Journal of Helminthology

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Biocontrol potential of six *Heterorhabditis bacteriophora* strains isolated in the Azores Archipelago

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Research Paper

Cite this article: Beltrí R, Monteiro HR, Toubarro D, Simões N and Garriga A (2024). Biocontrol potential of six *Heterorhabditis bacteriophora* strains isolated in the Azores Archipelago. *Journal of Helminthology*, **98**, e43, 1–10
https://doi.org/10.1017/S0022149X24000336.

Received: 29 February 2024
Revised: 08 May 2024
Accepted: 08 May 2024

Keywords:
Entomopathogenic nematodes; biological control; native isolates; virulence; phylogeny

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Abstract

Entomopathogenic nematodes (EPNs) are closely associated with *Popillia japonica* and potentially used as their biological control agents, although field results proved inconsistent and evoked a continual pursuit of native EPNs more adapted to the environment. Therefore, we surveyed the Azorean Archipelago to isolate new strains of *Heterorhabditis bacteriophora* and to evaluate their virulence against the model organism *Galleria mellonella* under laboratory conditions. Six strains were obtained from pasture and coastal environments and both nematode and symbiont bacteria were molecularly identified. The bioassays revealed that Az172, Az186, and Az171 presented high virulence across the determination of a lethal dose (LD50) and short exposure time experiments with a comparable performance to Az29. After 72 hours, these virulent strains presented a mean determination of a lethal dose of 11 infective juveniles cm^{-2}, a lethal time (LT50) of 34 hours, and achieved 40% mortality after an initial exposure time of only 60 minutes. Az170 exhibited an intermediate performance, whereas Az179 and Az180 were classified as low virulent strains. However, both strains presented the highest reproductive potential with means of 1700 infective juveniles/mg of larvae. The bioassays of the native EPNs obtained revealed that these strains hold the potential to be used in biological control initiatives targeting *P. japonica* because of their high virulence and locally adapted to environmental conditions.

Introduction

The Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae), stands as a major insect pest native to Japan that reached mainland Europe in 2014 causing great damage in the Italian region (Glazer *et al.* 2022; Gotta *et al.* 2023). The larval stage exerts detrimental effects on grasslands and pastures, whereas adults feed on the foliage of agricultural crops and ornamental plants (Potter and Held 2002). Early investigations on this pest identified naturally occurring parasitism of *P. japonica* larva by entomopathogenic nematodes (EPNs) leading to the isolation of *Steinernema glaseri* (Rhabditida: Steinernematidae) (Poinar 1990). Indeed, EPNs are obligate insect parasites that belong mainly to the families Steinernematidae and Heterorhabditidae (order Rhabditida) (Alatorre-Rosas and Kaya 1990; Peters 1996). These nematodes form symbiotic associations with gram-negative bacteria of the genera *Xenorhabdus* and *Photorhabdus* respectively (Bird and Akhurst 1983; Griffin *et al.* 2005). Infective juveniles (IJs) penetrate the host via natural openings or by penetrating the cuticle and releasing the bacteria while developing to the adult stage (Snyder *et al.* 2007). The nematode-bacteria complex kills the host within 24 to 48 hours through septicaemia or toxaemia (Dowds and Peters 2002; Forst and Clarke 2002). Adult nematodes feed on bacteria-degraded insect tissues, generating subsequent generations of IJs that emerge from the depleted cadaver to seek out new hosts (Poinar 1990).

Our understanding of EPNs has progressively expanded enabling their use as effective biological control agents. EPNs offer a distinctive advantage because of their ability to find and infect soil-dwelling and cryptic environment pests, mirroring their natural habitat characteristics. The application of EPNs yielded favourable outcomes in managing soil pests such as *Diabrotica* spp. (Coleoptera: Chrysomelidae) and *Sphenophorus* spp. (Coleoptera: Curculionidae) (Jaffuel *et al.* 2020; Leite *et al.* 2023). Nevertheless, field applications of EPNs against *P. japonica* larvae have yielded inconsistent control over the years (Helmberger *et al.* 2018). Georgis and Gaugler (1991) suggested that factors like unsuitable nematode strains or adverse environmental conditions impeded an effective control of *P. japonica* during field trials. This led to focus on isolating new strains, seeking native EPNs better adapted to the local environment, and climatic, and edaphic soil conditions (Aryal *et al.* 2022a; Lacey *et al.* 2015). EPNs exhibit a ubiquitous global distribution, thriving in diverse landscapes ranging from agricultural fields to urban gardens (Aryal *et al.* 2022a; Campos-Herrera *et al.* 2007; Julià *et al.* 2023; Londoño-

Caicedo *et al.* 2023; San-Blas *et al.* 2019). This broad ecological adaptability positions EPNs as versatile and cosmopolitan agents with potential applicability in a wide array of environments. Besides, nematode strains can display large differences regarding their biological traits; thus, the selection of the best EPNs for one pest target entails the search for new isolates (Gulzar *et al.* 2020; Campos-Herrera *et al.* 2021). Evaluation of novel isolates must go beyond taxonomic classification and extend to defining their traits such as fitness, infection potential, and environmental stress, as explored in previous studies on EPN strains (Morton and García-del-Pino 2009; Khashaba *et al.* 2020; Aryal *et al.* 2022b). Virulence characterisation is fundamental to grasp the potentiality of these newfound isolates and serves to optimise the forthcoming assessments directed at various insect pests.

In the early 1970s, *P. japonica* reached the Azores Islands from America, firmly establishing itself in the archipelago (Simões *et al.* 1993). To assess the potential for biological control, a nematode sampling was undertaken across the islands with the isolation of several strains of *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) and *Steinernema carpocapsae* (Rhabditida: Steinernematidae) (Rosa *et al.* 2000). Although these strains demonstrated notable virulence against *P. japonica* larvae only in controlled experiments, they proved ineffective under field conditions (Simões *et al.* 1993). In 2015, the designation of *P. japonica* as a priority pest in Europe renewed interest in exploring biological control strategies (EPPO 2014). Subsequent investigations confirmed the superior virulence of *Heterorhabditis* spp. compared to *Steinernema* spp. against the larval stage of *P. japonica* under laboratory conditions and semi-field experiments (Renkema and Parent 2021; Torrini *et al.* 2020). The continuous search for new EPN strains adapted to the local environment is crucial for the efficacy of biocontrol measures against insect pests. In this sense, a recent soil survey across the Azorean archipelago was conducted to isolate novel *H. bacteriophora* strains. The present study focused on the isolation and molecular identification of Azorean native strains of *H. bacteriophora*, and the evaluation of the virulence and reproductive potential of the strains using *Galleria mellonella* L. (Lepidoptera: Galleridae) as model organism, with the goal of determining their potential use as biological control agents.

Material and methods

Soil sampling

A total of 164 soil samples were collected from 82 sites in all the islands of the Azorean Archipelago (Portugal) except Santa Maria Island. Ten sites were sampled on each island, with an additional two points on São Miguel Island from spring to autumn 2022. The collection sites were selected across each island in areas infested with *P. japonica* and two soil samples were collected in each site. Each soil sample weighed approximately 1 kg resulting from three subsamples taken with a garden shovel at a depth of 10–25 cm. Samples were placed in a polyethylene bag to prevent water loss and transported to the laboratory under cool conditions. Before EPN isolation, samples were sieved to remove stones, organic matter, and residues. Landscape features, geographical coordinates, and altitude were all recorded at each point using a GPS. The Azorean Archipelago lies between 0 to 2300 m above sea level, although all collection sites were obtained below 950 m. The prevailing conditions across the sampling area were a mean temperature of 13–24 °C and a mean rainfall of 800–1500 mm, recorded by the official stations. Due to the volcanic origin, the prevailing soil is Androsols with a pH of 5.4–6.

