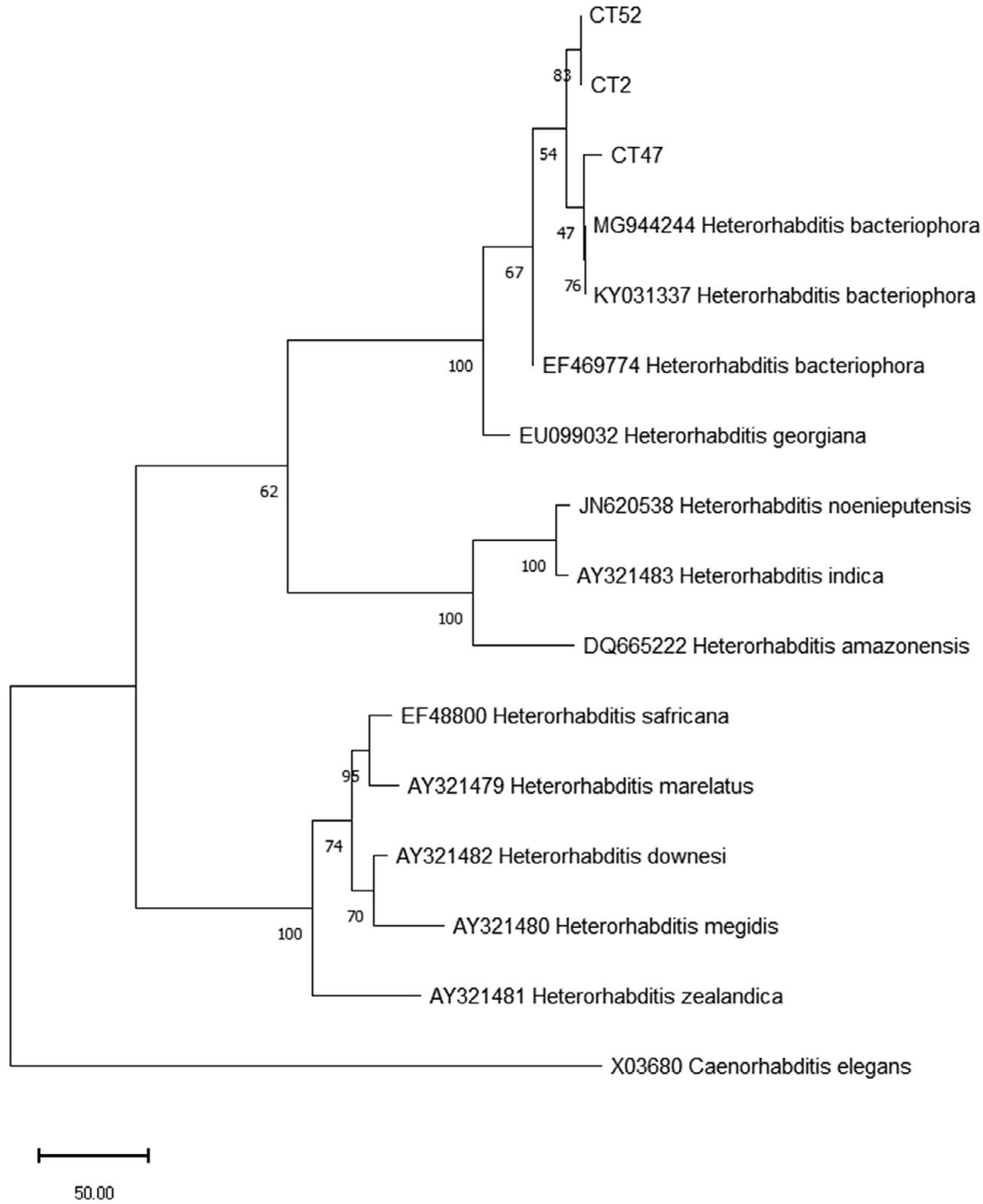

Figure 3. Phylogenetic relationship of isolated *H. bacteriophora* strains from Catalonia (CT2, CT47 and CT52) and published ITS-sequences of *Heterorhabditis* species using maximum parsimony tree. *C. elegans* was used as the outgroup. Numbers before species names correspond to GenBank accession numbers.

