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**Integrative taxonomy of *Meloidogyne graminicola* populations with different esterase phenotypes parasitizing rice in Brazil**

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## Summary

The rice root-knot nematode *Meloidogyne graminicola* has been reported in Southeast Asia, China, India, South Africa, United States, Brazil and other countries. Recent surveys in Southern Brazil showed high spread of *M. graminicola* in irrigated rice in Rio Grande do Sul, Santa Catarina and Paraná states and the presence of a species complex with predominance of *M. graminicola* (Est VS1=G1) and other variants showing close esterase phenotypes (Est G2 = R2, G3=R3). *Meloidogyne oryzae* (Est O1) and *M. ottersoni* (Est Ot0) were also part of its complex and were recently redescribed and detected on rice. The present study provides an integrative taxonomy approach of the typical and atypical populations of *M. graminicola* on the basis of morphological, morphometrical and molecular data. Considering morphological and morphometric features, the two atypical populations (Est G2 and G3) are in close agreement with the description of *M. graminicola*. Based on the molecular characterization, populations G1, G2 and G3 were successfully amplified by *M. graminicola* SCAR markers, although the specificity of these markers was questioned. Phylogenetic relationships complemented and confirmed the other studies. In Maximum likelihood analysis of ITS, D2D3 rRNA and COXII-16S rRNA sequences, all populations of *M. graminicola* from different esterase phenotypes clustered together with other *M. graminicola* populations, thus confirming that these enzyme phenotypes (G1, G2 and G3) are related to the same species. A high level of intraspecific variability was detected among all populations, but no correlation between genetic variability and geographic origins occurred.

**Keywords** – esterase phenotypes, COXII-16S rRNA, D2D3 rRNA, ITS, morphology, *Oryza sativa*, root-knot nematode.

## Introduction

Rice (*Oryza sativa* L.) is the third most important cereal crop in the world, just behind wheat and maize, and provides food security for more than half of the world's human population. It is the second most consumed food in the entire globe, and Brazil has a leading position among the largest rice producers (Chauhan *et al.*, 2017). The southern states of the country are the largest producers, responsible for more than 82% of all national production, with the predominance of flooded rice (Conab, 2019).

Several factors can interfere in rice crop productivity, including nematodes. More than 35 genera have been reported parasitizing rice plants, but few have been described causing significant damage (Khan, 2015). However, several authors reported the predominance in rice agrosystems of *M. graminicola* Golden & Birchfield, 1965, an obligate sedentary endoparasitic species that is well adapted to the flooded conditions of rice cultivation (De Waele & Elsen, 2007; Khan, 2015).

*Meloidogyne graminicola* was first isolated from barnyard grass *Echinochloa colonum* L. growing in a field at Baton Rouge, Louisiana, USA. Since then, *M. graminicola* has been identified in many countries, presenting a distribution of rice growing areas throughout South and South-East Asia and in the USA (Peng *et al.*, 2018), Latin America (Mantelin *et al.*, 2016) and Europe (e.g., Italy; Fanelli *et al.*, 2017).

In Brazil, Ribeiro *et al.* (1984) reported the occurrence of *Meloidogyne* sp. in Rio Grande do Sul (RS) on flooded rice, with suspected detection of *M. graminicola*. The first official report of *M. graminicola* in Brazil was made by Monteiro & Ferraz (1988) in Cyperacea collected in Presidente Prudente, São Paulo state. However, this species was only reported for the first time on rice in RS state in 1991 by Sperandio & Monteiro, in the municipality of Palmares do Sul. Recent surveys showed high spread of *M. graminicola* in irrigated rice in RS, Santa Catarina (SC) and Paraná (PR) states (Negretti *et al.*, 2017;

Mattos *et al.*, 2017) and the presence of a species complex associating the predominant *M. graminicola* species and other variants, including *M. oryzae* Mas, Sanders & Dede, 1978 and *M. ottersoni* (Thorne, 1969) Franklin, 1971. These two species were recently redescribed from SC state, Brazil by Mattos *et al.* (2018) and Leite *et al.* (2020), respectively.

