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Occurrence and Distribution of Root-Knot Nematodes *Meloidogyne* spp. in Serbia

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Abstract: This study examines the occurrence and distribution of root-knot nematodes (RKN), *Meloidogyne* spp., in Serbia through an official survey conducted from 2021 to 2023. A total of 241 plant and soil samples were collected from 25 districts across two regions: Northern Serbia (Vojvodina Province) and Central Serbia. RKN infestations were detected in 23.7% of the samples. Among the 57 identified populations, 5 *Meloidogyne* species were recorded: *M. incognita*, *M. hapla*, *M. luci*, *M. arenaria* and *M. javanica*. *Meloidogyne luci* was reported in Serbia for the first time, marking a significant finding for nematology in the region. This study highlights the importance of implementing effective pest management strategies to mitigate the agricultural impact of RKN in Serbia.

Keywords: *Meloidogyne* spp.; molecular identification; morphological identification; distribution; Serbia

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1. Introduction

Root-knot nematodes (RKNs) of the genus *Meloidogyne* (Göldi, 1892) are economically significant obligate polyphagous parasites that infect nearly all species of higher plants [1]. According to Blok et al. [2], annual vegetable crop losses attributed to RKN exceeds EUR 80 billion worldwide. These nematodes are distributed globally, attacking root systems and causing abnormal swelling known as galls, resulting in yield and quality reduction, particularly in vegetables and ornamental plants. Over 100 species have been described within this nematode genus [3]. Four species are considered major pests in agricultural systems: *M. incognita* (Kofoid and White, 1919) Chitwood, 1949; *M. javanica* (Treub, 1885) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; and *M. hapla* Chitwood, 1949 [4]. Among the described RKN species, *M. ethiopica* Whitehead, 1968, is listed on the A1 quarantine list [5], while six species (*M. chitwoodi* Golden, O'Bannon, Santo & Finley 1980; *M. enterolobii* Yang & Eisenback, 1983; *M. fallax* Karssen,1996; *M. graminicola* Golden & Birchfield, 1965; *M. luci* Carneiro et al., 2014; and *M. mali* Itoh, Ohshima & Ichinoe,1969) are included in the A2 quarantine list of the European Plant Protection Organisation (EPPO) to prevent further spread of these harmful pests in Europe [6].

The agricultural and food sector is one of the most significant contributors to the Serbian economy. Agricultural land in Serbia spans approximately 5.1 million hectares (ha), accounting for over two-thirds of the country's total area, with approximately 3.6

million ha classified as arable land [7], Aaccording to the Statistical Office of the Republic of Serbia [8].

Serbia is divided into two main regions—Northern Serbia (Vojvodina Province) and Central Serbia—comprising 30 districts in total based on political and administrative boundaries. The majority of arable land (67%) is allocated to cereal crops, 19% to industrial crops, 9% to fodder crops and 4% to other crops, including vegetables. Vegetable production is dominated by potato (*Solanum tuberosum* L.), followed by pepper (*Capsicum annuum* L.), onion (*Allium cepa* L.) and tomato (*Solanum lycopersicum* L.). Greenhouses and other protective structures account for 13% of the total area under vegetable cultivation

Only seven species of *Meloidogyne* have been reported in Serbia to date (Table 1). The first species initially identified as *Heterodera marioni* (nomen nudum) was first discovered in 1947 on cucumber (*Cucumissativus* L.) in a greenhouse located near Belgrade [9]. *Meloidogyne incognita*, *M. arenaria*, *M. hapla*, *M. javanica* and *M. incognita acrita* Chitwood, 1949 (a synonym for *M. incognita*) were detected on tomato, pepper, cucumber, lettuce (*Lactuca sativa* L.), celery (*Apium graveolens* L.), black nightshade (*Solanum nigrum* L.) and ornamental plants in greenhouses located in Central Serbia [10,11]. In the field, *M. hapla, M. incognita, M. arenaria* and *M. naasi* Franklin, 1965, were reported on sugar beet (*Beta vulgaris* L.), carrot (*Daucus carota* subsp. *sativus* Hoffm.), tobacco (*Nicotiana tabacum* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and sunflower (*Helianthus annuus* L.) in the central, western and southern regions of Serbia [11–14]. In the 1990s and 2000s, no systemic surveillance or new reports of RKN were recorded in the country. However, between 2014 and 2018, severe damage to potato crops (caused by *M. incognita* and *M. arenaria*) was recorded along the Serbian–Hungarian border [15,16]. *M. incognita* and *M. hapla* were also detected on tomato, carrot and parsnip (*Pastinaca sativa* L.) in greenhouses and fields near Leskovac, Veliko Gradište, Kruševac and Belgrade [17,18]. In addition, *M. arenaria* was identified on calla (*Calla palustris* L.) and the quarantine species *M. luci* was reported for the first time on tomato in a greenhouse in Northern Serbia [19,20].

Table 1. Species of *Meloidogyne* spp. detected in Serbia.

Species	References
M. hapla	[10,13,14,17,18]
M. arenaria	[10,11,13,16,19]
M. naasi	[10–12]
M. incognita	[11,13–15,17]
M. incognita acrita	[11,13]
M. javanica	[11,13]
M. luci	[20]

Serbia was part of the former Yugoslavia until the 1990s. RKNs were also investigated in other countries of the former Yugoslavia (Croatia, Bosnia and Herzegovina, North Macedonia, Slovenia and Montenegro). By 2024, seven RNK species had been detected across these countries: *M. arenaria*, *M. hapla* and *M. artiellia* Franklin, 1961, in Croatia [21]; *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica* in Bosnia and Herzegovina [22,23]; *M. incognita*, *M. arenaria*, *M. hapla* and *M. javanica* in North Macedonia [24,25]; *M. incognita*, *M. hapla*, *M. luci* and *M. arenaria* in Slovenia [26,27]; and *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla* and *M. ardenensis* Santos, 1968, in Montenegro [28].

