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Microsatellites reveal genetic diversity in *Rotylenchulus reniformis* populations

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Microsatellites reveal genetic diversity in *Rotylenchulus reniformis* populations

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Abstract: *Rotylenchulus reniformis* is the predominant parasitic nematode of cotton in the Mid South area of the United States. Although variable levels of infection and morphological differences have been reported for this nematode, genetic variability has been more elusive. We developed microsatellite-enriched libraries for *R. reniformis*, produced 1152 clones, assembled 694 contigs, detected 783 simple sequence repeats (SSR) and designed 192 SSR-markers. The markers were tested on six *R. reniformis* cultures from four states, Texas, Louisiana, Mississippi and Georgia, in the USA. Based on performance we selected 156 SSR markers for *R. reniformis* from which 88 were polymorphic across the six reniform nematode populations, showing as the most frequent motif the dinucleotide AG. The polymorphic information content of the markers ranged from 0.00 to 0.82, and the percentage of multiallelic loci of the isolates was between 40.9 and 45.1%. An interesting finding in this study was the genetic variability detected among the three Mississippi isolates, for which 22 SSR markers were polymorphic. We also tested the level of infection of these isolates on six cotton genotypes, where significant differences were found between the Texas and Georgia isolates. Coincidentally, 62 polymorphic markers were able to distinguish these two populations. Further studies will be necessary to establish possible connections, if any, between markers and level of pathogenicity of the nematode. The SSR markers developed here will be useful in the assessment of the genetic diversity of this nematode, could assist in management practices for control of reniform nematode, be used in breeding programs for crop resistance, and help in detecting the origin and spread of this nematode in the United States.

Key words: DNA fingerprinting, genetics, molecular biology, molecular markers, nematode, simple sequence repeats, SSR, STR, reniform nematode.

Rotylenchulus reniformis (Linford & Oliveira) was first described in Hawaii by Linford and Oliveira (1940). This species occurs in subtropical, tropical and some temperate soils worldwide (Robinson et al., 1997; Nakasono, 2004), having as hosts at least 314 plant species in 77 families (Robinson et al., 1997), 56 of which are of agricultural importance. In the Mid South area of the United States, *R. reniformis* is the predominant phytoparasitic nematode on upland cotton (*Gossypium hirsutum* L.). The most recent loss report (Blasingame et al., 2008) indicates that 2.0% of the crop was lost to this pathogen across the United States cotton belt in 2007, with higher losses of 4.0%, 9.0%, and 8.5% in the mid south states of Louisiana, Mississippi, and Alabama, respectively. Fewer and smaller bolls are produced on infected cotton plants, and lint percentage is reduced (Jones et al., 1959; Cook and Namken, 1994; Lawrence and McLean, 2001).

In general, to develop effective control practices of plant pathogens and to achieve long lasting resistance to pathogens through breeding programs, the genetic variability of both host and pathogen need to be known (Araya, 2003; Werlemark et al., 2006; Silva et al., 2008). Most plant pests and diseases are polycyclic, as the parasite passes through more than one generation on the same plant (Tellier and Brown, 2008), thus specific pathogen genotypes can overcome the host resistance in a relatively short time (Brown, 1996). Current management practices to control the reniform nematode emphasize nematicide use and rotation to non-host crops to reduce losses (Robinson, 2007; Starr et al., 2007). Host plant resistance, while preferred by growers, is not currently available in commercial cotton though efforts are underway to identify resistance genes and transfer them into upland cotton (Robinson, 2007; Robinson et al., 2007; Starr et al., 2007; Sacks and Robinson, 2009). Little is known about genetic variability in reniform nematode populations, and the potential of the genetic variability to affect the identification, utility, and durability of resistance is not understood. It has been demonstrated that genetic variability can impact long term management efforts for nematode species damaging to other crops, including soybean cyst nematode (*Heterodera glycines* Ichinohe) (Riggs et al., 1981; Niblack et al., 2002), Columbia root-knot nematode (*Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley) (Van der Beek et al., 1999), and peanut root-knot nematode (*Meloidogyne arenaria* (Neal) Chitwood) (Noe, 1992).

