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Research Papers

## Beyond the primary host: survival of *Heterodera schachtii* (Nematoda, Heteroderidae) through alternative hosts

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**Summary.** Plant-parasitic nematodes are an underestimated cause of crop yield losses. Cyst nematodes, particularly *Heterodera* spp., are important pests of sugar beet, cereals, and soybean. Presence of these nematodes was investigated in a plot near Lisbon (Golegã), where problems with cyst nematode infestations had been detected. *Heterodera* cysts were extracted and isolated from soil samples, and were identified morphologically and using PCR and DNA sequencing. Morphological identifications were difficult, but molecular analyses confirmed the presence of *H. schachtii*, linking this with the plot's history. This nematode had remained viable in the soil for more than 15 years, despite absence of sugar beet, the primary host.

**Keywords.** Cyst nematodes resilience, population dynamics, host range, morphological identification, molecular identification.

### INTRODUCTION

Nematodes in soil are commonly classified into functional groups based on their feeding strategies which reflect their roles in soil ecosystems (Yeates *et al.*, 1993; Decraemer and Hunt, 2013). The plant-parasitic nematodes predominantly occupy upper soil layers, where organic matter and plant roots are abundant. Their stylets inject enzymes into plant cells to extract cell contents for feeding (Camacho *et al.*, 2020).

Cyst nematodes are economically important (Camacho *et al.*, 2017), contributing to yield losses in agricultural crops (Sikora *et al.*, 2023). Their

damage is intensified by the interactions with biotic and abiotic factors including soil pathogens, low soil fertility, reduced soil biodiversity, and climate variability (Sikora *et al.*, 2023). The most important cyst nematode species belong to the genera *Heterodera* and *Globodera*, which are endoparasites of plant roots (Lilley *et al.*, 2005; Smiley and Nicol, 2009). Moreover, eggs and juvenile forms can survive in the soil within cysts for long periods, potentially lasting several years, until a susceptible host is present in the area (Lilley *et al.*, 2005; Hunt, 2008; Smiley and Nicol, 2009). Although it is difficult to predict how long sugar beet cyst nematodes may survive in soil without hosts, a small percentage of eggs within each cyst can survive for 12 years under fallow conditions (Khan *et al.*, 2021). This longevity is particularly important because it limits the effectiveness of control methods and reduces the growth of specific crops.

*Heterodera* life cycles begin with eggs and progress through four juvenile stages, each separated by moults, before reaching adult stages (male or female) (Lilley *et al.*, 2005; Hunt, 2008). Each female can contain 100 to 600 eggs enclosed in a lemon-shaped cyst (Hunt, 2008; Smiley and Nicol, 2009). Nematodes of this genus complete their life cycles in 21 to 25 days (Inagaki and Tsutsumi, 1971), and can have more than one generation per year (Turner and Subbotin, 2013), due to the short life cycle, adaptability to warm climates, and reduced dormancy requirements, and thus cause damage in crop fields. The most important *Heterodera* species are *Heterodera glycines* Ichinohe (soybean cyst nematode), *Heterodera avenae* Wollenweber (cereal cyst nematode), *Heterodera schachtii* Schmidt (sugar beet cyst nematode) and *Heterodera zea* Koshy, Swarup & Sethi (corn cyst nematode) (Lilley *et al.*, 2005; Moens *et al.*, 2018).

Traditionally, *Heterodera* species are identified using cyst and juvenile morphology and morphometrics (Golden, 1986; Rivoal *et al.*, 2003). This is time-consuming and requires knowledge and experience (Yan and Smiley, 2009). However, morphological identification is not always reliable, making it important to confirm results through molecular identification methods (Seesao *et al.*, 2016; Camacho *et al.*, 2017), and several DNA-based methods are used to study nematodes biodiversity. These include amplification of barcoding sequences by conventional PCR followed by DNA sequencing. The most used barcoding sequences are the LSU (28S rDNA), SSU (18S rDNA), the full ITS-rDNA region and mtCOI gene (Nisa *et al.*, 2022; Camacho *et al.*, 2025). Given these challenges, the primary goal of the present study was to investigate the presence and survival of *Heterodera schachtii* cyst nematodes in agricultural fields in the

Golegã region of Portugal, due to their historical occurrence in soils where sugar beet had been cultivated for approximately 15 years.