The identification of *Meloidogyne* species is crucial for developing effective management practises. Analysing the morphological characteristics of RKNs in combination with biochemical or molecular techniques has proven to be more dependable than relying on morphology alone, as morphological characteristics are often similar and may vary between individuals and species.

Currently, there is a lack of comprehensive data on the occurrence, geographical distribution and host plant records of RKN species in Serbia. Therefore, the aim of this study was to update the knowledge on the occurrence and distribution of *Meloidogyne* species in greenhouses and open fields in Serbia, as part of the official survey on quarantine RKNs conducted from 2021 to 2023. Accurate identification of RKNs using morphological (perineal pattern morphology) and molecular methods is a critical first step towards establishing an effective programme for the control of *Meloidogyne* species in the country.

2. Materials and Methods

2.1. Sampling

The official *Meloidogyne* survey was carried out by the Serbian Plant Protection Directorate of the Ministry of Agriculture, Forestry and Water Management (MAFWM) between June 2021 and October 2023 as part of the Plant Health Measures Programme. Below-ground plant material (roots and tubers) with soil was collected from greenhouses and fields by 27 agricultural advisory services in two regions across 25 districts (7 in Northern Serbia (Vojvodina Province) and 18 in Central Serbia). Samples of several symptomatic and asymptomatic root systems were collected from tomato, pepper, cucumber, melon (*Cucumis melo* L.), ornamental plants (calla, gladiolus (*Gladiolus communis* L.), potato, rose (*Rosa cinnamomea* L.), carrot, parsley (*Petroselinum crispum* (Mill.) Fuss), parsnip, horseradish (*Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.), cabbage (*Brassica oleracea* L.) and black nightshade (*Solanum nigrum* L.) and were sampled equally across both regions (40 samples), totalling approximately 80 samples collected annually (Figure 1, Table 2). Sampling instructions were prepared by the laboratory for nematode diagnostics. Sample numbers were planned based on previous data on vegetable and ornamental production in 25 districts and were to be collected by the Serbian Plant Protection Directorate. Some samples were collected based on prior reports of infestation, while others were selected randomly. In addition to plant material, soil samples were collected from the same locations. Soil samples, with an approximate volume of 1500 mL per greenhouse or field, were taken using a shovel from a depth of 15–20 cm. This was intended to facilitate nematode reproduction in cases where unidentified RKN populations were present. The sampling tools were cleaned and disinfected with ethanol after each use. The samples were placed in plastic bags, individually coded and labelled with information on the farmer, field or greenhouse, plant host, cultivation conditions and location. The samples were stored in a refrigerator at approximately 4 °C and promptly transported to the laboratory for nematode analysis.

Table 2. Number of samples collected according to growing conditions in Serbia from 2021 to 2023.

Cultivation Conditions	2021	2022	2023
Greenhouse	28	48	30
Field	55	29	51
Total per year	83	77	81

Figure 1. (**A**) Samples were collected between 2021 and 2023 from the regions of Northern and Central Serbia, highlighted in blue with the border depicted by a bold black line between Northern Serbia (7 districts) and Central Serbia (18 districts). Samples were not collected in 5 districts, highlighted in red on this map. Map is from https://en.wikipedia.org/wiki/Administrative_districts_of_Serbia (accessed on 8 January 2025). (**B**) Symptoms caused by *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, on potato (*Solanum tuberosum* L.) *and M. arenaria* (Neal, 1889) Chitwood, 1949, on carrot (*Daucus carota* subsp. *sativus* Hoffm.).

2.2. Nematode Extraction

Fresh plant material (roots and tubers) was analysed for the presence of galls. Galled roots were washed free of soil and the gall index (GI) was determined on a scale of 0–10 [29]. Females and egg masses were extracted from the roots by dissecting the tissue with needles with a stereomicroscope (Nikon SMZ 800N, Tokyo, Japan) at 40× magnification for morphological and molecular analysis. The remaining plant material and soil infected with RKN were heat-treated in an autoclave after nematode extraction.

2.3. Morphological Analysis

The morphological identification of the females was carried out by observing the perineal patterns. The perineal patterns were prepared following the procedures outlined by Hartman and Sasser [30]. The posterior part of the female was dissected using a surgical steel blade in 45% lactic acid, after which the body contents were removed and the cleaned cuticle was transferred to a drop of glycerol on a clean slide for permanent mounting. The morphology of the perineal patterns was examined with a microscope (Leica DM 1000 LED, Wetzlar, Germany) at 400× magnification and photographed using a digital camera (ICC50 W Camera—HDMI, Wetzlar, Germany). Species identification was performed based on the criteria established by Chitwood, 1949, according to Karssen [31].

