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Enzymatic and Molecular Identification of *Meloidogyne* Species in Tomato Orchards in Paraguay

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Abstract: Tomato is a major crop in Paraguay, where it provides a source of employment and income for households. Tomato production can be affected by root-knot nematodes, especially *Meloidogyne* spp. The unequivocal identification of *Meloidogyne* spp. in Paraguay has not been conducted yet. This study aims to identify *Meloidogyne* species in eight tomato production districts of this country by biochemical and molecular techniques. Females of *Meloidogyne* spp. were extracted from tomato roots and characterized using esterase isozyme phenotypes. In addition, DNA was extracted from nematode eggs, and species-specific SCARs (sequence-characterized amplified regions) were used to confirm the diagnosis. Nematodes were detected in 100% of studied samples (prevalence), of which *M. incognita* (Est: I2, Rm: 1.1;1.2) and *M. javanica* (Est: J3, Rm: 1.0, 1.20, 1.35) were present in 39.13% and 26.08% of samples, respectively. One population (8.69%) of *Meloidogyne* sp. presenting an atypical esterase profile (Rm: 1.0 and 1.3) was only detected in Julián Augusto Saldívar District. Mixed populations, mostly *M. incognita* and *M. javanica*, were observed in 26.08% of samples. The SCAR primers incK14F/incK14R amplified specific fragments for *M. incognita* (399 bp) and *M. javanica* (670 bp), confirming the enzymatic results. Here, we present the first study of root-knot nematode identification at the species level in Paraguay.

Keywords: esterase phenotype; *Meloidogyne incognita*; *Meloidogyne javanica*; root-knot nematode; SCAR markers

Citation: Resquín-Romero, G.; Mattos, V.S.; Monteiro, J.M.S.; Lopez-Nicora, H.D.; Amarilla, S.P.; Chamorro-Diaz, S.; Moral, J.; Carneiro, R.M.D.G. Enzymatic and Molecular Identification of *Meloidogyne* Species in Tomato Orchards in Paraguay. *Agronomy* **2023**, *13*, 670. https://doi.org/10.3390/agronomy13030670

Academic Editor: Salvatore Davino

Received: 6 October 2022
Revised: 25 January 2023
Accepted: 17 February 2023
Published: 25 February 2023

1. Introduction

Tomato is an important crop in Paraguay and occupies a prominent position in the food security of this country, providing employment and income for households. During 2015 and 2017, Paraguayan orchards produced 55 million kg of tomatoes in 1390 ha [1]. In this country, tomato is grown mainly by smallholder rural families, who participate in the production and commercialization of this crop. Unfortunately, many abiotic and biotic factors can adversely impact tomato production, including plant-parasitic nematodes. Root-knot nematodes (RKN) are among the most economically significant tomato pests; remarkably, the genus *Meloidogyne* is distributed worldwide [2–5].

RKN are sedentary parasites that attack the tomato root vascular system, leading to host nutrient deprivation and impaired water transport, causing aboveground symptoms of stunting, wilting, chlorosis, and reduced crop yields. Integrated pest management practices remain an effective strategy to maintain RKN population densities below damage thresholds. However, smallholder tomato growers must first recognize the presence

of such a threat in their tomato production. Moreover, a correct diagnosis of the RKN species present in the orchard is essential for adequate pathogen management, including cultivar selection [4,6]. Because over 100 *Meloidogyne* species have been reported [7], their identification at the species level remains challenging for many researchers [6]. There are specific preventive strategies that can be included in a nematode management plan, such as the use of resistant plants to control different *Meloidogyne* species [8]. Both enzymatic and molecular techniques have been commonly used to improve the accuracy of RKN species diagnosis.

Esterase phenotyping has been used to identify *Meloidogyne* species and has been proven to be species-specific in many cases [9,10]. In addition, specific sequence characterized amplified region (SCAR) markers have been successfully developed to diagnose the dominant tropical root-knot nematodes associated with important crops such as tomato, coffee, guava, and grapevine; these nematodes include *M. javanica* [11], *M. arenaria* [11], *M. incognita* [12], *M. paranaensis*, *M. exigua* [12], *M. enterolobii* [13], *M. arabicida*, *M. izalcoensis* [14], and *M. ethiopica* [15].