Table 3. Number of soil samples with other identified species of free-living bacteriophage nematodes

Species	Teruel	Catalonia	Global
Rhabditis terricola	3	1	4
Oscheius sp.	3	0	3
Panagrolaimus sp.	2	1	3
Cruznema tripartitum	1	2	3
Pratylenchus goodeyi	0	2	2
Total	9	6	15

Discussion

This study represents the first survey aimed to isolate native EPN species and necromenic *Pristionchus* nematodes in truffle soils. Despite a relatively low prevalence of EPNs (7 of 164 samples; 4.3%), our findings are consistent with previous studies. Globally, EPN prevalence in soil samples has been reported to range from 0.7% to 50% (Hominick 2002). In Spain, Morton and Garcia del Pino (2009) observed an incidence of 5.2% in stone fruit orchards in Catalonia, which aligns with the results of our study. Similar results were obtained by Campos-Herrera et al. (2007) in soils of La Rioja (5.4%). In contrast, Garcia del Pino and Palomo (1996) recovered EPNs in 28.2% of cultivated soils and in 16.3% of woodlands in Catalonia.

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Table 4. Distribution of *Pristionchus* at different environmental variables

Categories (total samples)	Recovery frequency (%)	Positive samples	
		P. maupasi	*Pristionchus sp.*
Habitat type			
Productive plantation (110)	29.1	28 (71.8%)	4 (57.1%)
Null-low productive plantation (21)	33.3	6 (15.4%)	1 (14.3%)
Truffle forest (33)	21.2	5 (12.8%)	2 (28.6%)
Altitude (masl)			
500–800 (22)	18.2	2 (5.1%)	2 (28.6%)
800–1100 (90)	31.1	26 (66.7%)	2 (28.6%)
1100–1400 (41)	29.3	10 (25.6%)	2 (28.6%)
>1400 (5)	40	1 (2.6%)	1 (14.3%)

Table 5. Distribution of *Pristionchus* at different physical–chemical variables

Categories (total samples)	Recovery frequency (%)	Positive samples	
		P. maupasi	*Pristionchus sp.*
Soil pH			
7–7.5 (1)	0	0	0
7.5–8 (11)	27.3	2 (5.1%)	1 (14.3%)
8–8.5 (129)	21.7	24 (61.5%)	4 (57.1%)
≥8.5 (23)	65.2	13 (33.3%)	2 (28.6%)
Organic matter			
≤1 (7)	42.9	3 (7.7%)	0
1–2 (48)	22.9	10 (25.6%)	1 (14.3%)
2–3 (31)	38.7	11 (28.2%)	1 (14.3%)
3–4 (38)	28.9	8 (20.5%)	3 (42.9%)
4–5 (21)	9.5	2 (5.1%)	0
≥5 (19)	31.6	4 (10.3%)	2 (28.6%)
Texture			
Sandy loam (24)	29.2	7 (17.9%)	0
Loam (59)	20.3	12 (30.8%)	3 (50%)
Sandy clay loam (37)	29.7	11 (28.2%)	1 (16.7%)
Silt loam (4)	25	1 (2.6%)	0
Clay loam (36)	16.7	6 (15.4%)	2 (33.3%)
Sandy clay (1)	100	1 (2.6%)	0
Clay (3)	33.3	1 (2.6%)	0

Table 6. Distribution of *Pristionchus* at burnt and nonburnt areas

Categories (total samples)	Recovery frequency (%)	Positive samples	
		P. maupasi	*Pristionchus sp.*
Burnt area (21)	38.1	6 (85.7%)	2 (100%)
Nonburnt area (21)	4.8	1 (14.3%)	0 (0%)

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In our study, the diversity of EPNs was found to be low, with only two species isolated: *S. feltiae* and *H. bacteriophora*. These findings are consistent with previous studies conducted in Mediterranean countries, which have also reported low diversity of EPNs (Garcia del Pino and Palomo 1996; Campos-Herrera et al. 2007; Morton and Garcia del Pino 2009; Noujeim et al. 2011; Valadas et al. 2013; Tarasco et al. 2009, 2015; Benseddik et al. 2020). Four new strains of *S. feltiae* were isolated, being the most common species detected globally (57%) and in the region of Teruel (100%). In fact, *S. feltiae* is frequently reported as the dominant EPN species in Mediterranean countries, accounting for 38% in Italy (Tarasco et al. 2015), 55% (Campos-Herrera et al. 2007) and 70% in Spain (Garcia del Pino and Palomo, 1996), 75% in Portugal (Valadas et al. 2013), 71–85% in Turkey (Hazir et al. 2003; Yuksel and Canhilal 2019; Gümüş Askar et al. 2022) and 87% in Algeria (Tarasco et al. 2009). In the region of Catalonia, three strains of *H. bacteriophora* were identified and were the most common species detected (75%). This species was isolated in inland areas at lower altitudes (694–807 masl) compared to *S. feltiae* (899–1417 masl). Most studies have reported that *H. bacteriophora* is commonly found in maritime environments, where it tends to be more prevalent than steinernematids (Rosa et al. 2000; Emelianoff et al. 2008). However, our results align with other reports, suggesting that *H. bacteriophora* can also be the dominant species in inland areas (Benseddik et al. 2020).