2.4. Molecular Identification

The extracted egg masses or females were pooled in 150 µL of sterile water, and total DNA was isolated using the Quick-DNATM Fecal/Soil Microbe Miniprep kit, following the manufacturer's instructions. Molecular identification was carried out according to PCR protocols and primers as described in [32–36]. Specific regions were amplified by the PCR method using 1 µL of 10 µmol forward and reverse primers, 1 U of Taq polymerase (GoTaq Flexi DNA polymerase by Promega, Madison, WI, USA) and 5 ng of genomic DNA in 25 µL of the total PCR reaction mixture volume. Depending on the primers (Table 3), the PCR reaction was performed according to the programmes and conditions described in Supplementary Material 1 (Tables S1–S4). Amplified PCR products were visualised on 1% agarose gel and the following bands were expected depending on the primers used. In the first step, based on the PCR with primers 194/195, the species of the genus *Meloidogyne* could be separated into three groups with the following expected bands: *M. hapla* (yielding a 700 bp fragment); *M. incognita*, *M. arenaria* and *M. javanica* (720 bp); *M. enterolobii* (780 bp); and *M. chitwoodi* and *M. fallax* (between 1600 and 1700 bp). The use of these primers resulted in consistent amplifications of DNA extracted from females and egg masses. The samples were further analysed by the PCR method, with primers MI-F and MI-R specific for the species *M. incognita* (999 bp); primers Fjav and Rjav specific for the species *M. javanica* (720 bp); primers Far and Rar specific for the species *M. arenaria* (420 bp); and primers JMV1, JMV2, JMV hapla and JMV tropical specific for *M. chitwoodi* (540 bp), *M. fallax* (670 bp), *M. hapla* (440 bp) and *M. incognita* (615 bp). *Meloidogyne luci* was identified using group-specific PCR, species-specific PCR and sequence analysis. The affiliation of the nematode to the tropical RKN group and the *M. ethiopica* group was determined using two PCR reactions. Identification was confirmed by a species-specific PCR of *M. luci*, which yielded a band of approximately 770 bp. In addition, identification was confirmed by sequence analyses. The region of the mtDNA was amplified with primers C2F3 (GGTCAATGTTCAGAAATTTGTGG) and 1108 (TACCTTTGACCAATCACGCT), cloned, sequenced (acc. no. OQ211107) and compared with other sequences of *Meloidogyne* spp. from Genbank. To identify the nematode *M. luci* belonging to the tropical root-knot nematode group (i.e., clade I of *Meloidogyne* spp.), the forward primer C2F3 and a group-specific reverse primer Mt575R yielded a 621 bp long amplicon specific for the tropical root-knot nematode group. The next step was to determine whether the nematode belongs to the *M. ethiopica* group (i.e., a group of three species: *M. ethiopica*, *M. luci* and *M. inornata* Lordello, 1956) by PCR reaction. The reaction with primers Me309F and Me549R resulted in a 241 bp long amplicon specific to the *M. ethiopica* group. Finally, *M. luci* was identified by the PCR reaction. The reaction with the Mlf and Mlr primers resulted in a band of approximately 770 bp.

Table 3. Primers used for *Meloidogyne* spp. identification.

Primer Name	Primer Sequences (5′-3′)	Target Species/Group of Species	Expected Fragment Length (bp)
194	TTAACTTGCCAGATCGGACG	*Meloidogyne* spp.	700—*M. hapla* 720—*M. arenaria M. incognita*, *M. javanica* 780—*M. mayaguensis* 1600–1700—*M. chitwoodi* and *M. fallax*
195	TCTAATGAGCCGTACGC		
C2F3	GGTCAATGTTCAGAAATTTGTGG	tropical root-knot nematode group	621
Mt575	AGAACTTAAACTCTAAATAAC		
Me309	CTAATTTGGGTGAATTT	*M. ethiopica* group	241
Me549R	AATCAAAATCTTCTCCT		

Mlf	ACTCCTGCGACCTCATGGCATTTA	*M. luci*	770
Mlr	ACTCCTGCGAACACAACATTTACT		
Far	TCGGCGATAGAGGTAAATGAC	*M. arenaria*	420
Rar	TCGGCGATAGACACTACAAACT		
Fjav	GGTGCGCGATTGAACTGACG	*M. javanica*	720
Rjav	CAGGCCCTTCAGTGGAACTATAC		
MI-F	GTGAGGATTCAGCTCCCCCAG	*M. incognita*	999
MI-R	ACGAGGAACATACTTCTCCGTCC		
JMV1	GGATGGCGTGCTTTCAAC	*M. hapla;*	540
JMV2	TTTCCCCTTATGATGTTTACCC	*M. chitwoodi;*	670
JMV hapla	AAAAATCCCCTCGAAAAATCCACC	*M. fallax* and	440
JMV tropical	GCKGGTAATTAAGCTGTCA	*M. incognita*	615

2.5. Culturing of Meloidogyne Populations

Infected plant material with soil from three unidentified samples collected in 2021 was used for a bioassay with tomato seedlings in 2022. Portions of the plant material with soil were transferred to one-litre pots in which a susceptible tomato variety *S. lycopersicum* L. "Marathon" (Superior Company, Serbia) was planted as a host plant. The plants were grown in a greenhouse over a period of 110 days and watered as necessary. Subsequently, the roots of the plant were analysed for the presence of galls. The roots were dissected with a stereomicroscope and females and egg masses were extracted and stored at −20 °C for detailed molecular analysis to identify the RKN species. Morphological analysis was performed on fresh material.

2.6. Statistical and Meloidogyne spp. Community Analyses

To evaluate the differences in the frequency and abundance of *Meloidogyne* across regions and districts of Serbia, as well as the differences in the frequency of individual species of *Meloidogyne*, multiple proportion tests were performed using SPSS statistical software SPSS Statistics 30 (https://www.ibm.com/spss)/ https://www.ibm.com/products/spss-statistics (accessed on 8 January 2025). For one of the tests, only samples identified at the species level were included. Hypothesis tests were performed with a significance level of $\alpha = 0.05$. The occurrence and frequency of root-knot nematodes at both the genus and species levels were calculated using the following formulae [37].

