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Integrative Taxonomy of *Meloidogyne oryzae* (Nematoda: Meloidogyninae) parasitizing rice crops in Southern Brazil

Vanessa Silva da Mattos · Juvenil Enrique Cares · Cesar Bauer Gomes · Ana Cristina Meneses Mendes Gomes · Jessica da Mata dos Santos Monteiro · Guillermo Marcello Gomez · Philippe Castagnone-Sereno · Regina Maria Dechechi Gomes Carneiro

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Abstract A root-knot nematode parasitizing rice (*Oryza sativa* L.) in Santa Catarina state (Brazil) was identified as *Meloidogyne oryzae* Maas, Sanders and Dede, 1978 using different approaches. The specimens studied from this Brazilian population were compared with the type description of *M. oryzae* from Suriname, with additional morphological, biochemical and molecular characterization. The female has a longer stylet (15.0 μm) when compared with *M. graminicola* (11.2 μm) with irregularly shaped knobs, vulva offset and slightly protruding in posterior region. The lip region was distinct from first body annuli, and labial disc and the medial lips form an anchor-shaped structure. Perineal patterns were similar to *M. graminicola*. The male has a lip region offset and the presence of few short and irregular lines; medial lips divided, not fused with labial disc and stylet (18.2 μm) longer than in *M. graminicola* (16.8 μm). Second-stage juvenile (J2) tail (75.8 μm) was longer than in *M. graminicola* (70.9 μm) with a very long narrow hyaline portion (22 μm in *M. oryzae* and 17.9 μm in *M. graminicola*). Biochemically, it presented a distinct esterase profile (Est O1=R1), differentiating it from *M. graminicola* (Est VS1). The number of chromosomes was 3n = 50–56, and in DNA sequences of ITS1–5.8S–ITS2 rRNA the two populations of *M. oryzae* clustered together with other mitotic parthenogenetic species, differentiating them from *M. graminicola* with *n* = 18 chromosomes and clustered with meiotic species. Phylogenetic analysis using neutral markers (AFLP and RAPD) showed that both *M. oryzae* populations form a coherent, closely related cluster separately from *M. graminicola* isolates. This study represented the first detection of *M. oryzae* in Brazil and the second in the world after the species description in 1971.

Keywords AFLP · esterase phenotype · ITS · morphology · *Oryza sativa* · RAPD · root-knot nematode · taxonomy · tomato

V. S. da Mattos · J. E. Cares
Departamento de Fitopatologia, Universidade de Brasília, Brasília, DF 70910-900, Brazil

V. S. da Mattos · A. C. M. M. Gomes · J. d. d. Monteiro · G. M. Gomez · R. M. D. G. Carneiro (✉)
Embrapa Recursos Genéticos e Biotecnologia, C.P. 02372, Brasília, DF 70849-979, Brazil
e-mail: regina.carneiro@embrapa.br

C. B. Gomes
Embrapa Clima Temperado, C.P. 403, Pelotas, RS 96001-970, Brazil

P. Castagnone-Sereno
INRA, CNRS, UMR 1355-7254, Institut Sophia Agrobiotech, University Nice Sophia Antipolis, 06900 Sophia Antipolis, France

Introduction

Many genera of parasitic nematodes are associated with rice, among which are several root-knot nematode (RKN) species, *Meloidogyne* spp. Almost all the RKN species described in rice belong to the so-called 'graminis-group'. They are morphologically similar (Jepson 1987) and their enzymatic characterization is

difficult due to the large drawn-out bands and high enzymatic activity revealed on gels. Due to these similarities, some species may have been misidentified with other *Meloidogyne* spp. parasites of rice, especially with *M. graminicola* Golden and Birchfield 1965 (Negretti et al. 2017).

The rice RKN, *M. graminicola,* has emerged as the most widespread root-knot nematode in Brazil (Negretti et al. 2017), and worldwide in both subtropical and tropical regions. It is considered to be a major threat to the rice crop, particularly in Asia where changes in agricultural practices in response to climate change and socioeconomic conditions have led to a dramatic increase in *M. graminicola* and probably other species (Negretti et al. 2017; De Waele and Elsen 2007).

Meloidogyne oryzae Maas, Sanders and Dede, 1978 was first detected in 1971 in Suriname, causing damage to rice plants (Maas et al. 1978). Although valuable information using Light Microscopy (LM) was included in the original description (Maas et al. 1978), lack of Scanning Electron Microscopy (SEM) and biochemical/molecular data led to a limited characterization of the species. Although Jepson (1987) used SEM to provide additional information for the characterization of *M. oryzae*, little attention was given to this species for several years. More recently, *M. oryzae* was detected again in Suriname and French Guiana using only the esterase phenotype as a diagnostic character (Carneiro et al. 2000). Two similar non-specific esterase profiles have been already attributed to *M. oryzae*: VS1 (Esbenshade and Triantaphyllou 1985) and O1 (Carneiro et al. 2000). A new malate dehydrogenase profile MDH O3 (Rm 1.4, 1.6, 1.8) was also reported by the last authors. Although this second detection was performed using a topotype population from Suriname collected 20 years after the original description, the species-specific identity of this population was not confirmed by morphological approaches (Regina M.D.G. Carneiro unpublished). The same population was studied by Tigano et al. (2005) using 18 s rDNA sequences, and it clustered with meiotic parthenogenetic species in phylogenetic reconstruction, contradicting the fact that *M. oryzae* has 51–55 chromosomes and a mitotic parthenogenesis mode of reproduction (Esbenshade and Triantaphyllou 1985). This population from Suriname was studied later and presented ($n = 18$) chromosomes and an esterase phenotype similar to *M. graminicola* (Carneiro et al. 2000), confirming the suspicion of an erroneous identification (Negretti et al. 2017). Considering this additional information, *M. oryzae* had been detected only once in the world, in Suriname (1971), described by Maas et al. (1978) and later studied by Esbenshade and Triantaphyllou (1985) and Jepson (1987).

Meloidogyne species with an elevated perineum like *M. oryzae* were previously assigned to the genus *Hypsoperine.* This genus was proposed by Sledge and Golden in 1964 (Coetzee 1956) for *Hypsoperine graminis* as the type species and for *H. acronea* Coetzee 1956 (Araki 1992). These authors and Page (1985) believed that the new genus *Hypsoperine* occupied a position between *Heterodera* and *Meloidogyne* and presented females with eggs containing J1 and J2 and hatched J2 inside the bodies. However, the taxonomic status was in dispute for many years. Siddiqi synonymized *Hypsoperine* with *Meloidogyne* in 2000 (Siddiqi 2000). Recently, a phylogenetic tree inferred from 18S sequences placed *M. spartinae* (= *H. spartinae*) within the genus *Meloidogyne*, which showed that *Hypsoperine* was a junior synonym of *Meloidogyne* (Plantard et al. 2007).

A recent survey in the south of Brazil detected *Meloidogyne graminicola*, *M. javanica* (Treub, 1885) Chitwood, 1949 and three other *Meloidogyne* spp. populations with atypical esterase phenotypes causing damage in rice-fields of Rio Grande do Sul (RS) and Santa Catarina (SC) states (Negretti et al. 2017). Considering the morphological, morphometrical, enzymatic and molecular differences in *M. graminicola* and other *Meloidogyne* species from rice, the aim of this study was to perform an integrative taxonomic description based on LM, SEM, ITS, AFLP, RAPD and isozyme phenotyping, which remains essential for accurate diagnosis (Hunt and Handoo 2009) of the unknown *Meloidogyne* sp.1 root-knot nematode populations found in SC state. The second objective was to clarify the mode of reproduction of these populations, by studying the cytology of the parasite and giving an evolutionary perspective using phylogeny. We also reported here the first occurrence of *M. oryzae* in the southern region of Brazil, and its second detection in the world after the species description.