## MATERIALS AND METHODS

### *Location and establishment of study plots*

This study was carried out in agricultural fields in the Golegã region, central Portugal (39°23'07"N; 8°29'26"W). These fields had been cultivated over the previous 15 years with crops of wheat, corn, potatoes, and peas, as well as cover crops including brassicas, grasses, and legumes, either as single species or as mixtures, namely: (A) *Avena strigosa* and *Raphanus sativus* var. *oleiferus*; (B) *Avena strigosa*, *Brassica carinata*, *Brassica napus*, *Lathyrus sativus*, *Lolium multiflorum*, *Pisum sativum*, *Raphanus sativus* var. *longipinnatus*, *Raphanus sativus* var. *oleiferus*, *Sinapis alba*, *Trifolium* spp., and *Vicia sativa*; and (C) *Avena sativa*, *Lolium multiflorum*, *Trifolium* spp., and *Vicia sativa*. Although sugar beet was no longer cultivated in these fields, this crop had been grown until 2008.

### *Sampling and processing of soil samples*

Sampling was carried out in agricultural fields according to Annex II of DL 87/2010 (Portuguese Ministry of Agriculture, 2010). Twenty soil samples, each of approx. 1 L, were randomly collected from bare soil, across a zigzag pattern, and at depth of 20 to 25 cm, which corresponded to the depth of the rhizosphere. The top layer of soil was removed to avoid plant debris and possible contaminants. The samples were then stored in labelled plastic bags.

The samples were transported to the Nematology Laboratory of the Instituto Nacional de Investigação Agrária e Veterinária, I.P. - INIAV in Oeiras, Portugal, where they were kept at room temperature for 3 days to dry. The Fenwick can then was used to extract cyst nematodes from samples of maximum weight 400 g, according to EPP0 protocols PM7/119(1) (EPP0, 2013a) and PM7/40(3) (EPP0, 2013b). The samples were then assessed using a light microscope (Olympus Bx51) equipped with a Leica MC190 HD optical camera, and software version LAS V4.12 (Leica Microsystems). When lemon-shaped cysts characteristic of *Heterodera* were observed, they were isolated and stored at room temperature to be used in later analyses (Fenwick, 1940; EPP0, 2013b).

### *Morphological identification of Heterodera spp.*

Vulval cones were cut from isolated cysts with an ophthalmic scalpel, and released eggs and second-stage juveniles (J2) were mounted in sterile tap water. Morphological and morphometric characteristics of ten second-stage juveniles from each selected cyst were then assessed, and were compared with available descriptions (Subbotin *et al.*, 2010).

### *Molecular identification of Heterodera spp.*

**DNA extraction and amplification:** Viable juveniles from cysts analysed morphologically were used for DNA extractions using the QIAamp® DNA Mini Kit (Qiagen), following the manufacturer's instructions. The mtCOI gene, and the 18S rDNA and 28S rDNA regions were amplified as described in Table 1. PCR reactions were carried out using a Biometra TOne Gradient thermocycler (Biometra). Possible contaminations were evaluated by including non-template controls (NTC) without DNA.

Amplified products were loaded onto a 1.5% agarose gel in TAE, and then subjected to electrophoresis at 5 V cm<sup>-1</sup> in a Mupid One System (Nippon Genetics Europe). This system allows visualization and detection of DNA fragments during each run, using direct staining of DNA with Midori Green (Nippon Genetics Europe) together with safe Blues LEDs that do not degrade or mutate DNA.

**Sequencing and sequence quality control:** PCR products were enzymatically purified using ExoSAP-IT PCR Product Cleanup (Thermo Fisher), following the manufacturer's instructions (incubation for 15 min at 37°C, followed by 15 min at 85°C). Cycle sequencing was carried out with the ABI BigDye Cycle sequencing kit (Applied Biosystems) on an ABI Prism 3130XL capillary sequencer (Applied Biosystems), in both directions using the same PCR primers. Sanger sequencing was outsourced at the molecular biology laboratory of INIAV (Oeiras, Portugal).

Chromatograms were visualized and nucleotide sequences were edited and analysed using BioEdit v7.2.0 (Ibis Biosciences) and MEGA X version 10.2.6 (Pennsylvania State University). Unidirectional sequences were each considered successful when the sequence of the complementary primer was present at the 3'end, no double peaks were observed, and high fluorescence was measured along the entire sequence. The mtCOI sequence was translated using the translation table 5 for invertebrate mtDNA genetic code and aligned. Non-stop codons were not visualized.

When all the quality criteria were fulfilled, the primer sequences were trimmed, and a consensus sequence was generated. The resulting mtCOI, 18S rDNA and 28S rDNA consensus sequences were used to investigate, by a blast search the "core nucleotide database (core-nt)" in NCBI GenBank, to identify the most similar sequences within *Heterodera* species.

