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Development of Diagnostic SCAR Markers for *Meloidogyne graminicola*, *M. oryzae*, and *M. salasi* Associated with Irrigated Rice Fields in Americas

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Abstract

Root-knot nematodes (RKN) cause important production losses of rice (*Oryza sativa* L.) in the world. Together with *Meloidogyne graminicola* Golden and Birchfield 1965, *M. oryzae* Maas, Sanders and Dede, 1978 and *M. salasi* López, 1984 have been causing damages in irrigated rice fields in Central and South America. In addition, six other RKN species may occur in rice fields in other regions of the world. Correct identification of *Meloidogyne* spp. is difficult but essential for the management of rice RKNs. The objective of this study was to develop some species-specific molecular markers for the diagnosis of South American RKN rice-related species. Isozyme phenotypes indicated the occurrence of some RKN species in the Brazilian samples, namely *M. graminicola*, *M. oryzae*, *M. javanica*, and two cryptic species designated as *Meloidogyne* sp. 2 and *Meloidogyne* sp. 3. Random amplified polymorphic DNA (RAPD) analysis of 16 isolates revealed interspecific genetic polymorphism between *Meloidogyne* spp., but isolates belonging to the same species (i.e., sharing the same esterase phenotype) always clustered together, whatever the species considered. Specific SCAR markers of 230, 120, and 160 bp were developed for *M. graminicola*, *M. oryzae*, and *M. salasi*, respectively. These SCAR markers may be potential molecular tools for application in routine diagnostic procedures subject to their validation with other rice RKN field populations in the world.

Rice (*Oryza sativa* L.) is one of the world's most important staple food crops, and Brazil is among the 10 largest rice exporter countries, with a share of 1.7% in the international market (FAO 2017). The main Brazilian producer states are Rio Grande do Sul (RS) and Santa Catarina (SC) (CONAB 2017). Many nematode species have been found associated with rice (Bridge et al. 1990), among which the root-knot nematode (RKN) *Meloidogyne graminicola* Golden and Birchfield, 1965. This nematode causes production losses at a large scale (ranging from 11 to 80%) in irrigated rice systems in Asia and the Americas (Plowright and Bridge 1990; Soriano et al. 2000) and has been recently detected in North Italy (Fanelli et al. 2017). Because it may be responsible for intense crop damages, this nematode has been designated as a quarantine pest in many countries. In Brazil, *M. graminicola* has been detected since the 1980s (Monteiro and Barbosa Ferraz 1988). More recently, *M. oryzae* Maas, Sanders and Dede, 1978 was also detected in the SC state, causing damage to irrigated rice (Mattos et al. 2018; Negretti et al. 2017). *Meloidogyne salasi* López, 1984 is another RKN species present in Central and South America, which causes serious losses in rice fields in Costa Rica, Panama, and Venezuela (López 1984; Medina et al. 2009, 2011; Sancho and Salazar 1985). Recently, in a survey carried out in rice in Paraná state, Brazil, two cryptic species (based on their unusual esterase phenotypes) designated as *Meloidogyne* sp. 2 and *Meloidogyne* sp. 3, were the most prevalent, surpassing in importance *M. graminicola* (Soares 2017). All these RKN species reported to damage irrigated rice are difficult to identify at the species level, mainly owing to their morphological and morphometric similarities (Jepson 1987), although their correct identification is important for the implementation of adequate control strategies.

Neutral molecular markers as random amplified polymorphic DNA (RAPD) have been used to detect genetic variability within RKN species, and such methods are sensitive, rapid, and relatively simple, showing several loci scattered throughout the genome without requiring prior knowledge of the genetic information of the target species (e.g., Carneiro and Cofcewicz 2008; Carneiro et al. 1998; Castagnone-Sereno et al. 1994; Cenis 1993; dos Santos et al. 2012; Fargette et al. 2005; Randig et al. 2002). In addition, sequencing of differential DNA fragments resulting from these markers may be successfully used to design longer, species-specific polymerase chain reaction (PCR) primers and amplify sequence characterized amplified regions (SCARs) that do not face the problem of low reproducibility generally encountered with RAPDs. Such PCR-based detection tools used for RKN species are attractive because they are simple, fast, and can be used routinely on a large number of samples, particularly for quarantine purposes, without requiring prior multiplication of the target nematode, either living juveniles or females (Blok 2005; Blok and Powers 2009; Powers 2004; Randig et al. 2002; Tigano et al. 2010; Zijlstra 2000; Zijlstra et al. 2000). Recently, species-specific molecular markers have been developed for the identification of *M. graminicola* (Bellafiore et al. 2015; Htay et al. 2016), but the specificity of these markers has been questioned (Negretti et al. 2017).