Morphometric differences within *R. reniformis* have been documented in Japan (Nakasono, 2004), Brazil (Rosa et al., 2003; Soares et al., 2003, 2004), Africa (Germani, 1978) and the United States (Agudelo et al., 2001, 2005). Despite the variable morphology among *R. reniformis* populations, genetic variations have not always been obvious. Using amplification of the nuclear rRNA first internal transcribed spacer region (ITS1), only a parthenogenic population from sweet potato in Japan showed differences when compared to amphimictic *R. reniformis* of North and South America (Agudelo et al., 2005). In separate studies using ITS1 and 18S nuclear ribosomal DNA, Tilahun et al. (2003, 2008) have shown genetic variability in both DNA regions within populations of *R. reniformis* from Alabama, USA. Given the

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initial lack of correlation between phenotypic and genotypic variations, Agudelo et al. (2005) suggested that the development of microsatellites could provide a more reliable way to evaluate populations.

Microsatellites or simple sequence repeats (SSR), are short tandem DNA repeats, with 2 to 8-bp motifs, whereas motifs of 9-bp or longer are considered minisatellites (Richard et al., 2008). These repeats are widely spread throughout eukaryotic genomes (Anwar and Khan, 2005; Richard et al., 2008) and are ideal markers for a number of applications from genomic-assisted breeding in plants (Varshney et al., 2005) to detecting genetic disorders in humans (Richard et al., 2008). Microsatellites have become one of the most powerful genetic markers in biology, and they have shown to be useful in the characterization of plant pathogenic fungi, i.e., *Sclerotinia* (Sirjusingh and Kohn, 2001) and *Crinipellis* (Gramacho et al., 2007), as well as in plant pathogenic nematodes, *Meloidogyne* (De Luca et al., 2002) and *Globodera* (Thiéry and Mugniéry, 2000). Here we report the development of a large number of microsatellites for *R. reniformis* that can be used in population studies and can also assist in the genotyping/fingerprinting of isolates of this nematode for plant breeding and agronomic control programs.

Materials and Methods

Collection of nematode eggs for DNA analysis

Six amphimictic populations of *R. reniformis* were multiplied in the greenhouse using tomato (*Solanum lycopersicon* L. 'Rutgers') as the host. These populations originated from four southern U.S. states: Texas, Louisiana, Mississippi, and Georgia (Table 1). Nematode eggs were extracted from root tissue using a protocol similar to that described by Hussey and Barker (1973). Briefly, plant roots were carefully rinsed with tap water to remove as much soil as possible. Roots were swirled in a 1% sodium hypochlorite solution for three minutes, and then the suspension was poured onto nested 75- over 25-μm pore sieves, and rinsed with tap water to remove as many eggs as possible. Eggs retained on the 25-μm pore sieve were inspected and an additional removal of plant tissue and soil was performed using a sucrose gradient when necessary (Jenkins, 1964). The cleanup with sucrose was accomplished by centrifugal flotation for ten minutes with a 70% (w/v) sucrose solution. The supernatant was pipetted onto a 25-μm pore sieve, rinsed with deionized water and the eggs were transferred to a beaker for counting. To minimize possible contamination from non-parasitic nematodes occasionally found in soil, vermiform nematodes were removed by hand prior to DNA extraction. Approximately 10,000 eggs were divided into bead beater tubes (Fast Prep 2-ml tube; MP Biomedicals, Irvine, CA) with 1,000 to 2,000 eggs per tube. The tubes were centrifuged at 2,000 rpm (5810R, Eppendorf, Westbury, NY) for two minutes and the sample was allowed to settle for five minutes. Excess liquid was pipetted off the suspension leaving approximately 300 μl in the tube to avoid removing the eggs, followed by an addition of 750 μl buffer AP1 from Qiagen Maxi Kit (Qiagen, Valencia, CA) to facilitate distribution in aliquots and to have them in the appropriate buffer for DNA extraction. Tubes were stored at −80°C.

