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Detection and molecular characterization of the northern root-knot nematode, *Meloidogyne hapla*, infesting a tree tomato field in Ecuador

Pablo Llumiquinga[1] · Wladimir J. Enríquez[2,3] · Karina Proaño[3] · Patricio Gallegos[1] · Carlos Gutiérrez-Gutiérrez[1,4,5]

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Abstract

Meloidogyne hapla was detected for the sixth time in Ecuador; however this species was recorded for the first time infesting a tree tomato (*Solanum betaceum* Cav.) crop in this country. It represents the first record for this species in the Tungurahua province, Ecuador. These results confirm a wider geographical distribution pattern of this species in Ecuador and a wide range of host species. The identification was based on the morphology of female perineal pattern and molecular data. Contrasting morphological hypotheses with molecular data provided rapid and accurate identification of *M. hapla*.

Keywords Bayesian inference · D2–D3 expansion segments of the 28S rDNA · Cytochrome oxidase subunit II of mtDNA · Root-knot nematode · RKNs · *Solanum betaceum* Cav

Agriculture in Ecuador is a key sector of great social, territorial, cultural and economic relevance. Tree tomato or tamarillo (*Solanum betaceum* Cav.) is a native subtropical perennial plant from the Andean mountains in South America and is usually grown as a fruit crop in the Ecuadorian highlands (Feicán-Mejias et al. 2016).

Plant-parasitic nematodes (PPN) are recognized as one of the greatest threats to Solanaceous crops (e.i. eggplant, pepper, potato, tomato, tree tomato, naranjilla) causing serious damage in terms of yield production and crop quality (Perry et al. 2009; Trudgill et al. 2000; Jones et al. 2013; Kantor et al. 2022). Among these PPNs, *Meloidogyne* spp., commonly known as the root-knot nematodes (RKN), are ranked at the top in the list of economically plant pathogenic important PPNs in Ecuador (Calderon et al. 2022). To date, five *Meloidogyne* species, *M. arenaria* (Neal 1889) Chitwood, 1949, *M. graminicola* Golden & Birchfield 1965, *M. hapla* Chitwood, 1949, *M. incognita* (Kofoid & White, 1919) Chitwood, 1949 and *M. javanica* (Treub, 1885) Chitwood, 1949, have been identified in Ecuador, however only *M. incognita* has been reported infesting tree tomato in this country (Calderon et al. 2022).

Meloidogyne hapla, commonly known as the northern root-knot nematode, is an important soilborne pathogen of numerous agricultural crops such as vegetables (e.i. potato, carrot, tomato, onion), clover, alfalfa, ornamentals, tree tomato, kiwi, strawberries and grapevines in temperate regions and cold climates as higher altitude areas in the tropics (Knight et al. 1997; Trudgill et al. 2000; Prohens and Nuez 2001; Múnera 2004; Shokoohi and Mashela 2020; Kantor et al. 2022; Rusinque et al. 2022). In Ecuador, this species has been found infesting five crop plants (*Ipomeas*

✉ Carlos Gutiérrez-Gutiérrez
carlosg@uevora.pt

1 Dept. Protección Vegetal, Estación Experimental de Santa Catalina, Instituto Nacional Autonómico de Investigaciones Agropecuarias (INIAP), Panamericana Sur s/n, Ap., 1701-340 Machachi, Ecuador

2 Dept. de Nematología, Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD), Ministerio de Agricultura y Ganadería, Av. Interoceánica Km. 141/2, La Granja MAGAP, Tumbaco, Quito, Pichincha, Ecuador

3 Departamento de Ciencias de la Vida, Laboratorio de Biotecnología Vegetal, Universidad de las Fuerzas Armadas ESPE, Av. General Rumiñahui, P.O. BOX 171-5-231B, 171103 Sangolquí, Ecuador

4 NemaLab, MED – Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, Instituto de Investigação e Formação Avançada, Universidade de Évora, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal

5 Secretaría Nacional de Educación Superior, Ciencia, Tecnología e Innovación de Ecuador, SENESCYT, 9 de Octubre y Ramírez Dávalos, EC170517 Quito, Pichincha, Ecuador

sp., *Lactuca sativa* L., *Solanum tuberosum* L., *Capsium annum* L., *Rose* sp.) in four provinces (Carchi, Chimborazo, Loja, Pichincha) (Calderon et al. 2022).

