Single Nucleotide Polymorphism Analysis Using KASP Assay Reveals Genetic Variability in *Rotylenchulus reniformis*

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Abstract

This study employed single nucleotide polymorphisms (SNPs) to determine the genetic variability present in 26 isolates of *Rotylenchulus reniformis* from Louisiana, Mississippi, Arkansas, South Carolina, Georgia, Hawaii, and Alabama. Genomic DNA from reniform nematode was extracted and increased quantitatively using the process of whole genome amplification. More than 162 putative SNPs were identified, 31 of which were tested using a KASP kompetitive allele-specific PCR genotyping assay. Of the SNPs tested, 13, 17, and 19 SNPs revealed genetic variability within reniform nematode isolates from Louisiana, Mississippi, and

Arkansas, respectively. Seven SNPs elucidated genetic differences among isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. Eight SNPs determined genetic variability among individual isolates from South Carolina, Georgia, Hawaii, and Alabama. This study is the first to report genetic variability in geographic isolates of reniform nematode employing a SNP assay. This study also demonstrated that SNP markers can be used to evaluate isolates of *R. reniformis* and could be useful to assess their genetic diversity, origin, and distribution. Such information would be extremely useful in resistance breeding programs.

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) has established itself as the major plant parasitic nematode of cotton and soybean in the United States (Allen et al. 2017; Arias et al. 2009; Leach et al. 2012; Robinson 2007; Stetina and Young 2006). A significant amount of cotton yield is lost to reniform nematode in the Southern United States, with Louisiana, Alabama, and Mississippi sustaining the greatest damage of 3.5, 4, and 5%, respectively (Lawrence et al. 2018). Similarly, 0.5, 0.8, and 1% of soybean yield is lost to reniform nematode in Florida, Mississippi, and Louisiana, respectively (Allen et al. 2017).

Current practices of reniform nematode management are based primarily on the use of chemicals or crop rotation. Chemical control is no longer a preferred method of reniform nematode management because of health and environmental issues (Khanal et al. 2018a). Selection of a suitable crop for rotation can help reduce losses caused by the nematode; however, reniform nematode populations resurge quickly to a damaging level in a single crop growing season when a susceptible crop is planted (Robinson et al. 2007). Use of host plant resistance has been the most desirable, but reniform nematode-resistant cotton cultivars are not available (Khanal et al. 2018a). Some cotton breeding lines and soybean cultivars showing moderate to high levels of resistance to reniform nematode are available; however, their yield performance is not always similar when tested across different geographic locations (Robinson et al. 1997, 2004; Weaver et al.

2007; Yik and Birchfield 1984). It is well known that durable host plant resistance is mainly dependent on the amount of variability present in a pathogen (Niblack et al. 2002; Noe 1992; Riggs et al. 1981; Van der Beek et al. 1999). The inconsistency in suppression of the reproduction of reniform nematode in different geographic locations by some available resistant cultivars/breeding lines has been reported (Bell et al. 2015; Robinson et al. 1997, 2004; Wallace et al. 2013; Weaver et al. 2007; Yik and Birchfield 1984). This inconsistency in host reaction could have been caused by the existence of physiological and genetic variability in geographic isolates of reniform nematode. Utilization of molecular techniques helps better understand the genetic variability in reniform nematode populations, which ultimately would be useful in developing durable reniform nematode-resistant cultivars.

Molecular techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), intersimple sequence repeat (ISSR), simple sequence repeat (SSR), sequence-related amplified polymorphism, and single nucleotide polymorphisms (SNPs) have been used in the last 3 decades to study genetic variations and characterize many organisms (Grover and Sharma 2016; Li and Quiros 2001). The first generation of molecular markers such as RFLP, RAPD, and AFLP are not currently much used for genetic variability assays because they are complex, costly, and identify low rates of polymorphism (Gao et al. 2016; Yang et al. 2017). ISSR and SSR are the second generation of genomic markers and are useful in determining a higher polymorphism rate (Gao et al. 2016). However, ISSR and SSR markers are often considered cumbersome to use in high-throughput genotyping protocols and they may not be widely and evenly distributed in the genome (Salem et al. 2012). The third generation of markers, SNPs (Gao et al. 2016), are superior for use in studying genetic variability because they are abundant and widely distributed in the genome (Salem et al. 2012). The advent of next generation sequencing (NGS) technology has enabled more efficient study of genetic variation in an organism using SNP analysis (Gao et al. 2016). The utility of SNPs allows genomic sequences of an organism to be compared in a rapid, reliable, and highly efficient way (Graves et al. 2016; Yang et al. 2017). SNP analyses have been useful in discriminating species of many organisms, including fungi, bacteria, viruses, nematode, plants, and animals (Faga et al. 2001; Figueiredo et al. 2013; Gao et al. 2016; Linlokken et al. 2017; Lu et al. 2013; Morais et al. 2006; Ojeda et al. 2014; Rattei et al. 2007; Von Samson-Himmelstjerna et al. 2007; Yang et al. 2017). More

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importantly, SNPs are useful in discriminating among genotypes within a single species.

The existence of morphometric, physiological, and genetic variabilities within *R. reniformis* populations have been documented from different parts of the world, including Japan (Nakasono 2004), India (Dasgupta and Seshadri 1971), Africa (Germani 1978), Brazil (Rosa et al. 2003; Soares et al. 2003, 2004), and the United States (Agudelo et al. 2005; Arias et al. 2009; Khanal et al. 2018b; Kularathna et al. 2017; McGawley and Overstreet 1995; McGawley et al. 2010, 2011; Tilahun et al. 2008). The variability among geographic populations of *R. reniformis*, particularly genetic variability, has not always been obvious. A study conducted by Agudelo et al. (2005) showed no variation in the first internal transcribed spacer (ITS1) region of reniform nematode collected from 10 states in the United States. In contrast to the report by Agudelo et al. (2005), Tilahun et al. (2008) found substantial variation in ITS1 as well as in the 18S region of reniform nematode populations from Alabama. Regarding the lack of correlation between phenotypic and genotypic variation, Agudelo et al. (2005) suggested that the use of microsatellite markers could provide a more reliable way to evaluate populations. Studies conducted by Arias et al. (2009) and Leach et al. (2012) using microsatellite markers reported genetic variability in geographic isolates of reniform nematode. With the advent of NGS technology, it is possible to analyze whole genomic DNA of reniform nematode in much more detail using a robust molecular assay such as SNPs. Analysis of SNP data would be useful in determining genetic variability among and within geographic populations of reniform nematode. To date, there have not been any published reports of SNP molecular markers specific to reniform nematodes. Our goal in this study was to evaluate the utility of a selection of *R. reniformis*-specific SNPs in determining genetic variability among 26 isolates of reniform nematode at the molecular level.