In this study, the genetic variation and relationships of *Meloidogyne* spp. isolates representative of the species complex occurring on irrigated rice in southern Brazil were evaluated using RAPD neutral markers. Some of the amplified DNA fragments that were found to be specific for the major, described species parasites of rice in Central and South America (i.e., *M. graminicola*, *M. oryzae*, and *M. salasi*) were further cloned and transformed into SCAR markers to provide reliable tools for the specific diagnosis of these economically important pests.

Materials and Methods

Nematode isolates. The 16 rice nematode isolates analyzed in this study are listed in Table 1. Fourteen Brazilian isolates of *Meloidogyne* spp. were collected in irrigated rice crop fields, mostly in RS and SC states (Negretti et al. 2017), and further purified (Carneiro and Almeida 2001). Two other rice *Meloidogyne* isolates were added to the study as comparison: one *M. graminicola* isolate from the Philippines and one *M. salasi* isolate from Costa Rica (Table 1). All isolates were multiplied on rice plants (*O. sativa* cultivar BR-IRGA 410) under greenhouse conditions (28 to 30°C) for 3 months.

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Isoenzyme characterization. Esterase and malate dehydrogenase profiles of the 16 *Meloidogyne* spp. isolates were obtained as previously described in Carneiro et al. (1996) and Carneiro and Almeida (2001).

Nematode DNA extraction. Eggs were extracted from infected roots according to the method of Carneiro et al. (2004) and stored at –80°C. For each nematode isolate, total genomic DNA was extracted and purified from aliquots of 200 to 300 µl of eggs according to a protocol described by Randig et al. (2002). For SCAR assays, genomic DNA was also extracted from a single second-stage juvenile (J2) nematode and/or female following a method described by Castagnone-Sereno et al. (1995).

RAPD analysis. A batch of 25 random 10-mer oligonucleotide primers (Operon Technologies) was used for assessing the genetic diversity of the nematode isolates (Table 2). Amplification reactions were conducted with each primer on the DNA of the nematode isolates tested. PCR reactions were performed in a 13-µl final volume containing 1.3 µl of 10× PCR reaction buffer (Phoneutria Biotecnologia and Serviços), 4 µM primer (Operon Technologies), 2.5 mM deoxyribonucleotide triphosphate (Invitrogen), 1 U/µl Taq DNA polymerase (Phoneutria Biotecnology and Services), and 9 ng/µl *Meloidogyne* spp. total genomic DNA. Amplifications were 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, and 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), and visualized under ultraviolet light. All RAPD analyses were repeated at least two times, and only DNA fragments consistently present between replicates were recorded as present or absent directly from the gels. DNA fingerprints were converted into a 0 or 1 binary matrix, and relationships between isolates were determined using the neighbor-joining algorithm (Saitou and Nei 1987) implemented in PAUP* version 4b10 (Swofford 2002), considering the data as unordered with no weighting. To test the node support of the generated tree, the analysis was performed on 1,000 bootstrap replicates, and only values above 50% were considered.

Development of species-specific SCAR-PCR. RAPD fragments present only in *M. graminicola*, *M. oryzae*, or *M. salasi* were excised from the agarose gel and purified using the Wizard SV Gel and PCR Clean Up System (Promega). After cloning into the pGEM-T Easy vector (Promega) and transfer into *E. coli* DH10B by electroporation, two independent clones were sequenced for each RAPD fragment selected (GATC Biotech, Konstanz, Germany). From each consensus sequence, a pair of species-specific SCAR primers of 20 to 21 base pairs, spanning the corresponding RAPD primer sequences, was designed using PRIMER3 version 4.0 software (Rozen and Skaletsky 2000) and synthesized by Eurogentec (Seraing, Belgium) (Table 3).