### *Phylogenetic analyses*

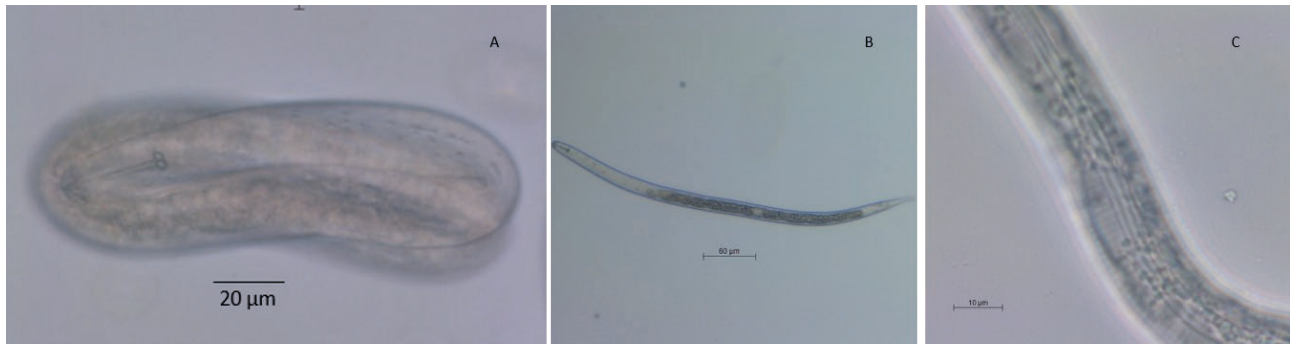
The nucleotide alignments were also used to construct distance trees. All sequences were aligned by CLUSTAW with default parameters. The pairwise aligned sequences were phylogenetically analysed by the Neighbor-Joining Tree-Built method and the Tamura-Nei Genetic Distance model employing MEGA X version 10.2.6 software (Pennsylvania State University). A bootstrap analysis with 1000 replications was also conducted to infer robustness of the phylogenetic trees. Sequences from *Globodera rostochiensis* were selected as the outgroup.

## RESULTS

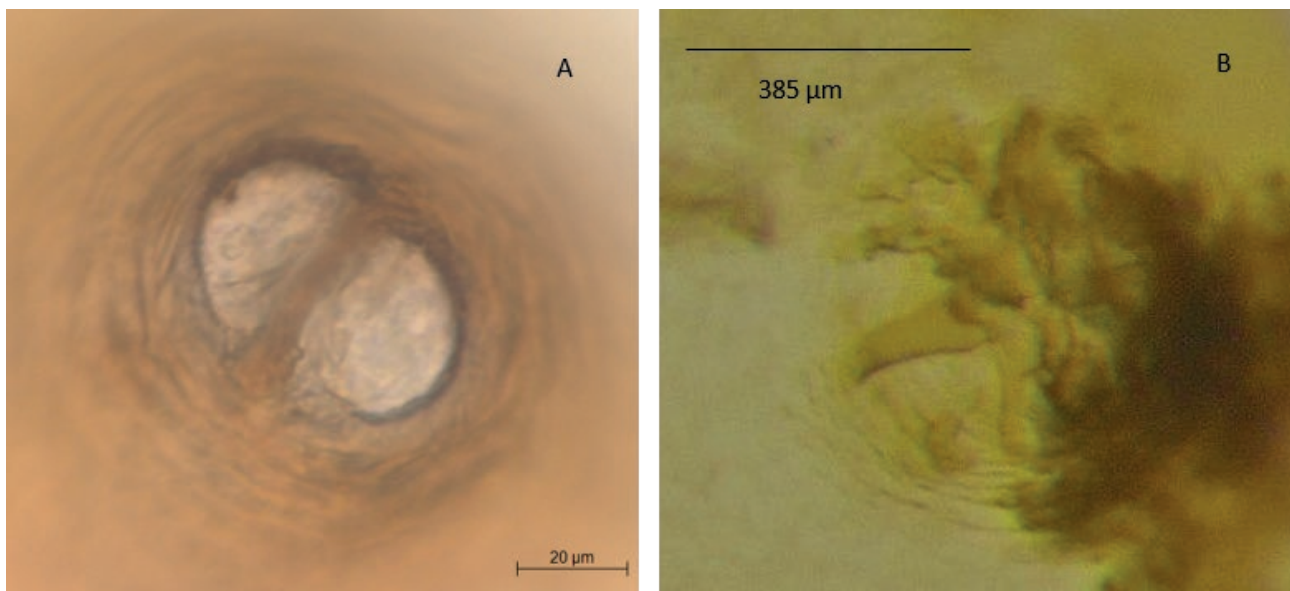
### *Morphological identification of Heterodera spp.*

Cysts were light to dark brown. By cutting the vulval cone of *Heterodera* cysts, it was possible to identify eggs (Figure 1 A), second-stage juveniles (Figure 1 B), and the vulval cone characteristics (Figure 2).