Materials and methods

Nematode populations

Stock pure cultures of *Meloidogyne* spp., including two populations of *Meloidogyne* sp.1 populations from SC rice-fields (Negretti et al. 2017) (Table 1) were

maintained on rice plants (*Oryza sativa* L.– 'BR-IRGA 410') in greenhouse conditions (25–28 °C). All morphological and biochemical studies were done with these cultures. Egg masses and females were handpicked from infected rice roots, and second-stage juveniles (J2) were hatched in moist chambers. Males were obtained by placing roots with egg masses in water, aerating them with an aquarium pump and periodically collecting the specimens from the water (Carneiro et al. 1998).

Morphological studies

Male and second-stage juvenile (J2) nematodes were killed in cold (5 °C) 2% formaldehyde and measured immediately under light microscope (LM). Perineal patterns were cut from live young females in 45% lactic acid and mounted in glycerin (Taylor and Netscher 1974). Male, J2, and female specimens were prepared for scanning electron microscopy (SEM) according to previously described methods (Eisenback and Hirschmann 1979, 1980; Eisenback et al. 1980). Specimens were viewed and photographed with a Zeiss DSM-962 scanning electron microscope. Both populations of *Meloidogyne* sp.1 were investigated.

Biochemical and cytogenetic studies

Electrophoresis of the two *Meloidogyne* sp.1 populations from rice was performed on 7% polyacrylamide gel slabs isozymes in a horizontal electrophoresis system, according to Carneiro and Almeida (2001). Both esterase (EST) and malate dehydrogenase (MDH) enzymes were investigated. *Meloidogyne javanica* (EST J3, MDH N1) was used as reference. Cytogenetic studies were carried out with a propionic orcein staining method (Triantaphyllou 1985b).

Molecular studies

For the phylogenetic analyses, the ITS1–5.8S–ITS2 region of rRNA (primer set: **5367**-5_-TTGATTAC GTCCCTGCCCTTT-3_ and **F195** -5_-TCCTCCGC TAAATGATATG-3_Schmitz et al. 1998) and the D2-D3fragment of the 28S rRNA gene (primer set: **D2A** -5_-ACAAGTACCGTGAGGGAAAGTTG-3_ and **D3B**- 5_-TCGGAAGGAACCAGCTACTA-3_; De Ley et al. 1999) were amplified according to Subbotin et al. (2000) conditions. PCR products were cleaned using the Wizard® SV Gel/PCR Clean-Up System (Promega) and cloned into the pGem-T® Easy Vector (Promega), following the manufacturer's instructions. Sequence alignments were performed using MEGA version 5.03 with default parameters (Tamura et al. 2011), with sequences obtained from the *Meloidogyne* spp. from rice (Table 1), and also with sequences of other *Meloidogyne* spp. retrieved from the NCBI database. A tree was generated using the Neighbour-Joining (NJ) algorithm (Saitou and Nei 1987) in PAUP* software version 4b10 (Swofford 2002). Sequences from *Pratylenchus pinguicaudatus* Rensch, 1924 Filipjev and Schuurmans Stekhoven, 1941 (KP995311.1) was used as outgroup. To test the node support of the generated trees, 1000 bootstrap replicates were performed and only values above 50% were considered.

As a different and complementary strategy to study the relationships between *Meloidogyne* species from rice, we further tested neutral AFLP and RAPD markers. For the analysis with RAPD markers, PCR reactions were performed according to Randig et al. (2002). Twenty-five 10-mer oligonucleotide primers (Operon Technologies) were used: OPA-12, OPAB-02, OPAS-08, OPAU-13, OPB-05, OPC-07, OPC-09, OPD-13, OPG-06, OPG-13, OPH-01, OPJ-17, OPK-20, OPL-20, OPM-20, OPN-10, OPP-05, OPR-03, OPR-07, OPR-12, OPW-05, OPW-06, OPX-16, OPY-06, OPZ-07. The amplification was performed on a PTC-100 thermocycler using the following settings: 5 min at 94 °C: 40 cycles of 30 s at 94 °C, 45 s at 36 °C, 2 min at 70 °C; and a final extension of 10 min at 70 °C (Randig et al. 2002). PCR products were separated by electrophoresis in a 1.5% (*w*/*v*) agarose gel, stained with ethidium bromide (0.3 μg ml^{-1}) and visualised under UV light. In the AFLP analysis, DNA digestion were executed following the method of Suazo and Hall (1999). A total of six random 22-mer primers (Integrated DNA Technologies) were used, consisting of the *Eco*RI adapter core sequence 5_- GACTGCGT ACCAATTCAGT-3_ plus the three_ selective nucleotides (ACT, ATT, GGC, CAG, CCT and TCG) PCR reactions were performed in a 25 μl final volume containing 1 μl (50 ng μl^{-1}) digested DNA, 2.5 μl 10× PCR buffer without magnesium chloride (Invitrogen), 1 μl 50 mM MgCl2,0.5 μl 10 mM dNTPs, 1 μl 10 μM primer and 0.3 μl *Taq*DNA polymerase (5 U μl^{-1}; Invitrogen). DNA was amplified using a PTC-100 thermocycler (MJ Research) under the following cycling parameters: 1 min at 95 °C: 37 cycles of 1 min at

Table 1 List of *Meloidogyne* spp. isolates used in this study: population code and origin

Population Code	Species	Origin (Municipality/State/Country)[a]
Mg G2	*Meloidogyne graminicola*	Guaramirim/SC/Brazil
Mg G4	*M. graminicola*	Camburiú/SC/Brazil
Mg G6	*M. graminicola*	Camburiú/SC/Brazil
Mg G8	*M. graminicola*	Capão do Leão/RS/Brazil
Mg G12	*M. graminicola*	Capão do Leão/RS/Brazil
Mg G13	*M. graminicola*	Rio do Sul/SC/Brazil
Mg P[b]	*M. gramnicola*	The Philippines
Msp 1–1	*Meloidogye* sp.1 (=*M. oryzae*)	Ilhota/SC/Brazil
Msp 1–2	*Meloidogye* sp.1 (=*M. oryzae*)	Camboriú/SC/Brazil
Msp 2	*Meloidogyne* sp. 2	Uruguaiana/RS/Brazil
Msp 3	*Meloidogyne* sp. 3	Uruguaiana/RS/Brazil
Ms[c]	*M. salasi*	Costa Rica

[a] Brazilian states: RS = Rio Grande do Sul and SC = Santa Catarina

[b] *M. graminicola* from The Philippines population was donated by Dr. Gerrit Karssen (University of Wageningen, The Netherlands)/ Dutch NPPO (code: E8256)

[c] *M. salasi* population was donated by Lorena Flores M.Sc (Universid de San José, Costa Rica)

94 °C, 1 min at 56 °C, 2.5 min at 72 °C; and a final extension of 10 min at 72 °C (Suazo and Hall 1999). PCR products were separated by electrophoresis in a 1.5% (w/v) agarose-synergel (0.7% agarose, 0.4% synergel; Diversified Biotech), stained with ethidium bromide (0.3 μg ml^{-1}) and photographed under UV light. The analysis was repeated twice. DNA fingerprints were converted into a 0–1 binary matrix and phylogenetic reconstruction was performed using the NJ algorithm (Saitou and Nei 1987) in PAUP v. 4b10 (Swofford 2002), considering the data as unordered with no weighting. To test the node support of the generated trees, the analysis was performed on 1000 bootstrap replicates and only values above 50% were considered. As the AFLP and RAPD markers could be considered to be independent characters, the two data sets were combined into a global NJ analysis, using the total evidence approach proposed by Huelsenbeck et al. (1996). For both analysis, one population of each *M. incognita* and *M. javanica* were included.