Several plant-parasitic nematode species have been reported in Paraguay [16–18]. However, these latter studies were conducted based only on the perineal pattern morphology of the nematodes: they allowed the identification of the species *M. incognita* and *M. javanica* affecting lettuce (*Lactuca sativa*) in the Central Department [19] and peanuts (*Arachis hypogaea*) in Conolinias Mennonitas, Chaco, Paraguay [17], respectively. However, relying only on morphological characteristics to identify root-knot nematode species can lead to misdiagnoses. The available literature on *Meloidogyne* species in Paraguay needs to be updated and clarified. The objective of this study was to identify Meloidogyne species from small-scale orchards in seven tomato-producing departments in Paraguay using enzymatic and molecular methods.

2. Materials and Methods

2.1. Sampling Collection and Nematode Extraction

From 2015 to 2017, eight tomato orchards were surveyed, and various plant roots with knot samples were collected. The studied orchards are distributed in seven departments of Paraguay. One or two districts were arbitrarily selected within each department, and within those districts, two or three orchards were chosen for sample collection. Samples were collected from the district of Julian Augusto Saldivar, Central Department (JAS); San Pedro de Ycuamandiyu, San Pedro (SPY); Tobati, Cordillera (TC); Yaguaron, Paraguari (YP); San Juan Bautista, Misiones (SJB); San Ignacio, Misiones (SIM); San Cosme y Damian, Itapua (SCD); and Coronel Oviedo, Caaguazu (CO) (Figure 1).

The studied orchards had been planted with tomatoes for at least five consecutive seasons. Three tomato plants exhibiting aboveground symptoms of nematode damage from each orchard were randomly selected and uprooted. If root galls were present, suggesting root-knot nematode infection, roots were collected, placed in labeled plastic sample bags, and transported to the laboratory in insulated containers. Upon arrival at the laboratory, samples were kept at 4 °C until processed.

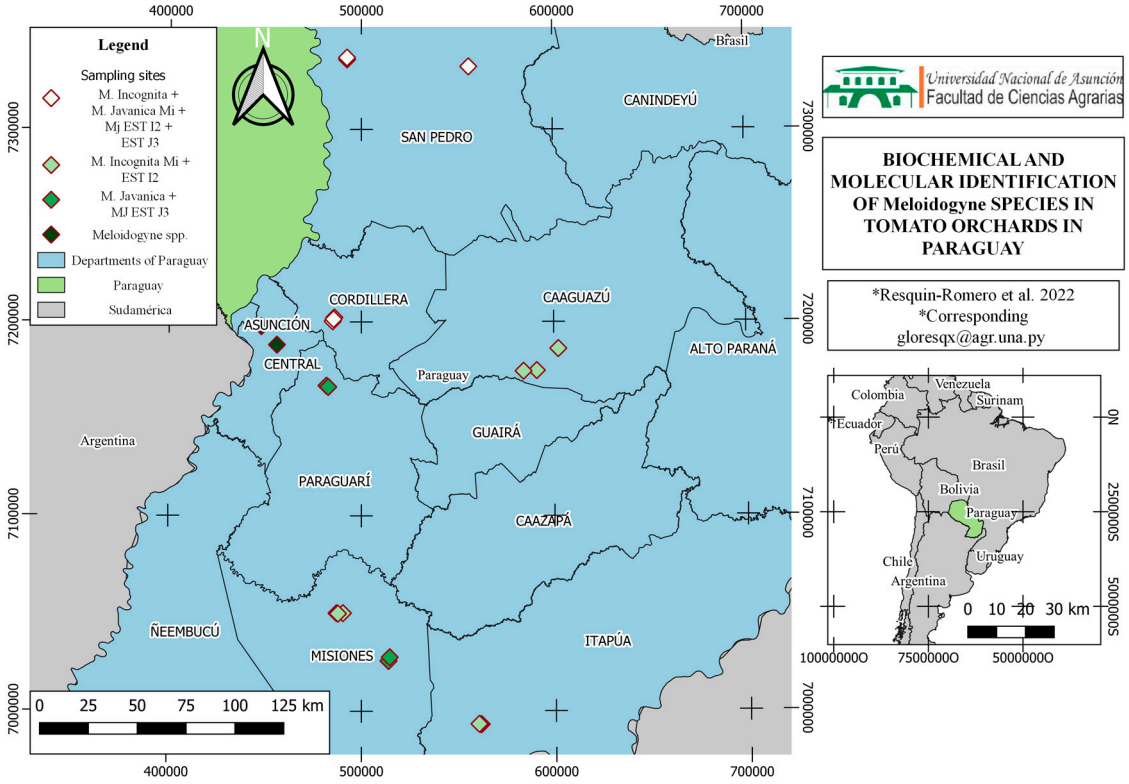


Figure 1. Illustrative map of location and collection of samples to identify species of Meloidogyne.