DNA extraction, SSR-enriched library construction and primer design

The Mississippi isolate *R. reniformis* RR01 was used for the generation of SSR-enriched libraries. DNA, approximately 300-500 ng was extracted from 10,000 eggs according to Harmon et al. (2006) and used for library construction. Eggs were suspended in 200 μl AP1 buffer (Qiagen, Valencia, CA) and placed in 2-ml FastPrep tubes (MP Biomedicals, Irvine, CA) containing eight 2.5-mm zirconia beads, two 5-mm stainless-steel beads and ~50 mg sand. For the disruption, we used a Mini BeadBeater-8 (Biospec Products, Bartlesville, OK) for three minutes at the homogenize setting. Disrupted eggs were processed with DNeasy Plant Maxi kit (Qiagen, Valencia, CA) for DNA extraction and SSR-enriched libraries were generated following the protocol of N.Techen (unpublished) briefly described here. DNA from *R. reniformis* was digested with restriction enzymes Alu I, Hae III, Dra I, Rsa I (New England Biolabs, Ipswich, MA) individually and in pairs of these enzymes. The restriction-digested DNA was separated by agarose gel electrophoresis; and fragments between 300 and 2000 bp were purified. The blunt-end DNA fragments were A-tailed with Taq-DNA Polymerase (Promega, Madison, WI) in the presence of dATP for 2 hrs, then ligated for 3 hrs at 16°C to the linker SSRLIB3 N.

Table 1. List of *Rotylenchulus reniformis* isolates, origin and host plant.

Isolate	Geographic origin	Population developed from	Original host
MSRR01	Mississippi (Elizabeth farm)	combination of 300 egg masses	cotton
MSRR03	Mississippi (Elizabeth farm)	one egg mass	cotton
MSRR04	Mississippi (Elizabeth farm)	one egg mass	cotton
LA	Louisiana	many individuals	cotton
TX	Texas	many individuals	unknown crop
GA	Georgia	many individuals	cotton

Techen (unpublished), made from oligos SSRLIBF3: 5′- CGGGAGAGCAAGGAAGGAGT-3′ and SSRLIBR3 5′Phos-CTCCTTCCTTGCTCTCTCCCGAAAA-3′. The ligated fragments were purified with MinElute (Qiagen, Valencia, CA) and amplified by 20 cycles of PCR using primer SSRLIBF3 and High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA) at: 94°C for 30 sec, 60°C for 30 sec and 68°C for 90 sec. The amplified products, approximately 1.5 μg DNA in 200 μl reaction, were hybridized to four groups of biotinylated oligo repeats: group 1 [$(AC)_{13}$, $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, $(ATCC)_5$], group 2 [$(AG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(ACT)_{12}$, $(ATC)_8$], group 3 [$(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] and group 4 [$(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$], primers were bought from Invitrogen (Carlsbad, CA). The final concentration of each oligo in the mix was 1 μM and 2 μl of each oligo mix were used in 50-μl hybridization reactions. Hybridizations were performed in a gradient thermocycler at 95°C for 10 min, followed by 3 hrs at the annealing temperature using a gradient block at (Group1: 56°C, Group 2 & 4: 50°C and Group 3: 53°C) and an extension step of 10 min at 68°C in the presence of High Fidelity Taq Polymerase (Invitrogen, Carlsbad, CA) as indicated in Hayden et al., (2002). That is, the hybridization is set up as a PCR reaction and the polymerase extends the hybridized molecules. Sequences containing repeats were captured using streptavidin-coated magnetic beads M-270 (Invitrogen, Carlsbad, CA) in a Labquake tube shaker/rotator (Barnstead/Thermoline, Dubuque, IA) at 22°C for 1 hr, modification of the method reported by Kijas et al. (1994). After binding, the beads were washed with 2xSSC, 1xSSC at ambient temperature and 0.5xSSC at 50°C for 5 min each. Elution of the DNA from the biotinylated oligos was done with 60 μl miliQ water at 96°C for 15 min, twice. The eluate was PCR amplified for 20 cycles as described in the ligation step, the PCR products were cloned in vector TOPO4 (Invitrogen, Carlsbad, CA) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled in contigs using DNAStar Lasergene7 (DNASTAR, Inc., Madison, WI) and visually checked. Repeats were searched using SSRFinder (Sharopova et al., 2002) and Sputnik (C. Abajian, http://espressosoftware.com/pages/sputnik.jsp). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) with stringent parameter conditions: Tm 63 optimum (60/65) min/max, length 24 optimum (20/28) min/max, 3′ GC clamp, and maximum overlap of repeat within the primer was 5 bp.