Results

Morphological studies

Meloidogyne sp.1 incites large, elongated galls on rice plants, often at the root tip, which may become hooked, similar to *M. graminicola* (Fig. 1a). Females are completely embedded in the root tissue, where eggs are laid. All egg masses are internal. Males are not very frequent, but when present, they are often found inside the root together with the female and second-stage juveniles. In tomato, small galls are observed, usually located just behind the root tip and internal egg masses.

Females The body shape is elongated and globular pear-shaped with an off-set neck; coarse annuli in neck. The anterior end of the body is usually in line with the posterior end. The vulva is located on a slight posterior protuberance formed especially by the vulva lips (Fig. 1b), and it is observed in most of the specimens. The perineal patterns are round to oval-shaped; the dorsal arch smoothly rounded; no lateral incisures or gap observed. Phasmids are small (not always visible). The ridges on the cuticle in the dorsal region form wavy broken or unbroken irregular lines around and between the phasmids. Striae rather coarse and no vulva lip striae. The perivulval area usually free of striae. A prominent fold covers the anus dorsally, while in most specimens the ventral side is marked by a distinct rather thick fold or at least an obvious regular line (Fig. 2). The lip region is low and lips slightly off-set (Fig. 3a). Under Scanning electron microscope (SEM), lip region is distinct from first body annuli, labial disc and the medial lips form an anchor-shaped structure, and lateral lips are

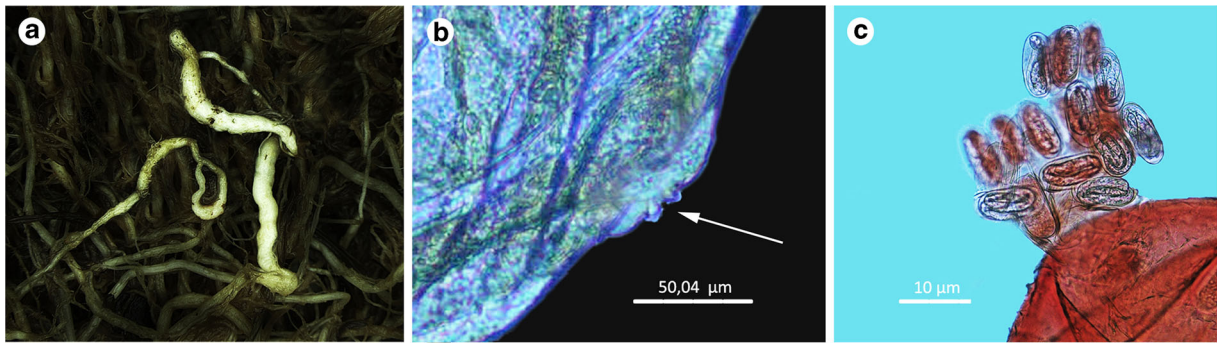

Fig. 1 Photomicrographs of *M. oryzae*: **a**- Galls formed by *M. oryzae* in rice roots; **b**-Protuberant vulva of *M. oryzae* female; **c**- Eggs containing J1 and J2 inside *M. oryzae* female body

not protuberant (Fig. 3b, c). First body annulus post-labial, apparently thicker than the subsequent ones. Excretory pore is located 2–3 stylet lengths behind the anterior region apex and anterior to the median bulb (Fig. 3a). The stylet conus sometimes curved dorsally (Fig. 3d, f). Stylet length 14–18 µm; knobs generally set off from the shaft and sometimes presenting an irregular shape. The distance of the DEGO to the base of the stylet is 4–6 µm (Fig. 3a, d-f, Table 2). Often, females containing eggs at different embryonic stages (including J1 and J2) and hatched J2 were observed within the female bodies (Fig. 1c).

Males Body vermiform, lip region is offset (Fig. 4a). Presence of few short, irregular lines in lip region (Fig. 4b). Under SEM the labial disk and medial lips are fused, medial lips appear anchor-shaped and separated from labial disk by shallow depression in most of the specimens. Lateral lips are completely absent. The stylet is well-developed with knobs small and rounded, backwardly sloping onto shaft, the anterior part (conus) slightly shorter than the posterior part (shaft) (Fig. 4c, d). The stylet length is 18–20 µm and DEGO is 4–6 µm. The lateral field occupies about 1/3 of the body width and consists of 4–8 incisures, aerolated at mid-body (Fig. 4f), narrowing anteriorly, and posteriorly until the end of the tail. Under SEM, it is possible to observe non-continuous lines or broken incisures on the lateral field. The tail is bluntly rounded with an unstriated

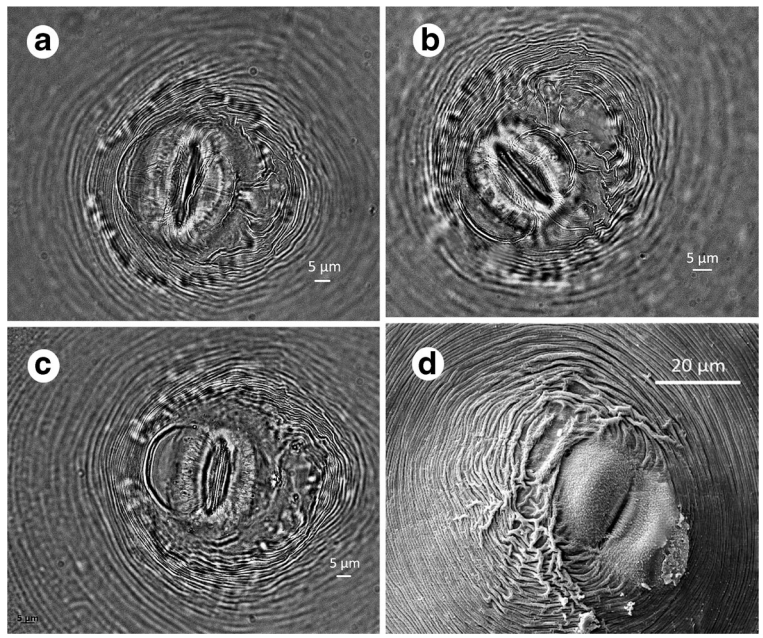

Fig. 2 Perineal patterns of *Meloidogyne oryzae* from Brazil. **a-d:** Light microscopy micrographs showing typical variation for this species