The measured prevalence of EPN is influenced by various factors, including sampling method, insect bait and environmental and soil characteristics such as habitat type, altitude, soil texture, moisture level, organic matter, pH and biotic factors (Garcia del Pino and Palomo 1996; Benseddik et al. 2020). In our study, we found that habitat type significantly influenced the recovery frequency of EPNs. Although productive plantations are subjected to more frequent irrigation compared to null-low productive plantations and truffle forests, EPNs were only isolated from the latter two habitats, and none were isolated from productive plantations. *T. melanosporum* is associated with the development of burnt area around the host tree where the herbaceous cover is scarce (Splivallo et al. 2011). This characteristic is more prominent in productive plantations, potentially leading to a loss of diversity among potential insect hosts (Campos-Herrera et al. 2007). Although highly intensive monoculture areas can produce outbreaks of insect pest species susceptible to EPNs infection (Campos-Herrera et al. 2007), the beetle *L. cinnamomeus* develops during the coldest period of the year when low temperatures are suboptimal for EPN infection (Julià et al. 2023). Moreover, the high recovery rates of the genus *Pristionchus* observed in burnt areas could indicate that the herbicides emitted by *T. melanosporum* may not have a nematicidal effect on nematodes, including EPNs. Thus, the greater presence of vegetation in null-low productive plantations and wild truffles could positively influence the presence of EPNs by providing increased diversity of potential insect hosts during spring and summer.

In our survey, EPNs were recovered from soils with moderate to high sand content. Previous studies observed that EPNs were commonly found in soils with high sand content (Stock et al. 1999; Valadas et al. 2013; Tarasco et al. 2015), suggesting that light textured soils improve the mobility and survival of EPNs (Stock et al. 1999) compared to heavy textured soils, which clay content could difficult the EPNs movement and affect their recovery (Mráček et al. 2005). Furthermore, organic matter content has also been observed to positively correlate with the occurrence of EPNs (Hominick and Briscoe 1990; Alumai et al. 2006; Canhilal and Carner 2006). Our results agree with these studies, in which both EPN species were significantly more prevalent in soils with higher organic matter. Additionally, all strains of *S. feltiae* and *H. bacteriophora* were isolated from alkaline soils because *T. melanosporum* requires soils with high pH levels, ranging from 7.0 to 8.9 (Jaillard et al. 2016). Our results agree with Campos-Herrera et al. (2007), who also isolated EPNs from alkaline soils, while Khatri-Chhetri et al. (2010) detected most of EPNs in acidic soils, particularly steinernermatids. Kung et al. (1990) reported that pH values within the range of 4–8 did not significantly affect the survival and pathogenicity of EPNs, suggesting that the pH tolerance of indigenous nematodes may vary depending on the region of isolation (Khathwayo et al. 2021). In fact, EPNs are believed to be adapted to the specific soil conditions of the region they were isolated (Kung et al. 1991).