Isolation of entomopathogenic nematodes

EPNs were isolated from soil samples using the insect baiting technique described by Bedding and Akhurst (1975). Each soil sample was processed by filling two plastic jars (500 mL, 12 cm in diameter) with soil. Four *G. mellonella* larvae were then placed on the soil surface in each jar. The plastic jars were incubated in darkness at 23 ± 1 °C, sealed, and inverted daily. Moisture levels were adjusted by adding water as needed during the baiting period. After 7 days of incubation, insect larvae were removed from the jars. If no EPNs were detected initially, the assay was repeated. Dead larvae exhibiting signs of nematode infection were individually transferred onto modified White traps (Kaya and Stock 1997) and maintained at 23°C ± 1 °C. The collected IJs underwent several cycles of exposure to fresh *G. mellonella* larvae to confirm pathogenicity and establish the new culture.

Nematodes and bacteria culture

Once EPNs were isolated, they were maintained as laboratory cultures according to the procedures of Woodring and Kaya (1988). EPNs were reared *in vivo* using larvae of *G. mellonella* as previously described and collected in a White trap (White 1927). Nematodes were stored in Ringer solution at 10 °C for 7–14 days. Before any experiment, IJs were acclimated at room temperature, checked for vitality, and adjusted to the required test dose.

The symbiotic bacteria were isolated from nematode-infected *G. mellonella*, following the protocol described in Rosa et al. (2002). Larvae were disinfected on the surface, and the haemolymph was streaked onto NBTA (nutrient agar supplemented with 5 g yeast extract, 25 mg bromothymol blue, and 40 mg triphenyl tetrazolium chloride per liter). After 48 hours' incubation at 27 °C in the dark, the cultures were transferred to 10 °C and subcultured weekly.

DNA extraction

The whole body of IJs was used to extract the DNA from both nematode and symbiont bacteria. Several thousand IJs were frozen at -80 °C and homogenized with a pestle for 30 seconds to break the cuticle. This was followed by a 3-hour incubation at 55 °C with protease K and lysis buffer while maintaining constant agitation at 700 rpm, and a subtle agitation every 30 minutes. After incubation, the DNA was obtained with the extraction kit Zymo Quick-DNA MiniPrep (Zymo Research Corporation) following the manufacturer's instructions. This process was repeated with three biological replicates for each isolate. The complete genomic DNA was sequenced using universal primers by the Next-Generation Sequencing (NGS) whole-genome sequencing technique via the Illumina platform and based on the mechanism of sequencing by synthesis. The quality of reads was assessed using FastQC and trimmed from adaptors and low-quality nucleotides (Andrews 2010). Then, Burrows-Wheeler Aligner software was used to filter and map the reads to the corresponding *Heterorhabditis* genome of reference (GCA_000223415) and the *Photorhabdus* genome of reference (GCF_001083805) (Li and Durbin 2009).

https://doi.org/10.1017/S0022149X24000336 Published online by Cambridge University Press

Phylogenetic analysis of nematode and bacteria

From the mapped reads to the genome, manual curation was done to extract the fragment containing the complete 18s - ITS - 28s region (1290 bp) from each nematode isolate. To obtain the reference sequences for the nematode phylogenetic analysis, the fragments of our isolates were submitted to Blastn for similarity and *Heterorhabditis* sequences were obtained altogether with *Oscheius tipulae* (Rhabditida: Rhabditidae) as an outgroup. Sequences were aligned using the Multiple Sequence Alignment of the ClustalW software. The phylogenetic tree was calculated with the Tamura-Nei + G model, using maximum likelihood with the MegaX software (Kumar *et al.* 2018; Tamura 1992). The robustness of the phylogenetic tree was evaluated by a bootstrap analysis of 10,000 replicates.

For the bacterial isolates, the 16s rRNA region (1545 bp) and the GyrB gene (partial cds 1136 bp) were manually selected from each isolate. Reference sequences of the genera *Photorhabdus* were obtained from NCBI Reference Sequences following the phylogeny proposed by Tailliez et al. (2010). *Escherichia coli* was used as an outgroup. Sequences for the two regions were aligned separately using the ClustalW software and then concatenated to be modelled as one. The phylogenetic tree was calculated using the maximum likelihood method with the Kimura 2-parameter + G+I model and evaluated by a bootstrap of 10,000 replicates (Kimura 1980).

Bioassays using G. mellonella larvae

To determine the virulence of the six strains, three different bioassays were performed using the last instar larvae of *G. mellonella*. All larvae proceeded from our laboratory culture and only healthy insects weighing 240 ± 20 mg were used in the experiments. Different batches of insects were used for each experimental replicate. Throughout the experiments, insect larvae were deemed dead if they exhibited: (i) a lack of movement and unresponsiveness to probing with a dissecting needle, (ii) loss of turgor, or (iii) displayed symptoms characteristic of EPN infection, such as brick-red coloration resulting from *Photorhabdus* proliferation (Kazimierczak *et al.* 2018). Besides, bioassays were performed alongside the native *H. bacteriophora* strain Az29 as a reference strain due to its well-described virulence (Rosa *et al.* 2002), thus serving as a positive control in experimental setups.

Determination of the dose–mortality relationship

The determination of the lethal dose (LD50), expressed in terms of IJ cm^{-2}, involved individual exposures of insects subjected to different doses of EPNs in 24-well plates. Each well, lined with two filter papers, housed one *G. mellonella* larva and the corresponding dose of IJs, administered in 100 µL of sterile tap water. Eight doses were used for each EPN strain corresponding to 5, 10, 20, 40, 80, 160, 320, and 640 IJs per insect larvae (equivalent to 3, 5, 11, 21 44, 88, 168, and 337 IJ cm^{-2}). Employing a randomised block design, plates were sealed with Parafilm and incubated in darkness at 23 ± 1 °C. Insect mortality was checked at 24, 48, 72, and 96 hours of exposure, based on examination of the insects´ condition as previously described. The assay consisted of four *G. mellonella* larvae per nematode strain and dose and was replicated twice. Negative control using sterile tap water instead of IJs was conducted with the same number of replicates and insect larvae.

Determination of the lethal time at fixed dose

The lethal time (LT50) was determined for each EPN strain by administering a fixed dose of IJs to *G. mellonella*. The assay was conducted in 24-well plates filled with two filter papers where insect larvae were individually exposed to 200 IJs suspended in 100 µL solution. Plates were sealed with Parafilm and placed in the dark at 23 ± 1 °C. Twelve hours after the application of IJs, we performed larval mortality checks every 2 hours until >80% of insect larval mortality was achieved in all experimental treatments. The experiment was conducted twice, with each repetition involving 24 *G. mellonella* larvae per EPN strain. Negative controls, comprising 24 *G. mellonella* larvae, were subjected to the same manipulation but received sterile tap water instead of IJs.

Short exposure assay

To test the ability of each EPN strain to infect in a short exposure time, we assess the *G. mellonella* larvae mortality after three exposure periods time: 20, 60, and 180 minutes. The infection was performed as described previously, individualising insect larvae in 24-well plates filled with two filter papers. Each well received a dose of 211 IJ cm^{-2} suspended in 100 µL of sterile tap water. After the designated exposure period, each insect larva was carefully removed from the test well and rinsed with diluted 70% ethanol and subsequently with sterilised distilled water to remove any attached nematodes. Rinsed larvae were then incubated at 23 ± 1 °C for an additional 48 hours to monitor the mortality induced by nematodes. Negative controls, comprising the same number of *G. mellonella* larvae per exposure time were subjected to the same manipulation but without exposure to IJs. A total of six insect larvae per EPN strain and exposure time was used and repeated twice.

Reproductive potential

An assessment to determine the reproductive potential of the EPN strains was conducted using *G. mellonella* larvae following the established infection methodology and dose for rearing EPNs (Bedding and Akhurst 1975; Woodring and Kaya 1988). Dead insect larvae were transferred individually to small White traps made with 5-mm Petri dishes. Emerging nematodes from the insect larvae cadaver were collected daily and stored in Ringer solution at 10 °C. After 1 week without further collection, the total number of EPN offspring was counted through serial dilution. There were six *G. mellonella* larvae replicates per nematode strain and the experiment was done twice.