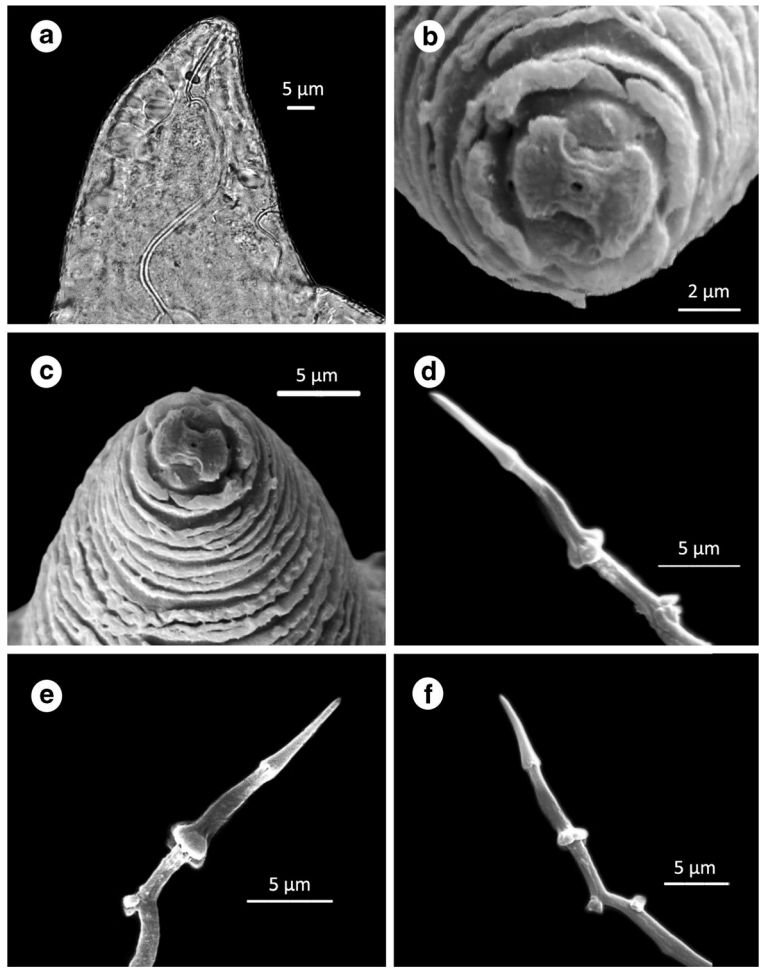

Fig. 3 F`emale of *Meloidogyne oryzae* from Brazil. **a**: Light microscopy micrographs of anterior region; **b and c**: Scanning electron microscopy (SEM) micrographs of anterior region; **d**, **e**, **f**: SEM micrographs of excised stylets

terminus (Fig. 4e). Gubernaculum 6–9 µm in length. Phasmids not observed.

Second stage juvenile The body is slender, vermiform, tapering gradually towards ends and clearly annulated. The lip is truncate and not offset (Fig. 5a), without annulations. Labial disc and medial lips are fused, dumbbell shaped (Fig. 5e). The stylet is very slender with small backward-sloping basal knobs (Fig. 5a). The stylet (11–14 µm) and the DEGO (3–5 µm) are longer when compared with *M. graminicola*: 11–12 µm and 2.8–3.4 µm. The lateral field consists of four areolated incisures (Fig. 5f). The tail is very long, conoid and thin, tapering to a long narrow hyaline portion, often appearing slightly clavate, with a clear unstriated terminal region and two or three cuticular constrictions (Fig. 5b-d). Phasmids posterior to anus very small, located in central incisures of lateral fields. The tail length is 66–85 µm and the hyaline terminus is 19–24 µm. The rectum is inflated in most of the specimens.

Biochemical studies

Meloidogyne graminicola and *Meloidogyne oryzae* (=*Meloidogyne* sp.1) presented species-specific esterase phenotypes (Fig. 6) forming large drawn-out bands with high enzymatic activity. *Meloidogyne graminicola* presented esterase phenotype VS1, Rm = 0.70 with a large band, extending from 0.65 to 0.75 and *M. oryzae* Est O1 (Rm = 1.02), another large band extending from 1.0 to 1.4. The same malate dehydrogenase phenotype (MDH N1a, RM: 1.4) was detected in both *M. graminicola* and *M. oryzae*.

Table 2 Morphometric data of *Meloidogyne oryzae* from Brazil. All measurements are in μm and in the form: mean ± s.d. (range) and coefficient of variation. Suriname populations from species description or Jepson 1987(*)

Character source	Female		Male		J2	
	Brazil	Suriname	Brasil	Suriname	Brazil	Suriname
N	30	7/25	20	22	30	60
L	490 ± 11.7 (420–630) 7.6	625 475–750	1226 ± 24.0 (1100–1520) 11.7	1667 1195–1915	444 ± 4.0 (420–500) 19.8	545 (500–615)
L/max. Body diam. (a)	1.6 ± 0.1 (1.0–2.0) 4.5	1.7	41.5 ± 1.0 (33–63.0) 6.8	56 44–68	27.6 ± 0.6 (23–41) 8.0	37 (30–45)
L/ tail length (c)	–	–	108 ± 3.0 (84.6–125) 7.8	–	5.9 ± 0.1 (5.0–6.5) 14.0	7.0 (6.8–8.6)
Max. body diam.	317 ± 12.0 (220–422) 4.7	361 250–432	31 ± 1.9 (27–40) 4.9	30 24–43	16 ± 0.4 (11–20) 8.0	15 (12–17)
Neck length	119 ± 3.18 (100–170) 6.9	107 80–136	–	–	–	–
Stylet length	15 ± 0.2 (14–18) 13.0	15 14–18/ 14.7* 14.5–15.0*	18.0 ± 0.4 (18–20) 11.4	19 19–20/18–20*	12.5 ± 0.2 (11–14) 14.5	14.2 14–15
Stylet knob Height	1.9 ± 0.1 (1–2) 6.0	–	3.1 ± 0.1 (3–4) 8.6	–	1.60.1 (1–2) 3.0	–
Stylet knob Width	3.5 ± 0.1 (3–4) 6.8	–	3.9 ± 0.1 (3–4) 10.5	–	2.6 ± 0.1 (2–3) 5.0	–
DGO	4.4 ± 0.1 (4–6) 7.0	7	4.5 ± 0.1 (4–6) 8.7	4–5/5.0* 4.5–6.0*	3.7 ± 0.1 (3–5) 6.9	–
Excretory pore to ant. End	29 ± 0.6 (21–35) 8.0	–	132 ± 0.1 (128–140) 30.0	–	70.5 ± 1.1 (66–76) 11.9	–
Vulva slit	27 ± 0.3 (25–30) 15.9	33 29–42	–	–	–	–
Anus to vulva (center distance)	19 ± 0.3 (15–21) 11.5	20 13–28	–	–	–	–
Interphasmid Distance	18 ± 0.3 (16–23)	21 15–29	–	–	–	–

Table 2 (continued)

Character source	Female		Male		J2	
	Brazil	Suriname	Brasil	Suriname	Brazil	Suriname
	9.0					
Spicule	–	–	30 ± 0.2 (29–32) 27.8	31 25–34	–	–
Gubernaculum	–	–	8.5 ± 0.2 (6–9) 9.0	8–10	–	–
Tail length	–	–	11.9 ± 0.3 (10–13) 9.7	–	75.8 ± 0.8 (66–85) 17.0	79.0/75.5* (70–90) 70.0–81.9*
Tail hyaline region	–	–	–	–	22 ± 0.3 (19–24) 12.0	21/22.9* (14–26) 19.8–27.9 *

Cytogenetic studies

Meloidogyne oryzae from Brazil reproduces by mitotic parthenogenesis and presented the triploid form (3n = 50–56 chromosomes).