Meloidogyne species have been detected numerous times in Ecuador (Calderon et al. 2022), however the majority of these identifications have been based on classical morphological analysis, mainly the morphology of females' perineal pattern (Calderon et al., 2022). Nowadays, genotyping by PCR-RFLP (Restriction Fragment Length Polymorphism) and sequencing approaches is a powerful level-species diagnostic tool for the genus *Meloidogyne* (Power and Harris, 1993; Powers et al. 2005; Calderon et al., 2022).

Among the symptoms in Solanaceus crops infected by *M. hapla* are usually the typical galls in secondary and even primary roots and a reduced plant root system volume (Perry et al. 2009). Due to high infection by this species, the production of Solanaceus crops is reduced (Perry et al. 2009). Today, chemical nematicides are in disuse because of their toxic effect on the environment, so alternative control methods for many Solanaceus crops are urgently needed. Therefore, a robust taxonomic approach based on the classical analysis of the female perineal patterns and molecular data is strongly advised in order to ensure accurate nematode identification at the species level, which is a crucial pre-requisite for designing effective management strategies against them.

During a nematode survey for RKNs in Ecuador was revealed plants showing decline inside a tree tomato field (sample code: 103–112) in Pillaro locality (Tungurahua province) (GPS coordinates: S 01° 10′ 86″ W 78° 33′ 30″). Tree tomato plants showed the typical symptoms of infection by RKNs characterized by a distorted root system and galls on secondary roots. Thus, the main objective of this work was to characterize and identify at species level this Ecuadorian population of *Meloidogyn*e spp. infesting a tree tomato field using a robust approach based on the morphology of the female perineal pattern and molecular data. Galls were dissected and the nematodes (J2s or second-juveniles stages) were extracted and quantified from soil according to Barker (Barker 1985). The identification was based on the morphology of the female perineal patterns (Eisenback and Triantaphyllou, 1991; Ghaderi and Karssen 2020) and molecular analysis based on sequencing of ribosomal DNA (rDNA) (Shokoohi and Mashela 2020) and restriction enzyme analysis of mitochondrial DNA (mtDNA) (Power and Harris 1993; Powers et al. 2005). Female perineal patterns were prepared according to standard procedures (Eisenback and Triantaphyllou 1991; Ghaderi and Karssen 2020). Root tissues were teased apart with a needle to remove adult females. The lip and neck regions of the female specimens were excised, and the posterior end was placed in 45% lactic acid until body contents are removed. Then, the perineal pattern was trimmed and transferred to a drop of glycerine for light micrographs using a light microscope (Olympus BX50, Hamburg, Germany) and an Olympus DP70 camera