The international literature reported evidence of high variability occurring within isolates of *M. graminicola* from India (Salalia *et al.*, 2017) and from Italy, the USA and China (Fanelli *et al.*, 2017). These authors agreed on the existence of two groups of *M. graminicola* (based on COXII-16S rRNA genetic analysis): group A, which clusters the populations from the USA and Italy, and group B, those from China. Pokharel *et al.* (2007), using ITS sequences as genetic markers, also detected two groups (I and II) in Nepalese populations of *M. graminicola*: group I, clustering with *M. trifoliophila* Bernard & Eisenback, 1997, and group II with *M. graminicola* from the USA; it should be noted that all the investigated populations attacked rice and wheat. Pokharel *et al.* (2010) identified a new race of *M. graminicola* from Florida, USA, which did not parasitize rice; however, this new race has been identified as *M. graminicola* based on the ITS region and morphological and morphometrical characters that are not species-specific. More recently, Salalia *et al.* (2017) and Bellafiore *et al.* (2015) observed great morphological variability among populations of *M. graminicola* from India and Vietnam, respectively, and concluded using an ITS marker that all the diverse isolates from rice belonged to same species, *M. graminicola*. In addition, Salalia *et al.* (2017) hypothesized the presence of cryptic species among Indian populations. Isoenzyme phenotyping remains essential for accurate diagnosis of root-knot nematodes (RKN), *Meloidogyne* spp. (Hunt & Handoo, 2009). Because of the associations between species and esterase phenotypes, Esbenshade & Triantaphyllou (1985) designated these phenotypes with a letter suggesting the particular nematode species, followed by a number indicating the number of major bands of enzymatic activity. The following phenotype-

nematode species associations were recognized: H 1 for *M. hapla*, I 1 for *M. incognita* and J3 for *M. javanica*. The esterase phenotype designated as VS 1 (very slow with one band) had a large drawn-out band of high enzymatic activity and was observed by Esbenshade & Triantaphyllou (1985) in one population of *M. oryzae*. The same phenotype, with slightly slower band (Est VS1), was detected also in a population of *M. graminicola* and two populations from Panama and Costa Rica of an undescribed species from rice, lately described as *Meloidogyne salasi* Lopez, 1984. Considering the VS1 phenotype did not characterize a single species, it remained the esterase phenotype of these three species. In a survey carried out by Negretti et al. (2017), four variants of VS1 were found; these authors characterized *M. graminicola* as Est VS1 and named the other with the first letter of the name of the crop (rice, R1, R2, R3 ) to characterize the detected cryptic species. More recently, Mattos et al. (2018) identified *M. oryzae* through integrative taxonomy and named the esterase phenotype of this species as Est O1 (= Est R1) name of the species *oryzae*, with one band. Considering that, four similar esterase phenotypes (drawn-out bands with high enzymatic activity) were detected in different populations of *Meloidogyne* spp. from rice in Brazil. The Est VS1=G1 phenotype ( $R_m=0.70$ , extending from 0.68 to 0.72) is related to *M. graminicola* (Negretti et al., 2017); the Est O1= R1 phenotype ( $R_m=1.02$ , extending from 1.0 to 1.04) is related to *M. oryzae* (Mattos et al., 2018); two additional esterase phenotypes that were detected in undescribed species, Est R2 ( $R_m=0.85, 0.90$ , extending from 0.80 to 0.95) in *Meloidogyne* sp. 2 and Est R3 ( $R_m 0.75, 0.77$  and 0.80, extending from 0.74 to 0.82) in *Meloidogyne* sp.3 (Negretti et al., 2017; Mattos et al., 2017). The populations from *Meloidogyne* sp.3 were detected on rice predominantly in PR and less frequently in RS and SC states, respectively (Mattos et al., 2017). The populations from *Meloidogyne* sp.2 were detected in all three states, often mixed with populations of *M. graminicola* or *M. oryzae* (Mattos et al., 2017; Negretti et al., 2017).

Considering the high diversity observed in various populations of *M. graminicola* worldwide (Mantelin *et al.*, 2016), this study aimed to conduct an integrative taxonomic identification of two *Meloidogyne* populations from rice exhibiting atypical esterase phenotypes (Est R2 and R3), using morphological, morphometrical and molecular approaches, and to compare them with typical populations of *M. graminicola* which presented a close enzyme phenotype (i.e., Est VS1=G1) and are constantly evolving in the same crop (rice) and field conditions.