Molecular studies

Phylogenetic analyses were used to complement the results obtained by morphological and isozyme approaches, by comparing sequences of the ITS1–5.8S–ITS2 rRNA segment from closely related species and other *Meloidogyne* spp. sequences obtained from the GenBank. All new sequences were deposited in GenBank and the accession numbers are presented in Fig. 7. Phylogenetic analysis obtained for the ITS1–5.8S–ITS2 rRNA segment showed that the two *M. oryzae* populations clustered together with other obligatory mitotic parthenogenetic species (Fig. 7). *Meloidogyne salasi* Lopez, 1984 clustered wih *M. graminicola* populations and the unknown species *Meloidogyne* sp.2 and *Meloidogyne* sp.3 originating from rice in southern Brazil. The species belonging to the 'graminis-group' (*M. graminicola*, *Meloidogyne* sp.2, *Meloidogyne* sp.3, *M. salasi* and *M. naasi* Franklin, 1965) clustered together and separated from *M. incognita, M. javanica* and *M. arenaria.* Analysis for D2-D3 fragment of 28S rRNA presents a phylogenetic tree with less variability; this result was not included because it was not informative.

A total of 6 AFLP and 26 RAPD primers were used to infer the genetic diversity within *Meloidogyne* spp. Both markers reproduced together 664 polymorphic fragments for all 12 *Meloidogyne* spp. populations studied (584 of RAPD and 80 of AFLP). Overall, there was a high level of polymorphism observed within the *M. graminicola* isolates (72.5%). For both Brazilian isolates of *M. oryzae*, 12.9% of polymorphism was recorded. The NJ trees (Fig. 8) were obtained after combining data from both markers together into one single analysis, and all species grouped together with high bootstrap and separately from each other. *Meloidogyne oryzae* populations clustered together in a single clade with 100% bootstrap support.

Diagnosis and relationships

In general, the morphology and morphometric data of the *Meloidogyne* sp.1 populations from Brazil

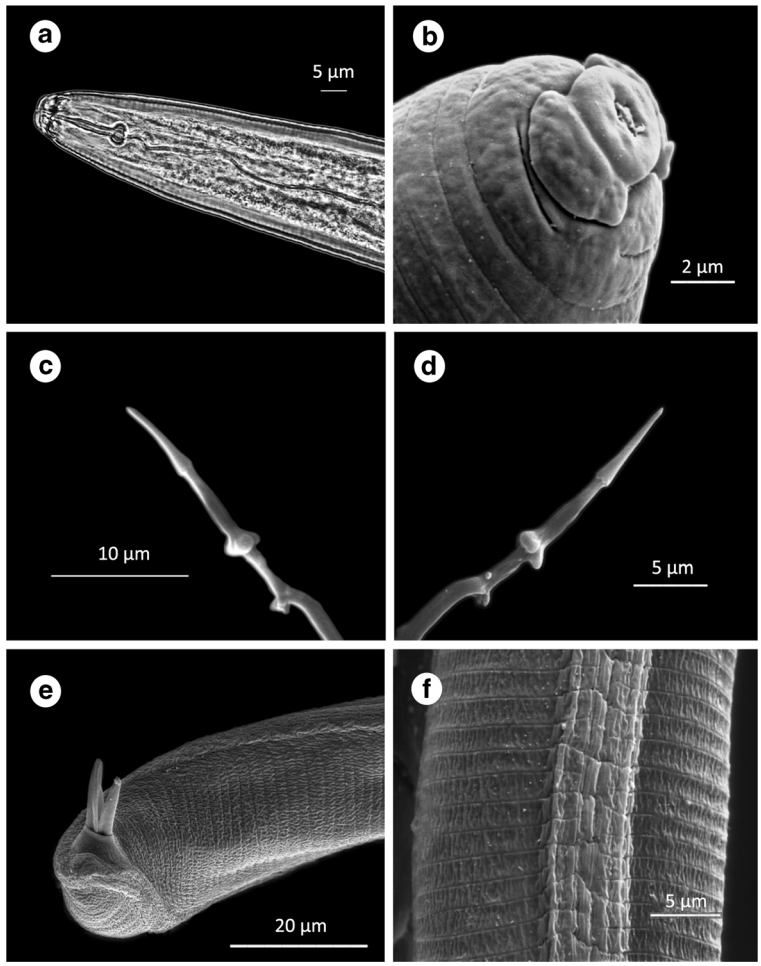

Fig. 4 Male of *Meloidogyne oryzae* from Brazil. **a:** Light microscopy (LM) micrograph of anterior region in lateral view; **b:** Scanning electron microscopy (SEM) micrographs of anterior region; **c:** SEM micrograph of posterior region showing spicules, **d**: SEM micrograph of excised stylet; **e: SEM micrograph of tail, f**: SEM micrographs of lateral field

were similar to the original description of *M. oryzae* (*Mo*) (Maas et al. 1978), with the expected variations that are frequent in the genus *Meloidogyne*. The populations from Brazil presented a smaller J2 and male length compared to the type population from Suriname, but the other measurements were in the range described for *Mo* (Table 2).

Meloidogyne oryzae resembles *M. graminicola (Mg).* The body shape and perineal patterns were similar with some minor variations, but differences are very subjective to differentiate these two species. *Mo* can be distinguished from *Mg* by the stylet and DEGO of females, which is longer (*Mo* = 14–18 µm) than in *Mg* (12.6–15.3 µm). The stylet knobs of *Mo* females are transversely ovoid, backwardly sloping onto shaft; the anterior part of female stylet (conus) is a little shorter than the posterior (shaft) (Fig. 3d-f). Different morphology was observed for *Mg:* the stylet knobs are set off from the shaft and the conus is longer than the shaft (Eisenback and Triantaphyllou 1991). In *Mo* female lip region is distinctly offset with a very visible and salient annulus; the opposite was observed for *Mg* (Golden and Birchfield 1965). The distance of the DEGO to the base of the stylet is 4–6 µm for *M. oryzae* and 3–4 µm for *M. graminicola* (Eisenback and Triantaphyllou 1991). The excretory pore in *Mo* is located 2–3 stylet lengths behind the anterior apex, and in *Mg* it is situated one and one half stylet lengths (Golden and Birchfield 1965) and, in both cases, anterior to the median bulb. The stylet length (18–20 µm) and DEGO (4–6 µm) for *Mo* and 16–17 µm and 3–4 µm, respectively, for *Mg* (Jepson 1987) can also differentiate these two species. Under SEM, the labial disk and medial lips of males are fused (*Mo* and *Mg),* medial lips appear anchor-shaped and separated from labial disk by shallow depression in *Mo* and forming a continuous anterior region cap in *Mg*