2.2. Morphological, Biochemical, and Molecular Characterization

Samples were processed in the laboratory of "Área de Protección Vegetal" (Facultad de Ciencias Agrarias, Universidad Nacional de Asunción) in Paraguay and the Laboratory of Nematology (EMBRAPA-CENARGEN, Brasília, DF) in Brazil. First, female nematodes were carefully excised from the plant tissue; perineal patterns were cut according to Hartman and Sasser (1985) [20] and cleaned with lactic acid. Finally, the perineal patterns were mounted in glycerin on glass slides, viewed, and photographed with a bright field light microscope equipped with AxioCam ICc1 digital camera and ZEN imaging software (Carl Zeiss, Germany).

2.3. Identification of Meloidogyne spp. by Esterase Phenotype

Additionally, single young female nematodes were extracted from tomato roots and identified by esterase phenotype according to the method described by Carneiro and Almeida (2001) [9]. Briefly, females were placed in glass microtubes containing 5 µL of extraction solution Sucrose/Triton X-100 (20 g saccharose and glycerol, 1cc Triton X-100, and 100 mL distilled water) and macerated with the use of a syringe Hamilton Syringe (volume 25 µL, needle size 22s ga (blunt tip), needle L 51 mm [2 in]). Electrophoresis was conducted in 7% polyacrylamide gels run in a horizontal CL18 Permatron gel tank. Isoenzymes were electrophoresed for 2 h at 4 °C and 80 volts. The 10 exemplars of *Meloidogyne javanica* (J3; Rm: 1.0, 1.3, and 1.4) were used as a gels reference.

2.4. DNA Extraction

Nematode genomic DNA was extracted from eggs collected from infected tomato roots using the method described in Carneiro et al. (2004) [21]. Cleaned eggs were concentrated and kept in sterile water suspension. Total genomic DNA was extracted and purified from 200 µL aliquots of egg suspension for each population following the method described by Randig et al. (2002) [12]. Species-specific SCAR primers (Table 1) were used individually or in multiplex polymerase chain reactions (PCR). All reactions were performed in 25 µL

volume, containing 2 µL of genomic DNA (3 ng·µL^{-1}), 1 µL (10 µM) of each primer, 4 µL of each dNTP (1.25 mM of dATP, dTTP, dGTP and dCTP; Invitrogen), 2.5 µL of 1× reaction buffer + $MgCl_2$ (Phoneutria Biotecnologia & Serviços, Belo Horizonte, Brazil), 1-unit Taq DNA polymerase (Phoneutria Biotecnologia & Serviços, Belo Horizonte, Brazil), and 14.25 µL purified water. The PCR reactions were run in a T100TM thermal cycler (Bio-Rad), with thermal conditions as follows: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 62 °C, 1 min at 70 °C, and a final extension step of 8 min at 70 °C. For multiplex reactions, the conditions used were as described by Silva et al. (2013) [22]. A universal pair of primers (18S nuclear rDNA primer (Mel F/R) was used to confirm the quality of the DNA extractions. The amplification products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide (0.3 µg·mL^{-1}), and visualized under UV light. Each sample was processed at least twice.

Table 1. Primers used in the reactions PCR-SCAR to identify *Meloidogyne* spp. from Paraguay.

Primer SCAR	Sequence (5′–3′)	Amplification (bp)	Reference	Target Species
inc-K14-F inc-K14-R	GGGATGTGTAAATGCTCCTG CCCGCTACACCCTCAACTTC	399	[12]	*M. incognita*
Fjav Rjav	GGTGCGCGATTGAACTGAGC CAGGCCCTTCAGTGGAACTATAC	670	[11]	*M. javanica*

2.5. Perineal Patterns

The perineal patterns were mounted in glycerine on glass slides, viewed, and photographed with a bright orchard light microscope equipped with an AxioCam ICc1 camera and ZEN imaging V 4.7 software (Zeiss, Oberkochen, Germany).