Statistical analysis

Survival data were fitted to a four-parameter logistic curve to determine LT50 and LD50 values. The estimated LD50 values at a particular time point and the LT50 at a fixed dose were compared between each pair of nematode strains using the extra sum-of-squares F test. For the short exposition and reproduction potential tests, the Kruskal-Wallis test was applied to detect significative differences among EPN strains, when the positive Dunn test was used to determine pairwise differences. Statistical analyses were conducted using GraphPad Prism 10 for Mac and RStudio, and the significance level was set at 0.05 (R Core Team 2017).

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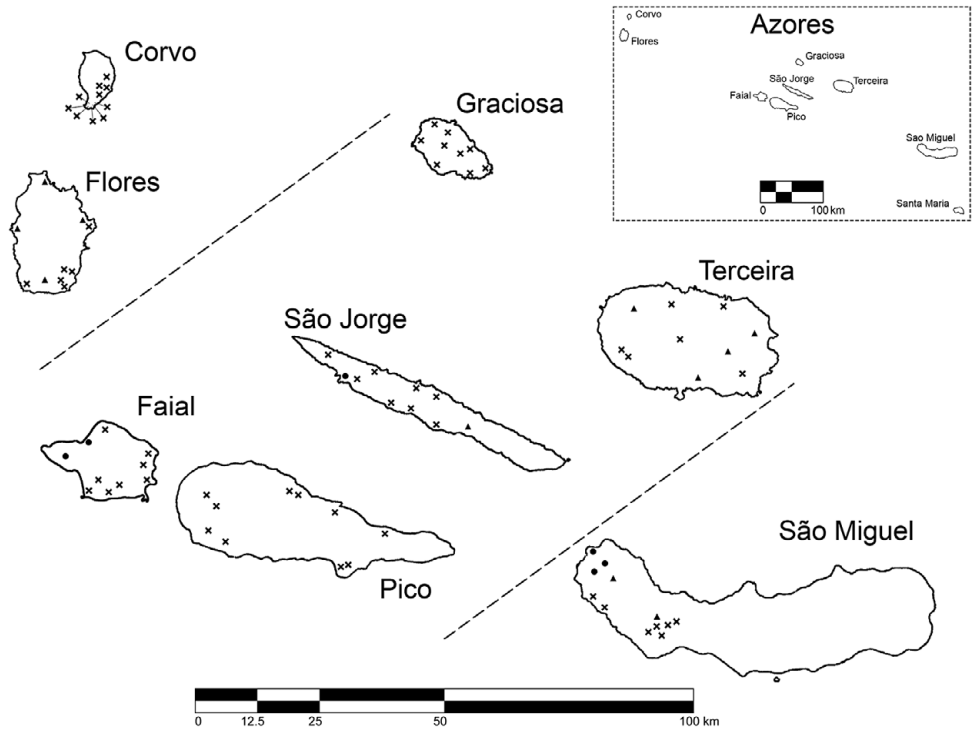

Figure 1. Geographical distribution of the sampling sites (x) in the Azores Islands with the identification of the positive samples for (▲) *Steinernema* sp. or (●) *H. bacteriophora.*

Table 1. Localisation of the isolated strains of *Heterorhabditis* sp. with GPS coordinates, altitude (in meters), and landscape

Strain	Location, Island	GPS	Altitude	Landscape
Az170	Ponta dos mosteiros, São Miguel	37°54'00.3"N 25°49'16.0"W	10	Coast
Az171	Miradouro da Lomba do Vasco, São Miguel	37°52'22.5"N 25°48'45.2"W	400	Pasture
Az172	Túnel das Sete Cidades, São Miguel	37°52'32.9"N 25°47'25.4"W	300	Pasture
Az179	Capelo, Faial	38°35'27.6"N 28°47'24.0"W	270	Pasture
Az180	Praia Da Fajã, Faial	38°36'41.1"N 28°44'36.1"W	35	Coast
Az186	Morro Grande, São Jorge	38°40'54.1"N 28°13'12.9"W	100	Scrubland

Results

Soil sampling

EPNs were recovered from 18 of the 164 sites sampled in the islands, accounting for 10.98% of positive samples (Fig 1). Among the isolated EPNs, we selected strains of *Heterorhabditis* sp. because of their significant role in the biological control of *P. japonica.* This particular nematode species was isolated from six different sites (3.7% of the total) across the islands of São Miguel, São Jorge, and Faial (Table 1). Besides, positive samples from the Islands of Terceira, Flores, and Graciosa contained unidentified EPNs. EPNs were found at various locations, ranging from sea level to 400 m of altitude, and spread across diverse landscapes. Three strains were isolated from pastures, the most common landscape type of the islands, characterised by loamy soils and grasslands. Two strains (Az170 and Az180) were obtained from a coastal landscape dominated by sandy soil and little vegetation. In São Jorge, a single positive sample was obtained from an area outside an urban zone composed of shrubs. All isolated strains of *H. bacteriophora* were maintained in continued culture at our facilities at 10 °C (Fig. S1).

Phylogenetical identification of EPNs

The six new isolates were phylogenetically identified with the regions 18s-ITS-28s for nematodes and with the concatenated 16s rRNA and GyrB gene for symbiotic bacteria. All nematode fragments presented over 99% similarity to *H. bacteriophora*, with a close phylogenetical relationship to *Heterorhabditis georgiana* (Rhabditida: Heterorhabditidae) (Fig. 2). Overall, we can firmly affirm that the new strains belong to *H. bacteriophora* with strong bootstrap support. Sequences for the isolated *H. bacteriophora* strains can be found in the following accession numbers of GenBank (PP738128, PP738129, PP738130, PP738131, PP738132, PP738133). Concerning the symbiont bacteria, the six isolates had over 97% similarity with *P. luminescens.* The phylogenetic analysis separated Az171 and Az186 bacteria with reliability (bootstrap value = 45) from the main node that includes Az180, Az172, and Az179 (Fig. 3). Az170 was clearly separated from the other isolates. Besides the minor differences pointed out by the analysis among bacterial strains, the phylogenetic tree located robustly all samples within the branch of *Photorhabdus luminescens*, specifically the subspecies *laumondii.* Sequences for the

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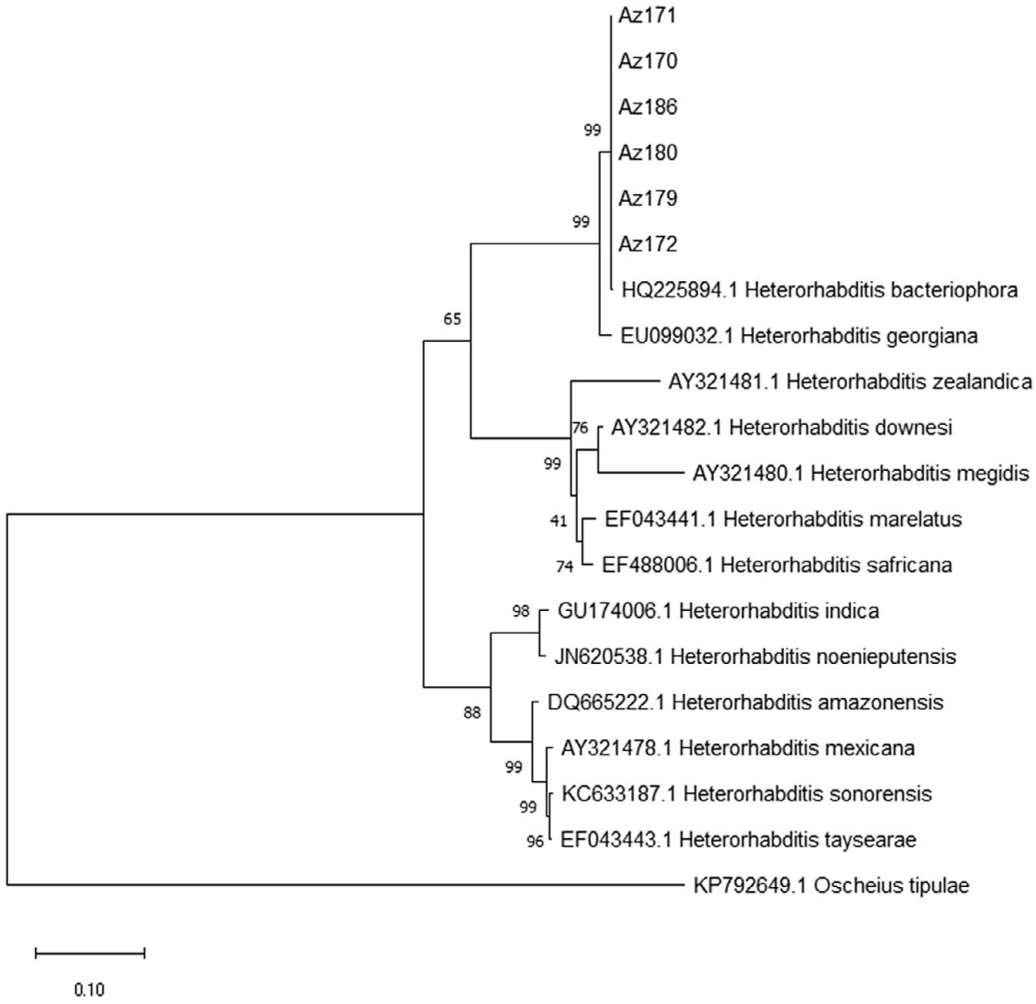