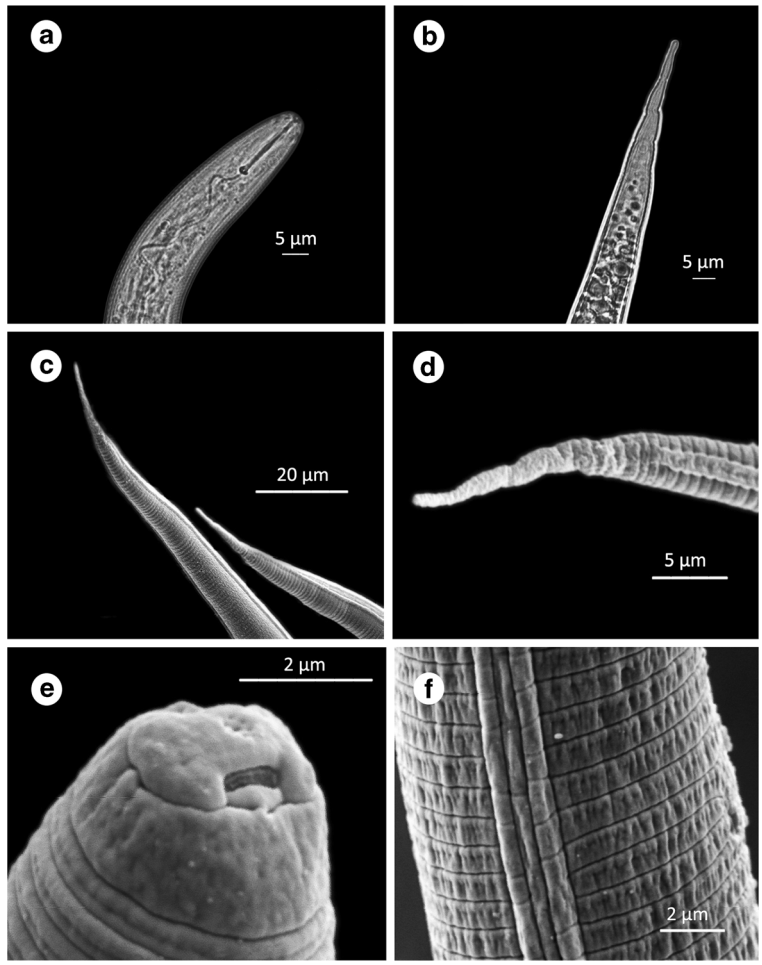

Fig. 5 Second-stage juvenile of *Meloidogyne oryzae* from Brazil. **a:** Light microscopy micrograph of anterior region; **b**: Light microscopy micrograph of tail; **c, d**: Scanning electron microscopy (SEM) micrographs of tail; **e:** SEM micrograph of anterior region; **f:** SEM micrograph of lateral field

(Jepson 1987). The male anterior region is usually not annulated in *Mg* (Eisenback and Triantaphyllou 1991) and annulated in *Mo.* In *Mo* males, the stylet is with knobs offset and slightly irregular, while in *Mg* they are pear shaped, and sloping backwards (Jepson 1987; Eisenback and Triantaphyllou 1991). Second-stage juveniles with average tail length in *Mo* (75.8 µm), longer than in *Mg* (70.9 µm). A very narrow hyaline tail portion

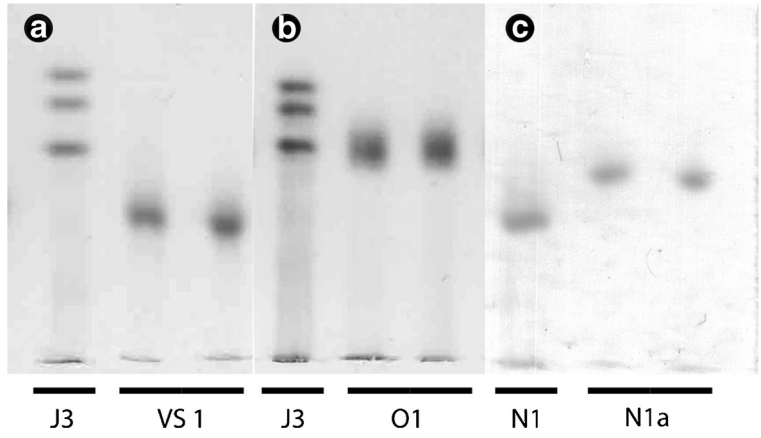

Fig. 6 Esterase (Est) and malate dehydrogenase (Mdh) phenotypes observed in *Meloidogyne* spp. populations studied: **a**: Est *M. graminicola* (VS1); **b**: Est of both populations of *M. oryzae* from Brazil (O1);**c**: Mdh for *M. graminicola* and *M. oryzae* from Brazil (N1a) *M. javanica* (Est J3 N1) was used as reference

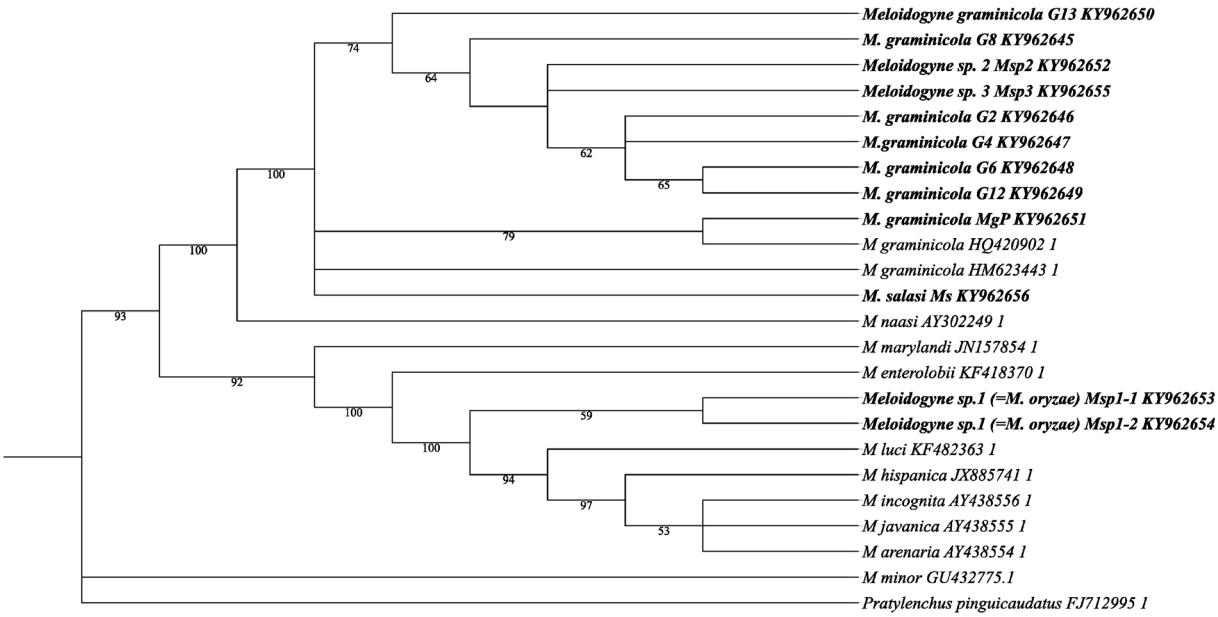

Fig. 7 Neighbour-joining trees showing phylogenetic relationships between *Meloidogyne* spp. from rice and other closely related species, based on ITS1–5.8S–ITS2 rRNA sequences (Numbers at nodes are bootstrap values (in %) for 1000 replicates

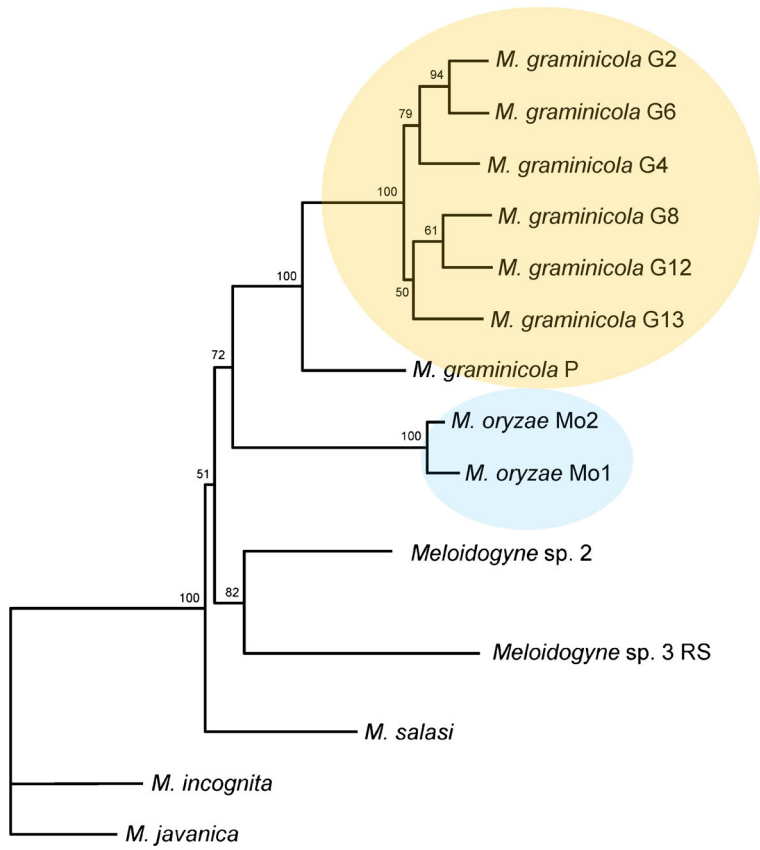

Fig. 8 Neighbour-joining trees showing phylogenetic relationships between *Meloidogyne* spp. from rice, based on the combined AFLP and RAPD data set