Occurrence of the genus or species = Number of samples with RKN infection × 100/Total number of samples analysed

Absolute frequency = Number of samples with species × 100/Number of samples collected

Relative frequency = Frequency of occurrence of the species × 100/Sum of the frequency of all *Meloidogyne* spp.

3. Results

3.1. Meloidogyne Detection

A total of 241 plant material samples were collected from greenhouses and fields in two regions of Serbia and analysed between 2021 and 2023. *Meloidogyne* spp. were detected in 57 samples, which corresponds to 23.7% of the total. Supplementary Material 2 (Table S5) includes data for each positive sample, detailing the host plant, district, gall index, primer pairs and identified RKN species for the period from 2021 to 2023.

3.2. Identification, Distribution and New Host Plant Records for Meloidogyne Species

The identification of *Meloidogyne* species, based on the analysis of perineal pattern morphology in combination with molecular methods, indicated that the most common species among the 57 RKN populations was *M. incognita*, followed by *M. hapla*, *M. luci* and *M. arenaria*. In the first year of the survey, two mixed *Meloidogyne* populations were detected (*M. hapla* and *M. incognita* and *M. hapla* and *M. javanica*). The analysis of perineal pattern morphology included the examination of the vulva/anus area, lateral lines, surrounding cuticle striae, tail terminus and phasmids [38]. Variability in the perineal pattern was observed among the identified species of the tropical RKN group (*M. arenaria*, *M. incognita* and *M. luci*). The perineal shape ranged from rounded to ovoid in *M. arenaria* and *M. incognita,* whereas it was oval to squarish in *M. luci*. Characteristic lateral lines were observed in *M. arenaria*, whereas they were absent or only weakly developed in *M. incognita* and *M. luci* (Figure 2).

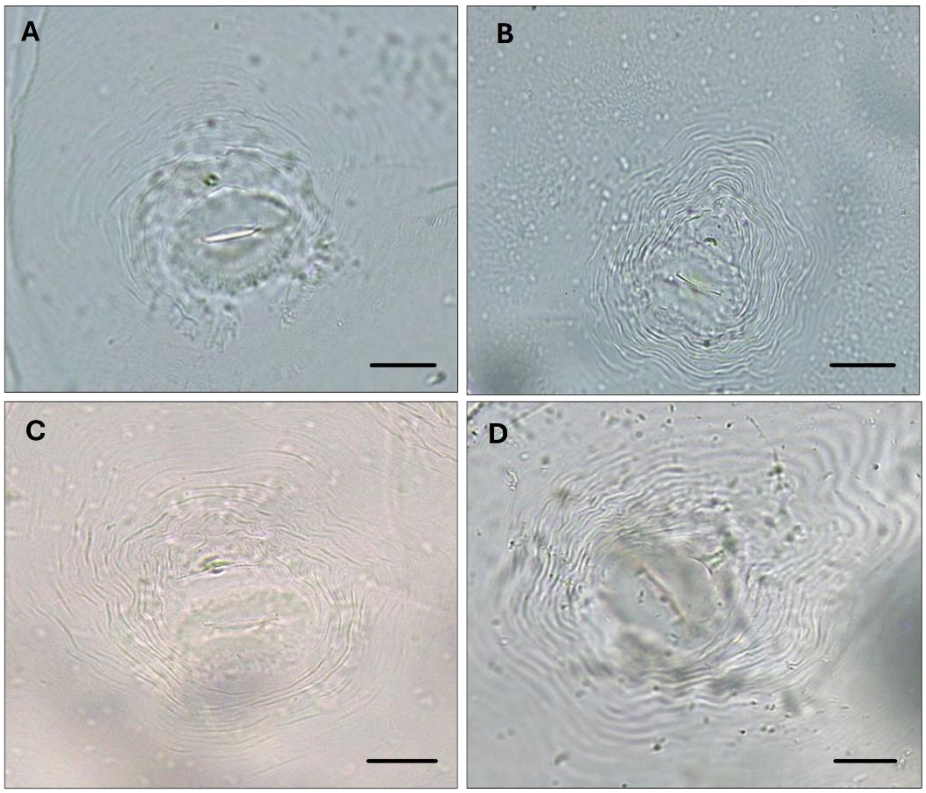

Figure 2. Perineal patterns in *Meloidogyne* specimens: (**A**) *M. hapla* Chitwood, 1949; (**B**) *M. incognita* (Kofoid and White, 1919) Chitwood, 1949; (**C**) *M. arenaria* (Neal, 1889) Chitwood, 1949; (**D**) *M. luci* Carneiro et al., 2014. Bar = 20 μm.

Meloidogyne spp. were confirmed in 57 samples using PCR analysis. The primers Far/Rar produced a DNA fragment size of 420 bp specific to *M. arenaria* in 3 samples, while the primer set MI-F/MI-R yielded a 999 bp fragment specific to *M. incognita* in 39 samples. Multiplex PCR analysis with JMV1/JMV2/JMVhapla primers resulted in a 440 bp DNA fragment size specific to *M. hapla* in eight populations. In addition, *M. luci* was confirmed in five samples using the primer set Mlf/Mlr. In the first year of the survey (2021), three *Meloidogyne* populations could not be identified. In 2022, these populations were identified as *M. luci* following a bioassay conducted in a greenhouse, combined with additional morphological and molecular analyses. The first population of *M. luci* was collected in 2021 in a greenhouse in Northern Serbia (West Bačka district) on tomatoes, marking the first report of this species in Serbia. The second population, also identified as *M. luci*, was found on cucumbers at the

same location. The third *M. luci* population was collected on cucumbers located in the Jablanica district. Morphological analysis of the perineal patterns initially suggested *M. incognita* but was later presumed to be *M. luci* based on a comparison with the originally described morphology of *M. luci*. The affiliation of the population to the tropical RKN group and the *M. ethiopica* group was determined using two PCR reactions. It was confirmed as *M. luci* through the detection of a species-specific 770 bp band and mtDNA sequence analyses. The sequence matched 100% with an unidentified *Meloidogyne* spp. from Serbia and 99.94% with sequences of *M. luci* populations from Slovenia, Greece and Iran. In the phylogenetic tree, all *M. luci* sequences, including the *Meloidogyne* sp. sequence (accession no. KM077449) from Serbia, are grouped into a single clade.

