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Invasion of *Popillia japonica* in Lombardy, Italy: Interactions with soil entomopathogenic nematodes and native grubs

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Abstract

1. The recent invasion of the Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) in northern Italy offered the opportunity to explore the entomopathogenic nematodes (EPNs) associated with the soil of hay meadows.
2. A total of 61 sites were considered for nematode sampling, and from 17 of them (27.9%) EPNs were isolated and further characterized with molecular and morphological techniques as well as with laboratory bioassays.
3. Two main species, *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae) were recorded with the same frequency together with a few other species (Rhabditida: *Oscheius* sp. and *Phasmarhabditis* sp.).
4. The newly isolated EPN populations were characterized for their activity (penetration rate) and infectivity to *P. japonica* grubs.
5. EPNs occurrence was related to the time of beetle invasion at each sampling site and there was evidence of a density-dependent response of the EPNs community to *P. japonica* density.
6. The invasion of *P. japonica* apparently did not significantly affect the occurrence of native grubs, although a tendency to a decline was observed and should be further investigated.

KEYWORDS
biological control, Coleoptera, invasive alien species, Rhabditida, Scarabaeidae, Turfgrasses

INTRODUCTION

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are ubiquitous soil organisms (Hominick et al., 1996). These nematodes live in obligate association with bacteria in the soil (Forst & Clarke, 2002) and kill a wide range of insects, among which soil-dwelling pests (Tarasco, Oreste, et al., 2015). EPNs are considered effective biological control agents and serve as an alternative to the chemical control of insect pests (Grewal et al., 2005). In the past three decades, many surveys were conducted in numerous countries and new

Itamar Glazer and Giacomo Santoiemma contributed equally to this study.

Heterorhabditid and Steinernematid species have been identified (Kaya et al., 2006). In Italy, Tarasco, Clausi, et al. (2015) and De Luca et al. (2015) have reported on the distribution and biodiversity of EPNs in the period 1990–2010. In pest infested areas, it was assumed that endemic populations of these biological control agents might offer an optimal regulation of the pests.

In 2014, the Japanese beetle *Popillia japonica* was reported in Northern Italy and EPNs were considered as mortality agents of the grubs in the soil (Marianelli et al., 2018). *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) proved to be effective against the larvae of *P. japonica* in laboratory and field trials. Effectiveness may depend on the larval instar, as the young larvae are generally more susceptible than the mature ones, especially after overwintering (Paoli et al., 2017; Torrini et al., 2020). In addition, Goda et al. (2020) explored the diversity of EPNs attacking *P. japonica* in the invasion area.

The area invaded by *P. japonica* in Italy is characterized by ancient hay meadows with high biodiversity (Della Rocca et al., 2021). Crop damage was never observed, likely because a natural regulation played by a number of factors, including EPNs (Torrini et al., 2020). The area is part of a riverine ecosystem (Ticino natural park) where there is a large use of surface irrigation water according to historical management techniques going back to the end of the 15th century and to the genius of Leonardo da Vinci (Taglialagamba, 2021). The invasion of *P. japonica* resulted in a reduction of hay yield where grub densities were high and asked for management measures (Santoiemma et al., 2021).

The ecological impacts of invasive alien species are multiple (Kenis et al., 2009). Invasive species may interfere with native species through competition (Carmo et al., 2018) or apparent competition, that is, when the relationships are mediated by shared natural enemies (Holt, 1977). A special case is represented when the invasive species is exploited by natural enemies of native herbivores, resulting in an alteration of the local regulation systems (Juliano et al., 2010). The latter possibility may occur if the natural enemies are generic and polyphagous and if they share the same habitat such as soil.

The soil-dwelling EPNs are rather polyphagous organisms associated with a large number of preys in different insect orders (Hominick et al., 1996). The invasion of *P. japonica* may have exposed the native communities of EPNs to an unusual abundance of hosts, which could have triggered a numerical response in the EPNs populations leading to host regulation. If this is welcomed by pest managers as a way to contain the damage to crops, it may have a negative impact on the community of insects living in the soil of the hay meadows, above all the native species of grubs.

The aim of this work is to explore if the invasion of *P. japonica* has altered the occurrence of EPNs in the hay meadows and if the density of native grub species has been affected. To address these questions, the EPNs occurring in hay meadows infested by *P. japonica* in Lombardy, Italy, were isolated and described, testing their responses to larvae of the laboratory standard reference *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) and of *P. japonica*. Commercial strains of *H. bacteriophora* and *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) were used for comparison. In addition, the species of native grubs were identified along a temporal and spatial gradient of *P. japonica* density measured by a grid of pheromone traps for adult beetles.

MATERIALS AND METHODS

Soil sampling for grubs and EPNs

From the beginning of the invasion in 2014, the plant protection service of the Lombardy Region is carrying out routine soil sampling in the infested and buffer areas to detect *P. japonica*. The area is now extending over 5000 km^2 and consists mainly of hay meadows mixed with agricultural and forest crops and human settlements. In March 2020, 61 sites (0.3 ha each) were considered for grub detection across the infested and buffer areas (Table S1 and Figure S1). At each site, 18 soil samples 20 × 20 × 20 cm were randomly taken and inspected on-site for grub presence. Grubs were separated from the soil and taken to a laboratory located in the infested area (Minoprio, Varese) for species identification according to Hůrka (1978) and EPPO (European and Mediterranean Plant Protection Organization) (2006). In addition, a portion of clean soil from each sample was taken to fill up a 1 L plastic container and taken to the same laboratory for EPNs' isolation.