## **Material and Methods**

### POPULATION OF NEMATODES AND ENZYMATIC CHARACTERIZATION

Populations of *M. graminicola* (Est VS1), *Meloidogyne* sp.2 (Est R2) and *Meloidogyne* sp.3 (Est R3), collected on flooded rice from three Brazilian states (RS, SC and PR; Table 1), were multiplied in rice plants (*Oryza sativa* L.- IRGA-410 and IRGA-424) in greenhouse conditions, with an average temperature of 25-28 °C. Populations were previously purified using esterase phenotypes following the methodology described by Carneiro & Almeida (2001), performed on 7% polyacrylamide gel slab isozymes in a horizontal electrophoresis system. *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (Est J3, MDH N1) was used as reference. Both esterase (Est) and malate dehydrogenase (Mdh) enzymes were investigated. Cytogenetic, morphological, morphometrical and molecular studies were done using pure cultures, and morphological and cytogenetic studies were performed using a subset of three populations (Table 1). Egg masses and females were obtained directly from the infected rice roots. Second-stage juveniles (J2) were hatched in humid chambers from egg-masses. Males were obtained placing the infected rice roots in aerated water, using an aquarium pump. The specimens were collected periodically from the water (every 48 hours) (Carneiro *et al.*, 1996).

## MORPHOLOGICAL AND MORPHOMETRIC STUDIES

Morphological and morphometric studies were performed with at least 20 specimens from each stage. Males and J2 were killed in cold 2% formaldehyde (at 5°C), measured and photographed immediately under light microscope. Morphometric studies were performed using the measurements suggested by Eisenback & Hunt (2009). The biometric studies were performed with adult females, cut in 45% lactic acid and their anterior region mounted with 2% formaldehyde at room temperature. The perineal cuts were mounted in glycerin (Taylor & Netscher, 1974). The photos and measurements were performed under a Zeiss - Axiophot microscope under white light. Females, male, J2 and stylets (females and males) were prepared for scanning electron microscopy (SEM), according to the methods proposed by Eisenback (1985). Samples were viewed and photographed with a scanning electron microscope JEOL-JSM-7001F.

## CYTOGENETIC STUDIES

Cytogenetic studies were carried out with a propionic orcein staining method (Triantaphyllou, 1985) using milky-white females extracted from roots.

## MOLECULAR CHARACTERISATION AND PHYLOGENETIC ANALYSES

DNA of *Meloidogyne* spp. from rice, originating from Brazil (Table 1) and previously identified by esterase phenotype, were extracted following the methodology described by Randig *et al.* (2002) and amplified using specific primers developed for *M. graminicola*: MgFW/MgRev (Bellafiore *et al.*, 2015), Mg-F3/Mg-R2 (Htay *et al.*, 2016) and GRAJ17F/GRAJ17F (Mattos *et al.*, 2019), according to each author's PCR instructions. For



this analysis, we also included one population of *M. oryzae*, *M. ottersoni* and *M. salasi* from our own RKN DNA collection at EMBRAPA (CENARGEN, Brasília, Brazil).

For the phylogenetic analyses, DNA of the *Meloidogyne* spp. populations was amplified and investigated using the primers specific for the ITS1- 5.8S-ITS2 region of rRNA (primer set: forward 5-TTGATTACGTCCCTGCCCTTT-3 and reverse 5-TCCTCCGCTAAATGATATG-3; Schmitz *et al.*, 1998), the D2-D3 fragment of the 28S rRNA gene (primer set: forward 5-ACAAAGTACCGTGAGGGAAAGTTG-3 and reverse 5-TCGGAAGGAACCAGCTACTA-3; De Ley *et al.*, 1999), and the region between the 3' portion of COXII and the 5' portion of 16S rRNA region (forward 5'-GGTCAATGTTTCAGAAATTTGTGG-3' and reverse 5'-TACCTTTGACCAATCACGCT-3'; Power & Harris, 1993), using the PCR conditions described by Subbotin *et al.* (2000). PCR products were cleaned using the Wizard<sup>®</sup> SV Gel/PCR Clean Up System (Promega) and cloned into the pGem-T<sup>®</sup> Easy Vector (Promega), following the manufacturer's instructions. Sequencing of the insert was carried out on two independent clones by Macrogen.

Sequences cloned in our laboratory were aligned using ClustalW in Mega 5.0.3 (Tamura *et al.*, 2011) and compared with other sequences retrieved from the NCBI database. The program MrModeltest (Nylander, 2004) implemented in PAUP (Phylogenetic Analysis Using Parsimony; v.4.0 b10) was used to identify the best-fit models of each analysis. The phylogenetic trees were generated based on Maximum Likelihood (ML) analysis in IQtree (Trifinopoulos *et al.*, 2016). The generated phylograms were bootstrapped 1,000 times and only values above 50% were considered.