Figure 2. Phylogenetic tree using maximum likelihood method of EPN isolates and other *Heterorhabditis* spp. based on the analysis of the region 18s-ITS-28s, with *O. tipulae* as outgroup. Bootstrap values are indicated in each node after 10,000 replicates. GenBank accession numbers are given in front of each entry. Scale bar represents the number of substitutions per site.

isolated *Photorhabdus* can be found in the following accession numbers of GenBank for 16s rRNA (PP738150, PP738151, PP738152, PP738153, PP738154, PP738155) and for GyrB gene (PP746494, PP746495, PP746496, PP746497, PP746498, PP746499).

Determination of the dose–mortality relationship

The bioassays included the comparison of the new strains alongside the Azorean reference Az29. The determination of LD50 revealed notable differences at the 72- and 96-hour intervals, serving as the initial metric to determine the potentiality of EPNs (Table 2). Az29 exhibited the minor LD50 at 48 hours with only 17.96 IJs and maintained the dose effect consistently over time. Among the new strains, Az171 showed the best performance against *G. mellonella*, which achieved LD50 values from 25 to 6.9 IJs cm^{-2}. Az172 and Az186 followed closely, showing no significant differences with Az171. Remarkably, these three strains exhibited an enhanced dose response effect after 72 hours, with marginal progress noted at 96 hours. In contrast, the strains Az179, Az170, and Az180 needed a higher dose to kill *G. mellonella* larvae, although these strains improved greatly the dose response when the experiment was extended to 96 hours. Especially, Az179 was the most favoured, presenting no differences with the top virulent strains at 96 hours. Therefore, Az170 and Az180 were the least virulent strains regarding the dose response.

Determination of the lethal time

The virulence of strains was also measured by the time of death of *G. mellonella* while strains exhibited significant differences among them, no mortality was registered in negative control larvae (Table 3). The reference strain Az29 exhibited the highest virulence of the experiment with an LT50 of 26.7 hours. Regarding the performance of the new strains, we split them into two main groups. One was composed by Az172, Az186, Az171, and Az170, which presented an intermediate LT50 from 35 to 39 hours. Az170 was the lowest among them, differing significantly from Az172 and Az186. The second group comprised strains with slower virulence, specifically Az179 and Az180, exhibiting LT50 values exceeding 40 hours. Particularly, there were statistically significant differences in the virulence between these strains manifesting the notably delayed virulence of Az180 compared to Az179.

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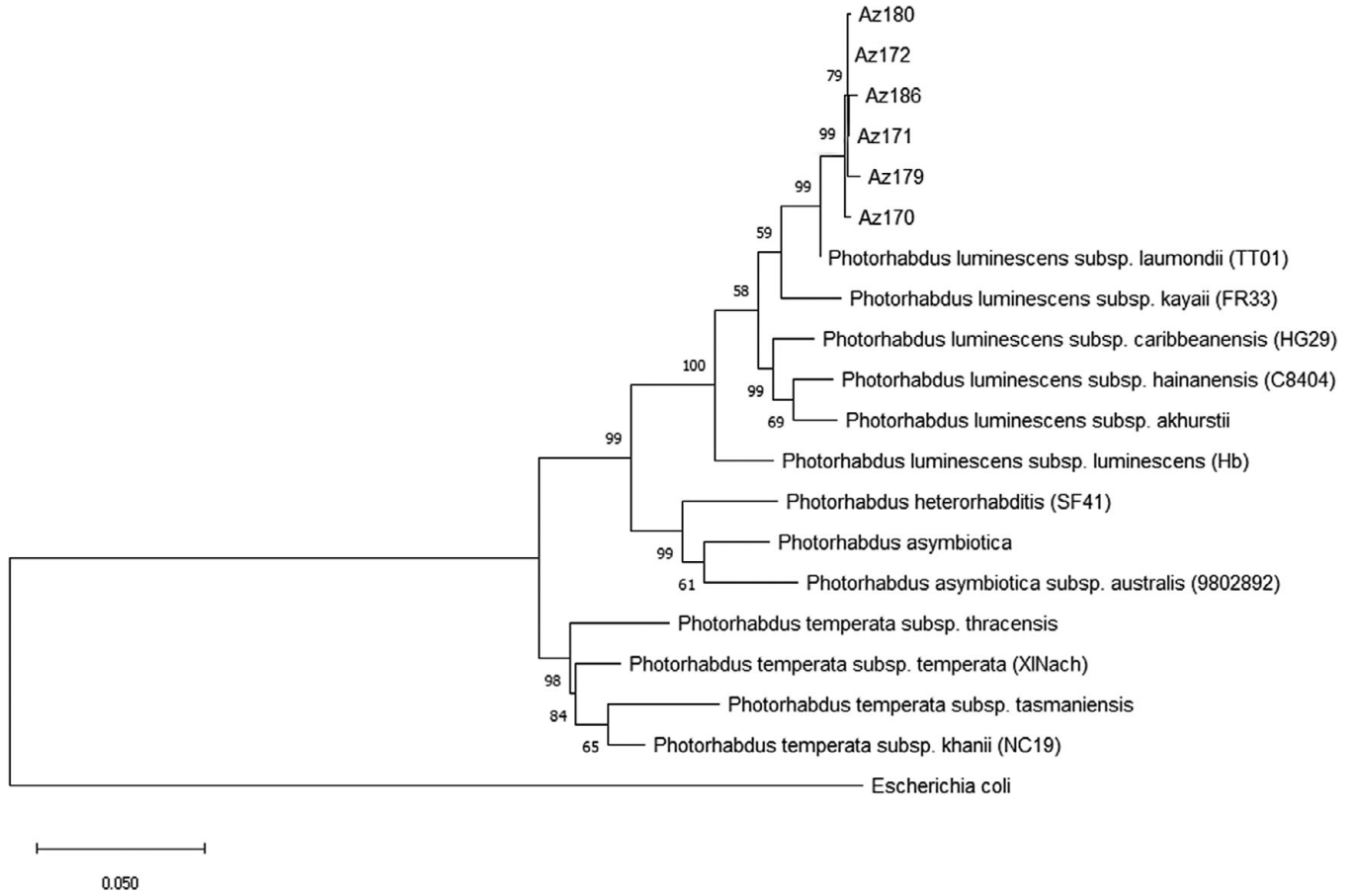

Figure 3. Phylogenetic tree using maximum likelihood method of bacteria isolates and other *Photorhabdus* spp. based on the analysis of the 16s rRNA and GyrB genes, with *E. coli* as outgroup. Bootstrap values are indicated in each node after 10,000 replicates. GenBank accession numbers are given in front of each entry. Scale bar represents the number of substitutions per site.