longer in *Mo* (22.0 μm) than in *Mg* (17.9 μm) is the most important morphological character to differentiate these two species and *Mo* from all other *Meloidogyne* species parasitizing rice (see Discussion). *Mo* also presents a longer stylet and the rectum dilated, in contrast with *Mg* (Eisenback and Triantaphyllou 1991). *Mo* presents a specific esterase phenotype profile (Est O1) in contrast with *Mg* (Est VS1) (Fig. 6). In cytogenetic studies, *Mo* presents 3n = 50–56 chromosomes and a mitotic parthenogenesis mode of reproduction, in contrast with *Mg,* which reproduces by facultative meiotic parthenogenesis and presents a haploid number of chromosomes (*n* = 18) (Eisenback and Triantaphyllou 1991).

Discussion

Accurate identification of nematodes is essential for understanding of host-parasite relationships and to implementing appropriate management options. The traditional methods of identification of RKN associated with rice are based on morphology (Jepson 1987; Eisenback and Triantaphyllou 1991), esterase phenotypes (Esbenshade and Triantaphyllou 1985; Carneiro and Almeida 2001) or molecular markers (Bellafiore et al. 2015).

Tail morphology of the second-stage juvenile of *M. oryzae* places it in Jepson's Group 11 (Jepson 1987) which now includes nine species that parasitize rice and present differential hyaline tail length: *M. graminicola* (17.9 μm), *M. graminis* (Sledge and Golden 1964) Whitehead,1968 (18.5 μm), *M. hainanensis* Liao and Feng, 1995 (15.3 μm), *M. lini* Yang, Hu and Xu, 1988 (~ 14.0 μm), *M. salasi* (19.7 μm), *M. tritricoryzae* Gaur, Saha and Khan, 1993 (17.6 μm), *M. sasseri* Handoo, Huettel and Golden 1995 (19.5 μm), *M. marylandi* Jepson and Golden, 1987 (11.8 μm), and finally the longest in *M. oryzae* (22.0 μm). Most of these species from rice were poorly described and very poorly characterized cytogenetically, enzymatically and molecularly, with the exception of *M. graminicola*, *M. graminis* and *M. marylandi.* Some morphological and biological characters are common in these species: female body elongated, vulva sometimes situated on a posterior protuberance, association with an amphimictic or meiotic parthenogenetic mode of reproduction, semi-endoparasitism, with males abundant; or mitotic parthenogenetic, with males rare and females deeply embedded in the host (Jepson 1987).

In a recent survey in southern Brazil, 56 populations of *Meloidogyne* spp. were detected in 48% of rice samples. *Meloidogyne* sp. 1 (now identified as *M. oryzae*) occurred in 62.5% of the samples, frequently in mixture with other species (Negretti et al. 2017), and *M. graminicola* was the most widespread. In this study, sequencing and phylogenetic analyses of internal transcribed spacer-rRNA (ITS) were performed to infer the phylogenetic relationship of these atypical *Meloidogyne* spp. populations. *Meloidogyne oryzae* grouped with the mitotic parthenogenetic species, while *M. graminicola* clustered with other meiotic parthenogenetic species, confirming the results obtained by Negretti et al. (2017).

In general, the morphology and morphometric data of *M. oryzae* populations from Brazil were similar to the original description of *M. oryzae* (Jepson 1987; Maas et al. 1978), with expected variations frequent in the genus *Meloidogyne*. The populations from Brazil presented a smaller J2 and male compared to the type population from Suriname. This variation in body length can occur in populations of the same species (Handoo et al. 1993). Some authors consider morphological features much more relevant in species characterization than morphometric characters (Jepson 1987; Eisenback and Triantaphyllou 1991). Considering these aspects, *M. oryzae* populations collected in Brazil were very close to the population of *M. oryzae* detected in Suriname.

Biochemically, *M. oryzae* and *M. graminicola* present distinct esterase profiles, with drawn-out bands in different positions. This phenotype was never clearly characterized before and seems to be new and species-specific for *M. oryzae*, (Esbenshade and Triantaphyllou 1985; Carneiro et al. 2000). These authors previously reported a high enzymatic activity in *M. oryzae* and *M. graminicola* esterase phenotypes, but showing close migration positions.

Meloidogyne oryzae is well adapted to irrigated rice system and also reproduces well on tomato (including the populations from Brazil), differently from *M. graminicola* (Golden and Birchfield 1965). The populations of *M. oryzae* studied in this work reproduce by mitotic parthenogenesis, present 51–52 chromosomes and clustered in NJ tree/ITS region with mitotic populations, confirming the results obtained by (Esbenshade and Triantaphyllou 1985) and (Negretti et al. 2017) and

corroborating the contradiction between the generally accepted phylogenic position of *M. oryzae* and its mitotic parthenogenetic mode of reproduction, as highlighted by Castagnone-Sereno et al. 2013.

Neutral molecular markers (AFLP and RAPD) analysis revealed the genetic diversity among the rice-related *Meloidogyne* species and sustained the separation between *M. graminicola* and *M. oryzae* and the existence of a species complex in rice as already detected in Negretti et al. 2017.

All the mitotic parthenogenetic forms have probably evolved from meiotic parthenogenetic ancestors or, less likely, from amphimitic ones, following suppression of the meiotic process during maturation of the oocytes. The variation in chromosome numbers observed among the mitotic parthenogenetic forms suggests several pathways of derivation. Species with about 51–56 chromosomes like *M. oryzae* could be considered as triploids, and they were most likely derived following hybridization of meiotic parthenogenetic forms like *M. graminicola,* involving, for example, the fertilization of an unreduced egg with 36 (18 + 18) chromosomes, with a normal sperm having 18 chromosomes (Triantaphyllou 1985a). This evolutionary phenomenon could explain the simultaneous occurrence of *M. oryzae* and *M. graminicola* in the same areas in the state of Santa Catarina (Negretti et al. 2017) and there is probably a speciation process occurring in these Brazilian rice fields.

Finally, our results clarified the taxonomic position of *M. oryzae*, and illustrated the interest of using integrative taxonomy for accurate species description (Padial et al. 2010; Pante et al. 2015). Indeed, detailed morphological, morphometric, cytological and molecular studies were performed to correctly characterize this nematode from rice fields. Precise identification of nematode species is important to elucidate future biological or agronomic studies in order to clarify the distribution and importance of this species in the field, and thus to plan efficient control measures for the management of this disease in the southern region of Brazil.