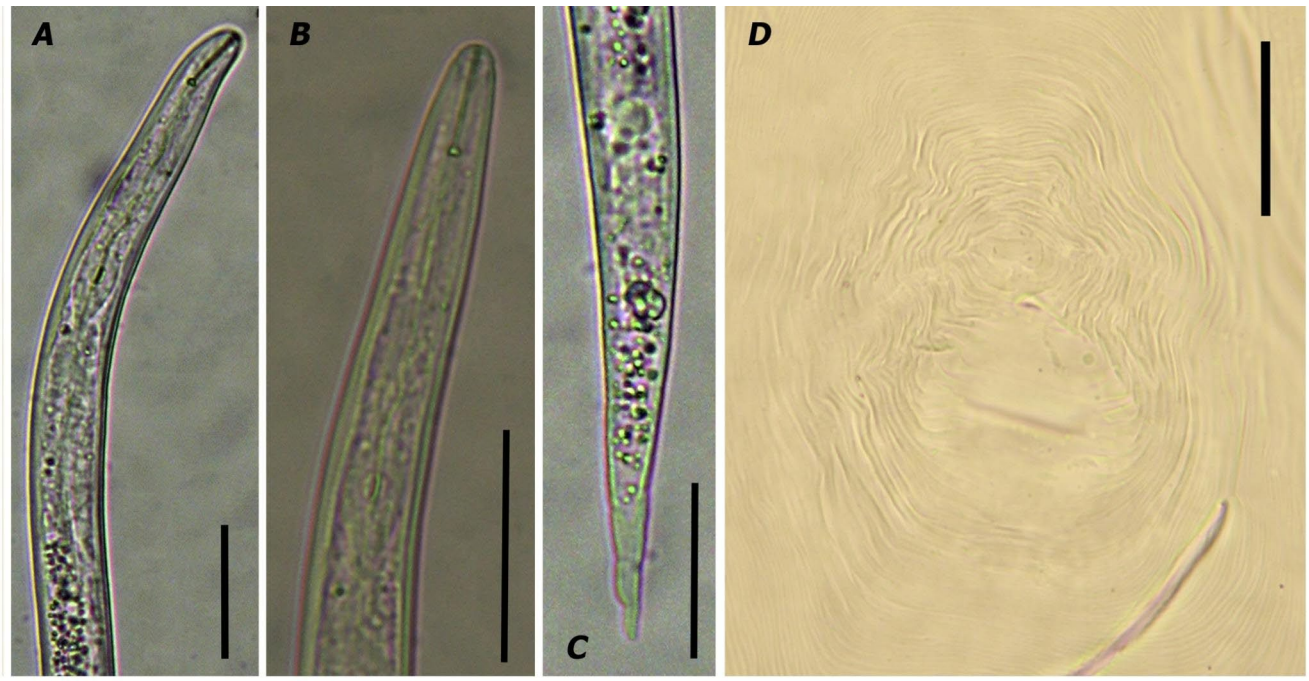

Fig. 1 Light micrographs of *Meloidogyne hapla* Chitwood, 1949: (A-C) Second-stage juvenile (J2): (A-B) pharyngeal region, and (C) tail region; (D) Female perineal pattern; (Scale bars A, B, C = 25 µm; D = 20 µm;)

Cell® software (Olympus Corp., Tokyo, Japan). A total of 10 female perineal patterns were examined for species identification. Microscopic slides of female perineal patterns (slide accession numbers PLE001– PLE005) have been deposited in the in the Nematode Collection of the Nematology Lab, Mediterranean Institute for Agriculture, Environment and Development, MED, University of Évora, Évora. Nematode population density was from 129 eggs and J2s per 100 cm^3 of soil. These alives J2s were placed in a drop of water on a glass slide and subsequently morphologically examined under a Zeiss light microscope to get a fast first preliminary taxonomic check at the genus level (Fig. 1A-C). The morphology of the female perineal pattern matched with those of the original description of *M. hapla* (Chitwood 1949; Eisenback 1985; Hunt and Handoo 2009) (Fig. 1D). These phenotypic data were contrasted with molecular data based on the sequencing of D2-D3 expansion segments of the 28S rDNA and molecular phylogenetic analysis using Bayesian inference (BI) as well as the genotyping of the COII fragment of mtDNA using PCR-RFLP analysis. For this study were implemented two molecular protocols using two pair primers: D2A/D3B primers for amplifying of the D2-D3 region of the 28 S rDNA (Shokoohi and Mashela 2020) and C2F3/1108 primers for amplifying of the COII fragment of mtDNA (Power and Harris 1993; Powers et al. 2005). Two D2-D3 sequences (OR387884- OR387885) from Ecuador were deposited on the Genbank database. The newly obtained D2-D3 of 28S sequences were compared with those of other nematodes sequences available in GenBank database using the BLAST homology search program (Altschul et al. 1990). Both D2-D3 sequences were identical (100%) (OR387884- OR387885) showing 99–100% homology with other sequences of *M. hapla* deposited in GenBank (e.i. MN475814; MN752204; MZ570969; OQ269671; OQ269684). These newly obtained D2–D3 expansion segments of 28S rRNA sequences from Ecuadorian population of RKN species found in this survey, together with the available sequences of *Meloidogyne* spp. obtained from the National Center for Biotechnology Information (NCBI) were used for phylogenetic analyses. The sequences were aligned using an online version of MAFFT v. 7 (Katoh et al. 2019) with default parameters. Sequence alignments were edited by Gblocks v. 0.91b (Castresana 2000) with less stringent selection Gblocks parameters (www.phylogeny.fr, accessed on 6 October 2023). Homogeneities of base frequencies and optional substitution models for 28S rRNA datasets were tested with Kakusan4 (Tanabe 2011). Bayesian inference (BI) tree of D2–D3 fragment of 28 S rRNA gene was constructed with MrBayes v. 3.2.1 (Ronquist et al. 2012). For BI analysis, GTR model with a gamma-shaped distribution was selected. Convergence of the MCMC chain and appropriate burn-in were assessed with Tracer 1.7.1 (Rambaut et al. 2018). BI analysis was run for 1,500,000 generations, sampling every 100th tree and discarding 'burn-in'for the first 25% of the sampled tree. The resulting trees were visualized and edited using FigTree v1.4.3 (Rambaut 2009). Molecular phylogenetic analysis using the Bayesian Inference (BI) method placed our *M. hapla* populations in a well-supported clade which included other word populations of this same species (Fig. 2). Thus, all these *M. hapla* populations form a cluster clearly separated from other morphologically related *Meloidogyne* species as *M. arenaria*, *M. chitwoodi* Golden, O'Bannon, Santo & Finley, 1980, *M. enterolobii* Yang & Eisenback, 1983, *M. fallax* Karssen, 1996, *M. incognita*, and *M. javanica* (Fig. 2). Additionally, *DraI* digestions of the 550 bp amplification product of the COII fragment of mtDNA generated a three-banded pattern (ca. 250 bp, 300 bp and 550 bp) (data not shown). This genotypic pattern from Ecuadorian populations of *M. hapla* agrees with the characteristic three-banded pattern of *M. hapla*; so that, like other word populations of *M. hapla*, our Ecuadorian population of *M. hapla* was characterized by two-banded pattern (ca. 250 bp and 300 bp) and an additional slightly visible undigested band (ca. 550 bp) (Power and Herris 1993; Powers et al. 2005).