Materials and Methods

Isolation and extraction of nematode. A total of 26 geographic isolates of reniform nematode (four from Louisiana, 12 from

Mississippi, six from Arkansas, and one each from South Carolina, Georgia, Hawaii, and Alabama) were used to evaluate putative SNPs and measure genetic variability (Table 1). Isolates LA1, LA2, LA3, LA4, MS1, MS2, MS3, and MS11 were from greenhouse populations derived from a single reniform nematode egg mass; isolates MS10, AR6, SC1, GA1, HI1, and AL1 were from curated greenhouse populations; and the remaining isolates were obtained from random field samples collected from the locations indicated in Table 1. From each isolate, individual or bulk reniform nematodes (gravid female with or without egg masses or vermiform life stages or combination of all life stages) were collected in 0.5-ml Eppendorf tubes. Gravid females with or without egg masses were excised from roots using a scalpel, tweezer, and stereoscopic microscope. Vermiform life stages were extracted from soil using the centrifugal/sugar flotation technique (Jenkins 1964). The extracted reniform nematodes were used for subsequent DNA extraction.

Genomic DNA extraction from nematode. DNA from single gravid females (Table 1) was prepared as part of previous research (Arias et al. 2009, 2011). For the four Louisiana samples and the three bulk (not from single egg masses) Mississippi samples (Table 1), DNA was extracted using a Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI). We added 500 μ l of cetyltrimethylammonium bromide buffer, 30 μ l of proteinase K (20 mg/ μ l), 2 μ l of RNase A (10 mg/ml; catalog number EN0531), and 2 μ l of lysozyme (500 ng/ μ l) to Eppendorf tubes containing the nematodes in a water solution. The tubes were briefly and vigorously vortexed. The tubes were then incubated for 2 h at 60°C with gentle shaking at 350 rpm. The tubes were then vortexed for 5 s to mix the solution. The solution obtained was processed using the Maxwell 16 FFS Nucleic Acid Extraction System (custom catalog number X9431; Promega). The supernatant, which is essentially genomic DNA, obtained at the end of process was transferred to labeled 1.5-ml Eppendorf tubes. The genomic DNA was quantified on a Synergy H1 microplate spectrophotometer (BioTek, Winooski, VT) at 260 nm ultraviolet absorption. DNA from the reniform nematode was isolated and whole genome amplified.

Table 1. Reniform nematode isolate identification number (ID), geographic origin, history, and source of DNA used for single nucleotide polymorphism analysis

Isolate ID	Origin of isolate ^a	Isolate history	Source of DNA ^b
LA1	West Carroll Parish, Louisiana	Greenhouse population derived from a single egg mass	Bulk females
LA2	Tensas Parish, Louisiana	Greenhouse population derived from a single egg mass	Bulk females
LA3	Rapides Parish, Louisiana	Greenhouse population derived from a single egg mass	Bulk females
LA4	Morehouse Parish, Louisiana	Greenhouse population derived from a single egg mass	Bulk females
MS1 ^c	Washington County, Mississippi	Greenhouse population derived from a single egg mass	Bulk females
MS2 ^c	Washington County, Mississippi	Greenhouse population derived from a single egg mass	Bulk vermiform
MS3 ^c	Washington County, Mississippi	Greenhouse population derived from a single egg mass	Bulk egg
MS4	Washington County, Mississippi	Field sample	Single female
MS5	Washington County, Mississippi	Field sample	Single female
MS6	Sunflower County, Mississippi	Field sample	Single female
MS7	Humphreys County, Mississippi	Field sample	Single female
MS8	Holmes County, Mississippi	Field sample	Single female
MS9	Holmes County, Mississippi	Field sample	Single female
MS10	Mississippi	Greenhouse population	Single female
MS11	Washington County, Mississippi	Greenhouse population derived from a single egg mass	Single female
MS12	Washington County, Mississippi	Field sample	Single female
AR1	St. Francis County, Arkansas	Field sample	Single female
AR2	St. Francis County, Arkansas	Field sample	Single female
AR3	Crawford County, Arkansas	Field sample	Single female
AR4	Crawford County, Arkansas	Field sample	Single female
AR5	St. Francis County, Arkansas	Field sample	Single female
AR6	Arkansas	Greenhouse population	Single female
SC1	South Carolina	Greenhouse population	Single female
GA1	Georgia	Greenhouse population	Single female
HI1	Hawaii	Greenhouse population	Single female
AL1	Alabama	Greenhouse population	Single female

^a Geographic location represented by the isolate.

^b Bulk females = 100 to 400 gravid females; bulk vermiform = 50,800 vermiform life stages; bulk egg = 66,000 nematode eggs; and single female = 1 gravid female.

^c MS1, MS2, and MS3 are from the same greenhouse population.

Whole genome amplification of genomic DNA. In order to conduct multiple SNP analyses, a larger amount of genomic DNA was required. A sufficient amount of genomic DNA for each reniform nematode sample was obtained by the process of whole genome amplification (WGA) as described by Arias et al. (2011) using GenomePlex Complete Whole Genome Amplification kits (catalog number WGA2; Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. The initial WGA for the single female isolates (Table 1) was prepared as described by Arias et al. (2011). The genomic DNA isolations for the bulk samples served as the DNA template for WGA. The procedure consisted of three broad steps: fragmentation, library preparation, and amplification. Briefly, for step 1 (fragmentation), 1 μ l of 10 $\times$ fragmentation buffer and 10 μ l of DNA (1 ng/ μ l) were pipetted in a 200- μ l polymerase chain reaction (PCR) tube. The tube was placed in a PTC-200 thermal cycler (MJ Research, Waltham, MA) at 95°C for 4 min. The sample was immediately cooled by placing the tube on ice for 2 min followed by a brief centrifugation to consolidate the contents. For step 2 (library preparation), 2 μ l of 1 $\times$ library preparation buffer and 1 μ l of library stabilization solution were added to the tube from step 1 and thoroughly vortexed. The tube was consolidated by centrifugation and placed in a PTC-200 thermal cycler at 95°C for 2 min. The sample was cooled again by placing the tube on ice for 2 min, then consolidated by centrifugation and returned to ice. One microliter of the library preparation enzyme was added to the tube, thoroughly vortexed, and briefly centrifuged. The tube was placed in a PTC-200 thermal cycler and incubated with the following conditions: 16°C for 20 min, 24°C for 20 min, 37°C for 20 min, and 75°C for 5 min. Tubes were