Isolation of EPNs from soil

Isolation of EPNs was done selectively using a baiting technique as described before for *G. mellonella* larvae (Tarasco, Oreste, et al., 2015). One trap with four mature larvae was inserted in each soil sample and incubated at 25°C and 40% of relative humidity for one week. At the end of incubation, the mortality of larvae in the traps was recorded. Dead larvae were further incubated in 'white traps' at 25°C to collect emerging infective juveniles of EPNs from the insect cadavers, as described by Kaya and Stock (1997). Emerging infective juveniles were collected in water and stored in a flask tissue culture at 8°C, and further recovery of them was conducted as described by Levy et al. (2020).

Characterization of EPNs

Morphological identification of the species was measured on 10 individuals per sample. Morphometric of main diagnostic characters of both infective juveniles and adults of *H. bacteriophora* and *S. carpocapsae* was taken at the light microscope equipped with Leica® DFC425 camera and LAS (Leica Application Suite) software, from individuals on temporary water-agar mounts (Esser, 1986; Troccoli, 2002). It was then compared with data from Nguyen and Hunt (2007) and assessed to fit with previous descriptions of these species.

Molecular characterization methods

Fifteen individual nematodes for each sample were processed and total DNA was extracted as described by De Luca et al. (2004). Specimens were handpicked and singly placed on a glass slide in 3 μL of the lysis buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, 15 mM $MgCl_2$, 0.1%

Triton X100, 0.01% gelatine with 90 μg/mL proteinase K) and then cut into small pieces by using a sterilized syringe needle under a dissecting microscope. The samples were incubated at 65°C for 1 h and then at 95°C for 15 min to deactivate the proteinase K. The following sets of primers were used for amplification of two DNA fragments in the present study: ITS (Internal Transcribed Spacer) containing regions using the forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Subbotin et al., 2001); the D2-D3 expansion domains of 28S rRNA using the primer set D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Nunn, 1992); the 18S rDNA using the 18SnF (5′-TGGATAACTGTGGTAATTCTAGAGC-3′) and 18SnR (5′-TTACGACTTTTGCCCGGTTC-3′); the portion of the mitochondrial cytochrome oxidase c subunit 1 (*mt*COI) gene was amplified with the primer set: COI-F1 (5′-CCTACTATGATTGGTGGTTTTGGTAATTG-3′) and COI-R2 (5′-GTAGCAGCAGTAAAATAAGCACG-3′) (Kanzaki & Futai, 2002). Amplification conditions were an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C/50 s, 55°C/50 s 72°C/1 min with a final extension time of 7 min at 72°C. All products were examined by standard electrophoresis on a 1% agarose gel. PCR (Polymerase Chain Reaction) products from two individual nematodes for each population were purified for sequencing, using the protocol listed by the manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned into pCR 2.1-TOPO plasmid and TOP10 competent cells were transformed using the TOPO-TA cloning kit (Invitrogen). Both strands of each clone were sequenced using M13 forward and M13 reverse primers. A Basic Local Alignment Search Tool (BLAST) search at National Center for Biotechnology Information (NCBI) was performed to confirm their nematode origins and species (Altschul et al., 1997). The newly obtained sequences were submitted to GenBank with the following accession numbers: OD921255-OD921257 for the ITS of *H. bacteriophora*, OD921258-OD921261 for the ITS of *S. carpocapsae*; OD921254 for the D2-D3 expansion domains of the 28S rRNA gene of *H. bacteriophora*.

PCR-RFLP (Restriction Fragment Length Polymorphism)

Ten μL of each product, containing the ITS region, from two individual nematodes of *H. bacteriophora* and *S. carpocapsae* species were digested with the following restriction enzymes: *Alu* I (Roche), *Ava* II, *Bam* HI, *Dde* I (Roche), *Hinf* I (Roche), and *Rsa* I (Roche) (5 U of enzyme for each digestion) at 37°C overnight. The digested DNA fragments were loaded onto 2.5% agarose gel and visualized by red staining gel. All gel images were stored digitally. The exact lengths of each restriction fragment from the PCR products were obtained by virtual digestion of the sequences using WEBCUTTER 2.0 (www.firstmarket.com/cutter/cut2.html).

Infectivity of EPNs

Infectivity was evaluated on *G. mellonella* by dose-response and penetration assays (Glazer & Lewis, 1998). Dose-response assay of *G. mellonella* consisted of exposing four mature larvae to 0, 50, 200, 400 or 1600 EPN infective juveniles from each strain (i.e., a species detected in the soil sample collected in a specific site) in 5 cm Petri dishes lined with moist filter paper (0.5 mL water suspension per dish). The dishes were incubated at 25°C for 72 h, and then insect viability was determined. Host penetration assay consisted of exposing ca. 1000 EPN infective juveniles from each strain to five last instar larvae of *G. mellonella* in 5 cm Petri dishes lined with moist filter paper for 48 h at 25°C, to ensure sufficient time for nematodes penetration. The insects were washed in tap water to remove any nematodes from the surface of their bodies. Then they were dissected under a stereomicroscope in a 5 cm Petri dish containing 10 mL of tap water. The number of invading nematodes was determined. Each treatment, for both dose-response and host penetration assays, consisted of three replicates (one dish = one replicate) and the experiment was repeated twice for each natural strain and three times for the commercial strains.

Individual first-instar larvae of *P. japonica* were exposed in 5 cm Petri dishes containing moist soil (7% wt/wt) to seven of the isolated EPN populations that demonstrated high infectivity to *G. mellonella* (>85% mortality after 48 h exposure to 400 EPN infective juveniles) (Table S1: *H. bacteriophora* type—THS, SV6, SVF and *S. carpocapsae* type—SRV, SXA, TDN, TUR-Exp). A total of 28 larvae were assigned to each treatment, including the control and commercial strains. The nematodes were applied in 1 mL water at a concentration of 1000 EPN infective juveniles per dish. The dishes were placed at 25°C for 10 days and then the mortality was recorded. Further assays on *P. japonica* and native grubs were not carried out due to the limited grub availability.