Table 2. LD_{50} and 95% confidence interval measured as IJs cm^{-2} for the three time points of the new strains alongside the reference Az29; the assignment of letter groups (a-c) is based on statistically significant differences among the strains

Strain	LD_{50} IJs/cm^2 (95% CI)	Sig. Group	LD_{50} IJs/cm^2 (95% CI)	Sig. Group	LD_{50} IJs/cm^2 (95% CI)	Sig. Group
	48 h		72 h		96 h	
Az171	25.91 (14.27–48.98)	a	8.37 (3.89–14.73)	a	6.98 (2.80–12.83)	a
Az172	23.35 (6.83–64.83)	a, b	8.98 (3.38–18.09)	a, b	7.16 (3.95–11.43)	a
Az186	50.64 (21.15–173.40)	a, b	13.31 (8.10–21.72)	a, b	11.49 (7.22–17.37)	a, b
Az29	17.96 (8.50–35.18)	a	13.54 (6.68–25.17)	a, b	11.74 (3.92–25.58)	a, b
Az179	76.59 (31.29–279.60)	b, c	25.51 (11.15–67.44)	b, c	13.60 (6.92–26.06)	a, b
Az170	73.20 (39.87–144.90)	b	45.12 (25.29–82.06)	c	24.15 (13.16–43.00)	b, c
Az180	69.15 (39.42–126.30)	b	48.08 (25.39–95.23)	c	35.28 (17.44–71.71)	c

Short exposure assay

In the exposure assay, we observed a positive correlation between exposure time and *G. mellonella* mortality. All *H. bacteriophora* strains caused a larval mortality below 30% when exposition lasted only 20 minutes, pointing to no differences among them. In contrast, from 60 minutes of exposure to 180 minutes, strains presented significant differences regarding their virulence (Fig 4). Mortality of *G. mellonella* increased rapidly to 50% with the 60-minute treatment of Az29, Az172, and Az186. The highest mortality rate was

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Table 3. LT_{50} and 95% confidence interval measured in hours of the new strains alongside the reference Az29; ahe assignment of letter groups (a-f) is based on statistically significant differences among the strains

Strain	LT_{50} (h) (95% CI)	Sig. Group
Az29	26.73 (26.05–27.39)	a
Az172	35.69 (34.50–36.93)	b
Az186	37.28 (35.43–39.22)	b, c
Az171	37.89 (36.31–39.51)	c, d
Az170	39.77 (38.42–41.15)	d
Az179	43.61 (41.11–46.29)	e
Az180	49.24 (47.26–51.52)	f

achieved by Az186, reaching 92% mortality at 180 minutes of exposure. Moreover, Az29, Az172, and Az171 reached 75% *G. mellonella* mortality at 180 minutes, whereas Az170 only killed 58% of larvae. Az180 and Az179 exhibited the lowest virulence in these experiments, reaching a maximum of 35% mortality even at the longest exposure time. As was observed in the LD50 test, the infectivity performance of Az179 was dependent on a long exposition time to achieve a higher mortality rate on larvae. Besides, no mortality was registered in control larvae during the experiment.

Reproductive potential

The reproductive potential of EPNs differed greatly between larvae, highlighting the influence of various factors on offspring production, even in model insects like *G. mellonella* (Fig. 5). However, we observed significantly higher reproduction of the Az179 strain producing a mean of over 400,000 IJs per larva (equivalent to 1701 IJs/mg of larvae). In contrast, Az172 and Az186 presented the lowest production, with only 480 and 778 IJs/mg, respectively. Strains Az29, Az171, Az170, and Az180 exhibited intermediate reproductive potential with a mean offspring of 1050 IJs/mg. Particularly, Az180 showed a wider range of offspring production from 364 to 2364 IJs/mg.

Discussion

The isolation of new EPN strains enlarges the chances of efficient biological control methods while helping to preserve the native biodiversity and ensure adaptability to regional conditions. In our survey across eight islands of the Archipelago of Azores, the total recovered EPNs represented 10.9% of the samples, which aligns with the average reported nematode recovery rates from cultivated and natural spots, including 11.8% in Oregon (Liu and Berry 1995), 5% in Spain (Campos-Herrera *et al.* 2007; Julià *et al.* 2023), 6.6% in Italy (Tarasco *et al.* 2015), or 6.8% in Hawaiian Islands (Hara *et al.* 1991). From the total of our isolates, 7.3% were unidentified steinernematid species, consistent with the trend of higher steinernematid recovery compared to heterorhabditids (Hominick 2002). The observed prevalence of *H. bacteriophora* in Azorean soils was similar to the previous occurrence study that showed a range from 1.2 to 4.6 positive samples depending on the island (Rosa *et al.* 2000). Because of the archipelago's nature, *H. bacteriophora* was found across islands, as it is commonly found in maritime environments (Emelianoff *et al.* 2008). In agreement with the previous survey, São Miguel, Faial, and São Jorge exhibited the highest occurrence of *H. bacteriophora* predominantly in pastures and coastal landscapes at lower altitudes.

The molecular identification revealed all novel strains as *H. bacteriophora* as it was suggested by the reddish colour during the infection of *G. mellonella* larvae. In particular, the tight relation between *H. bacteriophora* and *H. georgiana* observed in the analysis was already pointed out in other phylogenetic studies producing often unreliable nodes because of their proximity (Aryal *et al.* 2022a). For bacterial identification, phylogeny performed only with 16s rRNA gene often produces inconclusive results. Thus, we selected the GyrB gene to improve the analysis as suggested for the phylogenetical classification of *Photorhabdus* species (Shapiro-Ilan *et al.* 2009; Tailliez *et al.* 2010). With this procedure, the new isolates were robustly identified as *Photorhabdus luminiscens* subsp. *laumondii*. Interestingly, *H. bacteriophora* Az29 and Az186 were isolated from practically the same area but 30 years apart. However, Az29 harbours the bacterium *P. temperata* subsp. *thracensis*, distinct from the symbiont associated with Az186.

Recently isolated EPN strains can exhibit novel biological traits such as increased virulence, broad host range, or abundant offspring generation. Thus, it is crucial to evaluate through bioassays their abilities first under laboratory conditions. Virulence of the novel strains was evaluated with the tests of LD50, LT50, and short exposure, altogether with the assessment of the reproductive potential. The isolates were able to infect *G. mellonella* at high rates with the bioassays, although differences in their performance were observed. Therefore, we classified the strains from high to low

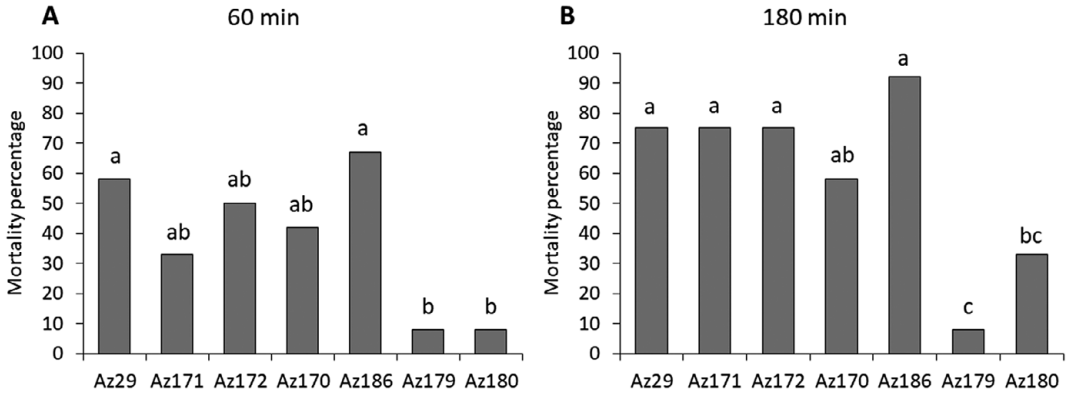

Figure 4. Mean mortality percentage of *G. mellonella* larvae obtained by the EPN strains after 60 minutes of exposure (A) and 180 minutes (B). The assignment of letter groups (a-c) is based on statistically significant differences among the strains.