Second-stage juveniles were vermiform, with annulated bodies that tapered at both ends (Figure 1 B). Mean body length was 429 ( $\pm$  18)  $\mu$ m, the heads were offset, and the cuticles were regularly annulated, each with four lateral fields extending from near the head to the tail (Figure 1 C). Stylets were well developed of mean length 23 ( $\pm$  1)  $\mu$ m, with anterior concave knobs. The tails gradually tapered towards finely rounded termini, and were mean length 49 ( $\pm$  2)  $\mu$ m, with hyaline parts of mean length 25 ( $\pm$  1)  $\mu$ m. The vulvas were ambifenestrate and each divided into two semifenestrae by a vulval bridge (Figure 2 A), and within the cone, remains of a vagina was attached to side walls by a well-developed underbridge and a number of irregularly arranged, dark brown bullae situated a short distance beneath vulval bridge (Figure 2 B). By comparing the data reported by Subbotin *et al.* (2010), these nematodes were identified as *Heterodera schachtii*.



**Figure 1.** A: Egg of *Heterodera* nematode containing a fully developed second-stage juvenile, ready to emerge. B: Fully developed second-stage juvenile. C: Juvenile cuticle regularly annulated with four lateral fields (C).



**Figure 2.** A: *Heterodera* vulval cone, vulva ambifenestrata divided into two semifenestratae by the vulval bridge. B: The cone has bullae and a well-developed underbridge.

#### *Molecular identification of Heterodera schachtii*

Using the procedure previously described, all primer pairs allowed amplification in the tested samples. Assembled sequences were compared with the GenBank database to confirm nematode identity. For the mtCOI sequence, all hits had coverage of 99%, that allowed identification of the *H. schachtii*. The nucleotide sequences had 100% similarity with other *H. schachtii* sequences from the Iberian region (Sevilla, Spain -MW345380-MW345391; Faro and Leiria, Portugal -PQ462045-PQ462046). For the 18S rDNA sequence, all hits had coverage of 99%, that allowed identification of *H. schachtii*. The nucleotide sequences had 100% similarity with *H. schachtii* sequences from Gent University,

Belgium (EU306355). For the 28S rDNA sequence, all hits had coverage of 96%, that allowed identification of *H. schachtii*. The nucleotide sequences had 99.72% similarity with *H. schachtii* sequences from Jeongseon, South Korea (MN720062).

The sequences were deposited at NCBI database under accession numbers PQ462055 (for mtCOI), PV351709 (for 18S rDNA), and PV351741 (for 28S rDNA), and were also used to obtain the phylogenetic tree at NCBI platform through the Neighbour Joining method as a confirmation step of identification (data not shown).

The phylogenetic relationship of the present study specimen with other *Heterodera* species based on the mtCOI gene region (Figure 3) was within the *H. schachtii* clade, and was highly supported by a 99% Bootstrap