Statistical analyses

To evaluate the distribution of *P. japonica* in the infested area, a negative binomial model with a log link-function was used. The response variable was the number of *P. japonica* grubs collected from each sampling site. The continuous explanatory variable was the number of years until 2020 since *P. japonica* detection in each sampling site (i.e., 2015 = 5, 2016 = 4, 2017 = 3, 2018 = 2, 2019 = 1), based on the grid of pheromone traps deployed by the plant protection service (Gilioli et al., 2021). To evaluate the distribution of native grubs and the putative competition with *P. japonica* grubs in the infested area, a negative binomial model with a log link-function was used. The response variable was the number of native grubs collected in each sampling site. The continuous explanatory variables were the number of years since *P. japonica* detection in each sampling site, the number of *P. japonica* grubs in each sampling site and their interaction. To evaluate the distribution of EPNs in the infested area, a binomial model with a logit link-function was used. The response variable was the presence of EPN species (pooled together to avoid the excess of zeros) in each sampling site. The continuous explanatory variables were the number of years since *P. japonica* detection in each sampling site, the number of *P. japonica* grubs in each sampling site and their

interaction. In all models, the quadratic term of the number of years since *P. japonica* detection was added in order to detect possible non-linear trends of grub and EPN densities over time. There was no evidence of spatial autocorrelation of model residuals, tested using Moran's I function.

For infectivity assays, to avoid model over-parameterization EPN strains were pooled into four groups according to the species after EPN identification: commercial and natural *H. bacteriophora*, commercial and natural *S. carpocapsae*. To evaluate the infectivity of *G. mellonella* exposed to different nematode concentrations, a binomial mixed model with a logit link-function was used. The response variable was the mortality rate of larvae per Petri dish. The categorical explanatory variables were the nematode concentration (five levels), the nematode groups (four levels) and their interaction. The experiment identity was included as a random factor. To evaluate the host penetration on *G. mellonella*, a negative binomial mixed model with a log link-function was used. The response variable was the number of invading nematodes per Petri dish. The categorical explanatory variable was the nematode group (four levels). The experiment identity was included as a random factor. To evaluate the infectivity to *P. japonica*, a binomial model with a logit link-function was used. The response variable was the survival rate of larvae per treatment. The categorical explanatory variable was the nematode group (five levels: four groups plus untreated control). Pairwise multiple comparisons among nematode groups were performed using post-hoc tests with Holm correction of *p*-values. All the analyses were performed in R (R Core Team, 2021). Generalized linear (mixed) models were run using the 'MASS' (Ripley et al., 2021) and 'lme4' (Bates et al., 2021) packages, and validated using the 'car' (Fox et al., 2021), 'pROC' (Robin et al., 2021) and 'DHARMa' (Hartig, 2021) packages. Moran's I analysis was run using the 'ape' package (Paradis et al., 2021). Pairwise multiple comparisons were run using the 'emmeans' package (Lenth et al., 2021).

RESULTS

Occurrence of EPNs in relation to *P. japonica* invasion and native grub populations

Out of the total 61 soil samples, 17 (27.9%) turned positive for *Galleria* traps, 6 (9.8%) yielded Heterorhabditids, 6 (9.8%) Steinernematids, and 10 (16.4%) other types of insect phoretic nematodes belonging to Rhabditids (*Oscheius* sp. and *Phasmarhabditis* sp.) (Table S1). The molecular and morphological characterization of the strains revealed that six strains were identified as *H. bacteriophora*, six strains as *S. carpocapsae*, and the rest were other types of insect phoretic nematodes. In most of the positive samples, a single strain of nematode species was isolated. However, in the site where the invasion likely started (Turbigo, see arrow in Figure 1a), all EPNs species were detected.

In 2020, *P. japonica* grubs were detected in 39 out 61 soil samples (64%) and the abundance of Japanese beetle grubs was strongly related to the number of years since its detection, showing higher numbers in the early infested area, with a decreasing quadratic trend over time (Table 1a and Figure 2a). In 2020, 4 species of native grubs were found in 27 soil samples (44%): the most abundant was *Amphimallon solstitiale tropicum* (Gyllenhal) (96 individuals in 27 sites), followed by *Mimela junii junii* (Duftschmid) (7 individuals in 5 sites) and one individual each of *Amphimallon assimile* (Herbst) and *Aplidia transversa transversa* (Fabricius). Their abundance was not significantly related to the number of years since *P. japonica* detection and to *P. japonica* grub abundance (Table 1b), although a slight negative correlation was observed (Figure S2). The probability to detect EPNs was higher in the early infested area, with a decreasing linear trend over time (Table 1c and Figure 2b), and it showed no relation to *P. japonica* grub abundance. None of the tested interactions resulted significantly.