## Results

### ENZYMATIC CHARACTERIZATION

Similar esterase phenotypes with drawn-out bands exhibiting high enzymatic activity were detected in different populations of *Meloidogyne* spp. from rice in Brazil. The phenotype Est VS1=G1 ( $R_m=0.70$ , extending from 0.68 a 0.72) is related to the typical *M. graminicola* (Carneiro *et al.*, 2000) Two other close esterase phenotypes were detected in other populations (Est R2,  $R_m=0.88$ , extending from 0.80 to 0.95) and (Est R3,  $R_m= 0.77$ , extending from 0.74 to 0.82). These three populations with different esterase phenotypes were characterized afterwards by morphological and molecular approaches (below) and the populations were identified as variants of *M. graminicola* (Est G1, G2 and G3) (Table 1, Fig. 1). The populations from the three Est phenotypes presented the same N1a malate dehydrogenase (MDH) phenotype (Fig. 1).

### MORPHOLOGICAL AND MORPHOMETRIC STUDIES

The morphology and morphometric data of the three different esterase phenotype populations of *M. graminicola* studied here are similar to the original descriptions of *M. graminicola* (Golden & Birchfield, 1965; Mulk, 1976; Jepson, 1987; Eisenback & Triantaphyllou, 1991; Hunt & Handoo, 2009), with some variations frequent in the genus *Meloidogyne*, and expected considering the high variability observed in this species. The measurements are shown in Table 2, and the morphological features of females, males and J2 were based on the characters of the three populations G1, G2 and G3 (Table 1).

#### *Females*

The morphology of females is shown in Figures 2 and 3. The body is variable in shape and size, elongate or globular to pear shaped, translucent-white, neck variable short to long (58-154  $\mu\text{m}$ ). Perineal patterns rounded to dorsoventrally ovoid (Fig. 2 A-F), dorsal

arch usually low but may be high and rounded. The tail terminal area may be flat with shallow transverse striae in SEM (Fig. 2 A, B) or it may be raised and surrounded by numerous irregular striae in Light Microscopy (LM) (Fig. 2 C-F). Perivulval region usually free of striae and broken striae above the anus. No punctuations were observed. Lateral fields are not clearly defined, and phasmids are obscure. In SEM, posterior protuberance distinct (Fig. 2B) and small lines continuous bordering anal-vulval region. The perineal patterns of populations G1, G2 and G3 were very similar.

In LM, the excretory pore located two stylets below head end (Fig. 3 A). In SEM labial disc fused with medial lips and forms one continuous head cap, each medial lip frequently with some lobes in populations G1, G2 and G3 (Fig. 3 B, C, D). Lateral lips distinct, triangular. Amphidial apertures presenting rectangular slits. Labial region surrounded by a collar-shaped structure present in most specimens of G1 and G2 populations (Fig. 3 B-C), but absent in population G3 (Fig. 3 E). Stylet robust (11-14  $\mu\text{m}$ ) depending on population (Table 2), cone slightly curved dorsally and gradually increasing in diameter posteriorly. Shaft gradually widening posteriorly to near junction with stylet knobs, knobs rounded, sometimes bilobed and set off from the shaft (Fig. 3 D, F). Dorsal gland orifice distance (DGO) 3-5  $\mu\text{m}$  (Table 2). This description is valid for the three *M. graminicola* populations (Est G1, G2, G3) analyzed in this study.

### *Males*

Measurements of 20 males are listed in Table 2. Males were often found. Body vermiform variable in size, tapering anteriorly, tail conoid, sometimes with an expansion region in the end (Fig. 3 I), sometimes twisting 90°. Cuticular annulations coarse (Fig. 4 B, C, G, H). Head cap high, rounded; lip region distinct from the first body annule, with few annulations in SEM (Fig. 1 B, C) in the three populations (Est G1, G2, G3). Labial disc and medial lips fused and form one smooth, continuous head cap. The posterior edges of the

medial lips are rounded and extend onto the lip region. Labial disc slightly elevated above medial lips. Lateral lips may be demarked on the lip region by shallow grooves. Amphidial apertures elongate slits (Fig. 4 B, C). Stylet robust (15-20  $\mu\text{m}$ , 17  $\mu\text{m}$ ), cone pointed at the tip, widening at junction with shaft, shaft cylindrical. Knobs large oval-shaped tapering onto shaft, round posteriorly (Fig 4 D, E, F). Dorsal gland orifice (DGO) 3-5  $\mu\text{m}$  (4  $\mu\text{m}$ ), from stylet base. Lateral fields sometimes crenated composed of numerous (6-12) sometimes broken incisures (Fig. 4 G, H). Spicules and gubernaculum arcuate. This description is valid for the three *M. graminicola* populations (Est G1, G2, G3) presented in this study.