3. Results

3.1. Identification of Meloidogyne spp. by Esterase Phenotype

Overall, *Meloidogyne* species (RKN) were found (prevalence 100%) in all the samples analyzed from the San Pedro, Central, Paraguari, Misiones, Itapua, and Caaguazu of Paraguay (Table 2).

Table 2. *Meloidogyne* spp. identified by esterase phenotypes (EST) and SCAR markers in Paraguay.

Code	Origin Department	Samples		Identification of *Meloidogyne* spp.			
		District	Total, Samples	Species	Identification SCAR/Esterase (EST.)		Perennial Pattern Morphology
1	San Pedro	SPY	3	*M. incognita* + *M. javanica*	Mi + Mj	EST I2 + EST J3	*M. incognita* + *M. javanica*
2	Cordillera	TC	3	*M. incognita* + *M. javanica*	Mi + Mj	EST I2 + EST J3	*M. incognita* + *M. javanica*
3	Central	JAS	2	*Meloidogyne* sp.	no amplification	Atypical (EST 1.0 and 1.3)	*Meloidogyne* sp.
4	Paraguarí	YP	3	*M. javanica*	Mj	EST J3	*M. javanica*
5	Misiones	SJB	3	*M. incognita*	Mi	EST I2	*M. incognita*
6	Misiones	SIM	3	*M. javanica*	Mj	EST J3	*M. javanica*
7	Itapua	SCD	3	*M. incognita*	Mi	EST I2	*M. incognita*
8	Caaguazu	CO	3	*M. incognita*	Mi	EST I2	*M. incognita*

Species identification through enzymatic characterization by the esterase phenotype (Figure 2) method showed the presence of *M. incognita* (Est: I2, Rm: 1.1; 1.2) in 39.13% of surveyed orchards. Also, the species *M. javanica* (Est: J3, Rm: 1.0, 1.20, 1.35) and mix populations (*M. incognita* and *M. javanica*) were detected in 26.08% of the orchards. Interestingly, a species that presented an atypical esterase profile (Rm: 1.0 and 1.3) was

observed in 8.69% of the samples; these correspond to the Central Department—Julián Augusto Saldívar District (Table 2).

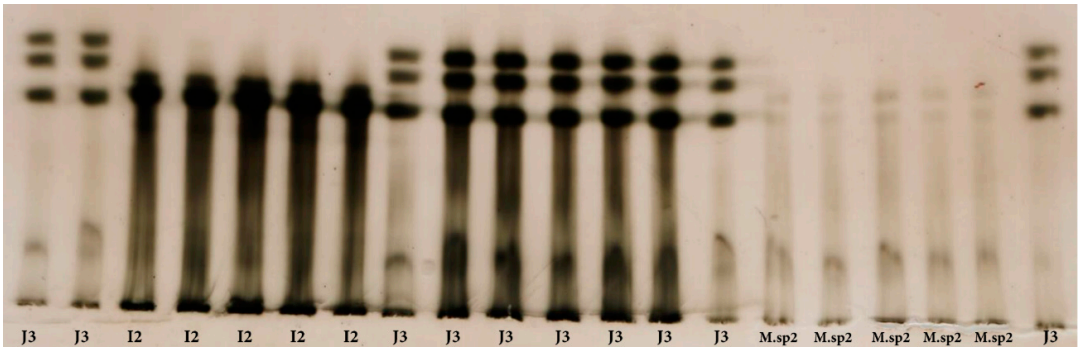


Figure 2. Three esterase phenotypes (EST) found in eight populations of *Meloidogyne* spp. collected in Paraguay. I2 = *Meloidogyne incognita*/J3 = *M. javanica*/M.sp.2 = *M.* sp. *M. javanica* (J3*) was used as reference in the gel.

3.2. Morphological, Biochemical, and Molecular Characterization

In order to confirm the esterase results, species-specific primers designed for *M. incognita* and *M. javanica* were tested in all the populations, including the atypical one (no amplification was detected—data not shown). The SCAR primers incK14F/incK14R [12] and Fjav/Rjav [11] amplified specific fragments for *M. incognita* (399 bp) and *M. javanica* (670 bp) confirming the enzymatic results. Mixed populations of *M. incognita* and *M. javanica* were also detected in a multiplex reaction (Figure 3). Although the perineal patterns were characteristic of *M. javanica* and *M. incognita* in our study, enzymatic and molecular approaches were essential for their identification (10). Unfortunately, population 3 (*Meloidogyne* sp.) could not be successfully reproduced in the greenhouse without further investigations. Here, we obtained RKN females of all samples, and perennial pattern morphology suggested the presence of *M. incognita* and *M. javanica*.