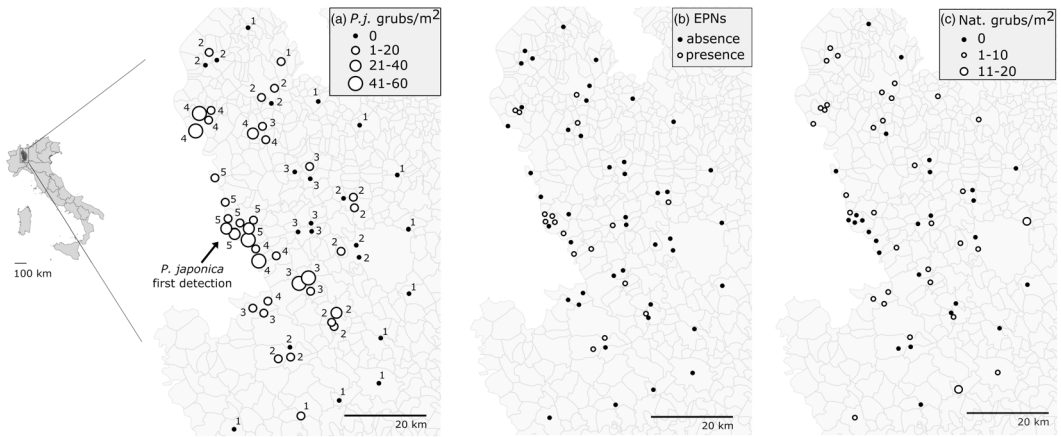

FIGURE 1 Occurrence of (a) *Popillia japonica* grubs (labels indicate the number of years since *Popillia japonica* detection), (b) entomopathogenic nematodes (EPNs) and (c) native grubs in soil samples taken in March 2020. Light grey polygons are the administrative boundaries of the municipalities of Lombardy region.

TABLE 1 Analysis of deviance tables from the generalized linear (mixed) models testing the response

	χ^2	d.f.	*p*-value
a) N. of *P.j.* grubs			
Years since *P.j.* detection	2.551	1	0.110
(Years since *P.j.* detection)^2	7.374	1	0.007
b) N. of native grubs			
N. of *P.j.* grubs	2.640	1	0.104
c) EPN occurence			
Years since *P.j.* detection	7.742	1	0.005
d) *G.m.* mortality			
EPN strain	7.508	3	0.057
EPN concentration	257.779	4	<0.001
e) N. of invading IJs			
EPN strain	47.181	3	<0.001
f) *P.j.* survival			
EPN strain	96.234	4	<0.001

Note: a) number of *Popillia japonica* (*P.j.*) grubs on number of years since *P.j.* detection (linear and quadratic term) (negative binomial model); b) number of native grubs on number of years since *P.j.* detection (linear and quadratic term), number of *P.j.* grubs and their interaction (negative binomial model); c) entomopathogenic nematodes (EPN) occurrence on number of years since *P.j.* detection (linear and quadratic term), number of *P.j.* grubs and their interaction (binomial model); d) mortality of *Galleria mellonella* (*G.m.*) larvae on different EPN strains and concentrations (binomial mixed model); e) number of infective juveniles (IJs) on different EPN strains invading *G.m.* larvae (negative binomial mixed model); f) survival of *P.j.* larvae on different EPN strains (binomial model). Only covariates from minimum adequate models are reported.

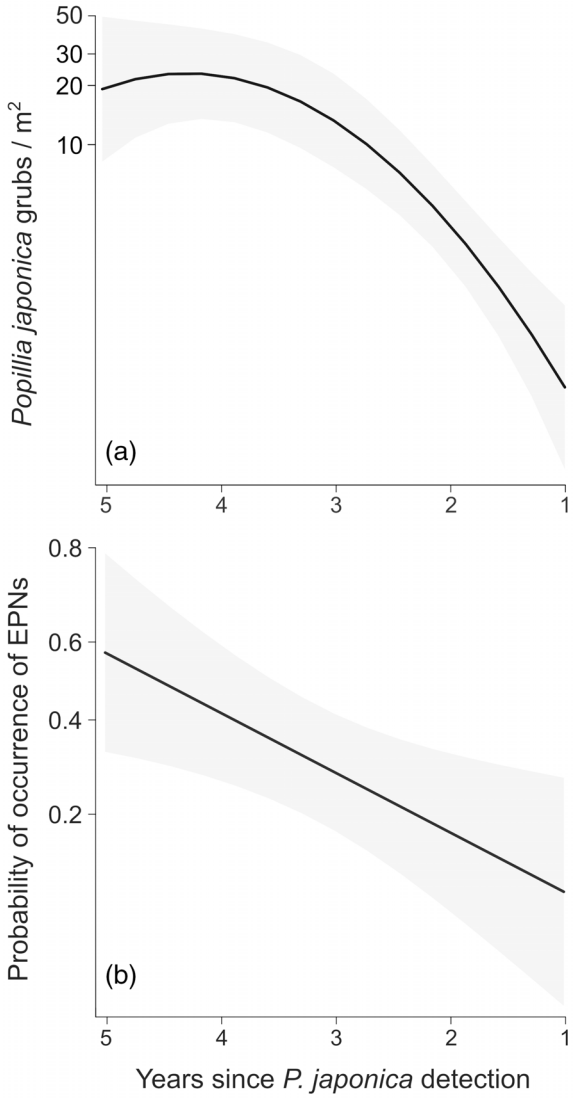

FIGURE 2 (a) Abundance of *Popillia japonica* grubs and (b) probability of occurrence of entomopathogenic nematodes (EPNs) in soil samples taken in March 2020, in relation to the number of years since *Popillia japonica* detection. Plots include model estimate (black line) and 95% confidence interval (grey shading) from the negative binomial and binomial model, respectively. Plots presenting partial residuals are available in Figure S4.