#### *Second-stage juveniles (J2).*

Measurements of 20 J2 are listed in Table 2. Body cylindrical vermiform (400-570  $\mu\text{m}$ ), tapering to an elongated tail (59-91  $\mu\text{m}$ ). Head with slight cephalic frameworks not offset from the body without post labial annules (Fig. 5 A, C). Stylet delicate (10-13  $\mu\text{m}$ , 11.3  $\mu\text{m}$ ), knobs rounded tapering onto shaft. DGO measures 2-4  $\mu\text{m}$  from stylet base. Metacarpus broadly oval, valve large and heavily sclerotized (Fig. 5 B). In SEM, labial disc and medial lips fused and form one smooth, continuous head cap, bow-tie shaped. Six depressed sensilla surrounded prestoma (Fig. 5 A, C). Amphid apertures large slit-like. The elongate lateral lips are in the same contour with lip region (Fig. 5 A, C). Variable broken lateral fields with 4 to 6 incisures (Fig. 5 I). Rectum not inflated. Tail is very long and thin, constricting at one, two or three places, tapering to a slender, terminal digitiform tip (Fig. 5 D-H). Hyaline region of tail long and narrow (14-24  $\mu\text{m}$ , 19.6  $\mu\text{m}$ ).

#### CYTOGENETIC STUDY

The reproduction of *M. graminicola* populations (Est G1, G2 and G3) was by meiotic parthenogenesis, being the haploid chromosome number  $n= 18$ .

## MOLECULAR CHARACTERISATION AND PHYLOGENETIC ANALYSIS

All the three SCAR markers tested were able to identify Est G1 populations, and two of them (MgFW/MgRev and Mg-F3/Mg-R2) also identified G2 and G3 populations (Figure 6). GRAJ17F/GRAJ17 primers were intentionally designed not to amplify populations with Est G2 (=R2) and Est G3 (=R3) phenotype (Mattos *et al.*, 2019), because G2 and G3 were initially considered as cryptic species (Negretti *et al.*, 2017). None of the markers was specific to *M. graminicola*, since primers MgFW/MgRev and Mg-F3/Mg-R2 also amplified *M. oryzae* populations, and primers MgFW/MgRev and GRAJ17F/GRAJ17R also amplified *M. ottersoni*, respectively. Conversely, no unspecific amplification was observed with *M. salasi* for the three tested markers, and the marker developed by Mattos *et al.* (2019) applies perfectly to this species.

At the nucleotide level, intraspecific variability among G1, G2 and G3 populations was very low, ranging from 1 to 4 nucleotides for ITS and D2D3 sequences. The COXII-16S rRNA region also showed low intraspecific variability, i.e., 1-2 nucleotides between G1 and G2, and 8-9 nucleotides among G1, G2 and G3, respectively (data not shown).

In ML trees (Fig. 7, 8, 9), all *M. graminicola* populations of the different esterase profiles clustered together with *M. ottersoni*, *M. salasi* and possibly *M. trifoliophila* (not tested with COXII) in a clade with high bootstrap support of 99% and 98% for ITS and COXII sequences, respectively and 84% for D2D3.

Although no clear correlation between geographic origin and phylogenetic clusters was observed, the Brazilian populations were closely related to *M. graminicola* from Italy (LT669809; LT669810; LT669811; LT669812) and USA (AY757884.1) (Fig. 7 and 9). These populations were previously classified as type I according to Pokharel *et al.* (2007) for ITS and as group A according to Fanelli *et al.* (2017) and Salalia *et al.* (2017) for COXII-16srDNA.

On a wider scale, *M. graminicola* populations appeared more closely related to meiotic RKN species, e.g., *M. trifoliophila*, *M. ottersoni* and *M. naasi* Franklin, 1965, than to mitotic species such as *M. incognita* (Kofoed & White, 1919) Chitwood, 1949 and *M. javanica*.

The position of *M. oryzae* (mitotic species) is unclear, since it clustered with *M. graminicola* in the D2D3 and COXII ML analyses, and was positioned outside the *M. graminicola* clade (i.e., with mitotic obligatory parthenogenetic species) in the ITS tree.