Meloidogyne spp. were detected in 11 out of 25 districts sampled in Serbia during the period between 2021 and 2023, with 5 districts located in Northern Serbia (Vojvodina Province) and 6 in Central Serbia. *M. incognita* was found in all districts where *Meloidogyne* spp. were detected: five in Northern Serbia (North Banat, South Banat, West Banat, South Banat, Srem) and six in Central Serbia (Mačva, Braničevo, Raška, Nišava, Jablanica, Pčinja). *M. hapla* was detected in six districts (North Banat, South Banat, South Bačka, Srem, Raška, Jablanica), *M. arenaria* in three districts (North Banat, South Banat, Braničevo) and *M. luci* in two districts (West Bačka, Jablanica). Two mixed *Meloidogyne* populations were detected: *M. hapla* and *M. javanica* in North Banat and Jablanica. Positive detections of *Meloidogyne* spp. were most frequent in Jablanica district and least frequent in Pčinja district. The districts with the highest species diversity were North Banat, Jablanica, West Bačka and South Banat, each with three species, followed by Srem, Braničevo and Raška, each with two species. Only one species was identified in South Bačka, Nišava, Mačva and Pčinja (Figure 3).

Statistical analysis confirmed that the abundance of *Meloidogyne* is not equal in all districts (p-value = 0.026 < 0.05). A more detailed statistical analysis revealed that the abundance of *Meloidogyne* in Pčinja district is significantly different (p-value < 0.05) from the West Bačka and South Bačka districts. Furthermore, Jablanica district significantly differs from the districts of Mačva, Braničevo and Raška. Statistically significant differences (p-value < 0.05) were observed in the abundance of *Meloidogyne* between the Jablanica and Pčinja districts.

In this survey, some host plants were analysed for the presence of RKN species for the first time in Serbia. *Meloidogyne* species were found parasitising eight different host plants, including three in fields and five in greenhouses. In the fields, *M. incognita* was detected on potatoes, while *M. arenaria* was found on carrots and parsley in two districts with sandy soils (Northern Banat and Braničevo). In greenhouses, *Meloidogyne* spp. were most frequently found on peppers, followed by cucumbers, tomatoes, calla and melons. The species with the highest number of host plants identified during this study was *M. incognita* (five), followed by *M. hapla* (four), *M. luci* (two) and *M. arenaria* (two). To our knowledge, this is the first report of *Meloidogyne* spp. infestation of parsley in fields and infestation of melons in greenhouses in the country (Table 4). However, *Meloidogyne* species were not detected on roses, black nightshade, cabbage, horseradish or gladiolus in the fields.