Characterization of EPN strains

All species of EPNs conformed to the morphological traits reported in the specific taxonomic literature. The DNA sequences of ITS and D2-D3 regions were determined for two specimens for each strain. The sequences of the entire ITS of *H. bacteriophora* were 1063 bp in length. RFLP profiles are reported in Figure S3A and are identical to those of other Italian strains (Susurluk et al., 2007). Low intraspecific variability was observed among different clones of the same strain and among strains, ranging from 0.12% to 0.72% (3–7 bp difference) from Lombardy. Inter-population variability among Lombardy populations with most sequences of *H. bacteriophora* present in the NCBI database (Altschul et al., 1997) ranged from 0.32% to 2.5% (1–25 bp difference). The sequencing of the D2-D3 expansion domains of the 28S rRNA gene of *H. bacteriophora* produced a sequence of 608 bp. The interpopulation variability of the partial D2-D3 sequence with the corresponding sequences present in the GenBank database ranged from 0% to 1% (0–4 bp difference). Amplification of the ITS region in *S. carpocapsae* produced a fragment of approximately 814 bp in length, based on sequencing, for all specimens. The restriction profiles of the ITS region are shown in Figure S3B. No intraspecific differences were found between Lombardy populations. The ITS sequence of *S. carpocapsae* showed an inter-population variability with the corresponding sequences of *S. carpocapsae* present in the database ranging from 0.2% to 2% (2–15 bp difference).

Infectivity of EPNs

In the *G. mellonella* assays, the mortality increased strongly with the EPN dose up to 400 EPN infective juveniles/mL and then a plateau close to total mortality was reached, irrespective of the EPN species and strains (Table 1d and Figure 3a). The penetration rate after the exposure of the EPN infective juveniles to the last instars of *G. mellonella* varied considerably. *Steinernema* invaded in higher

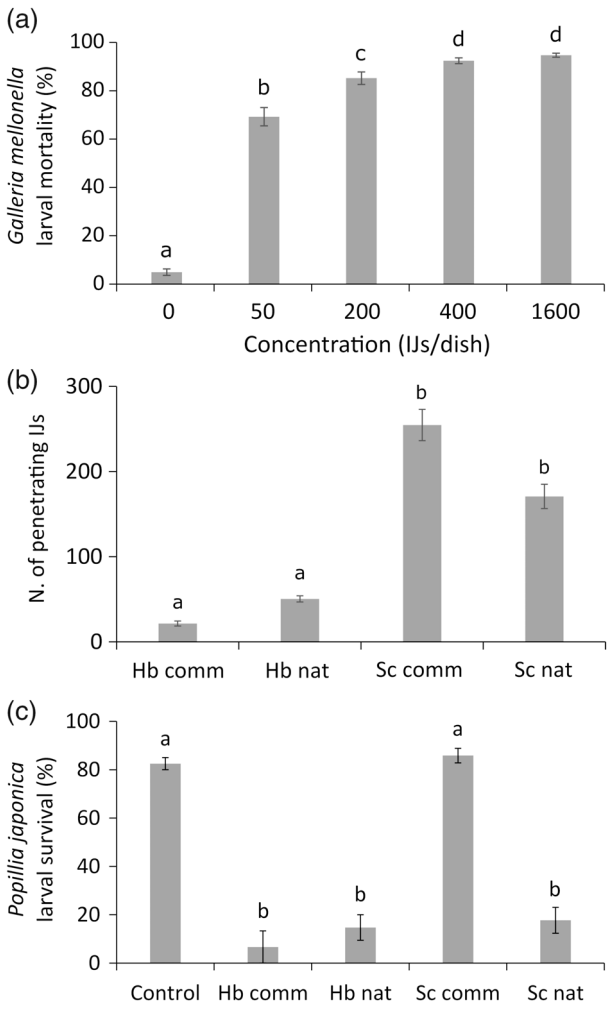

FIGURE 3 (a) Mortality of *Galleria mellonella* mature larvae when exposed to different concentrations of infective juveniles of entomopathogenic nematodes for all species and strains. (b) Number of infective juveniles (IJs) of commercial and natural strains of entomopathogenic nematodes that invaded the mature larvae of *G. mellonella*. (c) Survival of first-instar larvae of *Popillia japonica* after been individually exposed to commercial and natural strains of entomopathogenic nematodes for 10 days. Hb = *Heterorhabditis bacteriophora*, Sc = *Steinernema carpocapsae*, comm = commercial product, nat = natural strains. Error bars indicate standard errors. Different letters imply significant differences in pairwise comparison between treatments ($p < 0.05$).

numbers than *Heterorhabditis* for both commercial and natural strains (Table 1e and Figure 3b). In the *P. japonica* assays, commercial and natural strains of *H. bacteriophora* caused a high level of mortality compared to control while only the natural strains of *S. carpocapsae* showed to be effective (Table 1f and Figure 3c).

DISCUSSION

The results show that the invasion of *P. japonica* impacted on local EPNs but not on native grub populations. The rate of positive samples (>20%) is considerably higher than in previously reported surveys from different types of soils (5%–7%; Glazer et al., 1991; Iraki et al., 2000; Morton & Garcia del Pino, 2009) although it is in line with what found by Torrini et al. (2020) in Piedmont, a region bordering with Lombardy and sharing the same invasion history of the Japanese beetle. The density-dependence shown by the data may explain the higher rate, especially because of the prevalence of the EPNs in the areas of earlier colonization. The prompt response of EPNs can be associated with the occurrence of other soil-dwelling insects inhabiting the hay meadows of the region, which are characterized by a high diversity (Della Rocca et al., 2021).