removed from the thermal cycler and briefly centrifuged. For step 3 (amplification), the 15- μ l library sample obtained in step 2 was used for the subsequent amplification process by adding 7.5 μ l of 10 $\times$ Amplification Master Mix, 47.5 μ l of water (molecular biology grade), and 5 μ l of WGA DNA polymerase for a total volume of 75 μ l. The tube was thoroughly vortexed, briefly centrifuged, and placed in a PTC-200 thermal cycler for amplification. The thermal cycler was set with the following conditions: an initial denaturation at 95°C for 3 min, followed by 28 cycles of denaturation at 94°C for 15 s, and annealing/extension at 65°C for 5 min. A 5- μ l aliquot of the final product, WGA amplified DNA, was run out on a 1.5% agarose gel to confirm that the procedure was successful. The remaining volume of WGA DNA was purified using a GenElute PCR Clean-Up Kit from Sigma-Aldrich (catalog number NA1020).

The WGA amplified DNA from each reniform nematode isolate was quantified on a Synergy H1 microplate spectrophotometer and stored at -20°C. In order to obtain enough DNA for the subsequent SNP analyses, all WGA DNA samples (derived from bulk and single female isolates, Table 1) were reamplified using the GenomePlex WGA Reamplification Kit (catalog number WGA3; Sigma-Aldrich) following the same PCR amplification procedure (step 3) described above for the initial WGA. Reamplified DNA samples were purified using the GenElute kits and concentrations determined on the microplate spectrophotometer.

Identification of SNPs. Putative SNPs were derived from an analysis of sequence information from 26 reniform nematode genomes generated using Nextera-tagmented reductivity-amplified DNA (nextRAD; SNPsaurus, Eugene, OR) technology. SNPs were

Table 2. Summary of SNP identification number, contig, sequence position, fluorescence label for each SNP, guanine and cytosine content, and LGC Genomics reference number for the SNP-specific assays used in this study

SNP ID ^a	Contig	Sequence position	Allele FAM ^b	Allele HEX ^c	GC% FAM ^d	GC% HEX ^d	GC% common ^d	LGC Genomics number ^e
RREN_4410_3972	4,410	3,972	T	C	54.50	57.10	61.90	1140749440
RREN_1572_36933	1,572	36,933	T	C	45.80	52.00	59.10	1140749445
RREN_4410_3979	4,410	3,979	A	G	56.00	59.10	73.70	1140749464
RREN_43396_315	43,396	315	T	G	44.00	50.00	59.10	1140749374
RREN_523_19992	523	19,992	A	C	42.30	45.80	48.00	1140749391
RREN_367_3958	367	3,958	T	G	52.20	54.50	39.30	1140749398
RREN_4834_4618	4,834	4,618	A	G	42.30	45.80	48.00	1140749415
RREN_5033_5267	5,033	5,267	T	C	42.30	44.00	59.10	1140749422
RREN_845_36717	845	36,717	A	G	35.70	38.50	48.00	1140749439
RREN_3215_15723	3,215	15,723	T	C	42.30	44.00	37.90	1140749446
RREN_1660_513	1,660	513	T	C	30.00	35.70	33.30	0216484048
RREN_4410_3946	4,410	3,946	A	G	65.00	68.40	59.10	0216484047
RREN_7711_4758	7,711	4,758	T	C	42.30	45.80	48.00	0216484024
RREN_514_63176	514	63,176	A	G	54.50	57.10	59.10	0216484023
RREN_925_39379	925	39,379	C	G	33.30	31.00	48.00	0216484000
RREN_514_63173	514	63,173	T	G	59.10	65.00	54.20	0216483999
RREN_91287_201	91,287	201	A	C	44.00	45.80	33.30	0216483976
RREN_91287_193	91,287	193	T	C	44.00	45.80	37.90	0216484070
RREN_43396_339	43,396	339	A	C	29.00	30.00	59.10	0216484049
RREN_1990_6847	1,990	6,847	A	G	37.00	44.00	46.20	0216484046
RREN_20709_1089	20,709	1,089	A	C	33.30	37.00	37.90	0216484025
RREN_258_12977	258	12,977	A	G	52.00	52.20	48.00	0216484022
RREN_269_9935	269	9,935	A	G	24.20	30.00	46.20	0216484001
RREN_456_104249	456	104,249	T	G	34.50	37.00	48.00	0216483998
RREN_43396_325	43,396	325	T	G	37.00	40.70	59.10	0216483977
RREN_901_49990	901	49,990	A	G	30.00	31.00	37.90	0216484069
RREN_1886_12077	1,886	12,077	C	G	37.00	37.00	48.00	0216484050
RREN_16875_158	16,875	158	T	C	24.20	30.00	73.70	0216484045
RREN_251_23034	251	23,034	T	C	52.20	54.50	50.00	0216484026
RREN_1895_31360	1,895	31,360	A	T	44.00	44.00	33.30	0216484021
RREN_9137_320	9,137	320	T	C	35.70	42.30	37.90	0216484002

^a Single nucleotide polymorphisms (SNPs) were chosen such that they were not clustered together in the genome. ID = identification number.

^b Primer labeled with dye FAM.

^c Primer labeled with dye HEX.

^d Percentage of guanine (G) and cytosine (C) in a SNP sequence.

^e LGC Genomics reference number for each SNP-specific assay tested in this research.

specifically selected to be on different contigs (i.e., presumably at different genomic locations) as reported for the reniform genome (RREN 1.0) in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/assembly/GCA_001026735.1/). Flanking sequences of 162 putative SNPs are presented in Supplementary Table S1. None of these nextRAD-derived putative SNPs have been previously evaluated to determine whether they actually perform as intended. Therefore, a subset of 31 putative SNPs that represent the entire reniform nematode genome were designed and manufactured (LGC Genomics, Teddington, U.K.) to function as dual emission fluorescent KASP competitive allele-specific PCR primers (Table 2). These KASP primers enable the biallelic scoring of SNPs at specific loci. The 31 KASP SNP primers (Table 2) were evaluated against 26 reniform nematode isolates (Table 1).