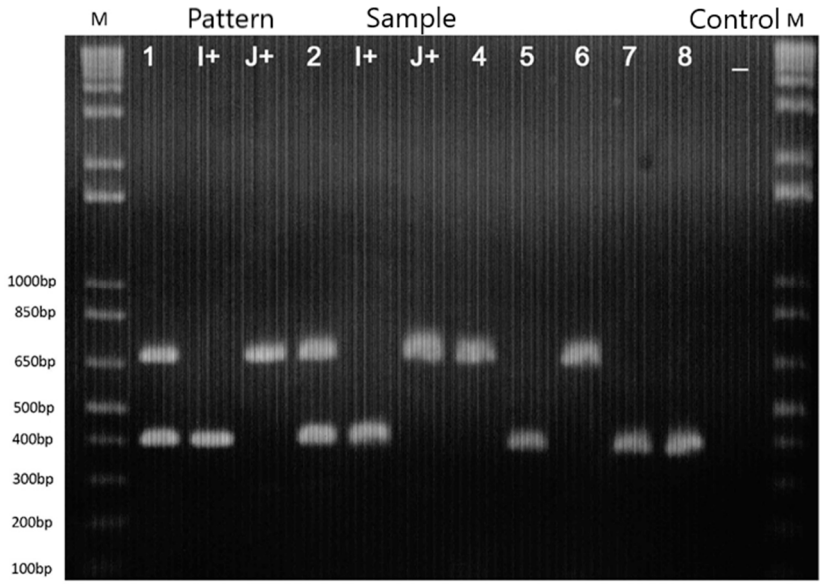
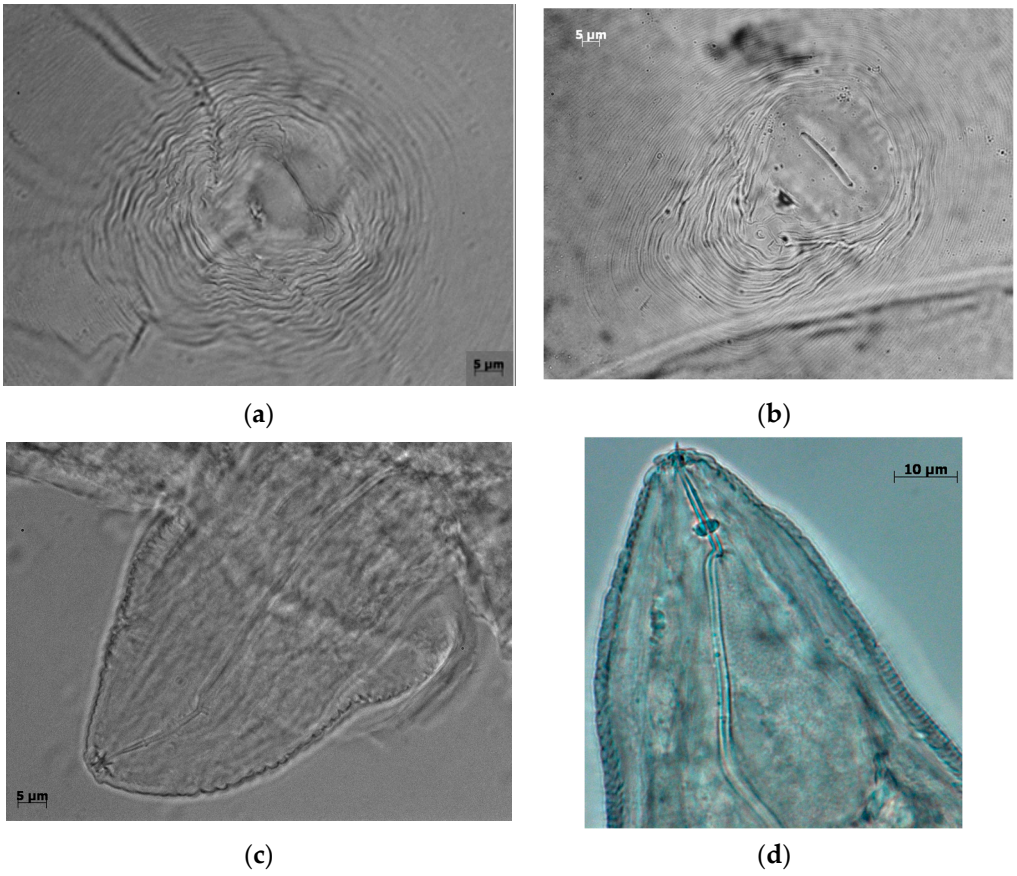