SCAR analysis. Reaction mixtures for SCAR-PCR were the same as those described for RAPD analysis. Amplifications were performed on a Biometra T3000 thermocycler with the following cycling conditions for *M. graminicola* (primers GRA-J17-1F/R): 5 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 52°C, and 1 min at 70°C; and a final extension of 8 min at 70°C. Cycling conditions for *M. oryzae* (primers ORYA12F/R) and *M. salasi* (primers SALR12-1F/R) were the same as those described for *M. graminicola*, except that the annealing temperature was 56°C. Reaction conditions of SCAR-PCR using single nematodes (J2, males and females) were the same as described above for the three nematode species, except that a total of 40 cycles, double volume of the *Taq* DNA polymerase, and primer final concentration at 10 µM were used. Because some of these species may be found in mixtures under natural infection, we also performed duplex SCAR analyses with primer combinations for *M. graminicola* and *M. oryzae* and *M. graminicola* and *M. salasi*, respectively. Duplex SCAR-PCR mixtures were prepared as described for *M. graminicola*, using 10 µM of each primer pair (GRA-J17-1F/R with ORYA12F/R or SALR12-1 F/R, respectively) and 6 ng of purified DNA from eggs or DNA extract from a single individual for each species as a template. Amplification products were resolved in a 1.7% agarose gel, stained with ethidium bromide solution (0.3 µg/ml), and visualized under ultraviolet light. The experiments were conducted at least three times.

Results

The *M. graminicola*, *M. oryzae*, *Meloidogyne* sp. 2, and *Meloidogyne* sp. 3 isolates obtained from rice field samples (Negretti et al. 2017) were purified and presented the expected specific esterase phenotypes: VS-1 (0.70, extending from 0.68 to 0.72), O1 (Rm: 1.02 extending from 1.0 to 1.04), R2 (Rm: 0.85 and 0.91, extending from 0.91 to 1.0), and R3 (Rm: 0.74 and 0.80 to 0.82), respectively. In addition, all isolates tested displayed the same malate dehydrogenase profile N1a (Rm: 1.4) (Fig. 1A–D and F), confirming some previous results (Negretti et al. 2017). *M. salasi* from Costa Rica presented the VS1-2 esterase profile (Rm: 0.64 extending from 0.60 to 0.70), slightly different from the *M. graminicola* profile, and the three-bands malate dehydrogenase S3 phenotype (Rm 1.4, 1.6, 1.8) (Fig. 1E and G).

Table 1. List of the *Meloidogyne* spp. isolates used in this study

Population code	Species	Origin (municipality/state/country)[a]	GenBank accession number[b]	
			ITS	D2-D3
G2	*Meloidogyne graminicola*	Guaramirim/SC/Brazil	KY962646	KY962657
G4	*M. graminicola*	Camboriú/SC/Brazil	KY962647	KY962658
G6	*M. graminicola*	Camboriú/SC/Brazil	KY962648	…
G8	*M. graminicola*	Capão do Leão/RS/Brazil	KY962645	KY962659
G12	*M. graminicola*	Capão do Leão/RS/Brazil	KY962649	KY962660
G13	*M. graminicola*	Rio do Sul/SC/Brazil	KY962650	…
P	*M. graminicola*	Philippines[c]	KY962651	KY962661
Mo1	*M. oryzae*	Ilhota/SC/Brazil	KY962653	
Mo2	*M. oryzae*	Camboriú/SC/Brazil	KY962654	KY962662
Msp2RS	*Meloidogyne* sp. 2	Uruguaiana/RS/Brazil	KY962652	KY962663
Msp2SC	*Meloidogyne* sp. 2	Camboriú/SC/Brazil	…	…
Msp3RS	*Meloidogyne* sp. 3	Uruguaiana/RS/Brazil	KY962655	KY962664
Msp3SC	*Meloidogyne* sp. 3	Camboriú/SC/Brazil	…	…
Ms	*M. salasi*	Costa Rica[d]	KY962656	KY962665
Mj	*M. javanica*	Camboriú/SC/Brazil	…	…
Mi	*M. incognita*[e]	Londrina/PR/Brazil	…	…

[a] Brazilian states: RS = Rio Grande do Sul, SC = Santa Catarina, and PR = Paraná.
[b] Populations previously studied by Mattos et al. (2018). ITS = internal transcribed spacer.
[c] *M. graminicola* population from the Philippines (Dutch NPPO: E8256) was donated by Gerrit Karssen (Plant Protection Service, Wageningen, the Netherlands).
[d] *M. salasi* population was donated by Lorena Flores (Universidad de San José, Costa Rica).
[e] *M. incognita* isolate belongs to Embrapa Recursos Genéticos e Biotecnologia's collection and it is from coffee.

