The Northern Root-Knot Nematode *Meloidogyne hapla*: New Host Records in Portugal

Leidy Rusinque, Filomena Nóbrega, Clara Serra and Maria L. Inácio

1. Introduction

Horticulture crops represent about 50% of the Portuguese agricultural added value. The most important crops are grapevines, oranges, apples, pears, cole crops, peaches, processing tomatoes and potatoes. Oramentals (cut flowers and potted plant production) in increasing areas represent about 600 ha [1].

Plant-parasitic nematodes (PPNs) are an important constraint to agricultural production as the losses they cause have been estimated from USD 175 billion per year [2,3].
However, the full extent of nematode damage is likely to be underestimated as many growers are unaware of their presence [4].

Root-knot nematodes (RKNs) are one of the oldest known parasitic nematodes of plants and considered serious pests of economically important crops [5]. The genus comprises more than 100 species, with many of them reported in Portugal: *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949; *Meloidogyne chitwoodi* Golden et al., 1980; *Meloidogyne enterolobii* Yang and Eisenback, 1983; *Meloidogyne hapla* Chitwood, 1949; *Meloidogyne hispanica* Hirschmann, 1986; *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949; *Meloidogyne javanica* (Trub, 1885) Chitwood, 1949; *Meloidogyne luci Carneiro et al., 2014; Meloidogyne lusitana Abrantes and Santos, 1991; and *Meloidogyne naasi* Franklin, 1965 [6–12]. The species, *M. arenaria, M. hapla, M. incognita* and *M. javanica*, are regarded as the most important, due to their worldwide distribution and polyphagia [2,4]. Typical symptoms of RKN attack include galling of the root system, stunting and yellowing, resulting in impaired root function which leads to a reduction in yield [13,14].

The northern RKN, *Meloidogyne hapla*, was first described from a potato field in Long Island, New York (Chitwood 1949) and is one of the most important species of RKNs occurring in temperate regions, as it can withstand the cold. Eggs and juveniles can survive field temperatures below 0 °C, with some studies recording survival down to −15 °C in soil for a prolonged period of time. The optimum temperature for penetration and development of *M. hapla* is in the range of 20–25 °C and a mean temperature of 28 °C may prevent its development [15–17]. *Meloidogyne hapla* is a sedentary, biotrophic parasite of plants that overwinters in soil or diseased roots, and multiplies by both sexual (amphimixis) and asexual (parthenogenesis) reproduction, in contrast to the most widespread, three other RKNs [18]. Unlike many other RKN species, *M. hapla* eggs and juveniles can survive field temperatures below 0 °C. However, there is no evidence of its inability to survive in hot temperatures [17]. It parasitizes nearly all temperate vegetables of economic importance, reducing considerably yield and even causing total crop losses [14,19]. Surveys of vineyards and potatoes conducted in the United States found *M. hapla* to be the most abundant RKN present in the fields [20]. In Portugal, *M. hapla* was first detected in 2008 in fig trees and a year later found parasitizing the potato; since then, no other detections have been reported [7,8].

Therefore, the aim of the present study was to morphologically and molecularly identify the isolates of the RKN found in the survey of different counties and crops across mainland Portugal and Madeira Island, namely in grapevines, potato and eucalyptus.

2. Materials and Methods

2.1. Sampling and Nematode Isolates

During 2019–2022, surveys of horticultural and ornamental fields were carried out, resulting in a total of 690 soil and root samples collected from various districts in Portugal. Soil sampling was carried out from the rhizosphere at about 15 cm in depth for horticultural crops and 70 cm for grapevines and trees. Each sample consisted of 5 to 8 cores, sampled in the vicinity of plants that presented symptoms, and in zigzag at roughly equal intervals in asymptomatic fields. Each composite soil sample was placed in a polyethylene bag and brought for analysis. A 400-mL subsample was taken from each composite sample and the nematodes were extracted according to the protocol PM 7/119 (1) [21]. The suspension was observed under a stereomicroscope (Nikon SMZ1500, Tokyo, Japan) and suspected specimens of *Meloidogyne* observed using a bright-field light microscope (Olympus BX-51, Hamburg, Germany) for confirmation. When second-stage juveniles (J2) of *Meloidogyne* were detected in the soil suspension, bioassays were carried out by planting tomato plants cv. Oxheart in the remaining soil from the sample and maintained in a quarantine greenhouse for two months to obtain different developmental stages for further studies. Roots (from sampling and bioassays) were observed under stereomicroscope. Egg masses were handpicked from infected roots and used to establish cultures of each isolate.
2.2. Morphological Characterization

Ten second-stage juveniles (J2) from each positive sample were placed in a drop of water on a glass slide, gently heat killed for morphological characterization using a bright-field light microscope (Olympus BX-51, Hamburg, Germany) and photographed with a digital camera (Leica MC190 HD, Wetzlar, Germany).