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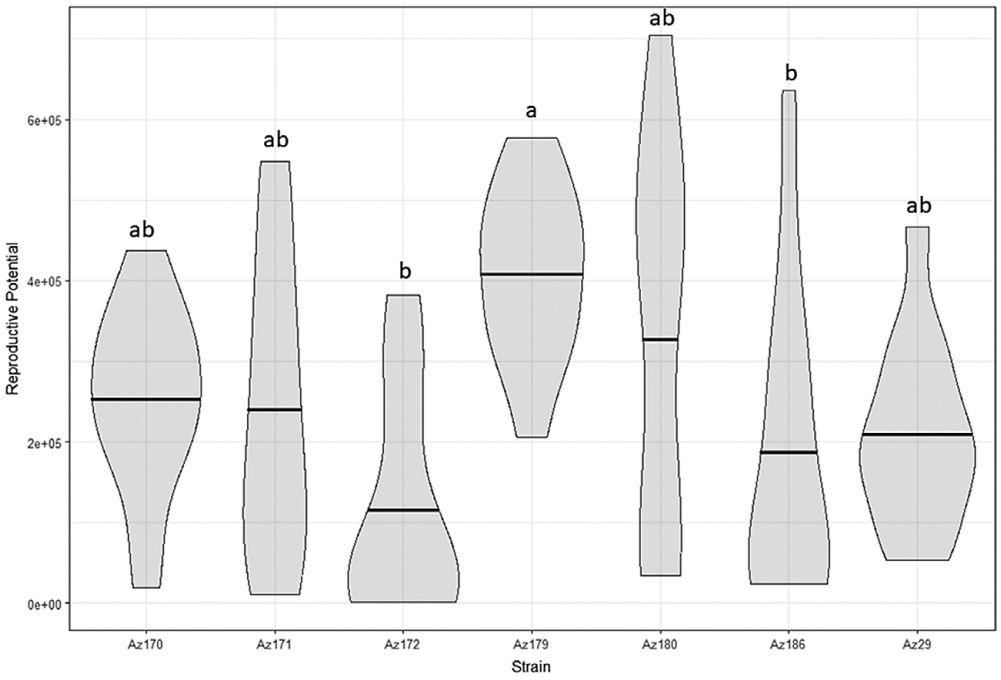

Figure 5. Violin plot showing the reproductive potential expressed as the total number of IJs produced per larvae of the new strains alongside the reference Az29. The assignment of letter groups (a-b) is based on statistically significant differences among the strains.

Table 4. Classification of the new *H. bacteriophora* strains performance from high to low regarding their virulence with the LD50, LT50, and short exposure (Expo.) test and the reproductive potential

Strains	Virulence			Reproductive potential
	LD50	LT50	Expo.	
Az29	High	High	High	Mid
Az172	High	High	High	Low
Az186	High	High	High	Low
Az171	High	Mid	High	Mid
Az170	Low	Mid	Mid	Mid
Az179	Mid	Low	Low	High
Az180	Low	Low	Low	High

following the measured parameters (Table 4). Az29 exhibited the highest virulence in the current experiments because it occurred when the EPN was first isolated (Rosa and Simões 2003). The high virulence of Az29 was followed by the new strains Az172, Az186, and Az171. Similar LD50 values were obtained by the Egyptian isolates of *H. bacteriophora*, whereas lower LD50 (2 to 6 IJs/larvae of *G. mellonella*) were achieved by the Thai EPNs isolates (Noosidum *et al.* 2010; Saleh *et al.* 2016). The LD50 results obtained with Az179 suggest that providing more exposure time or extended contact with IJs could reach high virulence. In contrast, when the dose was limited Az170 strain exhibited the worst performance. The strains displaying the highest virulence as determined by the LT50 were Az29, Az172, and Az186 consistently across all bioassays. The obtained LT50 values with *H. bacteriophora* strains were comparable to the obtained with *Steinernema feltiae* (Rhabditida: Steinernematidae) and *S. carpocapsae* strains against *G. mellonella* ranging from 12 to 48 hours (Campos-Herrera *et al.* 2007; Caroli *et al.* 1996). The capacity of EPNs to infect in short exposure periods is essential for efficient biological control of pests, with *Heterorhabitis* species showing the ability to infect diverse insects in short time experiments (Corrie *et al.* 2004; van Niekerk and Malan 2012). Notably, Az186 induced higher mortalities within short exposure times and Az170 demonstrated superior performance in these scenarios achieving almost 60% of mortality. Nevertheless, Az179 and Az180 suffered the most when exposure time was limited. The low general virulence exposed by the novel strain Az180 suggests the potential unsuitability of *G. mellonella* as its host.

Considering the weight of the *G. mellonella* larvae used, our strains produced between 1600 and 420 IJs per mg, similar to values obtained with strains of *H. bacteriophora* and *S. carpocapsae* (Campos-Herrera *et al.* 2007; Nouh 2016; Wang and Bedding 1996). The reproductive potential differs greatly among larvae, although our results suggest a correlation between IJs production and virulence. Because the most virulent strains Az186 and Az172 exhibited the lowest reproduction while the less virulent Az179 and Az180 achieved a production almost three times higher. This observation could hint at a specialised adaptation of the less virulent EPNs to increase their persistence with an enhanced reproductive capacity. Although this association has not been firmly elucidated, it is established that the reproductive potential of EPNs is liable to insect size and the number of IJs that enter the host (Bastidas *et al.* 2014; Caroli *et al.* 1996).

These isolates have the potential to become biological control agents for *P. japonica* as EPNs isolated from areas already infested are proposed to exhibit heightened virulence against the pest (Lacey *et al.* 2015; Glazer *et al.* 2022). Besides, these strains could display superior adaptability to the environmental and soil conditions for field applications. Native strains of *H. bacteriophora* from Italy exhibited an increased virulence in field trials proving the importance of using regional isolates for the integrated pest management strategies of *P. japonica* (Torrini et al. 2020). After this first

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assessment with *G. mellonella*, Azorean native strains showing high virulence traits will be tested against the pest aligning with the ongoing efforts to develop effective management strategies for *P. japonica*.

In conclusion, we isolated six native strains of *H. bacteriophora* with diverse virulence performances. With the bioassays, we revealed the potentiality of Az172, Az186, and Az171 whose performance is close to the well-known virulent Az29. Meanwhile, Az179 can be an interesting strain to use if an extended period of exposure can be assured because their reproductive potential could favour an enhanced persistence on field applications. Concerning Az180, the observed potentiality was rather low though future bioassays against other insects would clarify if the low virulence is related to the host suitability or a trait of the strain. Further experiments will be carried out to elucidate the efficacy of these strains against *P. japonica* as well as the behaviour and persistence of these EPNs under field conditions. Nevertheless, this preliminary evaluation serves as a foundation for directing further investigations aimed at assessing the potential of the novel Azorean strains for the control of *P. japonica* or other insect pests.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000336.

Acknowledgements. We thank the Serviços de Desenvolvimento Agrário dos Açores from the different islands for their cooperation in fieldwork and sampling. Besides, we thank the colleagues from the UAç-CBA laboratory that carried out the sampling as well: Jorge Frias, Mario Teixeira, Marina Oliveira, and Ángel Peñalver.

Author contribution. Conceptualisation and supervision: N.S. and A.G.; Investigation: R.B., H.M., and A.G.; Formal analysis: H.M.; Writing – original draft: R.B. and A.G.; Writing – review and editing: H.M., D.T., and N.S; Funding acquisition: N.S., D.T., and A.G. All authors read and approved the manuscript.