Figure 3. PCR amplification with primers for *M. incognita* (incK14F/incK14R) and *M. javanica* (Fjav/Rjav) for seven populations of *Meloidogyne* spp. collected in Central Paraguay. Samples' codes: 1, 2, 4, 5, 6, 7, and 8 (Table 2). I+ = positive control for *M. incognita*/J+ = positive control for *M. javanica*. M = molecular marker 1kb plus Invitrogen.

3.3. Perineal Patterns

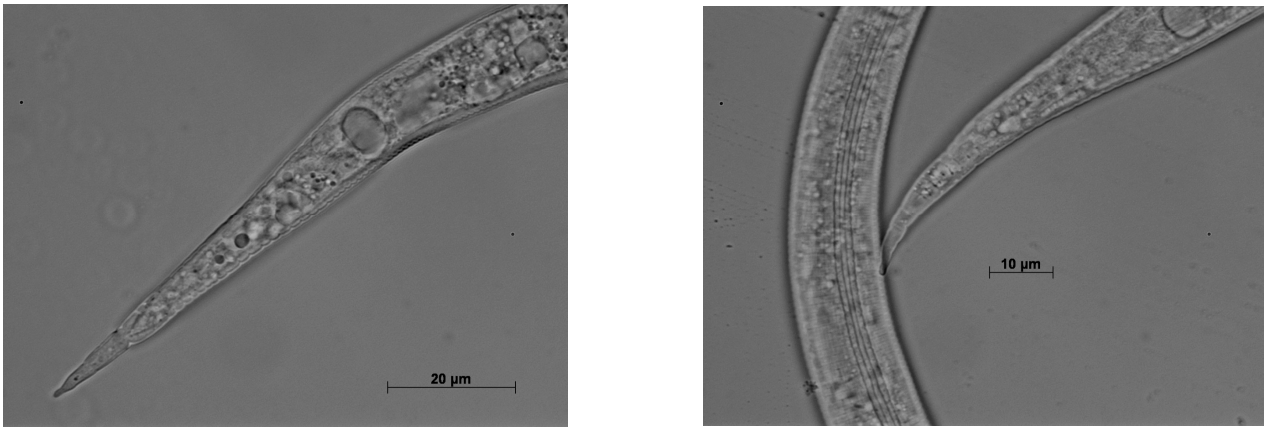
Root-knot nematode females were retrieved from all samples. Perennial pattern morphology suggested the presence of *M. incognita* and/or *M. javanica* (Figures 4 and 5).



(**a**) (**b**)

(**c**) (**d**)

Figure 4. Identification of species *Meloidogyne* based on the perineal pattern's morphology of females (**a**,**c**) *Meloidogyne javanica*, (**b**,**d**) *Meloidogyne incognita*.



(**a**) (**b**)

Figure 5. *Cont*.