2.3. Molecular Characterization

The species-specific primers, SCAR markers, and the ITS region were selected for molecular characterization of *Meloidogyne hapla* isolates found in the survey. The total DNA was extracted from a single juvenile using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The SCAR region was amplified in multiplex PCR using the primers JMV1 (5′-GGATGGCGTGCTTTCAAC-3′), JMV2 (5′-TTTCCCCCCTATGATGTTTACCC-3′) and JMVhapla (5′-AAAAATCCCCCTCGAAA AATCCACC3′), and the ITS region, using the forward primer, TW81 (5′-GT TTCCGTAGGT GAACCTGC-3′) and the reverse AB28 (5′-ATATGCTTAAGTTCAGGGT-3′) [22,23].

PCR reactions were performed in a 50-µL final volume mixture containing 25 µL of Supreme NZY Taq II 2× Green Master Mix (NZYTech, Lisboa, Portugal), 1 µL of isolated DNA and 0.2 µM of each primer in a Biometra TGradient thermocycler (Biometra, Göttin gen, Germany). Thermal cycling conditions were as described by [23,24]. PCR products were resolved by electrophoresis at 5 V.cm⁻¹, in agarose gel (1.5%) containing 0.5 µg/mL of ethidium bromide and 0.5 × of Tris-borate-EDTA (TBE) running buffer. Amplifications were visualized using the VersaDoc Imaging System (BioRad Laboratories, Hercules, CA, USA). PCR products were purified using the DNA Clean & Concentrator Kit (Zymo Research Corp, Irvine, CA, USA), according to the manufacturer’s instructions. Ampli cons were sequenced at the INIAV (Oeiras, Portugal) on an ABI PRISM 3730xl (Applied Biosystems, Walthman, MA, USA) DNA analyzer. The newly obtained sequences were manually checked, edited and assembled. The sequences were compared to those of *M. hapla* and other relevant sequences of *Meloidogyne* spp. available in the GenBank database using the BLAST homology search. The multiple alignment of the retrieved sequences was performed using ClustalW multiple alignment in BioEdit.

Phylogenetic analyses were conducted using MEGA 11 [25], the maximum likelihood (ML) and the Kimura 2-parameter model. The robustness of the ML tree was inferred using 1000 bootstrap replicates.

3. Results

3.1. Distribution

A total of 690 samples were collected from the south to the north of mainland Portugal and Madeira Island. From the total of samples collected, *Meloidogyne hapla* was detected in nine, corresponding to 1.5%. The detections were in four districts of the southern and northern regions and on Madeira Island. *Meloidogyne hapla* was not found in the central region. Three hosts were identified (potato, grapevine and eucalyptus) (Table 1) (Figure 1).

Table 1. Locations, dates and crops where *M. hapla* was found.

| Code   | Year | District  | County   | Crop       |
|--------|------|-----------|----------|------------|
| SV1943 | 2019 | Beja      | Odemira  | Grapevine  |
| SV55   | 2020 | Madeira   | Santana  | Grapevine  |
| SV1981 | 2020 | Beja      | Odemira  | Eucalyptus |
| SV1357 | 2021 | Viana de Castelo | Melgaço | Potato |
| SV2180 | 2021 | Porto     | Vila do Conde | Potato |
| SV352  | 2022 | Setúbal   | Montijo  | Grapevine  |
| SV12446| 2022 | Porto     | Amarante | Potato |
| SV12447| 2022 | Porto     | Amarante | Potato |
| SV13382| 2022| Porto     | Baião    | Potato |
3.2. Symptoms

The field symptoms (above- and below-ground) observed and the species of nematodes found in the soil are described in Table 2.