A total of 25 RAPD primers were used to evaluate the genetic diversity of the 16 *Meloidogyne* spp. isolates studied. In total, 584 DNA fragments were consistently amplified, and 208 were polymorphic (Fig. 2, Table 2). Overall, a variable level of intraspecific polymorphism was recorded according to the species considered (from 3.2 to 73.9% of polymorphic fragments; Table 3), but isolates belonging to the same species (esterase phenotype) always clustered together in the generated neighbor-joining tree, with high bootstrap support, whatever the species considered (Fig. 3). Within the *M. graminicola* cluster, the isolate from the Philippines was distinctly separated from those sampled in Brazil. Also, *M. salasi* appeared to be genetically distinct from the other species associated with rice and closer to *M. javanica* and *M. incognita*.

Among the 25 RAPD primers tested, three primers (J17, A12, and R12) were further selected based on the amplification of potential species-specific bands in *M. graminicola*, *M. oryzae*, and *M. salasi*, respectively. These DNA fragments were cloned, sequenced, and converted into SCAR markers, and the sequences were deposited in the NCBI database (GenBank accession nos. MH049592, MH049595, and MH049596, respectively). A BLAST search against the NCBI databases using these sequences resulted in no hit to any sequence deposited in the database (data not shown). As expected, simplex PCR reactions with the newly designed primers (i.e., GRAJ17-1F/R, ORYA12F/R, and SALR12-1F/R) resulted in species-specific fragments of 230, 120, and 160 bp for *M. graminicola*, *M. oryzae*, and *M. salasi*, respectively (Table 4, Fig. 4), whereas no amplification was detected for the other RKN species associated with rice (i.e., *Meloidogyne* sp. 2, *Meloidogyne* sp. 3, *M. javanica*, and *M. incognita*). In preliminary experiments, the same species-specific fragments were amplified using template DNA prepared from either a batch of nematodes or single individuals (J2 or females) (data not shown). Interestingly, these primer pairs were also successful in amplifying bands specific for their target species when used in duplex PCR reactions combining primers for *M. graminicola* with primers for either *M. oryzae* or *M. salasi* (Fig. 4).

Table 2. Code and sequence of the 25 random amplified polymorphic DNA (RAPD) oligonucleotide primers used with total number of reproducible amplified DNA fragments scored for the 16 *Meloidogyne* spp. analyzed

RAPD primer	Primer sequence (5′–3′)	Number of fragments
A12	TCGGCGATAG	31
AB02	GGAAACCCCT	11
AS08	GGCTGCCAGT	16
AU13	CCAAGCACAC	27
B05	TGCGCCCTTC	11
C07	GTCCCGACGA	11
C09	CTCACCGTCC	29
D13	GGGGTGACGA	27
G06	GTGCCTAACC	29
G13	CTCTCCGCCA	16
H01	GGTCGGAGAA	34
J17	ACGCCAGTTC	26
K20	GTGTCGCGAG	27
L20	TGGTGGACCA	27
M20	AGGTCTTGGG	24
N10	ACAACTGGGG	10
P05	CCCCGGTAAC	17
R03	ACACAGAGGG	18
R07	ACTGGCCTGA	35
R12	ACAGGTGCGT	29
W5	GGCGGATAAG	13
W6	AGGCCCGATG	55
X16	CTCTGTTCGG	23
Y06	AAGGCTCACC	13
Z07	CCAGGAGGAC	25

Table 3. Overview of random amplified polymorphic DNA (RAPD) data between *Meloidogyne* spp. isolates

Species	Number of RAPD fragments: Amplified	Number of RAPD fragments: Polymorphic (% in parentheses)
Meloidogyne graminicola	211	156 (73.9)
M. oryzae	110	11 (10.0)
Meloidogyne sp. 2	93	3 (3.2)
Meloidogyne sp. 3	132	15 (11.4)
M. salasi + *M. graminicola*	294	256 (87.0)
M. oryzae + *M. graminicola*	286	268 (93.7)