KASP genotyping assay. A total of 32 samples, which included four no-template controls and the 26 reniform nematode samples described above, were tested. The amplification reaction mix was prepared such that each reaction included 10 μ l of 1 $\times$ KASP Master Mix (LGC Genomics), 0.4 μ l of 1 $\times$ KASP Assay Mix (containing the allele-specific primers unique to each SNP, Table 2), and 9.6 μ l of WGA DNA (at a concentration of 12.5 ng μ l⁻¹). The PCR reactions were assembled in 96-well semiskirted PCR plates with white wells and clear frames (4ti-0951; 4titude Ltd., Wotton, Surrey, U.K.) using a Janus robot (PerkinElmer, Shelton, CT). The plate was sealed with qPCR adhesive seals (9095-10055; KBio, Beverly, MA) and placed in a PTC-200 thermocycler (Bio-Rad, Hercules, CA). PCR was conducted as follows: an initial denaturation at 94°C for 15 min; followed by 10 cycles of denaturation at 94°C for 20 s, and annealing/extension at 65°C for 1 min with a temperature reduction

Fig. 1. The single nucleotide polymorphism RREN_514_63173 distinguished four isolates of reniform nematode endemic in Louisiana, suggesting that genetic variability exists among the isolates. LA1, LA2, LA3, and LA4 represent the isolates of reniform nematode originated from West Carroll, Tensas, Rapides, and Morehouse Parish, respectively.

Table 3. Likely alleles as called by LightCycler 480 software after running an analysis of 31 single nucleotide polymorphisms (SNPs) on isolates from Mississippi, Arkansas, South Carolina, Georgia, Hawaii, Alabama, and Louisiana^a

SNP ID	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10	MS11	MS12
RREN_4410_3972	H	X	X	UNK	Y	H	X	X	X	X	UNK	X
RREN_1572_36933	H	H	H	H	H	Y	X	X	UNK	UNK	X	X
RREN_4410_3979	X	X	H	X	X	X	Y	H	X	Y	X	X
RREN_43396_315	—	—	X	—	—	—	X	X	UNK	X	—	—
RREN_523_19992	H	H	H	H	H	H	H	H	H	H	H	H
RREN_367_3958	Y	Y	Y	X	UNK	X	Y	Y	X	Y	X	X
RREN_4834_4618	H	H	—	X	Y	X	Y	Y	X	X	X	H
RREN_5033_5267	H	H	—	X	Y	H	X	X	H	UNK	UNK	X
RREN_845_36717	H	H	H	H	H	H	H	H	H	—	H	H
RREN_3215_15723	UNK	UNK	—	X	X	H	X	Y	UNK	H	H	Y
RREN_1660_513	H	H	—	H	H	H	X	X	H	UNK	H	UNK
RREN_4410_3946	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_7711_4758	Y	Y	X	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_514_63176	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_925_39379	H	H	H	Y	Y	H	H	Y	H	H	H	H
RREN_514_63173	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_91287_201	—	—	Y	—	—	—	Y	Y	—	Y	—	Y
RREN_91287_193	—	—	H	—	—	—	H	H	—	H	—	Y
RREN_43396_339	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
RREN_1990_6847	X	X	X	X	Y	X	X	X	Y	X	X	X
RREN_20709_1089	UNK	UNK	UNK	UNK	UNK	UNK	X	UNK	X	X	UNK	UNK
RREN_258_12977	X	X	X	X	X	X	X	X	X	X	X	X
RREN_269_9935	H	H	H	Y	UNK	X	Y	H	H	UNK	H	UNK
RREN_456_104249	X	X	X	X	X	X	X	X	X	X	X	X
RREN_43396_325	—	—	Y	—	—	—	Y	H	—	Y	—	—
RREN_901_49990	X	X	X	Y	X	H	UNK	X	H	Y	H	UNK
RREN_1886_12077	X	X	X	X	X	X	X	X	X	X	X	X
RREN_16875_158	—	—	Y	—	—	—	Y	Y	—	Y	—	—
RREN_251_23034	Y	Y	UNK	UNK	Y	H	UNK	UNK	H	UNK	Y	UNK
RREN_1895_31360	H	H	H	H	Y	Y	H	Y	H	Y	H	H
RREN_9137_320	H	H	H	Y	UNK	Y	H	UNK	H	Y	UNK	Y

(Continued on next page)

^a Single nucleotide polymorphisms (SNPs) were chosen such that they were not clustered together in the genome. Samples originated from locations in Mississippi (MS), Arkansas (AR), South Carolina (SC), Georgia (GA), Hawaii (HI), Alabama (AL), and Louisiana (LA). X, Y, and H represent LightCycler 480 software calls for FAM, HEX, or both fluorescent labels, respectively. UNK indicates that the specific allele could not be determined, and dashes indicate the failure of the SNP assay on that particular isolate. ID = identification number.

of -0.8°C per cycle; and a subsequent 26 cycles of denaturation at 94°C for 20 s, and annealing/extension at 57°C for 1 min. After amplification of the assay, the specific fluorescent emissions were read on a real-time PCR machine (LightCycler 480; Roche Diagnostics Corporation, Indianapolis, IN) and analyzed using proprietary Roche software (LightCycler 480 software version 1.5.1.62SP3) to determine which allele (or both alleles) was present in each sample. In the software, one allele was denoted as “X” and the other as “Y” based on emission wavelengths of the KASP assay. Mixtures of both alleles were denoted as “H” for heterozygotes. In some cases, the software was unable to determine the specific allele(s) present in a sample and the allele call was denoted as “UNK” (unknown).

Results

The procedures of WGA and WGA reamplification successfully produced sufficient quantities and quality of DNA from all 26 isolates of reniform nematode for SNP analysis. Of the 31 SNPs tested, 26 amplified genomic DNA of reniform nematode isolates from different geographic locations, while five SNPs, for the most part, failed to successfully amplify. As an example, RREN_514_63173 amplified and distinguished four isolates of reniform nematode that originated from Louisiana (Fig. 1). A summary of results from the KASP genotyping assays is presented in Table 3.

In the case of four reniform nematode isolates from Louisiana, 25 SNPs amplified all of the isolates, whereas six SNPs failed to amplify any of the isolates. Of the 25 positive SNPs, seven appeared to be monomorphic, while five identified only both alleles (i.e., heterozygous DNA) in all samples. The remaining 13 SNPs identified allelic variants (i.e., genetic differences) among the reniform nematode isolates from Louisiana.

For the 12 nematode isolates from Mississippi, five of the SNPs assays were inconclusive because some of them failed to amplify. Of the remaining 26 SNPs, seven were monomorphic, while two detected heterozygous loci in all isolates. The other 17 SNPs identified allelic variants among the reniform nematode isolates from Mississippi.