**Table 1.** PCR conditions, master mix used: Supreme NZYTaq II 2× Green Master Mix (NZYTech).

Primers (Reference)	Primers sequences	Amplicon size	Thermal cycling conditions	Reaction mix
<b>18S rDNA gene region</b>				
988F/1912R and 1813F/2646R (Hu <i>et al.</i> , 2002)	988F: 5'-CTC AAA GAT TAA GCC ATG C-3' 1912R: 5'-TTT ACG GTC AGA ACT AGG G-3' 1813F: 5'-CTG CGT GAG AGG TGA AAT-3' 2646R: 5'-GCT ACC TTG TTA CGA CTT TT-3'	98 bp and 880 bp, resulting a 1730 bp	Initial denaturation of 94°C for 5 min 53 cycles (94°C for 30 s, 45°C for 30 s and 72°C for 70 s) 35 cycles (94°C for 30 s, 54°C for 30 s and 72°C for 70 s) Final extension of 72°C for 10 min	– 5 µL of template DNA – 12.5 µL of Master Mix (NZYTech) – 1.5 µL of each primer – 4.5 µL of water
<b>28S rDNA region</b>				
D2A/D3B (Holterman <i>et al.</i> , 2006)	D2A: 5'-ACA AGT ACC GTG AGG GAA AGT TG-3' D3B: 5'-TCG GAA GGA ACC AGC TAC TA-3'	780 bp	Initial denaturation of 95°C for 10 min 40 cycles (95°C for 30 s, 60°C for 45 s and 72°C for 45 s) Final extension of 72°C for 10 min	– 2 µL of template DNA – 12.5 µL of Master Mix – 0.75 µL of each primer – 9 µL of water
<b>Mitochondrial cytochrome oxidase subunit one (mtCOI) gene region</b>				
JB3/ JB5 (Ley <i>et al.</i> , 1999)	JB3: 5'- TTT TTT GGG CAT CCT GAG GTT TAT -3' JB5: 5'- AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG -3'	447 bp	Initial denaturation of 98°C for 1 min 40 cycles (8°C for 10 s, 41°C for 20 s and 72°C for 30 seg) Final extension of 72°C for 10 min	– 5 µL of template DNA – 12.5 µL of Master Mix – 1.5 µL of each primer – 4.5 µL of water

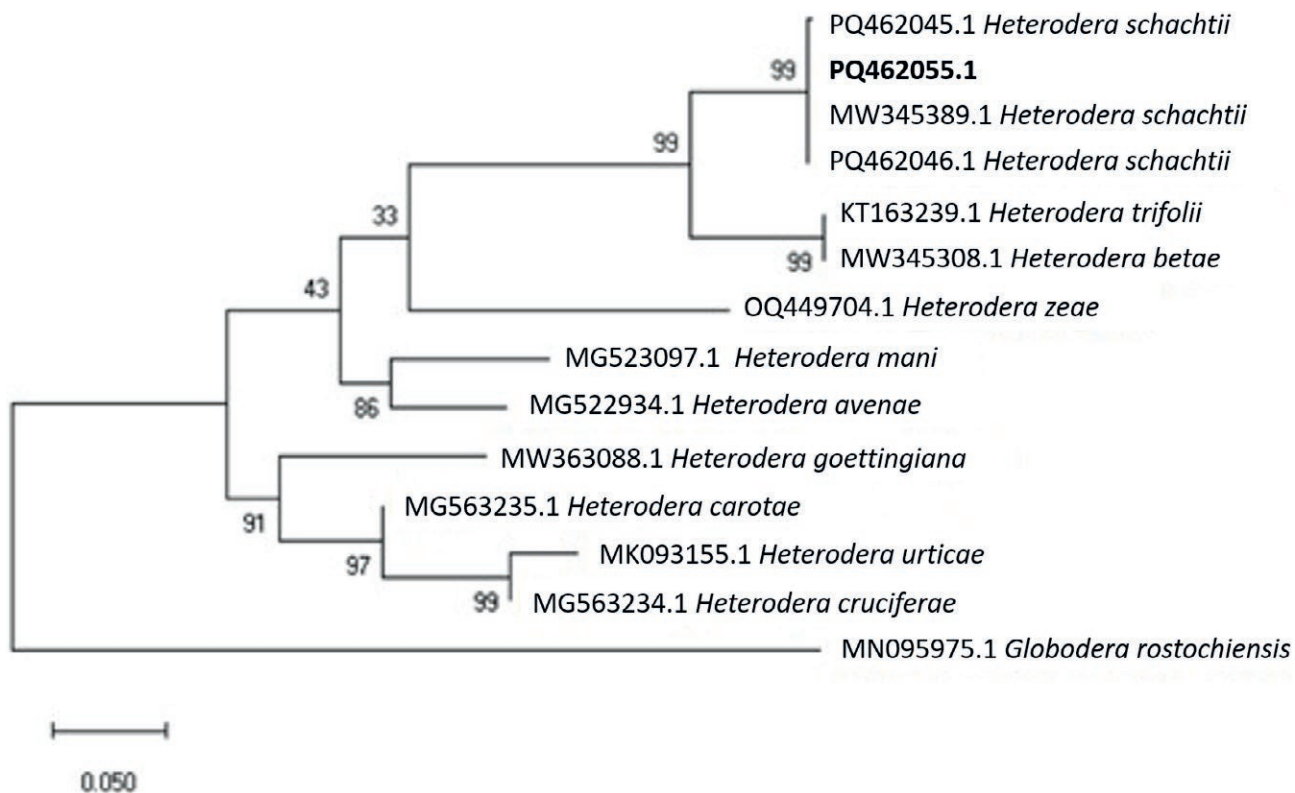
value. Similar situations were observed for the 18S rDNA and 28S rDNA trees (Figures 4 and 5).