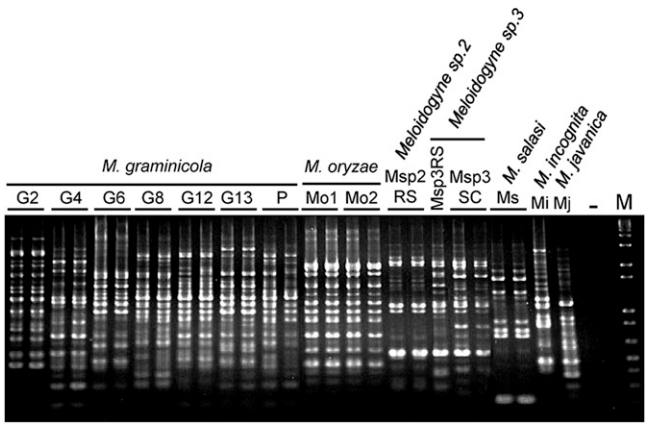

Fig. 2. Genetic diversity of *Meloidogyne* spp., analyzed with random amplified polymorphic DNA neutral markers (primer R12). Isolate codes are listed in Table 1. M = 1 kb DNA Plus Ladder.

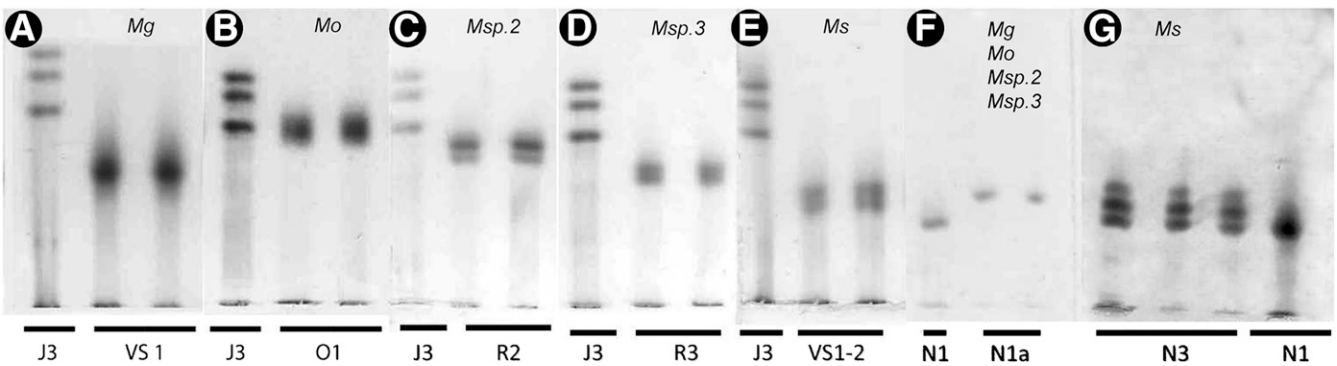

Fig. 1. Esterase (Est) and malate dehydrogenase (Mdh) phenotypes observed in the *Meloidogyne* spp. isolates studied: **A,** Est *M. graminicola* (VS1); **B,** Est of both populations of *M. oryzae* from Brazil (O1); **C,** Est of *Meloidogyne* sp. 2 (R2); **D,** Est of *Meloidogyne* sp. 3 (R3); **E,** Est *M. salasi* (VS1-2); **F,** Mdh N1a for *M. graminicola*, *M. oryzae*, *Meloidogyne* sp. 2, and *Meloidogyne* sp. 3; and **G,** Mdh for *M. salasi* (N3). For A–E, *M. javanica* (Est J3N1) was used as a reference.

Discussion

Isoenzyme characterization revealed the occurrence of a complex of RKN species associated with irrigated rice in southern Brazil (Negretti et al. 2017) and allowed the purification and characterization of four known species (*M. graminicola*, *M. incognita*, *M. javanica*, and *M. oryzae*) and also the detection of two atypical phenotypes, probably corresponding to two cryptic species *Meloidogyne* sp. 2 and *Meloidogyne* sp. 3.