All 31 SNPs amplified the six isolates from Arkansas. Three SNPs were monomorphic, while the other three detected heterozygous loci in all isolates from Arkansas. From the remaining 25 SNPs, 19 identified allelic variants, meaning they were able to detect genetic differences in reniform nematode isolates from Arkansas.

In this study, we included one isolate accession each from South Carolina, Georgia, Hawaii, and Alabama. For these isolates, five SNPs failed to amplify genomic DNA; of the remaining 26 SNPs, eight identified allelic differences among the isolates from these four geographical areas. Because only a single isolate was available from these areas, genetic differences from within these locations could not be elucidated.

Of the 26 SNPs tested, seven were able to identify genetic differences between and among the isolates of reniform nematode from Louisiana, Mississippi, and Arkansas. These seven SNPs were RREN_4410_3972, RREN_1572_36933, RREN_4834_4618, RREN_5033_5267, RREN_269_9935, RREN_901_49990, and RREN_251_23034.

Discussion

To date, no SNPs have been reported in reniform nematode. In this study, we identified 162 reniform nematode-specific SNPs and developed a selection of 31 KASP assays to evaluate the SNPs. The results showed that the assays could detect differences among the

Table 3. (Continued from previous page)

AR1	AR2	AR3	AR4	AR5	AR6	SC1	GA1	HI1	AL1	LA1	LA2	LA3	LA4
H	X	UNK	H	X	H	UNK	H	Y	H	H	H	X	X
—	—	Y	Y	X	Y	UNK	H	H	H	H	Y	Y	Y
H	H	X	X	X	X	X	X	X	X	X	X	X	X
H	H	X	X	X	—	—	—	X	—	—	—	—	—
H	H	H	H	H	H	H	H	H	H	H	H	H	H
Y	UNK	Y	X	UNK	X	X	Y	Y	Y	Y	UNK	Y	Y
Y	Y	Y	H	Y	X	X	X	Y	X	X	H	H	H
UNK	UNK	H	Y	H	UNK	UNK	X	Y	UNK	X	Y	X	H
H	H	H	H	H	H	H	H	H	H	H	H	H	H
H	H	UNK	H	UNK	H	Y	UNK	Y	UNK	X	H	Y	H
Y	X	Y	H	UNK	H	H	H	H	H	H	H	H	H
Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
H	H	Y	Y	X	Y	Y	Y	Y	Y	Y	Y	Y	Y
Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	X	Y
—	—	H	H	Y	H	H	H	H	H	H	H	H	H
Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	H	X	Y
Y	Y	Y	—	—	—	Y	—	—	—	—	—	—	—
H	H	H	H	—	—	—	—	—	—	—	—	—	—
Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	—	—	—	—
H	UNK	X	X	X	X	UNK	X	X	X	X	X	X	X
Y	UNK	Y	Y	X	Y	UNK	X	Y	UNK	H	X	X	X
Y	X	X	X	X	X	X	X	X	X	X	X	X	X
Y	Y	X	Y	UNK	Y	H	Y	H	H	Y	H	H	Y
H	X	X	X	X	X	X	X	X	X	X	X	X	X
Y	Y	H	—	H	—	—	—	—	—	—	—	—	—
H	H	Y	H	X	X	X	X	X	UNK	Y	Y	Y	X
Y	X	X	X	X	H	X	X	X	X	X	X	X	X
Y	Y	Y	—	Y	—	—	—	—	—	—	—	—	—
Y	Y	X	X	UNK	UNK	X	H	UNK	H	X	X	X	H
H	H	H	H	H	H	H	H	H	H	H	H	Y	Y
UNK	Y	—	UNK	UNK	Y	Y	Y	UNK	H	H	H	H	H

geographic isolates of reniform nematode. Additionally, we demonstrated that the KASP SNP primers could work on whole genome amplified DNA.

The existence of genetic variability in *Caenorhabditis elegans* and various plant parasitic nematodes, including but not limited to cyst nematode (Caswell-Chen et al. 1992; Folkertsma et al. 1994; Kalinski and Huettel 1988; Silva et al. 2000), root-knot nematode (Guirao et al. 1995; Khanal et al. 2016; Semblat et al. 1999; Tigano et al. 2010), rice white tip nematode (Figueiredo et al. 2013), and reniform nematode (Agudelo et al. 2005; Arias et al. 2009; Tilahun et al. 2008), has been documented. Previous identifications, however, were based on use of first- and second-generation markers. This study is the first to report genetic variability among geographic isolates of reniform nematode using SNP analysis.

Most of the SNP assays we tested identified the two SNP alleles among isolates as well as heterozygotes within isolates. However, some SNP assays only identified one allele or the other among the isolates evaluated. Monomorphic SNPs across isolates likely indicate a lack of genetic diversity at that genomic location. Increasing the number of different genotypes (i.e., isolates) tested would most likely increase the frequency of all alleles detected. Heterozygous SNP assay results may indicate a mixture of nematode genotypes (e.g., in bulk samples or from gravid females with eggs resulting from single or multiple inseminations). Another possibility is that the single nematode from which the WGA DNA was derived was not homozygous at every genomic location.

Five of the 31 SNP assays evaluated did not perform as expected because of poor amplification. Better amplification could possibly be achieved by optimizing PCR conditions specifically for each assay. Although some SNPs failed to amplify, 26 of 31 SNPs tested amplified reniform nematode DNA, giving a success rate of 83.9%. This rate of success is comparable to similar studies conducted in plants with success rates of 78.5 to 88.4% (Cockram et al. 2012; Graves et al. 2016; Saxena et al. 2012; Semagn et al. 2014). Assuming a similar success rate for the putative SNPs not tested, most of those SNPs shown in Supplementary Table S2 should function.

Prior to this research, no reniform nematode-specific SNPs have been made available. The sequence information for each SNP can be used to prepare reniform nematode-specific SNP assays. For the 31 SNPs tested in this study using KASP assays, the sequence information can be used to design other types of SNP assays. Alternatively, the LGC Genomics reference number provided in Table 2 can be used to directly order the KASP assays directly from LGC Genomics.