Fingerprinting

Using stringent conditions in Primer3 software we designed 192 primers on the flanking regions of the repeats and tested all of them on six *R. reniformis* populations. To determine the possible cross amplification of the common crops or plant hosts of *R. reniformis*, we tested the 192 SSR markers on tomato, soybean (*Glycine max* L.), sweet potato (*Ipomoea batatas* (L.) Lam) and upland cotton (*Gossypium hirsutum*). Forward SSR primers were 5′ tailed with the sequence 5′-CAGTTTTCCCAGTCACGAC-3′ to permit product labeling, and reverse primers were tailed at the 5′end with the sequence 5′-GTTT-3′ to promote non-template adenylation (Brownstein et al., 1996). Primer 5′-CAGTTTTCCCAGTCACGAC-3′ labeled with 6-carboxy-fluorescein (FAM) (IDT-Technologies, Coralville, IA) was used for amplification of 10-ng DNA using Titanium Taq DNA Polymerase (Clontech, Mountain View, CA) in 5 μL reactions on an M&J thermal cycler (BioRad, Hercules, CA) at 95°C for 1 min, 60°C for 1 min (2 cycles), 95°C for 30 sec, 60°C for 30 sec, 68°C for 30 sec (27 cycles) and a final extension at 68°C for 4 min. Fluorescently-labeled PCR fragments were analyzed on an ABI 3730XL DNA Analyzer and data processed using GeneMapper v. 3.7 (both from Applied Biosystems, Foster City, CA). Presence of alleles was converted to a binary matrix. The *R. reniformis* isolates were clustered using the unweighted paired group method and arithmetic averages (UPGMA), algorithm implemented in the SAHN program of NTSYSpc v. 2.2 (Exeter Software, Setauket, NY). The confidence levels for the dendrograms were assessed by bootstrap resampling (5000 replicates) (Felsenstein 1985; Efron et al., 1996) using WINBOOT (downloaded from www.irri.org/software) last accessed November 2008.

Polymorphism information content, percentage of multiallelic loci and Unique Pattern Informative Combinations (UPIC)

The polymorphism information content (PIC) for each marker was calculated according to Botstein et al., (1980), according to the formula:

$$PIC = \overset{n}{\underset{i=1}{1}} - \overset{n}{\underset{i=1}{\Sigma}} p_i^2 - \overset{n}{\underset{j=i+1}{\Sigma}} \Sigma 2p_i^2 p_j^2$$

where p_i is the frequency of the ith allele, j is the jth line (DNA sample or taxonomic unit) and n is the number of alleles for the marker. Percentage of multiallelic loci was calculated for each isolate across all the SSR markers tested. We have also calculated Unique Pattern Informative Combinations (UPIC) to determine the set of SSR markers derived from our analysis that will be the most informative for future studies. All coefficients, PIC, percentage of multiallelic loci, and UPIC values were calculated using UPIC Perl scripts (Arias et al., 2009).

Pathogenicity Test of Nematode Isolates on Cotton Varieties

Four of the *R. reniformis* isolates listed in Table 1 were used to analyze possible differences in level of pathogenicity of nematode isolates and the response of cotton varieties. Two treatments were combined in

a factorial arrangement and assigned in a completely randomized design in a greenhouse. The first factor was nematode isolates and four populations were tested. One treatment was one of four nematode populations representing TX, LA, MS (MSRR04) or GA. The second factor was cotton genotype, which ranged from resistant to susceptible to reniform nematode: *Gossypium arboreum* L. (A2-190) (resistant), *G. barbadense* L. (TEX 110) (resistant), *G. hirsutum* (FiberMax 960 BGRR)* (susceptible), *G. hirsutum* (TEX 19; 21-25), *G. hirsutum* (TEX 1347; 24-23), and *G. hirsutum* (TEX 1348; 25-03). The TEX 19, TEX 1347, and TEX 1348 lines are selections from day-neutral Texas race stock accessions chosen because they showed slightly improved levels of resistance to reniform nematode (Young et al., 2004). Each of the 24 treatment combinations was replicated five times, and the experiment was conducted twice. Preliminary analysis indicated no significant interactions involving run, so data from both runs of the experiment were combined for final analysis. Analysis of variance was completed using the MIXED procedure of SAS (SAS Institute, Cary, NC), and differences of least squares means were used to identify differences among cotton genotypes and among nematode populations.