However, although the specific distinction based on esterase phenotypes is generally efficient for the latter RKN species (Carneiro et al. 2000; Esbenshade and Triantaphyllou 1985), some risk of misidentification cannot be excluded. Indeed, little variation was observed between the esterase profiles of *M. graminicola* and *Meloidogyne* sp. 3, or when including *M. salasi* in the analyses.

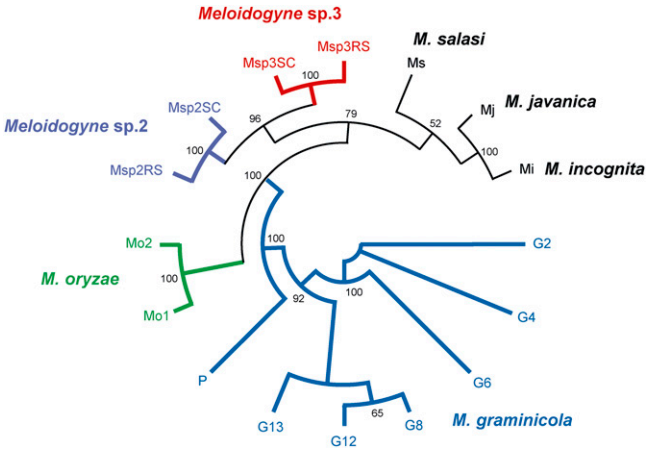

Fig. 3. Neighbor-joining analysis showing the relationship of *Meloidogyne* spp. isolates. The tree was built using the neighbor-joining algorithm implemented in PAUP* version 4b10, considering the data as unordered with no weighting. Bootstrap values (>50%) are shown based on 1,000 replicates.

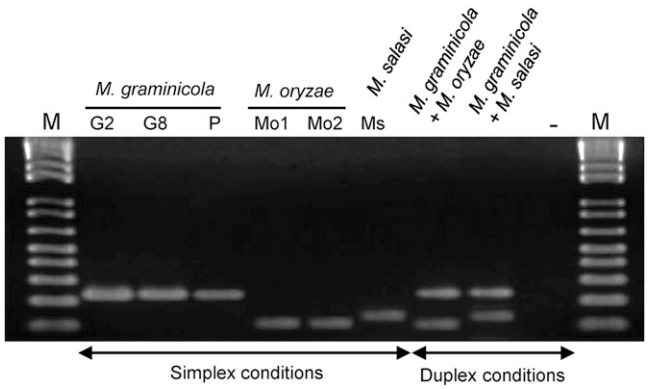

Fig. 4. Sequence characterized amplified regions–polymerase chain reaction amplification patterns for *Meloidogyne graminicola*, *M. oryzae*, and *M. salasi*, in simplex or duplex conditions.

Currently, this species has been found in Costa Rica, Panama, and Venezuela (López 1985; Medina et al. 2005), with a high incidence on rice crops (Medina et al. 2011; Sancho et al. 1987), but not in Brazil. However, under greenhouse conditions, a higher aggressiveness of *M. salasi* on the irrigated rice cultivar BR-IRGA 410 was observed compared with *M. graminicola* and *M. oryzae* (Vanessa Mattos, *unpublished*).

Accurate identification of RKN is an essential prerequisite for the implementation of successful management strategies based on the deployment of plant resistance. Whereas the characterization through isoenzyme phenotypes is restricted to *Meloidogyne* females (Carneiro and Cofcewicz 2008), PCR-based methods are more suitable for routine diagnosis. Indeed, PCR is rapid and can be used with a large number of samples, because the identification of species can be performed using a single nematode, whatever its developmental stage. Recently, a species-specific SCAR marker developed for *M. graminicola* (Bellafiore et al. 2015) had its specificity questioned (Negretti et al. 2017). Another molecular marker based on the internal transcribed spacer (ITS) region, developed by Htay et al. (2016), was tested on RKN species collected in rice fields in Brazil, but despite successful amplification for all *M. graminicola* populations, a nonspecific signal was also detected for the cryptic species *Meloidogyne* sp. 3 (data not shown). Although both markers were developed based on sequences isolated from *M. graminicola* and compared with other *Meloidogyne* spp. sequences deposited in the NCBI GenBank database, such a lack of specificity may be because unfortunately RKN species associated with rice are poorly studied at the genetic level.