Table 2. Symptoms observed in plants infected with *Meloidogyne hapla* and plant parasitic nematodes (PPNs) found in soil.

| Crop            | Above-Ground Symptoms (Figure 2) | Below-Ground Symptoms (Figure 3) | PPNs Present in Soil                                                                 |
|-----------------|----------------------------------|----------------------------------|-------------------------------------------------------------------------------------|
| Grapevine       | Drying of the bunches, Yellowing  | Presence of galls and egg masses | 70 J2 of *Meloidogyne* / 400 mL. No other active PPNs were found in the soil extractions |
|                 | Poor growth                      |                                  | 70 J2 of *Meloidogyne* / 400 mL. No other active PPNs were found in the soil extractions |
| Eucalyptus      | Stunting, Dead plants            | —                                | 80 J2 of *Meloidogyne* / 400 mL. No other active PPNs were found in the soil extractions |
| Potato          | Poor growth                      | Presence of galls and egg masses | 80 J2 of *Meloidogyne* / 400 mL. No other active PPNs were found in the soil extractions |

Figure 1. Counties and districts where *Meloidogyne hapla* was detected.

Figure 2. Above-ground symptoms of *Meloidogyne hapla* attack. (A,B): eucalyptus; (C): potato; (D): grapevine.
3.3. Morphological Characterization

Morphological characterization from the recovered second-stage juveniles was performed and was in agreement with previous descriptions of the genus (Figure 4) [24,26,27]. The second-stage juveniles were vermiiform, slender, and clearly annulated. The head region was slightly set off from body. The stylet was delicate and narrow, and the dorsal gland orifice (DGO) long, compared to the other three most common species. The knobs were rounded and appeared set off from the shaft. The excretory pore was distinct and hemizonid anterior or adjacent to excretory pore. The tail was long and slender with a narrow, tapering terminus that had several distinct annulations; the tail delimitation was not very clear in *M. hapla*. The hyaline tail terminus of *M. hapla* is highly variable and can go from deformed with an irregular shape to regular V shapes.

3.4. Molecular Characterization

The PCR amplification of species-specific SCAR markers yielded a single fragment of 440 bp as expected (Figure 5). The amplification of ITS (TW81/AB28) yielded a single fragment of about 550 bp. The nucleotide sequences obtained in this study were deposited into the GenBank database (NCBI) under the accession numbers, OP364010, OP364011, OP364012, OP364013, OP364014, OP364015, OP364016, OP364017 and OP364018. A BLAST search of the nucleotide sequences showed a similarity ranging from 99.8% to 100% with the sequences of *M. hapla* available in the database (Table 3).

**Figure 3.** Below-ground symptoms of *Meloidogyne hapla* attack (galls). (A,B): potato; (C): grapevine.

**Figure 4.** *Meloidogyne hapla* light microscope observations. Second-stage juvenile: (A) whole specimen; (B) anterior region; (C) tail region; (D) hyaline part (bar = 20 µm).
The molecular phylogenetic analysis is presented in Figure 6. The phylogram A revealed one clade, supported by a bootstrap value of 65%, that included only isolates of *M. hapla* from different hosts and geographical origins including Portugal. The phylogram B revealed three distinct clades: one represented by isolates of *M. hapla* from a different host and locations including Portugal, supported by a bootstrap value of 90%; a second one containing isolates of *M. chitwoodi* and *M. fallax* (bootstrap value of 97%); and a third clade with isolates of tropical species of *Meloidogyne* (bootstrap value of 98%).
4. Discussion

Nematodes are often overlooked because of the ambiguity of the symptoms they cause, and the difficulty in detecting and identifying them. Moreover, the impact the RKN has in agricultural areas reinforces the need for accurate diagnosis at the species level. Conventionally, the identification of Meloidogyne species has been based on morphological characters of second-stage juveniles, perineal patterns of adult females and isozyme phenotypes. Isozymes are highly reliable, but have drawbacks, as it requires a specific developmental stage (adult females) as well as considerable skills. Many different DNA-based methods have been used to identify M. hapla, among which include species-specific SCAR primers and the ITS region; these methods allow to identify the species in a timely and efficient manner. Since the eradication of nematodes is a complex task, the identification of the Meloidogyne spp. in the area of interest is of primary importance to implement management strategies that minimize damage. This will allow for the adoption of preventive practices such as the appropriate deployment of cultivars.
The presence of *M. hapla* has extended throughout the country, from detections in two districts in 2009 to five in the following four years (2019–2022). Such occurrences and increased detection draw attention to its potential to adversely affect economically important horticultural and ornamental crops. These detections were in northern and southern coastal counties—places where the climate is influenced by the Atlantic Ocean. Districts located up north are cooler and rainy, while the south becomes gradually warmer and sunnier; however, the Atlantic moderates the temperature, providing adequate conditions for *M. hapla* development and survival, as average temperatures throughout the year vary from 5 °C to 25 °C.