The native grub community did not appear to be significantly affected by the invasion of *P. japonica*, although a tendency to a decline was observed. The lack of significance could be linked both to methodological (e.g., too few locations or years) and ecological reasons (e.g., yearly stochastic factors influencing the presence of native grubs or potential reaction lags to the invasion of *P. japonica*). The lower frequency of native grubs in the areas of early invasion might also be explained by direct competition with *P. japonica* for access to grassroots as well as apparent competition mediated by natural enemies (Holt, 1977), with special reference to EPNs. Direct competition is known to occur between invasive and native species in a few cases (Kenis et al., 2009) and apparent competition involving invasive and native herbivores is even less frequent (but see Redman & Scriber, 2000; Barros et al., 2020). EPNs were never reported, to the best of our knowledge, as being involved in the apparent competition between invasive and native herbivores. Our data do not allow us to say which one of the two regulatory systems works. Further research at a smaller scale is required to ascertain whether direct or apparent competition is involved and to compare the susceptibility to EPNs of invasive and native species. Indeed, EPNs could have a different virulence on native grubs compared to that observed on *P. japonica*; however, Grewal et al. (2002) reported a similar response of native and non-native species of grubs to various EPNs.

Two species of EPNs, *H. bacteriophora* and *S. carpocapsae* were identified at a morphological and molecular level. Their ITS sequences are identical to Piedmont's ones (Torrini et al., 2020). In addition, the restriction patterns of *S. carpocapsae* of Lombardy (Figure S3B) were very similar to those found in *S. carpocapsae* collected from soil in various parts of the world (Reid et al., 1997; Susurluk & Toprak, 2006; Torrini et al., 2014). The only difference between *S. carpocapsae* strains from Veneto and Lombardy was the presence of two extra-polymorphic bands with the *Alu* I enzyme in the Veneto strain. While in the study by Torrini et al. (2020) in Piedmont Steinernematids were largely dominant, they are as common as Heterorhabditids in Lombardy. The difference can be explained by a lower frequency of hay meadows in Piedmont (about 50% of the sites) while in Lombardy they represent the totality of the sites. As a further confirmation of this interpretation, a third species (*Steinernema feltiae*) was found only in Piedmont from forest habitats that were not considered in Lombardy. Moreover, *S. feltiae* was only recovered from non-cultivated sites by Campos-Herrera et al. (2019).

In the infectivity tests, all isolates displayed high infectivity to the last instars of *G. mellonella* as shown in previous studies (Caroli et al., 1996; Glazer, 1991; Ricci et al., 1996). The dose-response bioassay was not sensitive enough to find differences among strains

(Glazer & Lewis, 1998) while the penetration rate assay was successful in measuring the ability of the EPN infective juveniles of different strains to reach the insect and penetrate in the hemolymph, overcoming the defence systems of the organism (Glazer & Lewis, 1998). Our results demonstrated that Steinernematid strains invade in higher numbers than Heterorhabditid strains. These findings are in agreement with those of previous studies (Caroli et al., 1996; Ricci et al., 1996). Heterorhabditid isolates displayed high infectivity levels in the first instars of *P. japonica*, as observed in other studies (Koppenhöfer et al., 2006, 2015; Torrini et al., 2020). Interesting enough, several Steinernematid strains showed similar high infective capability. This may be attributed to the design of the bioassay (Petridish arena), which eliminated the requirement to search for the insect. Steinernematids showed lower mortality than Heterorhabditids also in semi-field tests carried out with *P. japonica* in the invasion area of Piedmont (Torrini et al., 2020).

In conclusion, EPNs play an important role as natural regulators of *P. japonica*, as their density-dependent response is contributing to the containment of the damage in hay meadows of high ecological importance in the protected area of the Ticino natural park. The benefit of natural regulation should be compared, however, with the cost represented by the negative impact on native grub species. This aspect needs further consideration to understand the precise mechanism and take the possible mitigation actions. Whether EPNs should be used as biocontrol agents with field applications of commercial strains is still a matter of debate. On one side, EPNs could have negative impacts on the community of soil-dwelling insects could be worse, although previous studies proved that the impact of EPNs on the non-target fauna is negligible (Bathon, 1996; Poinar Jr & Grewal, 2012; Smits, 1996). On the other side, the use of EPNs could anticipate the regulation response of native species observed in the present study.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are partly available in the supplementary material of this article. The complete dataset is available from the corresponding author upon reasonable request.

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REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A., Zhang, J., Zhang, Z., Miller, W. et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389–3402.

Barros, M.E.N., Lima, D.B., Mendes, J.A., Gondim Jr, M.G.C. & Da Silva Melo, J.W. (2020) The establishment of an invasive pest mite, *Raoiella indica*, affects mite abundance and diversity on coconut plants. *Systematic and Applied Acarology*, 25, 881–894.

Bates, D., Maechler, M., Bolker, B., Walker, S. (2021) Linear mixed-effects models using 'Eigen' and S4. *R package 'lme4'*, version 1.1-27.1.

Bathon, H. (1996) Impact of entomopathogenic nematodes on non-target hosts. *Biocontrol Science and Technology*, 6, 421–434.

Campos-Herrera, R., Blanco-Pérez, R., Bueno-Pallero, F.A., Duarte, A., Nolasco, G., Sommer, R.J. et al. (2019) Vegetation drives assemblages of entomopathogenic nematodes and other soil organisms: evidence from the Algarve, Portugal. *Soil Biology and Biochemistry*, 128, 150–163.

Carmo, R.F.R., Vasconcelos, S.D., Brundage, A.L. & Tomberlin, J.K. (2018) How do invasive species affect native species? Experimental evidence from a carrion blowfly (Diptera: Calliphoridae) system. *Ecological Entomology*, 43, 483–493.

Caroli, L., Glazer, I. & Gaugler, R. (1996) Entomopathogenic nematode infectivity assay: comparison of penetration rate into different hosts. *Biocontrol Science and Technology*, 6, 227–233.