Financial support. This work was supported by the International Research Project-Integrated Pest Management of the invasive Japanese Beetle, Popillia japonica (IPM-Popillia; grant Nr. H2020-EU.3.2.1.1/ID: 861852); the Fundação Ciência e Tecnologia (FCT) with the project AGWAS-EPNs (2022.06153. PTDC). R.B. was hired by the IPM-Popillia project as a research assistant. A.G. received a postdoctoral grant from the M. Universidades—Margarita Salas. H.M. received the support of the FCT Pluriannual Program for the Funding of R&D Units (UIDB/05292/2020 and UIDP/05292/2020) granted to CBA.

Competing interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability. All sequences used for the phylogenetical identification can be found in the GenBank NCBI Database; see the Results section for the corresponding accession numbers.

References

Alatorre-Rosas, R, and Kaya, HK (1990) Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. *Journal of Invertebrate Pathology*, **55**, 179–188.

Andrews, S (2010) FastQC: a quality control tool for high throughput sequence data [Online] http://www.bioinformatics.babraham.ac.uk/projects/.

Aryal, S, Nielsen, UN, Sumaya, NH, De Faveri, S, Wilson, C, and Riegler, M (2022a) Isolation and molecular characterization of five entomopathogenic nematode species and their bacterial symbionts from eastern Australia. *BioControl*, **67**, 63–74. doi:10.1007/s10526-021-10105-7.

Aryal, S, Nielsen, UN, Sumaya, NH, Wilson, C, and Riegler M (2022b) Virulence, penetration rate and reproductive potential of entomopathogenic nematodes from eastern Australia in Queensland fruit fly, *Bactrocera tryoni*. *Biological Control*, **169**, 104871. doi: 10.1016/j.biocontrol.2022.104871.

Bastidas, B, Portillo, E, and San-Blas, E (2014) Size does matter: the life cycle of *Steinernema* spp. in micro-insect hosts. *Journal of Invertebrate Pathology*, **121**, 46–55. doi:10.1016/j.jip.2014.06.010.

Bedding, RA, and Akhurst, RJ (1975) A simple technique for the detection o f insect paristic rhabditid nematodes in soil. *Nematologica*, **21**, 109–110. doi: 10.1163/187529275X00419.

Bird, A, and Akhurst, R (1983) The nature of the intestinal vesicle in nematodes of the family Steinernematidae. *International Journal of Parasitology*, **13**, 599–606.

Campos-Herrera, R, Escuer, M, Labrador, S, Robertson, L, Barrios, L, and Gutiérrez, C (2007) Distribution of the entomopathogenic nematodes from La Rioja (Northern Spain). *Journal of Invertebrate Pathology*, **95**, 125–139. doi:10.1016/j.jip.2007.02.003.

Campos-Herrera, R, Vicente-Díez, I, Galeano, M, Chelka, M, Del Mar González-Trujillo M, Puelles M, Labarga D, Pou A, Calvo, J, and Belda, JE (2021) Intraspecific virulence of entomopathogenic nematodes against the pests *Frankliniella occidentalis* (Thysanoptera: Thripidae) and *Tuta absoluta* (Lepidoptera: Gelechiidae). *Journal of Nematology*, **53**, e2021–102. doi: 10.21307/jofnem-2021-102.

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

Corrie, AY, Grewal, PS, and Taylor, RA J (2004) Rapid age-related changes in infection behavior of entomopathogenic nematodes. *Journal of Parasitology*, **90**, 1229–1234.

Dowds, BCA, and Peters, A (2002) Virulence mechanisms. In R. Gaugler, ed., *Entomopathogenic nematodology*, CABI, New York, 79–98.

Emelianoff, V, Le Brun, N, Pagès, S, Stock SP, Tailliez P, Moulia C, and Sicard, M (2008) Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Hérault and Gard (Southern France). *Journal of Invertebrate Pathology*, **98**, 211–217. doi:10.1016/j.jip.2008.01.006.

EPPO, R (2014) Harmful organisms in the European Union: Annual report 2014. *EPPO Reporting Service.*

Forst, S, and Clarke, DJ (2002) Bacteria-nematode symbiosis. In R. Gaugler, ed., *Entomopathogenic nematodology*, Entomopathogenic nematology. CABI, New York, 57–77.

Georgis, R, and Gaugler, R (1991) Predictability in biological control using entomopathogenic nematodes. *Journal of Economic Entomology*, **84**, 713–720.

Glazer, I, Santoiemma, G, Battisti, A, De Luca, F, Fanelli, E, Troccoli A, Tarasco E, Sacchi S, Bianchi A, Gilloki G, Gherardo, P, and Mori, N (2022) Invasion of *Popillia japonica* in Lombardy, Italy: interactions with soil entomopathogenic nematodes and native grubs. *Agricultural and Forest Entomology*, **24**, 600–608. doi:10.1111/afe.12524.

Gotta, P, Ciampitti, M, Cavagna, B, Bosio, G, Gilloli G, Alma A, Battisti, A, Mori, N, Mazza, G, Torrini, G, Paoli F, Santoiemma, G, Simonetto, A, Lessio, F, Sperandio, G, Giacometto, E, Bianchi, A, Roversi PF, and Marianelli, L (2023) *Popillia japonica* – Italian outbreak management. *Frontiers in Insect Science*, **3**, 1–12. doi:10.3389/finsc.2023.1175138.

Griffin, CT, Boemare, NE, and Lewis, EE (2005) Biology and behaviour. In P. S. Grewal, R. U. Ehlers, and D. I. Shapiro-Ilan, eds., *Nematodes as Biological Control*, CABI International, 47–64.

Gulzar, S, Usman, M, Wakil, W, Gulcu, B, Hazir, C, Karagoz, M, Hazir, S, and Shapiro-Ilan, DI (2020) Environmental tolerance of entomopathogenic nematodes differs among nematodes arising from host cadavers versus aqueous suspension. *Journal of Invertebrate Pathology*, **175**, 107452. doi: 10.1016/j.jip.2020.107452.

Hara, A, Gaugler, R, Kaya, HK, and Le-Beck, L (1991) Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiian Islands. *Environmental Entomology*, **20**, 211–216.

Helmberger, MS, Thaler, JS, Shields, EJ, and Wickings, KG (2018) Entomopathogenic nematode performance against *Popillia japonica* (Coleoptera: Scarabaeidae) in school athletic turf: effects of traffic and soil properties. *Biological Control*, **126**, 177–184. doi:10.1016/j.biocontrol.2018.08.010.

Hominick, WM (2002) Biogeography. In R. Gaugler, ed., *Entomopathogenic nematodology*, CABI Publishing, Wallingford, Gran Bretaña, 115–143.

Jaffuel, G, Sbaiti, I, and Turlings, T (2020) Encapsulated entomopathogenic nematodes can protect maize plants from *Diabrotica balteata* larvae. *Insects*, **11**, 27. doi:10.3390/insects11010027.

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**. doi:10.1017/S0022149X23000615.

Kaya, HK, and Stock, SP (1997) Techniques in insect nematology. In L. A. Lacey, ed., *Manual of Techniques in Insect Pathology*, Academic Press, 281–324.

Kazimierczak, W, Lis, M, Skrzypek, T, and Kreft, A (2018) Comparison of the methods applicable for the pathogenicity assessment of entomopathogenic nematodes. *BioControl*, **63**, 289–298. doi:10.1007/s10526-017-9856-2.

Khashaba, EHK, Moghaieb, REA, Abd-El-Azim, AM, and Ibrahim, SAM (2020) Isolation, identification of entomopathogenic nematodes, and preliminary study of their virulence against the great wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). *Egyptian Journal of Biological Pest Control*, **30**, 35. doi: 10.1186/s41938-020-00257-6.