The high sequence similarity and consistent phylogenetic results provide robust molecular confirmation of the species identity. These results are consistent with the conclusions of Huston *et al.* (2022) and Camacho *et al.*, (2025), who demonstrated the reliability and utility of standard gene sequence barcodes, including mtCOI, 18S rDNA, and 28S rDNA regions, for accurate identification and differentiation of cyst nematodes within *Heterodera*.

## DISCUSSION

Morphological data showed that the cysts obtained in this study were of *Heterodera schachtii*. However, because morphological identification of nematodes is not always reliable, confirmation using molecular methods is recommended (Seesao *et al.*, 2016 and Camacho *et al.*, 2017)). In the present case, the morphological and molecular analyses confirmed the identification of *H. schachtii*, with JB3-JB5 being the best primer pair for *H. schachtii* identification. This is in agreement with previous research that highlights the reliability of standard gene sequence barcodes for identification of *Heterodera* species (Huston *et al.*, 2022, Camacho *et al.*, 2025).

*Heterodera schachtii* is a parasite of several plant families, including *Brassicaceae*, *Chenopodiaceae* and *Cruciferae* (Raski, 1950), with sugar beet being its primary host (Mwamula *et al.*, 2019). Given the history of the plot assessed in the present study and known hosts of *H. schachtii*, presence of these nematodes was likely. The present results show that cysts of *H. schachtii* remained viable after more than 15 years without cultivation of sugar beet, the main host crop this nematode. However, during this period, the plot was intermittently covered by many different plants, including associations with brassicas, particularly forage radish (*Raphanus sativus* var. *longipinnatus* and *Raphanus sativus* var. *oleiferus*), as well as plants used for green manuring, and weeds (*Avena sativa*, *Avena strigosa*, *Brassica carinata*, *Brassica napus*, *Lathyrus sativus*, *Lolium multiflorum*, *Pisum sativum*, *Sinapis alba*, *Trifolium* spp. and *Vicia sativa*). Among the plant species that have been cultivated in the studied plot, several have been described as hosts of *H. schachtii* (Goodey *et al.*, 1965). Although the *R. sativus* cultivars used are resistant to *H. schachtii* (Eberlein *et al.*, 2020), and were grown as a trap crop to reduce nematode densities, other brassicas may have contributed to persistence of the population. However, it is not possible to retrospectively determine whether any weed species harboured *H. schachtii* populations, or whether the bras-



**Figure 3.** Phylogenetic tree for *Heterodera* spp. based on sequence alignment of the mitochondrial cytochrome oxidase subunit one (mtCOI) gene. The analysis involved 14 nucleotide sequences, including the PQ462055.1 sequence, identified as *Heterodera schachtii*. *Globodera rostochiensis* was used as the outgroup taxon. The condensed phylogenetic tree was generated using the Neighbor-Joining method with 1,000 bootstrap replications. Bootstrap values are indicated at the nodes.

sicas used as cover crops, despite being considered nematode-resistant, may have sustained them. Previous studies allowing for greater control of variables, have been time restricted. This highlights the relevance of the present work, as it provides new insights into the prevalence of these nematodes in agricultural fields that have been without primary hosts for more than 15 years. Besides, no brassicas were included in the crop rotations over the past 3 years, yet *Heterodera* populations remained viable in the soil.