Seven SNPs did appear to identify nonheterozygous differences among the reniform nematode isolates from Louisiana, indicating that there are identifiable molecular differences between the isolates. Khanal et al. (2018b) found significant differences in reproduction and pathogenicity among the reniform nematode isolates from greenhouse and microplot experiments that employed cotton as a host. The LA4 isolate (from Morehouse parish) had the greatest reproduction and pathogenicity on cotton, whereas the LA3 isolate (from Rapides parish) had the least. A parallel research conducted by Kularathna et al. (2017) involving the same isolates of reniform nematode and soybean as host found that the LA4 isolate had the least reproduction and the greatest pathogenicity, whereas the LA1 isolate (from West Carroll parish) had the greatest reproduction and the least pathogenicity. This study involving SNP analysis also indicated that significant variability does occur in isolates of reniform nematode from Louisiana. Six SNPs were found to be effective in distinguishing LA4 and LA3 isolates. Similarly, eight SNPs were found to be effective in distinguishing LA4 and LA1 isolates. Salem et al. (2012) reported that some SNPs are associated with biological functions in an organism. Because the isolates having different levels of reproduction and pathogenicity were also found to be genetically different in the SNP analysis, some of the SNPs are possibly associated with reproduction and pathogenicity functions. The association of SNPs with biological functions in reniform nematode should be explored in future experiments. Although a reference sequence for reniform nematode is available (RREN 1.0 assembly at NCBI), the sequence

has not been fully annotated. As the sequence annotation becomes more complete, genes in the areas around the SNPs that identified genetic differences in this study can be investigated for their potential relationships with reproduction and pathogenicity functions previously reported (Khanal et al. 2018b; Kularathna et al. 2017).

Mostly the SNPs functioned as designed and identified molecular differences among a broad range of nematode samples. Several SNPs were polymorphic not only for reniform nematode isolates within a single geographic location but also across several locations. This suggests that a significant amount of genetic variability exists in reniform nematode isolates from within and among geographic locations. Variability in reproduction and pathogenicity in geographically distinct reniform nematode isolates was reported previously in microplot studies involving cotton and soybean as hosts (Khanal et al. 2018b; Kularathna et al. 2017; McGawley et al. 2010, 2011). The results from this study further supported the existence of variability in geographic isolates of reniform nematode. This study also identified seven such SNPs that detected genetic differences between and among the geographic isolates of reniform nematode, indicating that these SNPs could be valuable markers to distinguish reniform nematode isolates in larger geographic areas. A greater number of SNPs and utilization of a greater number of reniform nematode isolates would provide more robust data and improve the ability of the markers to distinguish among the reniform nematode isolates. It was also observed that some SNPs polymorphic in one geographic isolate of *R. reniformis* were not always polymorphic in other geographic isolates. For example, RREN_514_63173 was polymorphic for reniform nematode isolates from Louisiana but it was monomorphic on reniform nematode from other geographic locations. Finding additional such SNPs would be useful in determining which geographic isolates of reniform nematode are more genetically diverse. Evaluation of a greater number of SNPs and isolates could lead to the development of a SNP panel that could be used to categorize reniform isolates from any location.

Several studies were conducted in the past to understand the amount of genetic variability in geographic isolates of reniform nematode, although with contrasting results. Agudelo et al. (2005) did not find any differences in the ITS1 marker of reniform nematode from the United States, Brazil, Colombia, and Honduras except for a population from Japan. In contrast to the findings of Agudelo et al. (2005), Tilahun et al. (2008) found a fairly substantial nucleotide difference in the ITS1 marker of the reniform nematode collected from Alabama. Microsatellite analyses have also been employed to determine genetic variability in populations of reniform nematode (Arias et al. 2009; Leach et al. 2012). Although microsatellite analysis is useful in determining polymorphism in a pathogen, SNPs are much more efficient and powerful markers (Salem et al. 2012). Results from the SNP analysis utilized in this research provide additional evidence for the existence of genetic variability within and among geographic isolates of *R. reniformis*. The SNP markers developed in this study will be useful in the assessment of the genetic diversity, origin, and subsequent distribution of this nematode. Additionally, once SNPs associated with genes for pathogenicity in reniform nematode are found, plant breeders can identify the corresponding genes for resistance in the host. Markers for the resistance genes can be used to help breeders develop resistant germplasm.

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Literature Cited

- Agudelo, P., Robbins, R. T., Stewart, J. M., and Szalanski, A. L. 2005. Intraspecific variability of *Rotylenchulus reniformis* from cotton-growing regions in the United States. *J. Nematol.* 37:105-114.
- Allen, T. W., Bradley, C. A., Damicone, J. P., Dufault, N. S., Fasje, T. R., Hollier, C. A., Isakeit, T., Kemerait, R. C., Kleczewski, N. M., Kratochvil, R. J., Mehl, H. L., Mueller, J. D., Overstreet, C., Price, P. P., Sikora, E. J., Spurlock, T. N.,