This RKN species was originally described infesting a potato field in Long Island, New York (Chitwood 1949) and later was reported in soil samples around plant roots in cultivated and uncultivated environments worldwide (Trudgill et al. 2000; Kantor et al. 2022; Rusinque et al. 2022). Our findings represent the sixth records for *M. hapla* for Ecuador, however it represent first record for this species in the Tungurahua province. To our knowledge these results represent the first record of this species parasitizing tree tomato crop in Ecuador. We confirm a wider geographical distribution of this species in Ecuador and a wide range of host species. The identification of *M. hapla* is relevant because it may represent a threat for tree tomato production in Ecuador.

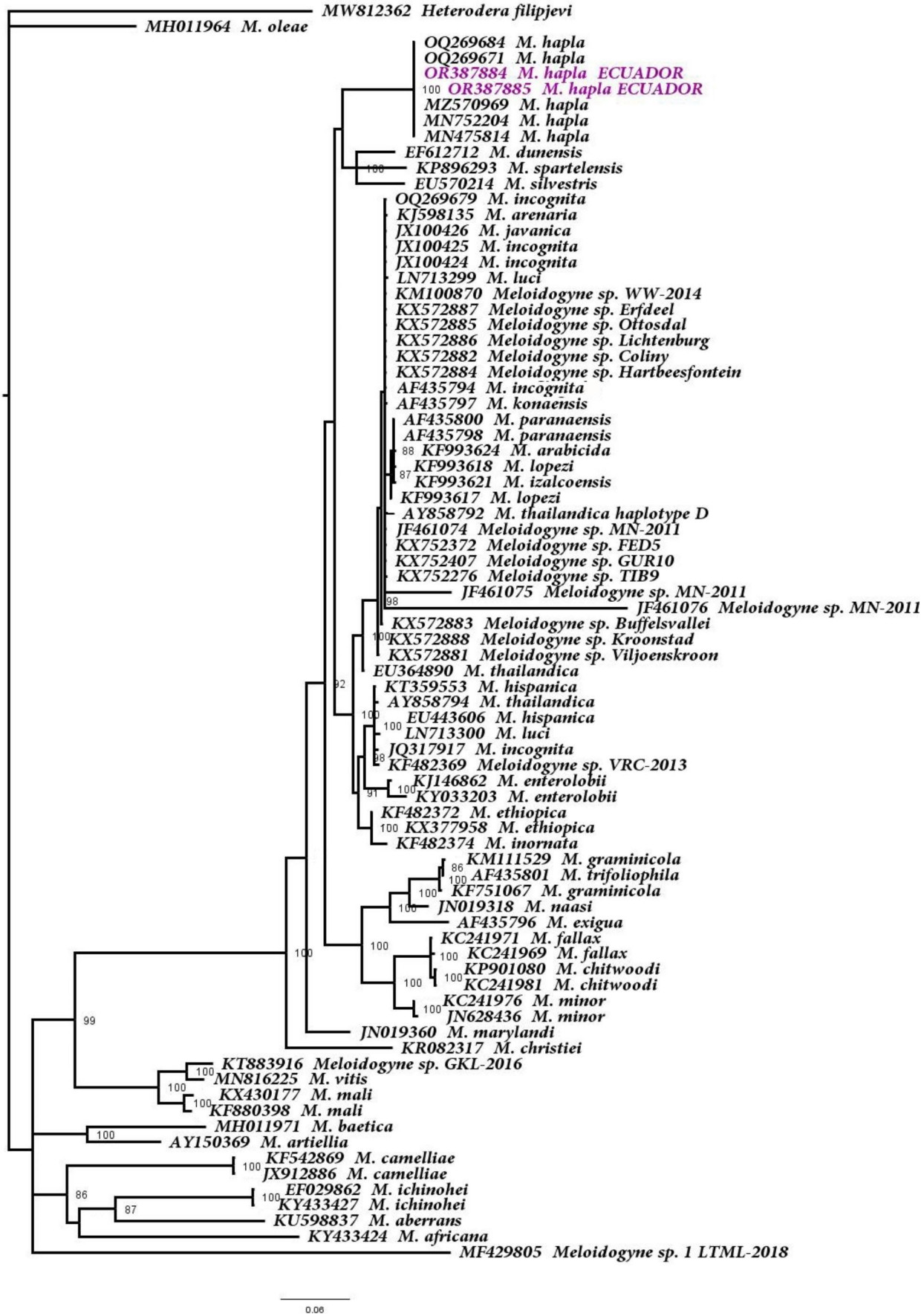
MW812362 Heterodera filipjevi
MH011964 M. oleae
OQ269684 M. hapla
OQ269671 M. hapla
OR387884 M. hapla ECUADOR
OR387885 M. hapla ECUADOR
MZ570969 M. hapla
MN752204 M. hapla
MN475814 M. hapla
EF612712 M. dunensis
KP896293 M. spartelensis
EU570214 M. silvestris
OQ269679 M. incognita
KJ598135 M. arenaria
JX100426 M. javanica
JX100425 M. incognita
JX100424 M. incognita
LN713299 M. luci
KM100870 Meloidogyne sp. WW-2014
KX572887 Meloidogyne sp. Erfdeel
KX572885 Meloidogyne sp. Ottosdal
KX572886 Meloidogyne sp. Lichtenburg
KX572882 Meloidogyne sp. Coliny
KX572884 Meloidogyne sp. Hartbeesfontein
AF435794 M. incognita
AF435797 M. konaensis
AF435800 M. paranaensis
AF435798 M. paranaensis
KF993624 M. arabicida
KF993618 M. lopezi
KF993621 M. izalcoensis
KF993617 M. lopezi
AY858792 M. thailandica haplotype D
JF461074 Meloidogyne sp. MN-2011
KX752372 Meloidogyne sp. FED5
KX752407 Meloidogyne sp. GUR10