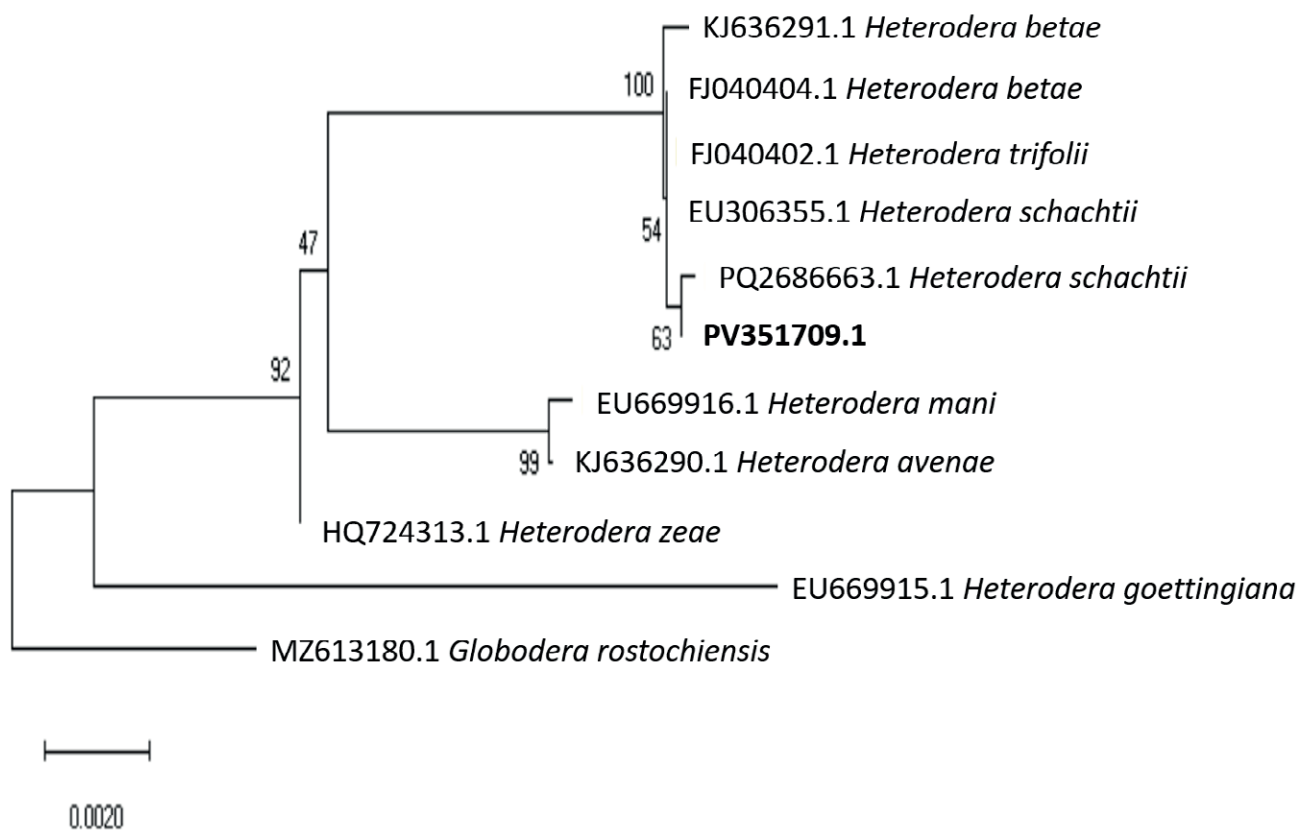
In the study of Westphal and Becker (2001), neither resistant nor susceptible radish cultivars allowed *H. schachtii* development. Therefore, it is concluded that for management of these nematodes in infested fields, brassicas can be used as cover crops unless resistant varieties to *H. schachtii* are selected.

*Heterodera* eggs within cysts can remain viable in soil, but dormant for several years (Lilley *et al.*, 2005; Smiley *et al.*, 2009; Hunt, 2008). However, previous studies have not provided specific observations on duration of cyst viability in the absence of primary hosts. The present study documents the prolonged survival of

lemon-shaped cysts of *H. schachtii*, in the absence of the nematode's primary host. This underscores the challenges for control of these nematodes, as their extended longevity in soil diminishes the effectiveness of inoculum reduction methods such as fallowing and crop rotations. Therefore, alternative strategies such as using trap crops or resistant cultivars are likely to be the most promising approaches for managing problems caused by *H. schachtii*.

#### AUTHOR CONTRIBUTIONS

Conceptualization, ML.I. and MJ.C.; methodology, E.A., ML.I., AP.R. and MJ.C.; software, E.A. and MJ.C.; validation, D.A.; E.A. ML.I., AP.R., and MJ.C.; formal analysis, D.A.; E.A., and MJ.C.; resources, E.A. and ML.I.; data curation, E.A. and ML.I.; writing—original draft preparation, D.A.; ML.I. and MJ.C.; writing—review and editing, D.A.; ML.I., E.A., ML.I., AP.R. and MJ.C.; visualization, D.A.; ML.I., E.A., ML.I., AP.R. and MJ.C.; supervision, ML.I., AP.R. and MJ.C.; project



**Figure 4.** Phylogenetic tree for *Heterodera* spp. based on the sequence alignment of 18S rDNA gene region. The analysis involved 11 nucleotide sequences, including the PV351709.1 sequence, identified as *Heterodera schachtii*. *Globodera rostochiensis* was used as the outgroup taxon. The condensed phylogenetic tree was generated using the Neighbor-Joining method with 1,000 bootstrap replications. Bootstrap values are indicated at the nodes.

administration, M.L.I.; funding acquisition, M.L.I. and E.A. All authors have read and agreed to the published version of the manuscript.