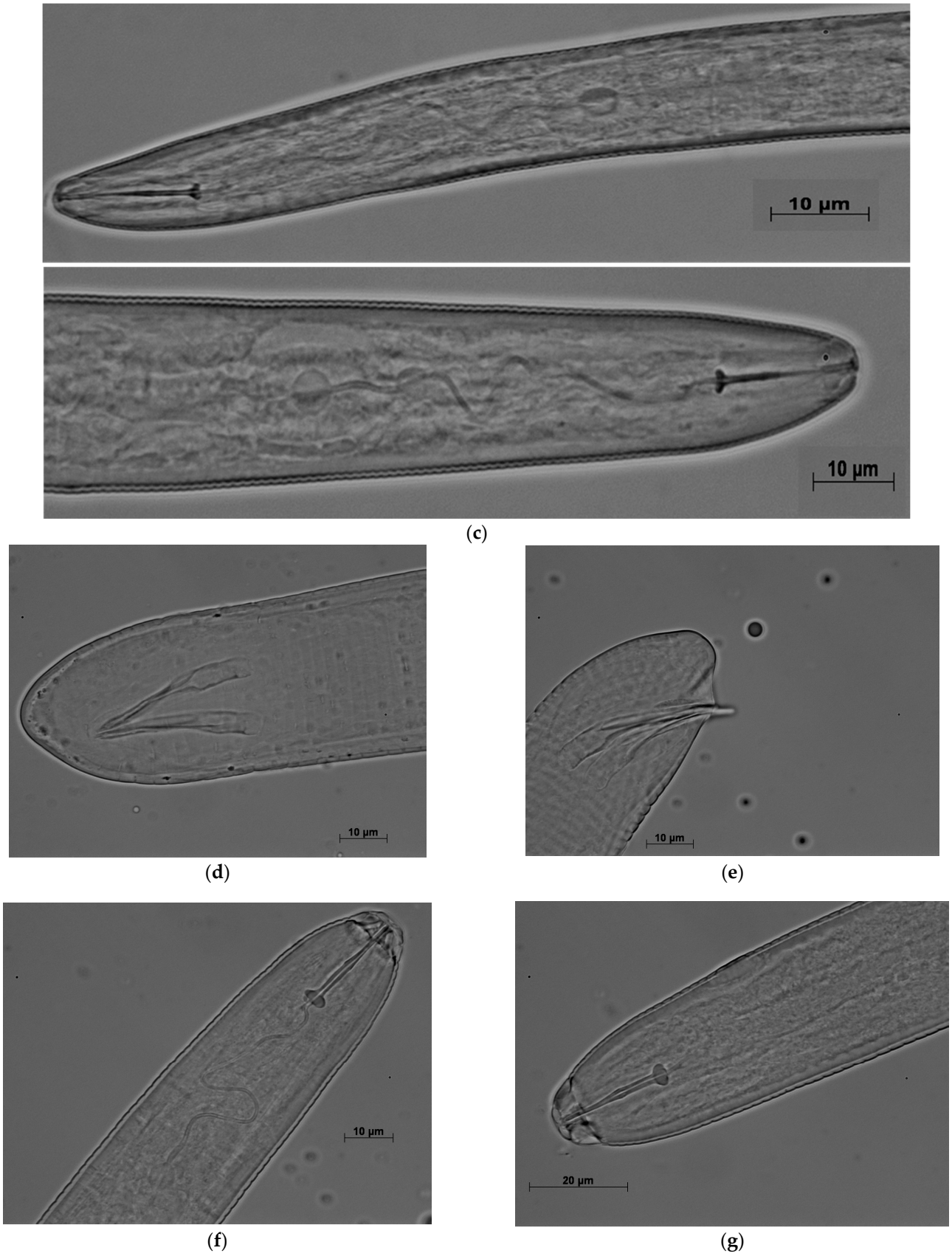


Figure 5. Identification of species *Meloidogyne* based on the morphology of second instar larvae and male adults (**a**,**b**), juvenile of second instar larvae (**c**–**e**) and male adults of *Meloidogyne incognita* (**f**,**g**).

4. Discussion

Yield reduction in small-scale tomato growers due to root-knot nematode (RKN) damage in Paraguay is of growing concern. Although the producers can indirectly recognize nematode presence in the orchards, they need to identify the species, limiting them from implementing adequate management strategies. On the other hand, biochemical and molecular techniques currently improve the accuracy of RKN species identification. Therefore, this study aimed to identify *Meloidogyne* species from small-scale orchards in seven tomato-producing Departments in Paraguay.

Unfortunately, a 100% prevalence of *Meloidogyne* spp. was displayed in all the samples analyzed from the San Pedro, Central, Paraguari, Misiones, Itapua, and Caaguazu of Paraguay. Our results agree with the recent report of Lopez-Nicora et al. (2022) [23], who described the genus *Meloidogyne* spp. as the most abundant nematode in vegetable orchards from Paraguay. Besides, it highlights that the population densities of *Meloidogyne* spp. in Misiones, Alto Paraná, Central, Paraguarí, and Caaguazú probably affect tomato production in Paraguay. Additionally, according to Jones et al. (2013) [24], *Meloidogyne* spp. is one of the three most significant nematodes due to the strong negative impact it causes on the economy. Likewise, the American Phytopathology Society estimated a 14% loss in crop yield, equivalent to approximately 125 billion dollars per year [25,26].