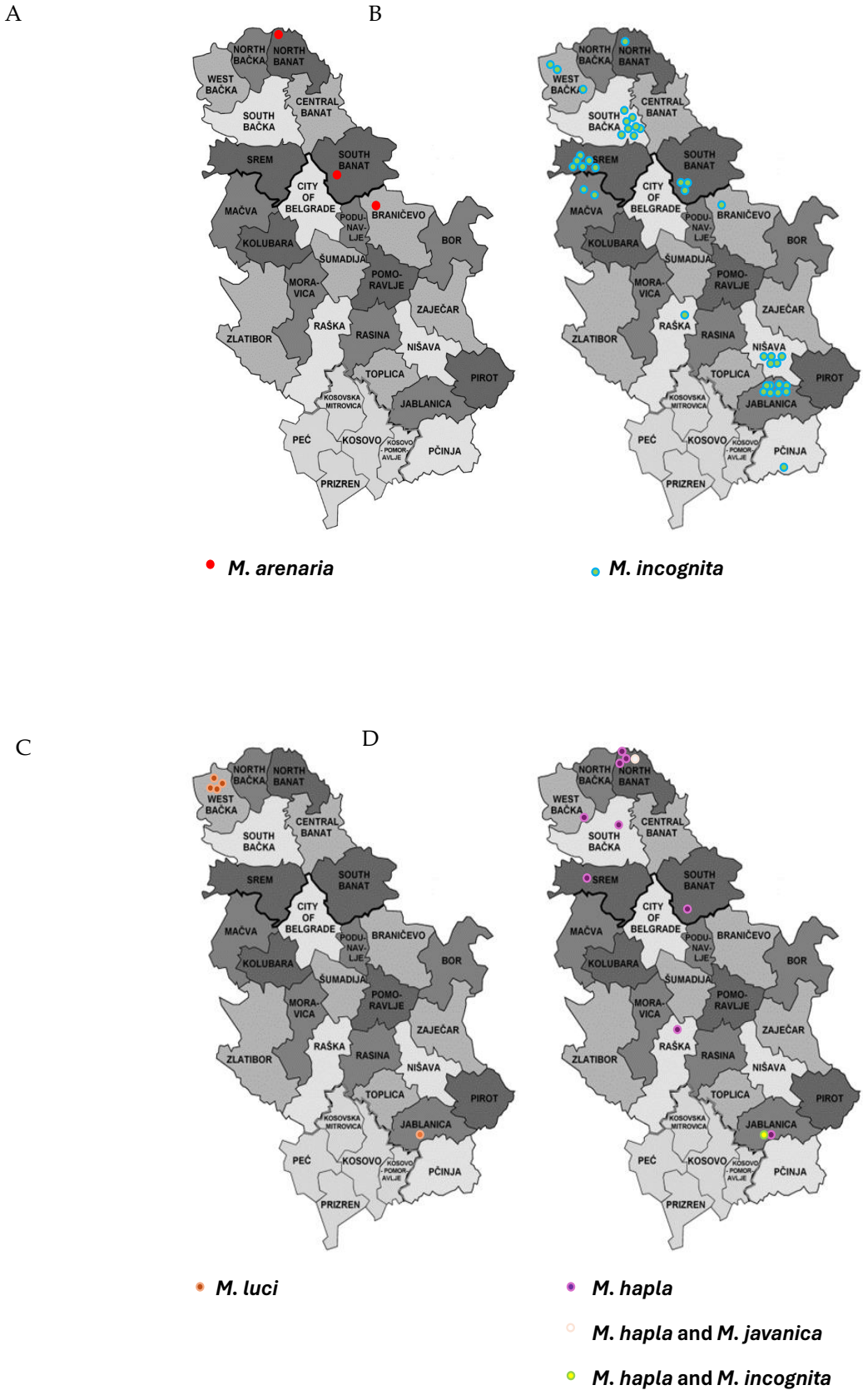

Figure 3. Distribution of *Meloidogyne* sp. in Serbia. (**A**) *M. arenaria*, (**B**) *M. incognita*, (**C**) *M. luci*, (**D**) *M. hapla* and mixed populations. Each dot on the map represents a single population.

The map is from https://en.wikipedia.org/wiki/Administrative_districts_of_Serbia (accessed on 8 January 2025).

Table 4. Districts and host plants associated with *Meloidogyne* species identified in Serbia in the period 2021–2023.

Species	Region	District	Host Plant	Cultivation Conditions
M. arenaria	Northern Serbia	South Banat	Calla (*C. palustris*)	Greenhouse
		North Banat	Carrot (*D. carota* subsp. *sativus*)	Field
	Central Serbia	Braničevo		
M. incognita	Northern Serbia	North Banat	Potato (*S. tuberosum*)	Field
		South Banat	Calla (*C. palustris*)	Greenhouse
		West Bačka		
		South Bačka		
		Srem		
	Central Serbia	Mačva	Pepper (*C. annuum*) Tomato (*S. lycopersicum*) Cucumber (*C. sativus*)	
		Braničevo		
		Raška		
		Nišava		
		Jablanica		
		Pčinja		
M. luci	Northern Serbia	West Bačka	Tomato (*S. lycopersicum*) Cucumber (*C. sativus*)	Greenhouse
	Central Serbia	Jablanica		
M. hapla	Northern Serbia	North Banat	Carrot (*D. carota* subsp. *sativus*) Parsley (*P. crispum*)	Field
		South Banat	Pepper (*C. annuum*) Tomato (*S. lycopersicum*) Melon (*C. melo*)	Greenhouse
		South Bačka		
		Srem		
	Central Serbia	Raška		
		Jablanica		
M. hapla and *M. javanica*	Northern Serbia	North Banat	Carrot (*D. carota* subsp. *sativus*)	Field
M. hapla and *M. incognita*	Central Serbia	Jablanica	Tomato (*S. lycopersicum*)	Greenhouse

3.3. Meloidogyne spp. Community Analyses

Of the 57 samples infested with *Meloidogyne* spp. from the survey areas (Northern Serbia and Central Serbia), 3 samples were infested with *M. arenaria*, 39 with *M. incognita*, 5 with *M. luci*, 8 with *M. hapla* and 2 with *Meloidogyne* mixed populations (one with *M. hapla* and *M. javanica* and one with *M. hapla* and *M. incognita*) (Table 5). *M. incognita* was the most abundant species in both regions infested by *Meloidogyne* spp. The occurrence of *M. incognita* was 16.2%, followed by *M. hapla* (3.3%), *M. luci* (2.1%), *M. arenaria* (1.4%) and the two mixed populations *(M. hapla* and *M. javanica* and *M. hapla* and *M. incognita*), each with the same percentage (0.4%). *M. incognita* was the most prevalent species in both survey regions, with absolute and relative frequencies of 68.4% and 70.9%, respectively. This was followed by *M. hapla* (14.0% and 14.5%), *M. luci* (8.8% and 9.1%) and *M. arenaria* (5.3% and 5.5%), as observed in the absolute and relative frequency values, respectively. The occurrence of *Meloidogyne* spp. was higher in Northern Serbia (28.1%) compared to Central Serbia (19.2%). Additionally, the percentage incidence of *M. arenaria, M. incognita*, *M.*

luci and *M. hapla* was higher in Northern Serbia than in Central Serbia. However, the observed differences were not substantial. Comparing the absolute and relative frequencies of all identified species in this survey, only the values for *M. incognita* were higher in Central Serbia (73.9% and 81.0%) compared to Northern Serbia (64.7% and 64.7%).

Table 5. Community analyses of *Meloidogyne* spp. in surveyed areas of Serbia.