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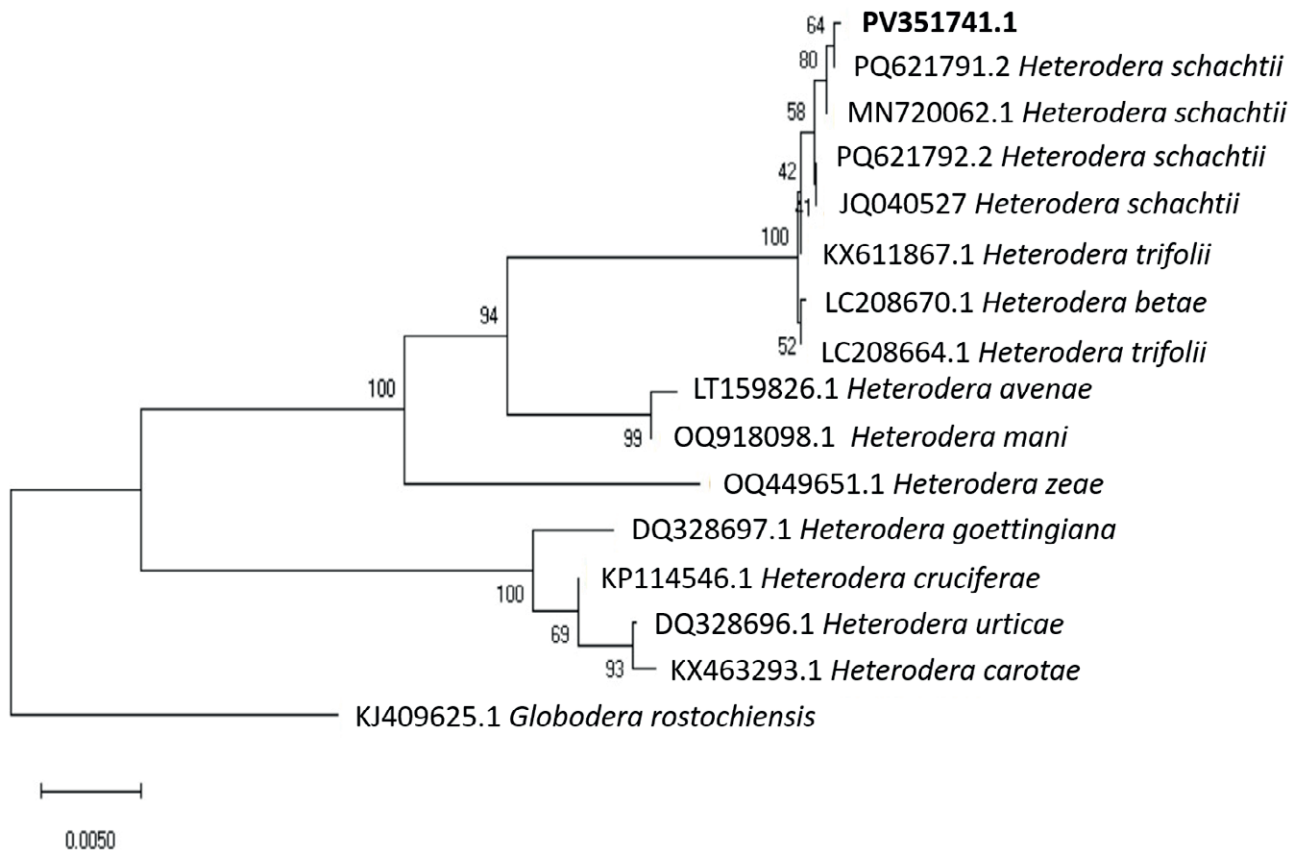
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**Figure 5.** Phylogenetic tree for *Heterodera* spp. based on the sequence alignment of 28S rDNA gene region. The analysis involved 16 nucleotide sequences, including the PV351741.1 sequence, identified as *Heterodera schachtii*. *Globodera rostochiensis* was used as the outgroup taxon. The condensed phylogenetic tree was generated using the Neighbor-Joining method with 1,000 bootstrap replications. Bootstrap values are indicated at the nodes.

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