Kimura, M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kumar, S, Stecher, G, Li, M, Knyaz, C, and Tamura, K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, **35**, 1547–1549. doi:10.1093/molbev/msy096.

Lacey, LA, Grzywacz, D, Shapiro-Ilan, DI, Frutos, R, Brownbridge, M, and Goettel, MS (2015) Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology*, **132**, 1–41. doi:10.1016/j.jip.2015.07.009.

Leite, LG, Chacon-Orozco, JG, Shapiro-Ilan, DI, Baldo, FB, and Cardoso, JFM (2023) Effects of temperature for optimizing production and storage of *Steinernema rarum* in a novel biphasic process, and efficacy of the nematode against *Sphenophorus levis*. *Biological Control*, **187**, 105381. doi: 10.1016/j.biocontrol.2023.105381.

Li, H, and Durbin, R (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics*, **25**, 1754–1760. doi:10.1093/bioinformatics/btp324.

Liu, J, and Berry, E (1995) Natural distribution of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) in Oregon soils. *Environmental Entomology*, **24**, 159–163.

Londoño-Caicedo, JM, Uribe-Londoño, M, Buitrago-Bitar, MA, Cortés, AJ, and Muñoz-Flórez, JE (2023) Molecular identification and phylogenetic diversity of native entomopathogenic nematodes, and their bacterial endosymbionts, isolated from banana and plantain crops in Western Colombia. *Agronomy*, **13**. doi:10.3390/agronomy13051373.

Morton, A, and García-del-Pino, F (2009) Ecological characterization of entomopathogenic nematodes isolated in stone fruit orchard soils of Mediterranean areas. *Journal of Invertebrate Pathology*, **102**, 203–213. doi: 10.1016/j.jip.2009.08.002.

Noosidum, A, Hodson, AK, Lewis, EE, and Chandrapatva, A (2010) Characterization of new entomopathogenic nematodes from Thailand: foraging behavior and virulence to the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). *Journal of Nematology*, **42**, 281–291.

Nouh, GM (2016) Impact of sandy soil on persistence, host finding and pathogenicity of entomopathogenic nematodes. *Egyptian Journal of Biological Pest Control*, **26**, 293–297.

Peters, A (1996) The natural host range of *Steinernema* and *Heterorhabditis* spp. and their impact on insect populations. *Biocontrol Science and Technology*, **6**, 389–402. doi:10.1080/09583159631361.

Poinar, G (1990) Biology and taxonomy of Steinernematidae and Heterorhabditidae. In R. Gaugler and H. K. Kaya, eds., *Entomopathogenic nematodes in biological control*, Florida: Boca Raton: CRC Press, 23–62.

Potter, D., and Held, D. (2002) Biology and management of the Japanese beetle. *Annual Review of Entomology*, **47**, 175–205.

R Core Team (2017) R: a language and environment for statistical computing.

Renkema, JM, and Parent, JP (2021) Mulches used in highbush blueberry and entomopathogenic nematodes affect mortality rates of third-instar *Popillia japonica*. *Insects*, **12**. doi:10.3390/insects12100907.

Rosa, J., Cabral, C, and Simões, N (2002) Differences between the pathogenic processes induced by *Steinernema* and *Heterorhabditis* (Nemata: Rhabditida) in *Pseudaletia unipuncta* (Insecta: Lepidoptera). *Journal of Invertebrate Pathology*, **80**, 46–54. doi:10.1016/S0022-2011(02)00038-1.

Rosa, JS, Bonifassi, E, Amaral, J, Lacey, LA, Simões, N, and Laumond, C (2000) Natural occurrence of entomopathogenic nematodes (Rhabditida: *Steinernema, Heterorhabditis*) in the Azores. *Journal of Nematology*, **32**, 215–222.

Rosa, JS, and Simões, N (2003) Evaluation of twenty-eight strains of *Heterorhabditis bacteriophora* isolated in Azores for biocontrol of the armyworm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). *Biological Control*, **29**, 409–417. doi:10.1016/j.biocontrol.2003.07.004.

Saleh, ME, Metwally, HM, El-Behery, H, Moawad, SS, and Mahmoud, YA (2016) Existence of entomopathogenic nematodes in fruit orchards and their virulence to *Galleria mellonella* L. *Egyptian Journal of Biological Pest Control*, **26**, 595–599.

San-Blas, E, Campos-Herrera, R, Dolinski, C, Monteiro, C, Andaló, V, Leite, LG, Rodríguez, MG, Morales-Montero, P, Sáenz-Aponte, A, Cedano, C, López-Nunez, JC, Del Valle, E, Doucet, M, Lax, P, Navarro, PD, Báez, F, Llumiquinga, P, Ruiz-Vage, J, Guerra-Moreno, A, and Stock, SP (2019) Entomopathogenic nematology in Latin America: a brief history, current research and future prospects. *Journal of Invertebrate Pathology*, **165**, 22–45. doi:10.1016/j.jip.2019.03.010.

Shapiro-Ilan, DI, Mbata, GN, Nguyen, KB, Peat, SM, Blackburn, D, and Adams, BJ (2009) Characterization of biocontrol traits in the entomopathogenic nematode *Heterorhabditis georgiana* (Kesha strain), and phylogenetic analysis of the nematode's symbiotic bacteria. *Biological Control*, **51**, 377–387. doi:10.1016/j.biocontrol.2009.07.009.

Simões, N, Laumond, C, and Bonifassi, E (1993) Effectiveness of *Steinernema* spp. and *Heterorhabditis bacteriophora* against *Popillia japonica* in the Azores. *Journal of Nematology*, **25**, 480–5.

Snyder, H, Stock, SP, Kim, S-K, Flores-Lara, Y, and Forst, S (2007) New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode host, *Steinernema carpocapsae*. *Applied and Environmental Microbiology*, **73**, 5338–5346. doi:10.1128/AEM.02947-06.

Tailliez, P, Laroui, C, Ginibre, N, Paule, A, Pagès, S, and Boemare, N (2010) Phylogeny of *Photorhabdus* and *Xenorhabdus* based on universally conserved protein-coding sequences and implications for the taxonomy of these two genera. Proposal of new taxa: *X. vietnamensis* sp. nov., *P. luminescens* subsp. *caribbeanensis* subsp. nov., P. l. *International Journal of Systematic and Evolutionary Microbiology*, **60**, 1921–1937. doi:10.1099/ijs.0.014308-0.

Tamura, K (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, **9**, 678–687.

Tarasco, E, Clausi, M, Rappazzo, G, Panzavolta, T, Curto, G, Sorino, R, Oreste, M, Longo, A, Leone, D, Tiberi, R, Vinciguerra, MT, and Triggiani, O (2015) Biodiversity of entomopathogenic nematodes in Italy. *Journal of Helminthology*, **89**, 359–366. doi:10.1017/S0022149X14000194.

Torrini, G, Paoli, F, Mazza, G, Simoncini, S, Benvenuti, C, Strangi, A, Tarasco, E, Barzanti, GP, Bosio, G, Cutino, I, Roversi, PF, and Marianelli, L (2020) Evaluation of indigenous entomopathogenic nematodes as potential biocontrol agents against *Popillia japonica* (Coleoptera: Scarabaeidae) in Northern Italy. *Insects*, **11**, 1–15. doi:10.3390/insects11110804.

van Niekerk, S, and Malan, AP (2012) Potential of South African entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) for control of the citrus mealybug, *Planococcus citri* (Pseudococcidae). *Journal of Invertebrate Pathology*, **111**, 166–174. doi:10.1016/j.jip.2012.07.023.

Wang, J, and Bedding, RA (1996) Population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in the larvae of *Galleria mellonella*. *Fundamental and Applied Nematology*, **19**, 363–367.

White, GF (1927) A method for obtaining infective nematode larvae from cultures. *Science*, 302–303.

Woodring, JL, and Kaya, HK (1988) Steinernematid and heterorhabditid nematodes: a handbook of biology and techniques. *Southern Cooperative Series Bulletin (USA).*