De Luca, F., Clausi, M., Troccoli, A., Curto, G., Rappazzo, G. & Tarasco, E. (2015) Entomopathogenic nematodes in Italy: occurrence and use in microbial control strategies. In: Campos-Herrera, R. (Ed.) *Nematode pathogenesis of insect and other pests*. Cham: Springer, pp. 431–449.

De Luca, F., Reyes, A., Grunder, J., Kunz, P., Agostinelli, A., De Giorgi, C. et al. (2004) Characterization and sequence variation in the rDNA region of six nematode species of the genus *Longidorus* (Nematoda). *Journal of Nematology*, 36, 147.

Della Rocca, F., Stefanelli, S., Cardarelli, E., Bogliani, G. & Bracco, F. (2021) Contribution to the knowledge of the arthropods community inhabiting the winter-flooded meadows (marcite) of northern Italy. *Biodiversity Data Journal*, 9, e57889.

EPPO. (2006) Diagnostic *Popillia japonica*. *OEPP/EPPO Bulletin*, 36, 447–450.

Esser, R.P. (1986) A water agar en face technique. *Proceedings of the Helminthological Society of Washington*, 53, 254–255.

Forst, S. & Clarke, D. (2002) Bacteria-nematode symbiosis. In: Gaugler, R. (Ed.) *Entomopathogenic nematology*. Wallingford, Oxfordshire: CABI Publishing, pp. 57–78.

Fox, J., Weisber, S., Price, B., Adler, D., Bates, D., Baud-Bovy, G. et al. (2021) Companion to applied regression. *R package 'car'*, version 3.0-11.

Gilioli, G., Sperandio, G., Simonetto, A., Colturato, M., Battisti, A., Mori, N. et al. (2022) Modelling diapause termination and phenology of the Japanese beetle, *Popillia japonica*. *Journal of Pest Science*, 95, 869-880.

Glazer, I. (1991) Invasion rate as a measure of infectivity of steinernematid and heterorhabditid nematodes to insects. *Journal of Invertebrate Pathology*, 59, 90–94.

Glazer, I. & Lewis, E.E. (1998) Bioassays for entomopathogenic nematodes. In: Navon, A. (Ed.) *Bioassays for entomopathogens and nematodes*. Wallingford: CABI Publishing, pp. 274–293.

Glazer, I., Liran, N. & Steinberger, Y. (1991) A survey of entomopathogenic nematodes (Rhabditida) in the Negev desert. *Phytoparasitica*, 19, 291–300.

Goda, N., Mirzaei, M. & Brunetti, M. (2020) Potentially entomopathogenic nematode isolated from *Popillia japonica*: bioassay, molecular characterization and the associated microbiota. *Bulletin of Insectology*, 73, 295–301.

Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (2005) *Nematodes as biocontrol agents*. Wallingford: CABI Publishing, p. 505.

Grewal, P.S., Grewal, S.K., Malik, V.S. & Klein, M.G. (2002) Differences in susceptibility of introduced and native white grub species to entomopathogenic nematodes from various geographic localities. *Biological Control*, 24, 230–237.

Hartig, F. (2021) Residual diagnostics for hierarchical (multi-level/mixed) regression models. *R package 'DHARMa'*, version 0.4.3.

Holt, R.D. (1977) Predation, apparent competition and the structure of prey communities. *Theoretical Population Biology*, 12, 197–229.

Hominick, W.M., Reid, A.P., Bohan, D.A. & Briscoe, B.R. (1996) Entomopathogenic nematodes: biodiversity, geographical distribution and the convention on biological diversity. *Biocontrol Science and Technology*, 6, 317–332.

Hůrka, K. (1978) Scarabaeidae. In: Klausnitzer, B. (Ed.) *Ordung Coleoptera (larven)*. Berlin: Akademie, pp. 103–113.

Iraki, N., Salah, N., Sansour, M.A., Segal, D., Glazer, I., Johnigk, S.A. et al. (2000) Isolation and characterization of two entomopathogenic nematode strains, *Heterorhabditis indica* (Nematoda, Rhabditida), from the West Bank, Palestinian territories. *Journal of Applied Entomology*, 124, 375–380.

Juliano, S.A., Lounibos, L.P., Nishimura, N. & Greene, K. (2010) Your worst enemy could be your best friend: predator contributions to invasion resistance and persistence of natives. *Oecologia*, 162, 709–718.

Kanzaki, N. & Futai, K. (2002) A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology*, 4, 35–41.

Kaya, H.K., Aguillera, M.M., Alumai, A., Choo, H.Y., de la Torre, M., Fodor, A. et al. (2006) Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biological Control*, 38, 134–155.

Kaya, H.K. & Stock, S.P. (1997) Techniques in insect nematology. In: Lacey, L.A. (Ed.) *Manual of techniques in insect pathology*. New York, NY: Academic Press, pp. 281–324.

Kenis, M., Auger-Rozenberg, M.-A., Roques, A., Timms, L., Péré, C., Cock, M.J.W. et al. (2009) Ecological effects of invasive alien insects. *Biological Invasions*, 11, 21–45.

Koppenhöfer, A.M., Grewal, P.S. & Fuzy, E.M. (2006) Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. *Biological Control*, 38, 397–404.

Koppenhöfer, A.M., Kostromytska, O.S., McGraw, B.A. & Ebssa, L. (2015) Entomopathogenic nematodes in turfgrass: ecology and management of important insect pests in North America. In: Campos-Herrera, R. (Ed.) *Nematode pathogenesis of insects and other pests*. Switzerland: Springer, pp. 309–328.