Biotic factors, such as FLBNs species, may also affect the presence of EPNs (Stuart et al. 2015). Previous studies observed that these nematodes are able to interfere and compete with EPNs species for host resources. Laboratory experiments observed that *Pellioditis* sp. can compete with and even displace EPNs species that had previously killed the insect host (Duncan et al. 2003). Another study demonstrated the ability of *Acrobeloides maximum* and *Rhabditis rainaispecies* to interfere with the development of various species of EPNs inside weevil *Diaprepes abbreviatus* hosts, reducing the production of new progeny (Campos-Herrera et al. 2012). The genus *Pristionchus* sp. is known to be associated with different substrates, such as soil, humus, compost, moss, around roots of several species, rotten wood, stems and fruits, and decomposing fungi (Sudhaus and Fürst von Lieven 2003; Félix et al. 2018). In our study, *Pristionchus* nematodes, particularly *P. maupasi*, were frequently found in truffle soils (28% of all samples) and on the fruit bodies of these truffles (100%). These results agree with Kilian et al. (2022), who also observed the presence of unidentified nematodes around the fruit bodies of *T. melanosporum*. Additionally, we detected the presence of this nematode under the elytra of *L. cinnamomeus* (45%), which is the first report that has confirmed the necromenic/phoretic relationship between *P. maupasi* and *L. cinnamomeus*. Previous studies reported that some *Pristionchus* species can prey on other nematodes due to the mouth dimorphism developed by these species (Serobyan et al. 2014; Wilecki et al. 2015). Moreover, we also observed predatory behaviour in *P. maupasi* towards EPNs under laboratory conditions (unpublished data). The co-occurrence of several other FLBN species with *P. maupasi* in truffle soil could indicate that these nematodes may serve as a food source in addition to bacteria. Therefore, the presence of *P. maupasi* may be a factor that has contributed to the low prevalence of EPNs in truffle soils.

In our study, *P. maupasi* was not significantly affected by environmental and soil variables. Félix et al. (2018) observed that both dauer and feeding stages of various *Pristionchus* species were frequently and abundantly present on different rotting vegetal matter and decomposing fungi. They also highlight the need for more detailed studies to confirm whether the beetles that have been associated with *Pristionchus* spp. visit places with rotting vegetal matter, considering the seasonality of the beetle's life cycle. In our study, *L. cinnamomeus* is a beetle that develops during the coldest period of the year, as it is adapted to the life cycle of *T. melanosporum* (Martin-Santafé et al. 2014). Despite the low temperatures, *P. maupasi* nematodes were not only isolated from truffle soils but also from *T. melanosporum* fruit bodies and the elytra of adult *L. cinnamomeus* beetles during this period. Adults of *L. cinnamomeus* frequently move between truffles to reproduce (Martin-Santafé 2020), visiting the fruit bodies where *Pristionchus*

https://doi.org/10.1017/S0022149X23000615 Published online by Cambridge University Press

populations are present. Therefore, our results confirm the phoretic/necromenic relationship between these organisms. This is supported by the higher presence of *Pristionchus* in burnt areas (38.1%) than in nonburnt areas (4.8%). These results are consistent with previous studies that have observed scarab beetle species associated with *Pristionchus* feeding as adults on mature and rotting vegetal matter, such as *Geotrupes stercorosus* and *Exomala orientalis* (Cambefort 1991; Choo et al. 2002; Herrmann et al. 2006).

To sum up, this study has revealed the natural occurrence of *S. feltiae* and *H. bacteriophora* in truffle soils but at relatively low frequency, suggesting these EPNs might have specific adaptations to local conditions, which make them potential candidates for the development of novel biological pest control agents. However, their absence in productive plantations, where the herbaceous cover around the host tree is reduced due to *T. melanosporum*, might impact EPNs by limiting the diversity of available insect hosts. The high recovery frequency of *P. maupasi* indicates that this nematode is closely associated with truffles, having a phoretic/necromenic relationship with the beetle *L. cinnamomeus*. Moreover, its presence may affect the occurrence of natural populations of other nematodes, including EPNs, through competition and predation. It is possible that the low presence of EPNs in truffle soils hinders their ability to naturally regulate the population of *L. cinnamomeus*. However, the survival of EPNs in truffle soils during the application of inundative biological control should not pose a problem. Therefore, the potential of isolated EPNs as control agents needs to be assessed through field assays against *L. cinnamomeus*.

Acknowledgements. We would like to thank truffle producters of la Asociación de Truficultores de Teruel (ATRUTER) and l'Associació de Productors de Tòfona de Catalunya (PROTOCAT) for letting us survey their truffle plantations. We also wish to thank Victor Pérez Fortea for helping us during the soil sampling.

Financial support. This work was supported by Proyecto UAB/ATRUTER 202/2021 Asociación de Truficultores de Teruel (ATRUTER): Aislamiento e identificación de nematodos entomopatógenos en fincas truferas de Teruel and by Proyecto I+D+I CIBR – FITE 2021 (CITA) Gobierno de Aragón: Nuevas estrategias de control del escarabajo de la trufa en Teruel.

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