Cotton plants were prepared for inoculation as follows: two cotton seeds were sown into 7.6-cm-diam. clay pots containing approximately 300 g of a mixture of 1 part steam pasteurized field soil (Dundee fine sandy loam soil; fine-silty, mixed, active, thermic Typic Endoaqualfs) and 2 parts sand. After emergence, plants were thinned to 1 per pot. One day after thinning, the soil in each pot was infested with 3,000 vermiform reniform nematodes suspended in 3 ml water. Plants were watered daily as needed with deionized water. Swollen females attached to the roots were counted 35 d after inoculation. Roots were removed from the soil by gentle agitation in tap water, then stained with red food coloring (Thies et al., 2002) as described by Stetina and Young (2006). To compensate for differences in the size of root systems, results were expressed as females per gram of root.

Results

Simple sequence repeats found

SSR-enriched libraries of *Rotylenchulus reniformis* were made using four groups of biotinylated oligo repeats. From those libraries, 1152 clones were sequenced, and the sequences assembled in 694 contigs where 783 repeats were detected by SSRFinder and Sputnik combined when using minimum repeat length 8 bp, and 20 bp for the minimum length of repeat-flanking region. Sequences of 690 contigs were submitted to GenBank with accession numbers (FJ905934 to FJ906620), four of the 694 contigs could have been derived from the plant host DNA, tomato, as the primers amplified fragments within the expected size range for the nematode and therefore were not submitted. Three minisatellites with motif lengths of 18, 20 and 40 bp were found among the repeats, these motifs were: AGGGTGATCGGGATGGGC, GGAAAGTGATCAGATGGCTT, and TCACTCACTCCT CTGACTCACTCTTACTCTCTTACAGCAC, within contigs 258, 368 and 390 respectively. Primer sequences designed for *R. reniformis* DNA are reported along with their corresponding motifs in Table 2. To simplify the recording of the repeat motifs, repeats that were circular permutations and reverse complements of each other were grouped together as one type, *i.e.*, AAC, ACA, CAA, GTT, TGT and TTG were recorded as AAC. Using this notation, 52 non-redundant repeat motifs were isolated from the *R. reniformis*-SSR-enriched libraries. From those 52 motifs, the 10 most abundant ones had frequencies from 480 to 7 (Fig. 1A). With the groups of oligos we used to make the SSR-enriched libraries, the most frequent motifs detected were AG, AAC, AC and AGG (Fig. 1A) and among those with low frequency (data not shown) we found the rare motifs CG, CCG and AGGGG. Repeats with frequencies lower than seven were not included in the plot. Frequencies of the isolated repeats decreased as their length increased from di- to tetranucleotides, and only few motifs were longer than four bp (Fig. 1B). Out of 192 markers tested, 23 did not amplify or produced a very weak amplification of the *R. reniformis* DNA tested.

The majority of the markers did not produce amplicons when tested on tomato, soybean and upland cotton, and in the few cases that resulted in amplification, the fragments did not match *R. reniformis* amplicons. Only exception was marker 207_a that showed in cotton an amplicon similar to. *R. reniformis*.

Markers that amplified R. reniformis

Based on the quality of electropherograms in GeneMapper and presence across samples, we selected 156 markers that had PCR products across the *R. reniformis* samples tested. From the 156 markers, 119 showed amplicons in all six DNA samples tested and 24 in five samples; the rest amplified four samples or less. A total of 88 markers were polymorphic across the samples tested and the range of alleles detected by each individual marker was between one and ten (Fig. 2B). A total of 390 alleles were detected in these 156 loci of *R. reniformis*, with an average of 200 alleles per isolate and 1.7 alleles per marker.