- Thiessen, L., Wiebold, W. J., and Young, H. 2017. Southern United States soybean disease loss estimates for 2017. Pages 3-8 in: Proceedings of the 44th Southern Soybean Disease Workers Meeting, Pensacola, FL.
- Arias, R. S., Stetina, S. R., and Scheffler, B. E. 2011. Comparison of whole-genome amplifications for microsatellite genotyping of *Rotylenchulus reniformis*. *Electron. J. Biotechnol.* 14:14.
- Arias, R. S., Stetina, S. R., Tonos, J. L., Scheffler, L. A., and Scheffler, B. E. 2009. Microsatellites reveal genetic diversity in *Rotylenchulus reniformis* populations. *J. Nematol.* 41:146-156.
- Bell, A. A., Robinson, A. F., Quintana, J., Duke, S. E., Starr, J. L., Stelly, D. M., Zheng, X., Prom, S., Saladino, V., Gutiérrez, O. A., Stetina, S. R., and Nichols, R. L. 2015. Registration of BARBREN-713 germplasm line of upland cotton resistant to reniform and root-knot nematodes. *J. Plant Regist.* 9:89-93.
- Caswell-Chen, E. P., Williamson, V. M., and Wu, F. F. 1992. Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *J. Nematol.* 24:343-351.
- Cockram, J., Jones, H., Norris, C., and O'Sullivan, D. M. 2012. Evaluation of diagnostic molecular markers for DUS phenotypic assessment in the cereal crop, barley (*Hordeum vulgare* ssp. *vulgare* L.). *Theor. Appl. Genet.* 125: 1735-1749.
- Dasgupta, D. R., and Seshadri, A. R. 1971. Races of the reniform nematode, *Rotylenchulus reniformis* Lindford and Oliveira, 1940. *Indian J. Nematol.* 1: 21-24.
- Faga, B., Maury, W., Bruckner, D. A., and Grose, C. 2001. Identification and mapping of single nucleotide polymorphisms in the varicella-zoster virus genome. *Virology* 280:1-6.
- Figueiredo, J., Simoes, M. J., Gomes, P., Barroso, C., Pinho, D., Conceicao, L., Fonseca, L., Abrantes, I., Pinheiro, M., and Egas, C. 2013. Assessment of the geographic origins of pinewood nematode isolates via single nucleotide polymorphism in effector genes. *PLoS One* 8:e83542.
- Folkertsma, R. T., Van Der Voort, J. N. A. M. R., Van Gent-Pelzer, M. P. E., Groot, K. E., Van Den Bos, W. J., Schots, A., Bakker, J., and Gommers, F. J. 1994. Inter- and intraspecific variation between populations of *Globodera rostochiensis* and *G. pallida* revealed by random amplified polymorphic DNA. *Phytopathology* 84:807-811.
- Gao, L., Jia, J., and Kong, X. 2016. A SNP-based molecular barcode for characterization of common wheat. *PLoS One* 11:e0150947.
- Germani, G. 1978. Caractères morpho-biométriques de trois espèces ouest-africaines de *Rotylenchulus* Linford & Oliveira 1940 (Nematoda: Tylenchida). *Rev. Nematol.* 1:241-250.
- Graves, H., Rayburn, A. L., Gonzalez-Hernandez, J. L., Nah, G., Do-Soon, K., and Lee, D. K. 2016. Validating DNA polymorphisms using KASP assay in prairie cordgrass (*Spartina pectinata* Link) populations in the U.S. *Front. Plant Sci.* 6: 1271.
- Grover, A., and Sharma, P. C. 2016. Development and use of molecular markers: Past and present. *Crit. Rev. Biotechnol.* 36:290-302.
- Guirao, P., Moya, A., and Cenis, J. L. 1995. Optimal use of random amplified polymorphic DNA in estimating the genetic relationship of four major *Meloidogyne* spp. *Phytopathology* 85:547-551.
- Jenkins, W. R. 1964. A rapid centrifugal-flotation technique for separating nematodes. *Plant Dis. Rep.* 48:692.
- Kalinski, A., and Huettel, R. N. 1988. DNA restriction fragment length polymorphism in races of the soybean cyst nematode, *Heterodera glycines*. *J. Nematol.* 20:532-538.
- Khanal, C., McGawley, E. C., Overstreet, C., and Stetina, S. R. 2018a. The elusive search for reniform nematode resistance in cotton. *Phytopathology* 108: 532-541.
- Khanal, C., McGawley, E. C., Overstreet, C., Stetina, S. R., Myers, G. O., Kularathna, M. T., McInnes, B., and Godoy, F. M. C. 2018b. Reproduction and pathogenicity of endemic populations of *Rotylenchulus reniformis* on cotton. *Nematropica* 48:68-81.
- Khanal, C., Robbins, R. T., Fiske, T. R., Szalanski, A. L., McGawley, E. C., and Overstreet, C. 2016. Identification and haplotype designation of *Meloidogyne* spp. of Arkansas using molecular diagnostics. *Nematropica* 46:261-270.
- Kularathna, M. T., Overstreet, C., McGawley, E. C., Stetina, S., Khanal, C., Godoy, F. M. C., and Xavier-Mis, D. M. 2017. Reproduction and pathogenicity of native isolates of *Rotylenchulus reniformis*, from Louisiana on soybean. *J. Nematol.* 49:508 (abstract).
- Lawrence, K., Hagan, A., Norton, R., Hu, J., Fiske, T., Hutmacher, R., Mueller, J., Small, I., Grabau, Z., Kemerait, R., Overstreet, C., Price, P., Lawrence, G., Allen, T., Atwell, S., Idowu, J., Boman, R., Goodson, J., Kelly, H., Woodward, J. E., Wheeler, T. A., and Mehl, H. L. 2018. Cotton disease loss estimate committee report, 2017. Pages 150-152 in: Proc. Beltwide Cotton Conf., Dallas, TX. Cordova, TN: National Cotton Council.
- Leach, M., Agudelo, P., and Lawton-Rauh, A. 2012. Genetic variability of *Rotylenchulus reniformis*. *Plant Dis.* 96:30-36.
- Li, G., and Quiros, C. F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in *Brassica*. *Theor. Appl. Genet.* 103:455-461.
- Linlokken, A. N., Haugen, T. O., Kent, M. P., and Lien, S. 2017. Genetic differences between wild and hatchery-bred brown trout (*Salmo trutta* L.) in single nucleotide polymorphisms linked to selective traits. *Ecol. Evol.* 7: 4963-4972.
- Lu, S., Edwards, M. C., and Friesen, T. L. 2013. Genetic variation of single nucleotide polymorphisms identified at the mating type locus correlates with form-specific disease phenotype in the barley net blotch fungus *Pyrenophora teres*. *Eur. J. Plant Pathol.* 135:49-65.
- McGawley, E. C., and Overstreet, C. 1995. Reproduction and pathological variation in populations of *Rotylenchulus reniformis*. *J. Nematol.* 27:508 (abstract).
- McGawley, E. C., Overstreet, C., and Pontif, M. J. 2011. Variation in reproduction and pathogenicity of geographic isolates of *Rotylenchulus reniformis* on soybean. *Nematropica* 41:12-22.
- McGawley, E. C., Pontif, M. J., and Overstreet, C. 2010. Variation in reproduction and pathogenicity of geographic isolates of *Rotylenchulus reniformis* on cotton. *Nematropica* 40:275-288.
- Morais, S., Marco-Moles, R., Puchades, R., and Maquieira, A. 2006. DNA microarraying on compact disc surfaces. Application to the analysis of single nucleotide polymorphisms in Plum pox virus. *Chem. Commun. (Camb.)* 22: 2368-2370.
- Nakasono, K. 2004. Studies on morphological and physio-ecological variations of the reniform nematode, *Rotylenchulus reniformis* Lindford and Oliveira, 1940 with an emphasis on differential geographical distribution of amphimictic and parthenogenetic populations in Japan. *J. Nematol.* 36:356-420.
- Niblack, T. L., Arelli, P. R., Noel, G. R., Opperman, C. H., Orf, J. H., Schmitt, D. P., Shannon, J. G., and Tylka, G. L. 2002. A revised classification scheme for genetically diverse populations of *Heterodera glycines*. *J. Nematol.* 34: 279-282.
- Noe, J. P. 1992. Variability among populations of *Meloidogyne arenaria*. *J. Nematol.* 24:404-414.
- Ojeda, D. I., Dhillon, B., Tsui, C. K. M., and Hamelin, R. C. 2014. Single-nucleotide polymorphism discovery in *Leptographium longiclavatum*, a mountain pine beetle-associated symbiotic fungus, using whole-genome resequencing. *Mol. Ecol. Resour.* 14:401-410.
- Rattei, T., Ott, S., Gutacker, M., Rupp, J., Maass, M., Schreiber, S., Solbach, W., Wirth, T., and Gieffers, J. 2007. Genetic diversity of the obligate intracellular bacterium *Chlamydophila pneumoniae* by genome-wide analysis of single nucleotide polymorphisms: Evidence for highly clonal population structure. *BMC Genomics* 8:355.
- Riggs, R. D., Hamblen, M. L., and Rakes, L. 1981. Intra-species variation in reactions to hosts in *Heterodera glycines* populations. *J. Nematol.* 13:171-179.
- Robinson, A. F. 2007. Reniform in U.S. cotton: When, where, why, and some remedies. *Annu. Rev. Phytopathol.* 45:263-288.
- Robinson, A. F., Bell, A. A., Dighe, N., Menz, M. A., Nichols, R. L., and Stelly, D. M. 2007. Introgression of resistance to nematode *Rotylenchulus reniformis* into upland cotton (*Gossypium hirsutum*) from *G. longicalyx*. *Crop Sci.* 47: 1865-1877.
- Robinson, A. F., Bridges, A. C., and Percival, A. E. 2004. New sources of resistance to the reniform (*Rotylenchulus reniformis*) and root-knot (*Meloidogyne incognita*) nematode in upland (*Gossypium hirsutum* L.) and sea island (*G. barbadense* L.) cotton. *J. Cotton Sci.* 8:191-197.
- Robinson, A. F., Inerra, R. N., Caswell-Chen, E. P., Vovlas, N., and Troccoli, A. 1997. *Rotylenchulus* species: Identification, distribution, host ranges, and crop plant resistance. *Nematropica* 27:127-180.
- Rosa, R. C. T., Moura, R. M., Pedrosa, E. M. R., and Chaves, A. 2003. Ocorrência de *Rotylenchulus reniformis* em Cana de açúcar no Brasil. *Nematol. Bras.* 27: 9395.
- Salem, M., Vallejo, R. L., Leeds, T. D., Palti, Y., Liu, S., Sabbagh, A., Rexroad, C. E., 3rd, and Yao, J. 2012. RNA-Seq identifies SNP markers for growth traits in rainbow trout. *PLoS One* 7:e36264.
- Saxena, R. K., Penmetsa, R. V., Upadhyaya, H. D., Kumar, A., Carrasquilla-Garcia, N., Schlueter, J. A., Farmer, A., Whaley, A. M., Sarma, B. K., May, G. D., Cook, D. R., and Varshney, R. K. 2012. Large-scale development of cost-effective single-nucleotide polymorphism marker assays for genetic mapping in pigeon pea and comparative mapping in legumes. *DNA Res.* 19:449-461.
- Semagn, K., Babu, R., Hearne, S., and Olsen, M. 2014. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): Overview of the technology and its application in crop improvement. *Mol. Breed.* 33:1-14.
- Semblat, J. P., Bongiovanni, M., Wajnberg, E., Dalmasso, A., Abad, P., and Castagnone-Sereno, P. 1999. Virulence and molecular diversity of parthenogenetic root-knot nematodes, *Meloidogyne* spp. *Heredity* 84:81-89.
- Silva, A. T. D., Penna, J. C. V., Goulart, L. R., Santos, M. A. D., and Arantes, N. E. 2000. Genetic variability among and within races of *Heterodera glycines* Ichinohe assessed by RAPD markers. *Genet. Mol. Biol.* 23:223-229.
- Soares, P. L. M., dos Santos, J. M., and Ferraud, A. S. 2004. Estudo morfológico comparativo de 58 populações brasileiras de *Rotylenchulus reniformis* (Nemata: Rotylenchulinae). *Fitopatol. Bras.* 29:419-424.
- Soares, P. L. M., dos Santos, J. M., and Lehman, P. S. 2003. Estudo morfológico comparativo de populações de *Rotylenchulus reniformis* (Nemata: Rotylenchulinae) do Brasil. *Fitopatol. Bras.* 28:292-297.
- Stetina, S. R., and Young, L. D. 2006. Comparisons of female and egg assays to identify *Rotylenchulus reniformis* resistance in cotton. *J. Nematol.* 38:326-332.
- Tigano, M., de Siqueira, K., Castagnone-Sereno, P., Mulet, K., Queiroz, P., dos Santos, M., Teixeira, C., Almeida, M., Silva, J., and Carneiro, R. 2010.