To our knowledge, this is the first report of *Meloidogyne hapla* in the grapevine and eucalyptus in Portugal, adding valuable information to the current situation of this organism in the European Plant Protection Organization (EPPO) zone.

5. Conclusions

Root-knot nematodes are one of the most devastating plant parasitic nematodes, affecting the yield and quality of many crops. Their wide host range makes the management of RKNs extremely difficult; so, identifying the nematode species we are dealing with and understanding the spread, survival and damage potential, in this case of the *Meloidogyne hapla*, is the first step to managing it efficiently and effectively.

This study confirmed the presence of *M. hapla* in the country and provided evidence of its dispersion and ability to survive in different crops, contributing important data that can be used in integrated management programs.

**Author Contributions:** Conceptualization, L.R.; methodology, L.R., F.N. and M.L.I.; software, F.N.; validation, L.R., F.N., C.S. and M.L.I.; formal analysis, L.R., F.N. and M.L.I.; investigation, L.R.; data curation, L.R. and F.N.; writing—original draft preparation, L.R.; writing—review and editing, L.R., C.S., F.N. and M.L.I.; project administration, M.L.I.; funding acquisition, M.L.I. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by the Portuguese Foundation for Science and Technology (Fundação para a Ciência e a Tecnologia, FCT), through Fellowship 2020.05541.BD.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors would like to thank the technicians, Margarida Fontes, Nidia Laureano and Maria José Silva from the Laboratories of Nematology and Biochemistry, respectively.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

**References**

1. Cook, E. *Agriculture, Forestry and Fishery Statistics—2020 Edition*; Publications Office of the European Union: Luxemburg, 2020; pp. 208–209.
2. Elling, A.A. Major Emerging Problems with Minor *Meloidogyne* Species. *Phytopathology* 2013, 103, 1092–1102. [CrossRef] [PubMed]
3. Abad, P.; Gouzy, J.; Aury, J.M.; Castagnone-Sereno, P.; Danchin, E.G.J.; Deleury, E.; Perfus-Barbeoch, L.; Anthouard, V.; Artigue-nave, F.; Blok, V.C.; et al. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat. Biotechnol.* 2008, 26, 909–915. [CrossRef] [PubMed]
4. Jones, J.T.; Haegeman, A.; Danchin, E.G.J.; Gaur, H.S.; Helder, J.; Jones, M.G.K.; Kikuchi, T.; Manzanilla-López, R.; Palomares-Rius, J.E.; Wesemael, W.M.; et al. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol. Plant Pathol.* 2013, 14, 946–961. [CrossRef] [PubMed]
5. Lima, F.S.O.; Mattos, S.V.; Silva, S.E.; Carvalho, M.A.S.; Teixeira, R.A.; Silva, J.C.; Correa, V.R. Nematodes Affecting Potato and Sustainable Practices for Their Management. In *Potato—From Incas to All over the World*, 1st ed.; Yildiz, M., Ed.; IntechOpen: London, UK, 2018; Volume 1, pp. 107–121.
6. Karssen, G.; Wesemael, W.; Moens, M. Root-Knot Nematodes. In *Plant Nematology*, 2nd ed.; Perry, R.N., Moens, M., Eds.; CABI International: Wallingford, UK, 2013; pp. 73–108.
7. Abrantes, I.D.; dos Santos, M.V.; da Conceição, I.L.; Santos, M.D.; Vovlas, N. Root-knot and other plant-parasitic nematodes associated with fig trees in Portugal. *Nema. Mediterr.* 2008, 36, 131–136.

8. Da Conceição, I.L.; da Cunha, M.J.; Feio, G.; Correia, M.; dos Santos, M.C.V.; de O Abrantes, I.M.; de A Santos, M.S. Root-knot nematodes, *Meloidogyne* spp., on potato in Portugal. *Nematology* 2009, 11, 311–313.