Heterozygosity (%) and Polymorphism Information Content (PIC)

The percentages of multiallelic loci for the populations of *R. reniformis* tested were: 44.5% (MSRR01), 40.9% (MSRR03), 43.5% (MSRR04), 43.6% (GA), 44.9% (LA) and 45.1% (TX). The polymorphism information content (PIC) calculated for the 156 markers is plotted

TABLE 2. Markers that amplified DNA samples of *Rotylenchulus reniformis* isolates. Markers were selected by their performance in terms of peak quality and distribution across isolates. The melting temperature (Tm) for all primers ranged from 60 to 65°C. Marker names follow the notation Stv: Stoneville, RR: *Rotylenchulus reniformis*, Contig number as submitted to Genebank, location within contig: a, b, c, etc., and sk indicates repeats detected by Sputnik, the rest were detected by SSRFinder.

SSR-Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)	Repeat Motif
StvRR_3_a	AAGTCGTCGGTCCCTAAAACTAGC	GTTGGAGATCTGGTGAGGTTTTC	CGT
StvRR_6_a	TAACTCGGTTAGATCCAGTTTCGC	CACATCAATTAAAGCAACAAACGC	TGT
StvRR_7_b	GTAATGCACCGAATGGGCTG	AATGAGGGGAAAGATGACCAAAAC	TCAG
StvRR_12_a	CTTGAAGTGCTCGCACAAATAGTC	GATAAGGCTTTAATCCAGGTGGTTC	CTG
StvRR_14_a	AAACTTGGTAGTGCTGGACAGGAG	TTTCCCTTTCAGTTTCCACTTTTG	GAG
StvRR_17_b	TTTCTGGGTTAAAGGTACCCACAC	ATTCTTGTGGCTGATGTGTGATTG	ACA
StvRR_22_a	CTACACTCGCTCGCCACCAC	CAACACATCAGTTACTCCTGCTGC	AGA
StvRR_24_a	ATCTATGGACAAATCCCAAAGCAG	AAATTCTCTGGCTTTTCTACGCTG	ACA
StvRR_28_a	CAAAGAAATCCACAATCATGTTGAC	TTCTAAATGTCATGCTGCCACC	GA
StvRR_30_a	CGATCGGAGGAGAGATAGAGGG	TAATTAGCCGCTGATCAGTTAGCC	TC
StvRR_32_a	GTCAGAAAATTGCAAAGGAAGACG	TTGCACCGAATAATAAACTGTCTCC	TGA
StvRR_35_a	AGTCGTCATCATCGTTGTTCACTC	TTTATCCTCCCCATTCTCTCTTCC	GA
StvRR_36_a	AGGATCAGAAGTTCATTTGCCTTG	GGACACAGATCACTCTTGTTCGAG	TC
StvRR_38_a	TGGTCAGTCGAGTTCTTCATCATC	ACAAAACACCTGTTGTTGCATCTC	CAA
StvRR_41_a	AGATCGACTTCGTTGAGGCACTAC	CTTGCAGCGTTAACTTTCGTTTG	TTTA
StvRR_43_a	TCCGTATACTCAACTCATTCGCTG	AAGTTTTCGTGTGTCCTCTCTTGC	AGTG
StvRR_46_b	TTGAAGGAAGATGCAAATTTTAGAAAG	TTTTGCTTGTGTAATACCTCTGCG	TG
StvRR_47_a	TCTTGGGCATTGTCATGTTATTTTC	CGGTTGGAAATATCACCAATAAAGG	TTG
StvRR_48_a	TATCGCTACTACCCGTTGGAATTG	CTCTGATTCTCCTCCAATTTGCTC	GAT
StvRR_49_a	ATTGGAACGAAAAGCAAATTCTTG	CAGAAAGTGTGAGAGAACGAGACG	TC
StvRR_52_a	TGGACAATGCTTATGACGAGGAG	TACTCATGATCGGTAGACTCGCTG	CAG