KX752276 Meloidogyne sp. TIB9
JF461075 Meloidogyne sp. MN-2011
JF461076 Meloidogyne sp. MN-2011
KX572883 Meloidogyne sp. Buffelsvallei
KX572888 Meloidogyne sp. Kroonstad
KX572881 Meloidogyne sp. Viljoenskroon
EU364890 M. thailandica
KT359553 M. hispanica
AY858794 M. thailandica
EU443606 M. hispanica
LN713300 M. luci
JQ317917 M. incognita
KF482369 Meloidogyne sp. VRC-2013
KJ146862 M. enterolobii
KY033203 M. enterolobii
KF482372 M. ethiopica
KX377958 M. ethiopica
KF482374 M. inornata
KM111529 M. graminicola
AF435801 M. trifoliophila
KF751067 M. graminicola
JN019318 M. naasi
AF435796 M. exigua
KC241971 M. fallax
KC241969 M. fallax
KP901080 M. chitwoodi
KC241981 M. chitwoodi
KC241976 M. minor
JN628436 M. minor
JN019360 M. marylandi
KR082317 M. christiei
KT883916 Meloidogyne sp. GKL-2016
MN816225 M. vitis
KX430177 M. mali
KF880398 M. mali
MH011971 M. baetica
AY150369 M. artiellia
KF542869 M. camelliae
JX912886 M. camelliae
EF029862 M. ichinohei
KY433427 M. ichinohei
KU598837 M. aberrans
KY433424 M. africana
MF429805 Meloidogyne sp. 1 LTML-2018
0.06

Fig. 2 Phylogenetic relationships within the genus *Meloidogyne* Göldi, 1887. Bayesian 50% majority rule consensus trees as inferred from D2-D3 expansion segments of the 28S rDNA gene sequences alignments under the GTR model with a gamma-shaped distribution. Posterior probabilities of more than 0.70 are given for appropriate clades. Newly obtained sequences of *Meloidogyne hapla* Chitwood, 1949 are coloured in purple. Scale bar = expected changes per site

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Declarations

Conflict of interest All authors declare no conflict of interest.

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