	Total Survey Area	**Northern Serbia**	**Central Serbia**
Detection and identification (no. of samples)	241	121	120
Meloidogyne spp. detected	57	34	23
Meloidogyne spp. not detected	184	87	97
M. arenaria	3	2	1
M. incognita	39	22	17
M. luci	5	4	1
M. hapla	8	6	2
M. hapla and *M. javanica*	1	1	0
M. hapla and *M. incognita*	1	0	1
Occurrence (%)			
Meloidogyne spp. detected	23.7%	28.1%	19.2%
Meloidogyne spp. not detected	76.3%	71.9%	80.8%
M. arenaria	1.4%	1.7%	0.8%
M. incognita	16.2%	18.2%	14.1%
M. luci	2.1%	1.7%	0.8%
M. hapla	3.3%	5.0%	1.7%
M. hapla and *M. javanica*	0.4%	0.8%	0.8%
M. hapla and *M. incognita*	0.4%	0.8%	0.8%
Absolute frequency (%)			
M. arenaria	5.3%	5.9%	4.3%
M. incognita	68.4%	64.7%	73.9%
M. luci	8.8%	11.8%	4.3%
M. hapla	14.0%	17.6%	8.7%
Relative frequency (%)			
M. arenaria	5.5%	5.9%	4.8%
M. incognita	70.9%	64.7%	81.0%
M. luci	9.1%	11.8%	4.8%
M. hapla	14.5%	17.6%	9.5%

Statistical analysis of the different *Meloidogyne* species showed that the presence of *M. incognita* varied significantly (p-value = 0.000 < 0.05) compared to the rest of the species (*M. arenaria*, *M. luci* and *M. hapla*), indicating its dominance in Serbia. In contrast, the less frequent species *M. arenaria* did not differ significantly from *M. luci* or *M. hapla* (p-value > 0.05).

4. Discussion

This study represents a detailed and continuous survey of the occurrence of RKNs in Serbia, conducted as part of the official survey of quarantine RKN species from 2021 to 2023. The survey covered 25 districts across two regions of Serbia (Northern and Central Serbia). The results revealed that out of a total of 241 samples of vegetable and ornamental plant materials collected from greenhouses and fields, 57 samples were infested with *Meloidogyne* spp. Five RKN species (*M. arenaria*, *M. incognita*, *M. luci*, *M. javanica* and *M. hapla*) were identified in this survey out of seven species previously detected in Serbia. To

date, 25 species of root-knot nematodes have been recorded in Europe [39,40]. All species identified in this study have already been recorded in other countries in the region as well as in the countries of the former Yugoslavia. Our findings confirm previous reports on the occurrence of *M. arenaria*, *M. incognita* and *M. hapla* in six districts (North Banat, South Banat, Braničevo, Nišava, Jablanica and Pčinja) and their incidence in countries with a similar continental climate characterised by cold winters and hot summers, such as Bulgaria [41], Romania [42] and Hungary [43].

During this survey, the EPPO A2 quarantine species *M. luci* was detected for the first time in Serbia, in two districts (West Bačka and Jablanica), on tomato and cucumber. This species, belonging to the tropical RKN group, was first identified in Europe in 2003, in Slovenia, on tomato roots in a greenhouse in Dornberk [44], initially classified as *M. ethiopica*. Subsequently, *M. ethiopica* was later recorded in other countries within the EPPO region, including Greece [45], Italy [46] and Turkey [47]. In 2014, *M. luci was* described as a new species by Carneiro et al. [48], revealing its close relationship with *M. ethiopica*. The description of this sibling species prompted the re-evaluation of populations previously identified as *M. ethiopica* [49]. Research demonstrated that all examined populations of *M. ethiopica* from Slovenia, Italy, Greece and Turkey were, in fact, *M. luci* [50]. In addition, this species was later detected in Portugal, both on the mainland and in Azores, parasitising potato crops [51,52]. A comparison of the mtDNA sequence of *M. luci* obtained in this study with publicly available sequences revealed an identical match with a *Meloidogyne* population from Serbia that could not be identified at the species level in 2014. This suggests that *M. luci* may have been present in Serbia for at least a decade but remained unidentified due to limited biological material and the unavailability of species-specific PCR for *M. luci* at that time. It is also possible that *M. luci* is more widespread than currently recognised, as its detection is not part of regular phytosanitary surveys in many countries.

Species from the tropical RKN group cause significantly greater damage and yield losses in tropical than in temperate regions due to favourable environmental conditions. However, climate change seems to be influencing field distribution and the impact of these species in temperate regions of south-eastern Europe, including Serbia. Findings from a recent Euphresco MeloTrop project conducted between 2017 and 2020 on global warming and the distribution of tropical RKN species revealed 107 localities of RKN in France, Portugal, Serbia and Slovenia. The study showed that *M. incognita* was the most frequent species, followed by *M. arenaria*, *M. javanica*, *M. hispanica*, *M. luci* and *M. enterolobii*. [53]. In a separate RKN survey conducted in Portugal between 2017 and 2022, *M. incognita* and *M. javanica* were the most prevalent species, followed by *M. arenaria* and *M. hapla*. Rare species such as *M. enterolobii*, *M. hispanica*, *M. luci* and *M. naasi* were also detected. Climate change, with rising air temperatures and increased moisture, may lead to higher rates of RKN infection, development and reproduction, ultimately altering their geographic distribution and abundance [54]. Our results showed the presence of species from the tropical RKN group, including *M. incognita*, *M. arenaria* and one mixed population of *M. hapla* and *M. javanica*. The significant damage caused by these species in the field seems to be related to the sandy soil prevalent in the North Banat district. While these species are generally native to tropical and subtropical regions, they can also survive in fields with Mediterranean and continental European climates. Notably, their infectivity is maintained even after exposure to sub-freezing temperatures during winter, as observed with *M. luci* in Slovenia [55]. Global warming has resulted in the migration of tropical RKN species into new field environments and the emergence of previously undocumented RKN species in Europe. *Meloidogyne* infestations disrupt water and nutrient intake from the soil, making crops more vulnerable to increased temperatures and drought. Additionally, the increased occurrence of heat waves in southern Europe threatens the effectiveness of widely used host plant resistances, including the *Mi-1* gene, which is the most