## **Discussion**

The traditional methods of RKN identification are based on morphology (Jepson, 1987; Eisenback & Triantaphyllou, 1991; Hunt & Handoo, 2009), esterase phenotypes (Esbenshade & Triantaphyllou 1985; Carneiro *et al.*, 2000) and molecular markers (Blok & Powers, 2009). The identification and diagnosis of *Meloidogyne* spp. from rice have been a challenge due to conservative morphology, unclear species boundaries and complex taxonomy (Jepson, 1987), and a lack of SCAR markers for some species (Bellafiore *et al.*, 2015; Mattos *et al.*, 2018, 2019), which complicates the diagnosis. The RKN species found on rice have been generally classified as *M. graminicola*, based on incomplete morphometric characters, root symptoms (hook-like galls) and the ability of this species to cause infection in lowland rice (Pokharel *et al.*, 2007, Bellafiore *et al.*, 2015, Salalia *et al.*, 2017). However, detailed information on identity and variability of rice-related RKN is still lacking. Recently, Salalia *et al.* (2017), studying 14 populations belonging to the '*M. graminicola* group' from India, suggested that cryptic species may be occurring in a mixture within the rice RKN.

Tail morphology of *M. graminicola* J2 places it in Jepson's Group 11 (Jepson, 1987). This is the most well defined group within the genus *Meloidogyne*, which now includes 10

species that parasitize rice and present differential hyaline tail length: *M. graminicola* (19.7  $\mu\text{m}$ ), *M. graminis* (Sledge & Golden, 1964) Whitehead, 1968 (18.5  $\mu\text{m}$ ), *M. lini* Yang, Hu and Xu, 1988 (~ 14.0  $\mu\text{m}$ ), *M. hainanensis* Liao and Feng, 1995 (~15.3  $\mu\text{m}$ ), *M. salasi* (19.7  $\mu\text{m}$ ), *M. triticoryzae* Gaur, Saha and Khan, 1993 (17.6  $\mu\text{m}$ ), *M. marylandi* Jepson and Golden, 1987 (11.8  $\mu\text{m}$ ), *M. sasseri* Handoo, Huettel and Golden, 1995 (19.5  $\mu\text{m}$ ), *M. oryzae* (22.0  $\mu\text{m}$ ) (Mattos *et al.*, 2018) and finally *M. ottersoni* ( 20.5  $\mu\text{m}$  ) (Leite *et al.*, 2020). Most *Meloidogyne* species from rice (Group 11), including *M. graminicola*, were poorly described, as well as poorly characterized at the cytogenetic, enzymatic and molecular levels. Overall, some morphological and biological characters are common in these species: female body elongated, vulva sometimes situated on a posterior protuberance, semi-endoparasitism, with males abundant and females deeply embedded in the host (Jepson, 1987). An exception is made for *M. oryzae*, which reproduces by mitotic parthenogenesis and presents few males (Mattos *et al.*, 2018).

During a survey of *Meloidogyne* spp. carried out in Southern Brazil using isoenzyme esterase as a marker for species identification (Negretti *et al.*, 2017), four esterase phenotypes were identified occurring simultaneously in rice fields. *Meloidogyne oryzae* was identified first (Mattos *et al.*, 2018). Additionally, the G1 (=VS1) phenotype (Rm=0.70, extending from 0.68 to 0.72) was the most distributed and was related to *M. graminicola* (Carneiro *et al.*, 2000). The other two populations showed slight differences in migration (Est G2, Rm=0.88, extending from 0.80 to 0.95) and (Est G3, Rm= 0.77, extending from 0.74 to 0.82). They were analyzed by integrative taxonomy in this study and were identified as variants of *M. graminicola*, considering morphological, morphometrical and molecular approaches. Other species of *Meloidogyne* have shown variations in enzyme profiles. This is the case of *M. paranaensis*, which also presented three Est profiles designated as P1, P2 and P2a (Santos *et al.*, 2018; 2019). *Meloidogyne incognita* (Santos *et al.*, 2012) and *M. exigua*

Goeldi, 1887 (Muniz *et al.*, 2008) also presented more than one esterase profile for the same species.

In the present study, the three *M. graminicola* populations collected from rice-flooded areas in Brazil showed comparable morphology, morphometrics and molecular characteristics. The morphology of females of the three populations (Est G1, G2 and G3) showed variable body shapes, as observed for *M. graminicola* by Jepson (1987). The configuration of the perineal region, although similar in the three Brazilian populations, cannot be considered species-specific because it is common to other rice species such as *M. oryzae*, *M. salasi* and *M. lini*, among others.