In the present study, three putative species-specific SCAR markers were developed to identify important RKN species associated with irrigated rice (i.e., *M. graminicola*, *M. oryzae*, and *M. salasi*). The specificity of these markers was validated using 16 populations of seven RKN species from different rice fields of southern Brazil, as well as populations from the Philippines and Costa Rica. Furthermore, in relation to the current distribution of species, duplex PCR allowed the simultaneous detection of species occurring in mixtures in the template preparation: for Brazil and Suriname, *M. graminicola* and *M. oryzae* can occur together, but *M. salasi* has not been reported so far in these countries; for Costa Rica, Panama, and Venezuela, *M. graminicola* can occur together with *M. salasi*, but *M. oryzae* has not been reported in these countries. Therefore, these SCAR markers represent new molecular tools that may be used for routine diagnosis after optimization of the amplification conditions with individual nematodes and validation on a larger set of populations. In Brazil, surveys in rice fields have been exclusively restricted to the southern states of the country. However, the use of a simple PCR-based identification method may reveal the presence of such RKN species in other regions of Brazil, as well as in other countries. In turn, rice crop surveys can help validate the markers under field conditions, such as the research conducted by Carneiro et al. (2005), in which they validated the SCAR markers developed by Randig et al. (2002) using RKN Brazilian populations sampled in coffee fields.

As an alternative to the use of SCAR markers for diagnostics, barcoding (i.e., sequence-based identification) has been considered in RKN, using either nuclear or mitochondrial genes as molecular targets (Hodgetts et al. 2016; Janssen et al. 2016). However, although promising, this approach did not yet provide end users with a

Table 4. Characteristics of sequence characterized amplified regions (SCAR) markers developed for *Meloidogyne* spp. associated with irrigated rice

Species	SCAR primers	Primer sequence (5′–3′)	Length (bp)
Meloidogyne graminicola	GRAJ17-1F	TTCGACTCTGTACGAAAGCC	230
	GRAJ17-1R	CAAAAGTAACCGGACACTCTTTT	
M. oryzae	ORYA12 F	CCAGCATCCGCTGTTGTAT	120
	ORYA12 R	AACAGGCTCCAGGTGAAAAG	
M. salasi	SAL R12-1F	CAAAGAACGGGGTTTATTCG	160
	SAL R12-1R	GGTTATCCGAACTCCCCAAT	

universal system that could be used indifferently regardless of the species considered, and it exhibited some limitations in distinguishing between closely related RKN species (Holterman et al. 2009; Kiewnick et al. 2014). Conversely, it is often recommended to combine the information from at least two types of sequences to get a robust and reliable identification (Kiewnick et al. 2014), which could rapidly become a problem as soon as a routine diagnostic protocol is required. For most of the RKN samples used in this study, including *Meloidogyne* sp. 2 and sp. 3, ITS and D2-D3 rRNA sequences are available (Table 1; Mattos et al. 2018). Unfortunately, their analysis did not allow a clear, phylogenetic differentiation of the taxa considered, which precludes any further use of these sequences as possible barcoding tools (Mattos et al. 2018).

Overall, our results contribute a molecular method for routine diagnosis of three important RKN rice-parasitic species in the Americas, but further studies on the other *Meloidogyne* species related to irrigated rice are needed to develop a future complete "rice diagnostic kit." However, we deliberately decided here not to investigate specific SCAR markers for the cryptic species *Meloidogyne* sp. 2 and sp. 3, because we considered it too preliminary at this stage to conclude about any new specific status for these two lineages. Although the SCAR developed for either *M. graminicola*, *M. oryzae*, or *M. salasi* failed to produce any amplification with both of them, a previous preliminary neighbor-joining analysis conducted on ITS1-5.8S-ITS2 rRNA sequences clustered the same *Meloidogyne* sp. 2 and sp. 3 samples with *M. graminicola* (Negretti et al. 2017), thus suggesting some close phylogenetic relationships between them. Currently, morphological, caryological, and additional molecular studies are being conducted in the laboratory on *Meloidogyne* sp. 2 and sp. 3 samples, to draw a final conclusion about their specific status. In the likely case in which they indeed belong to new species, an integrative taxonomy approach (Blok and Powers 2009; Janssen et al. 2017; Tautz et al. 2003), including the development of specific SCAR markers, will be conducted further.

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