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References

Araki, M. (1992). Description of *Meloidogyne ichinohei* n. sp. (Nematoda:Meloidogynidae) from *Iris laevigata* in Japan. *Japanese Journal of Nematology, 22*, 11–20.

Bellafiore, S., Jougla, C., Chapuis, E., Besnard, G., Suong, M., Vu, P. N., De Whale, D., Gantet, P., & Thi, X. N. (2015). Intraspecific variability of the facultative meiotic parthenogenetic root-knot nematode (*Meloidogyne graminicola*) from rice fields in Vietnam. *Comptes Rendus Biologies, 338*, 471–483.

Carneiro, R. M. D. G., & Almeida, M. R. A. (2001). Técnica de eletroforese usada no estudo de enzimas dos nematoides de galhas para identificação de espécies. *Nematologia Brasileira, 25*(1), 35–44.

Carneiro, R. M. D. G., Castagnone-Sereno, P., & Dickson, D. W. (1998). Variability among four populations of *Meloidogyne javanica* from Brazil. *Fundamental and Applied Nematology, 21*, 319–326.

Carneiro, R. M. D. G., Almeida, M. R. A., & Quénéhervé, P. (2000). Enzyme phenotypes of *Meloidogyne* spp. populations. *Nematology, 2*, 645–654.

Castagnone-Sereno, P., Danchin, E. G., Perfus-Barbeoch, L., & Abad, P. (2013). Diversity and evolution of root-knot nematodes, genus *Meloidogyne*: new insights from the genomic era. *Annual Review of Phytopathology, 51*, 203–220.

Coetzee, V. (1956). *Meloidogyne acronea*, a new species of root-knot nematode. *Nature, 4515*, 899–900.

De Ley, P., Felix, M. A., Frisse, L. M., Nadler, S. A., Stember, P. W., & Thomas, W. K. (1999). Molecular and morphological characterisation of two reproductive species with mirrorimage anatomy (Nematoda: Cephalobidae). *Nematology, 1*, 591–612.

De Waele, D., & Elsen, A. (2007). Challenges in tropical plant nematology. *Annual Review of Phytopathology, 45*, 457–485.

Eisenback, J. D., & Hirschmann, H. (1979). Morphological comparison of second-stage juveniles of six populations of *Meloidogyne hapla* by SEM. *Journal of Nematology, 11*, 5–16.

Eisenback, J. D., & Hirschmann, H. (1980). Morphological comparison of *Meloidogyne* males by scanning electron microscope. *Journal of Nematology, 12*, 23–32.

Eisenback, J. D., & Triantaphyllou, H. H. (1991). Root-knot nematode: *Meloidogyne* spp. and races. In W. R. Nickle (Ed.), *Manual of agricultural nematology* (pp. 191–274). New York: Marcel Dekker.

Eisenback, J. D., Hirschmann, H., & Triantaphyllou, A. C. (1980). Morphological comparison of *Meloidogyne* female lip region structures, perineal patterns, and stylets. *Journal of Nematology, 12*, 300–313.

Esbenshade, P. R., & Triantaphyllou, A. C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology, 17*, 6–20.

Golden, A. M., & Birchfield, W. (1965). *Meloidogyne graminicola* (Heteroderidae), a new species of root-knot nematode from grass. *Proceedings of the Helminthological Society of Washington, 32*, 228–231.

Handoo, Z. A., Huettel, R. N., & Golden, A. M. (1993). Description and SEM observations of *Meloidogyne sasseri* n. sp. (Nematoda: Meloidogynidae), parasitizing beachgrasses. *Journal of Nematology, 25*(4), 628.

Huelsenbeck, J. P., Bull, J. J., & Cunningham, C. W. (1996). Combining data in phylogenetic analysis. *Trends in Ecology & Evolution, 11*(4), 152–158.

Hunt, D. J., & Handoo, Z. A. (2009). Taxonomy, identification and principal species. In R. N. Perry, M. Moens, & J. L. Starr (Eds.), *Root-Knot Nematodes* (pp. 55–97). Wallingford: CABI.

Jepson, S. B. (1987). *Identification of root-knot nematodes (Meloidogyne species)*. Wallingford: CAB International.

Maas, P. W. T., Sanders, H., & Dede, J. (1978). *Meloidogyne oryzae* n. sp. (Nematoda, Meloidogynidae) infesting irrigated rice in Surinam (South America). *Nematologica, 24*, 305–311.

Negretti, R. R., Gomes, C. B., Mattos, V. S., Somavilla, L., Manica-Berto, R., Agostinetto, D., Castagnone-Sereno, P., & Carneiro, R. M. D. G. (2017). Characterisation of a *Meloidogyne* species complex parasitising rice in southern Brazil. *Nematology, 19*, 403–412.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology, 7*(1), 16.

Page, S. L. J. (1985). *Meloidogyne acronea*. In: *CIH Descriptions of Plant-parasitic Nematodes*, Set 8, No.114. Commonwealth Agricultural Bureaux, Farnham Royal.

Pante, E., Schoelinck, C., & Puillandre, N. (2015). From integrative taxonomy to species description: one step beyond. *Systematic Biology, 64*, 152–160.

Plantard, O., Valette, S., & Gross, M. F. (2007). The root-knot nematode producing galls on *Spartina alterniflora* belongs to the genus *Meloidogyne*: Rejection of *Hypsoperine* and *Spartonema* spp. *Journal of Nematology, 39*, 127–132.

Randig, O., Bongiovanni, M., Carneiro, R. M. D. G. & Castagnone-Sereno, P. (2002). Genetic diversity of root-knot nematodes from Brazil and development of SCAR markers specific for the coffee-damaging species. *Genome, 45*, 862–870.

Saitou, N., & Nei, M. (1987). The Neighbor-Joining method, a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, 406–425.

Schmitz, B., Burgermeister, W., & Braasch, H. (1998). Molecular genetic classification of Central European *Meloidogyne chitwoodi* and *M. fallax* populations. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, 50*, 310–317.

Siddiqi, M. R. (2000). *Tylenchida: parasites of plants and insects* (2nd ed.). Wallingford: CABI.

Suazo, A. & Hall, H. G. (1999). Modification of the AFLP protocol applied to honey bee (Apis mellifera L.) DNA. *Biotechniques, 26*(4), 704–705.

Subbotin, S., Waeyenberge, A.L. & Moens, M. (2000). Identification of cyst forming nematodes of the genus Heterodera (Nematoda: Heteroderidae) based on the ribosomal DNA RFLPs. *Nematology, 2*, 153–164.

Swofford, D. L. (2002). *PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4b10*. Massachusetts: Sinauer Associates.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Taylor, D. P., & Netscher, C. (1974). An improved technique for preparing perineal patterns of *Meloidogyne* spp. *Nematologica, 20*, 268–269.

Tigano, M. S., Carneiro, R. M. D. G., Jeyaprakash, A., Dickson, D. W., & Adams, B. J. (2005). Phylogeny of *Meloidogyne* spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. *Nematology, 7*(6), 851–862.

Triantaphyllou, A. C. (1985a). Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes. In J. N. Sasser & C. C. Carter (Eds.), *An Advanced Treatise on Meloidogyne. Vol. I. Biology and Control* (pp. 113–126). Raleigh: North Carolina State University Graphics.

Triantaphyllou, A. C. (1985b). Cytological methods for the study of oogenesis and reproduction of root-knot nematodes. In K. R. Barker, C. C. Carter, & J. N. Sasser (Eds.), *An advanced treatise on Meloidogyne: Methodology* (Vol. 2, pp. 107–114). Raleigh: North Carolina State University Graphics.