Excised stylets and anterior region of female and males are important species-specific characters for the identification of *M. graminicola* (Jepson, 1987). The three populations studied here (Est G1, G2 and G3) presented stylets similar to those described by Jepson (1987) and Eisenback & Triantaphyllou (1991).

Female, male and J2 face views have been little explored in the international literature and are not included in the descriptions. The anterior regions of males were identical for the three populations G1, G2, and G3. The anterior region of *M. graminicola* females had never been studied before, using SEM, and proved to be very similar to *M. trifoliophyla* with lobes in the medial lips. Conversely, the presence of a collar-like structure in populations G1 and G2 had never been observed before. The face views of J2 in SEM (Est G1, G2 G3) were similar to the photograph published by Jepson (1987).

Biochemically, three distinct esterase profiles were observed in this study (Est G1, G2, and G3). The positions of the *M. graminicola* esterase bands are variable in different publications (Esbenshade & Triantaphyllou, 1985; Carneiro *et al.*, 2000) due to the drawn-out bands, which are difficult to measure (Rms). These three phenotypes had never been



clearly characterized before and could represent a new and species-specific character for *M. graminicola*.

Phylogenetically, the Brazilian populations of *M. graminicola* (Est G1, G2 and G3) clustered together with other *M. graminicola*. A low level of genetic distance was detected among *M. graminicola* populations for all the genetic regions analyzed, which may suggest a recent expansion of this species, in connection with studies on mitochondrial haplotypes that concluded that distant populations from Brazil and Southeast Asia share a common ancestral haplotype (Mantelin *et al.*, 2016; Fanelli *et al.*, 2017; Besnard *et al.*, 2019).

The separation between mitotic and meiotic parthenogenetic species in the ML trees reinforce the possible monophyletic origin of facultative meiotic species and highlights the heterogeneity of these species compared to mitotic parthenogenetic species (Castagnone-Sereno *et al.*, 2013; Fanelli *et al.*, 2017).

The presence of intraspecific variability in rDNA sequences was reported for *M. graminicola* (Pokharel *et al.*, 2007; Bellafiore *et al.*, 2015; Salalia *et al.*, 2017). However, Besnard *et al.* (2019) contested this information based on their comparative genomic study among 12 *M. graminicola* populations, assuming that the coexistence of divergent haplotypes in individuals and PCR-generated chimeric sequences could explain the high variation in ITS data reported earlier. It should also be emphasized that high morphometric variability was noticed in these previous works, raising the possibility of the existence of cryptic species in the studied samples, which was not observed in the present work.

Accurate identification of RKN species, including those associated with rice, is extremely important for planning management practices. For that purpose, intensive research efforts have been conducted to identify species-specific markers. Recently, three species-specific markers were proposed for *M. graminicola* (Bellafiore *et al.*, 2015; Htay *et al.*, 2016; Mattos *et al.*, 2018); unfortunately, they also amplify some populations of *M.*

*ottersoni* (Leite *et al.*, 2020) or are not able to detect G2 and G3 (Mattos *et al.*, 2018). Following their detection in rice fields in Southern Brazil, *M. oryzae* and *M. ottersoni* were characterized through integrative taxonomy (Mattos *et al.*, 2018; Leite *et al.*, 2020). These two species exhibited different morphology, esterase profiles and molecular sequences when compared with *M. graminicola*. However, due to their closely related characters and the similar root symptoms they induce, they could be misidentified in confusion with this species. The whole mitochondrial genome of *M. graminicola* has been recently sequenced (Besnard *et al.*, 2014; Sun *et al.*, 2014; Besnard *et al.*, 2019). As mitochondrial DNA evolves faster in Nematoda than in other taxa (Humphreys-Pereira & Elling, 2015; Lunt & Hyman, 1997), this region has shown an interesting diversity to separate *M. graminicola* from *M. ottersoni* (Leite *et al.*, 2020).