Lenth, R.V., Buerkner, P., Herve, M., Love, J., Miguez, F., Riebl, H. et al. (2021) Estimated marginal means, aka least-squares means. *'emmeans' R package*, version 1.7.1-1.

Levy, N., Faigenboim, A., Salame, L., Molina, C., Ehlers, R.-U., Glazer, I. et al. (2020) Characterization of the phenotypic and genotypic tolerance to abiotic stresses of natural populations of *Heterorhabditis bacteriophora*. *Scientific Reports*, 10, 10500.

Marianelli, L., Paoli, F., Torrini, G., Mazza, G., Benvenuti, C., Binazzi, F. et al. (2018) Entomopathogenic nematodes as potential biological control agents of *Popillia japonica* (Coleoptera, Scarabaeidae) in Piedmont Region (Italy). *Journal of Applied Entomology*, 142, 311–318.

Morton, A. & Garcia del Pino, F. (2009) Ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean area. *Journal of Invertebrate Pathology*, 103, 203–213.

Nguyen, K. & Hunt, D. (2007) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. In: Hunt, D.J. & Perry, R.N. (Eds.) *Nematology monographs and perspectives*. Netherlands: Brill, pp. 449–450.

Nunn, G.B. (1992) *Nematode molecular evolution: an investigation of evolutionary patterns among nematodes based upon DNA sequences*. Dissertation, University of Nottingham.

Paoli, F., Marianelli, L., Torrini, G., Mazza, G., Benvenuti, C., Bosio, G. et al. (2017) Differential susceptibility of *Popillia japonica* 3rd instars to *Heterorhabditis bacteriophora* (Italian strain) at three different seasons. *Biocontrol Science and Technology*, 27, 439–444.

Paradis, E., Blomberg, S., Bolker, B., Brown, J., Claude J, Cuong HS, Desper R, Didier G (2021) Analyses of phylogenetics and evolution. *R package 'ape'*, version 5.6.

Poinar, G.O., Jr. & Grewal, P.S. (2012) History of entomopathogenic nematology. *Journal of Nematology*, 44, 153.

R Core Team (2021) *R: a language and environment for statistical computing*. https://www.r-project.org/

Redman, A.M. & Scriber, J.M. (2000) Competition between the gypsy moth, *Lymantria dispar*, and the northern tiger swallowtail, *Papilio canadensis*: interactions mediated by host plant chemistry, pathogens and parasitoids. *Oecologia*, 125, 218–228.

Reid, A.P., Hominick, W.M. & Briscoe, B.R. (1997) Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology*, 37, 187–193.

Ricci, M., Glazer, I. & Gaugler, R. (1996) Entomopathogenic nematode infectivity assay: comparison of laboratory bioassays. *Biocontrol Science and Technology*, 6, 235–245.

Ripley, B., Venables, B., Bates, D.M., Hornik, K., Gebhardt, A. & Firth, D. (2021) Support functions and datasets for Venables and Ripley's MASS. *R package 'MASS'*, version 7.3-54.

Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek F, Sanchez JC, Müller M, Siegert S (2021) Display and analyze ROC curves. *R package 'pROC'*, version 1.18.0.

Santoiemma, G., Battisti, A., Gusella, G., Cortese, G., Tosi, L., Gilioli, G. et al. (2021) Chemical control of *Popillia japonica* adults on high-value crops and landscape plants of northern Italy. *Crop Protection*, 150, 105808.

Smits, P.H. (1996) Post-application persistence of entomopathogenic nematodes. *Biocontrol Science and Technology*, 6, 379–388.

Subbotin, S.A., Vierstraete, A., De Ley, P., Rowe, J., Waeyenberge, L., Moens, M. et al. (2001) Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution*, 21, 1–16.

Susurluk, A., Tarasco, E., Ehlers, R.-U. & Triggiani, O. (2007) Molecular characterisation of entomopathogenic nematodes isolated in Italy by PCR-RFLP analysis of the ITS region of the ribosomal DNA repeat unit. *Nematologia Mediterranea*, 35, 23–28.

Susurluk, I.A. & Toprak, U. (2006) Molecular identification of three entomopathogenic nematodes from Turkey by PCR-RFLP of the ITS regions. *Phytoparasitica*, 34, 17–20.

Taglialagamba, S. (2021) Hydraulics in renaissance science. In: Sgarbi, M. (Ed.) *Encyclopedia of renaissance philosophy*. Switzerland: Springer Nature.

Tarasco, E., Clausi, M., Rappazzo, G., Panzavolta, T., Curto, G., Sorino, R. et al. (2015) Biodiversity of entomopathogenic nematodes in Italy. *Journal of Helminthology*, 89, 359.

Tarasco, E., Oreste, M., Li, X. & Liu, Q. (2015) Infectivity of Mediterranean native entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from natural habitats in relation to temperature. *Redia*, 98, 109–114.

Torrini, G., Landi, S., Benvenuti, C., De Luca, F., Fanelli, E., Troccoli, A. et al. (2014) Morphological and molecular characterization of a *Steinernema carpocapsae* (Nematoda Steinernematidae) strain isolated in Veneto region (Italy). *Redia*, 97, 89–94.

Torrini, G., Paoli, F., Mazza, G., Simoncini, S., Benvenuti, C., Strangi, A. et al. (2020) Evaluation of indigenous entomopathogenic nematodes as potential biocontrol agents against *Popillia japonica* (Coleoptera: Scarabaeidae) in Northern Italy. *Insects*, 11, 804.

Troccoli, A. (2002) Aspetti pratici e comparativi di alcune metodologie tradizionali applicate all'identificazione dei nematodi da quarantena. *Nematologia Mediterranea*, 30, 107–110.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1 Supporting information.

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