A loss in yield in tomato crops infected with *M. incognita* is estimated between 12% and 41%, with population densities of 1000 to 5000 nematodes/plant [27]. Previous studies in Paraguay on plant parasitic nematodes in tomatoes have focused on controlling them in greenhouses [28]. A recent report aimed to determine the prevalence and abundance of phytoparasitic nematodes in orchards in 37 vegetable orchards in nine Paraguay Departments only described the nematode population at the genus level. These authors highlighted the need for and importance of characterizing the most prevalent plant-parasitic nematodes described in their study to species level [22,28].

It is known that the dominant Mi-1.2 gene in tomato confers resistance to the three most important RKN species *M. incognita*, *M. javanica*, and *M. arenaria*, and minor species—*M. ethiopica*, *M. hispanica*, and *M. luci*, infect various crops in Brazil (8). Hence, the importance of identifying the different species of Meloidogyne.

Despite the importance of the tomato crop and RKN for Paraguayan agriculture, unequivocal information on nematode identification and species distribution in Paraguay is only now becoming available. For example, *M. incognita* was previously reported in lettuce [19]; while *M. javanica* was only described as affecting peanuts [17]. The identification of *Meloidogyne* spp. in these studies has relied upon the characterization of adult female perineal patterns using several morphometric and morphological features of juveniles. However, it is possible to confuse *M. incognita* with other related species (e.g., *M. paranaensis*, *M. izalcoensis*, and *M. inornate*) attending the female perineal pattern [8,10]. Here, we obtained RKN females of all samples [20], and perennial pattern morphology suggested the presence of *M. incognita* and *M. javanica*. *Meloidogyne* is a genus of obligated plant parasites with species distributed worldwide, with the ability to infect almost every vascular plant, both under protected agriculture, in greenhouses or the field. Major *Meloidogyne* species are *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla* [24,29]. Although they have a broad host crop selection, the most economically important crops are soybean, cereals, tomato, potato, and other solanaceous and tubercules [9,28,30].

Mixed populations of *M. incognita* and *M. javanica* were also detected in a multiplex reaction and a population with unknown esterase phenotype (EST 1.0 and 1.3); unfortunately, further investigations could not be carried out. Although several reports coincide with our findings, future research, and studies may complement our results and provide a broader view of nematode diversity in Paraguay [10,25].

The study has displayed that esterase phenotypes make possible the identification of both *Meloidogyne* species and atypical populations. Furthermore, we confirmed the results obtained by esterase characterization using SCAR markers (INCK14 F/R (12); and Fjav/Rjav, [11] designed for *M. incognita* and *M. javanica*, respectively. This study

represents the first identification report of RKNs to species level using enzymatic (esterase phenotypes) and DNA-based molecular (PCR-SCAR) methods from small-scale tomato orchards in Paraguay. Finally, control of parasitic nematodes depends on detection ability and accurate diagnosis of nematode species to choose suitable and sustainable management methods. Therefore, research aimed at identifying nematode species using enzymatic and molecular techniques will have a tremendous positive impact on tomato crops in the future.

5. Conclusions

Our study is the first in Paraguay to identify root-knot nematode species using esterase phenotypes and SCAR markers.

Two species were identified *M. javanica* and *M. incognita*, with predominance in all the sampled localities, and an atypical *Meloidogyne* species.

Author Contributions: Conceptualization and designed the experiments, G.R.-R. and R.M.D.G.C.; methodology and conducted the experiments, G.R.-R., V.S.M. and J.M.S.M.; analyzed the data V.S.M., J.M.S.M. and S.C.-D., and data curation R.M.D.G.C.; writing—original draft preparation, G.R.-R., supervision, H.D.L.-N., J.M., S.P.A. and V.S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Ethical review and approval were waived for this study due to conducted test involving unprotected plants and microorganisms. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data Availability Statement: Data are available by contacting G.R.R.

Acknowledgments: The first author thanks the National Council of Science and Technology (CONACYT, Paraguay) for the supported travel costs for this research and the Laboratory of Nematology (EMBRAPA-CENARGEN) (Brasília, DF, Brazil) for identifying the *Meloidogyne* species. J.M. is a Ramon y Cajal fellowship (RYC2019-028404-I) launched by the Spanish government (MICIN). The funders had no role in the study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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