The genetic variability of *Meloidogyne graminicola* was also investigated using other molecular markers. For example, RAPD data showed a high level of variability for this species (Mattos *et al.*, 2018), similar to other facultative meiotic species like *M. exigua* (Muniz *et al.*, 2008). Variability higher than 60% was found among *M. graminicola* populations (with G1, G2 and G3 esterase phenotypes), which allowed them to be clearly separated from *M. salasi* and *M. oryzae* (Mattos *et al.*, (2018). However, it was doubtful at this stage whether G2 and G3 were variants of *M. graminicola* or cryptic species (Negretti *et al.*, 2017)

*Meloidogyne salasi* is morphologically (Lopez, 1984; Medina *et al.*, 2011) and enzymatically (Mattos *et al.*, 2018) similar to *M. graminicola*; however, this species was distinct according to RAPD markers, and a species-specific SCAR marker was made available for this species (Mattos *et al.*, 2018).

Altogether, our observations suggest that atypical esterase phenotypes previously detected in rice fields in southern Brazil (i.e., G1, G2, G3) correspond to *M. graminicola*

variants, which are spread in all the Southern states of Brazil, along with other RKN species as *M. oryzae* and *M. ottersoni*. In complement to previous works, this study now provides very clear knowledge of the occurrence of these three different species parasitizing rice in southern Brazil. A combination of tropical climate, high intensity of rice production and the ban of most of the pesticides used by Brazilian farmers could create favorable conditions for the presence of this RKN species and species variant complex. Further DNA species-specific markers still need to be developed on these *Meloidogyne* spp., as well as more agronomical/host range/resistance studies, in order to guarantee new control strategies for irrigated rice production.

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## Figure legends

**Fig. 1.** Esterase (a) phenotypes of *Meloidogyne graminicola* (Est G1 (=R1), G2 (=R2), G3 (=R3), and malate dehydrogenase (Mdh N1A). *Meloidogyne javanica* (J3, N1) was used as reference.

**Fig. 2.** *Meloidogyne graminicola* perineal patterns. A, B: Scanning electron microscopy (SEM), in B slight protuberance of vulval region; C-F: light microscopy (LM) micrographs. A, B, C: population G1, D: population G2, E, F: population G3.

**Fig. 3.** *Meloidogyne graminicola* females. A: Light microscopy (LM) micrograph of anterior region. Arrow showing excretory pore of female. B, C, E: Scanning electron microscopy (SEM) micrographs of anterior region, (B, population G3, C, population G2 and E, population G1); D, F: SEM of excised stylets of populations G2 and G3.

**Fig. 4.** *Meloidogyne graminicola* males. A, I: Light microscopy (LM) micrographs of anterior region (A) and tail (I), arrow indicate an expansion in the tail end. D, E, F: Scanning electron microscopy (SEM) micrographs of excised stylets. G, H: SEM of lateral fields. A, D, I: population G1; B, E, G: population G2; C, F, H: population G3.

**Fig. 5.** *Meloidogyne graminicola* second-stage juveniles. A, C: Scanning electron microscopy (SEM) micrographs of anterior region. B: Light microscopy (LM) micrographs of anterior region. D, E, F: SEM of tails in lateral views. G, H: LM of tails in ventral and lateral views. I: SEM of lateral fields. A, E, G, I: population G2; B, D: population G1; C, F, H: population G3.

**Fig. 6.** Gel representing the amplification of *Meloidogyne graminicola* (G1, G2 and G3) and other rice-related species (ORY=*M. oryzae*, OTT=*M. ottersoni* and SAL=*M. salasi*) for

designed species-specific primers: A. MgFW/MgRev (fragment size: 640 bp, Bellafiore *et al.*, 2015).

**Fig. 7.** Maximum Likelihood analysis of the phylogenetic relationships of *Meloidogyne graminicola* (G1, G2 and G3) and other *Meloidogyne* spp. populations based on the ITS1-5.8S-ITS2 rRNA sequences. Numbers to the left of the branches are bootstrap values for 1,000 replications. Accession numbers from GenBank are listed before the species names.

**Fig. 8.** Maximum Likelihood analysis of the phylogenetic relationships of *Meloidogyne graminicola* (G1, G2 and G3) and other *Meloidogyne* spp. populations based on the D2-D3 fragment of 28S rRNA sequences. Numbers to the left of the branches are bootstrap values for 1,000 replications. Accession numbers from GenBank are listed before the species names.

**Fig. 9.** Maximum Likelihood analysis of the phylogenetic relationships of *Meloidogyne graminicola* (G1, G2 and G3) and other *Meloidogyne* spp. populations based on the COXII-16S rRNA sequences. Numbers to the left of the branches are bootstrap values for 1,000 replications. Accession numbers from GenBank are listed before the species names.