important resistance gene against RKN in tomatoes. The prolonged use of a limited number of resistant plant varieties has facilitated the emergence of virulent RKN populations in fields across southern Europe. Current cultivars are insufficient to prevent the further spread of RKN, emphasising the urgent need for efficient and sustainable solutions to mitigate RKN-related crop losses. Addressing this issue will be a key focus of the NEMEMERGE project, funded by the European Union [56]. Our findings confirmed the presence of tropical RKN species in the field and the occurrence of a newly identified species (*M. luci*) during this survey.

Meloidogyne spp. are among the most widespread nematodes and pose a threat to the production of a wide range of crops worldwide. These pathogens can be transmitted through infected plants, soil, running water and agricultural machinery. Moreover, controlling and eradicating RKN, particularly mixed species populations, is extremely challenging. Consequently, the primary aim is to minimise crop damage by keeping nematode densities as low as possible. Efficient detection and accurate identification are the first crucial steps in RKN management, but they present their own set of challenges. Morphological identification based on the characteristics of females and juveniles is unreliable due to interspecies variability and overlapping traits among different species. For instance, several RKN species could be, and likely have been, misidentified as *M. incognita* based on *M. incognita*-type perineal patterns, which are present in several RKN species. Biochemical methods, such as isozyme pattern analysis, offer a reliable alternative; however, they require bioassays to obtain females at a specific developmental stage, which can be time-consuming [57]. For this reason, PCR analysis is a more practical method for routine and rapid diagnosis of RKN species. Historically, analysis of perineal morphology was the only method applied to identify RKNs in Serbia. To our knowledge, this survey was the first application of the PCR method for RKN species identification in Serbia. Using species-specific primers, we identified *M. incognita*, *M. arenaria* and *M. hapla* as well as *M. luci* through DNA amplification from egg masses or females. Additionally, the identity of *M. luci* was confirmed through sequencing.

Various control strategies can be employed against RKNs, such as chemical nematicides, resistant cultivars and cultural practises. Among chemical nematicides, carbamates and organophosphates have been the most commonly used. However, some active ingredients have been banned or regulated due to their harmful effects on human health and the environment. These substances have been partially replaced by newer chemicals, such as fluopyram and biological nematicides based on fungal and bacterial agents [58]. Host resistance has proven effective against RKNs, but resistant cultivars are not available for all crops and markets. Furthermore, certain RKN species are not controlled by *Mi* gene-based resistance, and in some cases, populations of RKN species that were initially controlled by *Mi* gene-based resistance have evolved virulent populations capable of overcoming this resistance [59].

Current control measures applied in Serbia against RKNs focus on agrotechnical measures in combination with the use of pesticides containing the active ingredient fluopyram, such as Velum® Prime, Bayer (registered for use from 2 August 2016 to 31 January 2025). This approach has proven effective in reducing *Meloidogyne* populations, as demonstrated in Slovenia [60]. In cases of infestation by quarantine RKN species, strict control measures must be implemented upon nematode detection. These measures include restrictions on plant material movement and interruption of susceptible host varieties until nematode densities fall below the detection threshold. These measures could also be helpful in the Western Bačka and Jablanica districts, where *M. luci* was detected, to prevent its further spread to other regions. The national surveillance programme for quarantine RKNs in Serbia continued in 2024 and will extend into 2025. The findings of this study on the occurrence and distribution of RKNs will assist decision-making bodies,

phytosanitary inspectors, farmers, scientists, the breeding sector and plant protection companies in implementing effective plant protection, quarantine and monitoring measures to prevent the further spread of these pests.

5. Conclusions

This study shows that *Meloidogyne* species pose a significant threat to agricultural production and confirms their high occurrence and widespread distribution in Serbia. *Meloidogyne* infestations were identified on eight different plant hosts, and to our knowledge, this is the first report of *Meloidogyne* parasitising parsley in the fields and melons in the greenhouses of Serbia. This survey showed that *M. incognita* is currently the predominant RKN species in the country. Additionally, *M. luci*, an EPPO A2 quarantine species, was also discovered locally for the first time, with limited occurrences. The detection of new *Meloidogyne* species in various districts and new plant host records indicate that these infestations are likely linked to trade activity. Climate change and rising temperatures could significantly increase the spread and damage caused by tropical RKN species to various agricultural crops in temperate regions of south-eastern Europe, including Serbia, in future. This study implemented routine and rapid identification of *Meloidogyne* species, which is essential for developing and applying appropriate control strategies in Serbia. The findings presented here relative to the *Meloidogyne* species identified locally will assist in the development and establishment of efficient and sustainable measures and surveys aiming to prevent the introduction and spread of these pests across Europe.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1: Supplementary Material 1. Supplementary Material Table S1: Amplification conditions with different primer pairs; Supplementary Material Table S2: Amplification conditions for identification of *Meloidogyne luci* with primers Mlf/Mlr; Supplementary Material Table S3: Amplification conditions for identification of *Meloidogyne ethiopica* group with primers Me309/Me549R; Supplementary Material Table S4: Amplification conditions for identification of tropical root-knot nematodes group with primers C2F3/Mt575. Supplementary Material 2. Supplementary Material Table S5: Sample data and PCR results for samples where *Meloidogyne* spp. was detected.

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