- Genetic diversity of the root-knot nematode *Meloidogyne enterolobii* and development of a SCAR marker for this guava-damaging species. *Plant Pathol.* 59:1054-1061.
- Tilahun, M., Soliman, K., Lawrence, K. S., Cseke, L. J., and Ochieng, J. W. 2008. Nuclear ribosomal DNA diversity of a cotton pest (*Rotylenchulus reniformis*) in the United States. *Afr. J. Biotechnol.* 7:3217-3224.
- Van der Beek, J. G., Maas, P. W. Th., Janssen, G. J. W., Zijlstra, C., and Van Silfhout, C. H. 1999. A pathotype system to describe intraspecific variation in pathogenicity of *Meloidogyne chitwoodi*. *J. Nematol.* 31:386-392.
- Von Samson-Himmelstjerna, G. V., Blackhall, W. J., McCarthy, J. S., and Skuce, P. J. 2007. Single nucleotide polymorphism (SNP) markers for benzimidazole resistance in veterinary nematodes. *Parasitology* 134:1077-1086.
- Wallace, T. P., Golden, B., Thaxton, P. M., Scheffler, J., Lawrence, K. S., and Weaver, D. 2013. Agronomic performance of *barbadense* and *longicalyx* derived breeding lines. Page 1005 in: *Proc. Beltwide Cotton Conf.*, San Antonio, TX. Cordova, TN: National Cotton Council.
- Weaver, D. B., Lawrence, K. S., and Van Santen, E. 2007. Reniform nematode resistance in upland cotton germplasm. *Crop Sci.* 47:19-24.
- Yang, C. H., Wu, K. C., Dahms, H. U., Chuang, L. Y., and Chang, H. W. 2017. Single nucleotide polymorphism barcoding of cytochrome c oxidase I sequences for discriminating 17 species of Cumbidae by decision tree algorithm. *Ecol. Evol.* 7:4717-4725.
- Yik, C. P., and Birchfield, W. 1984. Resistant germplasm in *Gossypium* species and related plants to *Rotylenchulus reniformis*. *J. Nematol.* 16:146-153.