9. Maleita, C.; Esteves, I.; Cardoso, J.M.S.; Cunha, M.J.; Carneiro, R.M.D.G.; Abrantes, I. *Meloidogyne luci*, a new root-knot nematode parasitizing potato in Portugal. *Plant Pathol.* 2018, 67, 366–376. [CrossRef] [PubMed]

10. Rusinque, L.; Nobrega, F.; Cordeiro, L.; Serra, C.; Inácio, M.L. First Detection of *Meloidogyne luci* (Nematoda: Meloidogynidae) Parasitizing Potato in the Azores, Portugal. *Plants* 2021, 10, 99. [CrossRef] [PubMed]

11. Santos, D.; Correia, A.; Abrantes, I.; Maleita, C. The quarantine root knot nematode *Meloidogyne enterolobii*—A potential threat to Portugal and Europe. *Plant Pathol.* 2019, 68, 1607–1615. [CrossRef] [PubMed]

12. Viera dos Santos, M.; Almeida, M.T.M.; Costa, S.R. First report of *Meloidogyne naasi* parasitizing turfgrass in Portugal. *J. Nematol.* 2020, 52, 1–4. [CrossRef] [PubMed]

13. Been, T.H.; Korthals, G.; Schomaker, C.H.; Zijlstra, C. *The Melostop Project: Sampling and Detection of Meloidogyne Chitwoodi and* *M. fallax*; Report 138; Plant Research International BV: Wageningen, The Netherlands, 2008.

14. Kantor, M.; Handoo, Z.; Kantor, C.; Carta, L. Top Ten Most Important U.S.-Regulated and Emerging Plant-Parasitic Nematodes. *Horticulturae* 2022, 8, 208. [CrossRef] [PubMed]

15. Vrain, T.; Barker, K. Influence of low temperature on development of *Meloidogyne incognita* and *M. hapla* eggs in egg masses. *J. Nematology* 1978, 10, 311–313.

16. Belair, G. Winter survival of the Northern root-knot nematode *Meloidogyne hapla* in soil. *Can. J. Plant Sci.* 1985, 65, 435–439. [CrossRef]

17. Wu, X.; Zhu, X.; Wang, Y.; Liu, X.; Chen, L.; Duan, Y. The cold tolerance of the northern root-knot nematode, *Meloidogyne hapla*. *PLoS ONE* 2018, 13, e0190531. [CrossRef] [PubMed]

18. Moens, M.; Perry, R.; Star, J. *Meloidogyne* Species—A Diverse Group of Novel and Important Plant Parasites. In *Root Knot Nematodes*, 1st ed.; Perry, R.N., Moens, M., Star, J., Eds.; CABI International: London, UK, 2009.

19. Desaeger, J. *Meloidogyne hapla*, the Northern Root-Knot Nematode, in Florida Strawberries and Associated Double-Cropped Vegetables. ENY-070; University of Florida Institute of Food and Agricultural Sciences: Gainesville, FL, USA, 2018; pp. 2–5.

20. Zasada, I.A.; Riga, E.; Pinkerton, J.N.; Wilson, J.H.; Schreiner, R.P. Plant-parasitic nematodes associated with grapevines, Vitis vinifera, in Washington and Idaho. *Am. J. Enol. Vitic.* 2012, 63, 522–528. [CrossRef] [PubMed]

21. Standard Protocol PM 7/119 (1); Nematode Extraction. EPPO: Paris, France, 2013.

22. Wishart, J.; Phillips, M.S.; Blok, V.C. Ribosomal Intergenic Spacer: A Polymerase Chain Reaction Diagnostic for *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla*. *Plant Pathol.* 2002, 92, 885–892. [CrossRef] [PubMed]

23. Joyce, S.A.; Reid, A.; Driver, F.; Curran, J. Application of Polymerase Chain Reaction (PCR) Methods to Identification of Entomopathogenic Nematodes. In *Genetics of Entomopathogenic Nematode-Bacterium Complexes, COST 812 Biotechnology*; Burnell, A.M., Ehlers, R.U., Masson, J.P., Eds.; European Commission: Brussels, Belgium, 1994; pp. 178–187.

24. Standard Protocol PM 7/41 (3); *Meloidogyne Chitwoodi* and *Meloidogyne Fallax*. EPPO: Paris, France, 2016.

25. Tamura, K.; Stecher, G.; Kumar, S. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 2021, 38, 3022–3027. [CrossRef] [PubMed]

26. Chitwood, B.G. ‘Root-knot nematodes’. Part 1. A revision of the genus *Meloidogyne Goeldi*, 1887. *Proc. Helminthol. Soc. Wash.* 1949, 16, 90–114.

27. Eisenback, J.D. Morphological comparisons of females, males, and second-stage juveniles of *Heterodera avenae*. *Fundam. Appl. Nematol.* 1993, 16, 259–271.