StvRR_56_a	TACAATGACAGACCCCGGAC	TATGGACAGGCGGAAGAGTG	CT
StvRR_59_a	GATGTCAATCGATGCTGTCTCTTC	CTTTGCATCGGAATAAACCTGAC	AGA
StvRR_63_a	ATTCACATGGCAGTGGATGAAAC	ATTTGTTTCTTGCTGGAGCACAAC	GTTC
StvRR_65_a	GGTTTATGCTTGGAACAGGTGAAC	TCAACCATAAGGTCGGGAATTTAC	TTGT
StvRR_66_a	ACATTGCATTCGATCTTTCTCTCC	ACGCGCAGTTATCGATTTTATTTG	CT
StvRR_67_a	ATGCACTCTCTCATCGTTCCTCTC	GCTAGAAGTGATGCGGATTTCG	TCT
StvRR_69_b	AAATCATCAGAGCAGGTAACGAGG	TCCTGATCACTTCACTTTTGATCG	GA
StvRR_70_a	GCTGTGTCTCACTCACCACACTC	GGTGAGACAGAAGGGTGAGAGG	TC
StvRR_72_b	AATGGCAGTGGACAGAAAGATCAG	ATCATCCGATCACTTCTTTGCTTC	AG
StvRR_78_a	TGTACTTTGAGAGGGACTTTTGGG	GCTGTTCCTCTTCTTGTTCCTCTC	GAA
StvRR_82_b	CTTGGACGACTCTATTCGGATTTC	GCACAAGTATGGTGATGCAGAAAG	TC
StvRR_83_a	AAACTGAAAATGAACGCAAGGATG	ATATGTCCCACTCAGTGTCCGC	ACGG
StvRR_85_a	TCAAAACATGTTAACAACCCAACG	GGGACCAGATCAGTTCAACACATAC	AT
StvRR_88_a	CCCCACACTCTGTCTTCTCTGC	TCATTGCCAAGAGGGAAATAAATG	CT
StvRR_89_a	GGAAGAGTTGAGTTGTTGTTTGGG	CCCGTGTCAAATCACAATTTTCTC	GAA
StvRR_92_a	AAAGTGCCAAATTCACAAGAGGAC	AAGCAAATTGTTTAGGTTTCGTCG	TCAG
StvRR_92_c	CGGATAATTGCCAAGGTGTATTTG	ATCAACAGCAACAACAACCTCAAC	GTT
StvRR_96_b	GAGATGAATGGAATGGATGGTTTG	ACCAAAGTGTTTGCCATCCCTC	GAAT
StvRR_97_b	AGGAGTCCGATCAGAACAGAGTTAATAG	GAATTGTCGCTCTCTAACCCACC	GA
StvRR_98_a	TCACGTGCTGATTCTTGTAAGTTG	AACTTGTTTCCAATTTGCTCACG	TATC
StvRR_100_a	TGAAGTATAGCCTTCCCTCCATTTC	TCGATCTCTAACACGAATGATTGC	TCA
StvRR_101_a	GGATGACAGAGGTGAGGAATTAGG	CTCATCACAACTTTTCTTCCTCCC	GA
StvRR_104_a	CTCTTCTCTTTCTCCTTTCCCTCC	GAATGGTAGAAATGCACAAAAGGG	TCT
StvRR_107_a	CGATCAGATCACTGTCCACCTTC	GGGAAATAATCAAATTAGGGGCTG	TCCA
StvRR_107_b	CATCTCACAAAGATCGGAAATGC	CGGAACACCGTTCATAAAACTTC	CGG
StvRR_108_a	GTCGGCAGTCCTAACTTCGTTC	TTCAATTGTCTCCTCTCCTGGC	CAGCAGC
StvRR_110_a	AATGATCGGTGATCCAGACGAG	GGTATCTTGACTATCCATCCCAGG	AG
StvRR_111_a	GAGTCGCAGGTCACCTTCTCTG	CCTCGGGTGATCTTCGTGAG	GTT
StvRR_113_c	AATAAAACAAAACACCTGCCGAATC	GAAATTTTGCAACGCAAATTCAAC	AT
StvRR_116_a	TTCATCCGAACCAAAACAATAAGG	ATCAACGGTTGTTAGTGGGGATAC	TTG
StvRR_117_a	ATTGGGATTTGGACTACTTGGAGG	ACGGCGAAACGACCCAAAAC	CATT
StvRR_118_a	TCAGCCACTCACTTTTACTCCTCC	AGTCCATGAACCGACGATGAAC	ATGA
StvRR_120_a	CTTTCTCGACTTCTCCCCTTTCTC	TGTGGGTTTGGATTTGGACTATTG	TC
StvRR_122_a	GAATTTTCAGGAAGACTCTGAGGATG	TTTTCTCATTGACACATCTCAACTCAC	GA
StvRR_126_a	CATTTCGGATCTTCTAATTCTCGC	TCATGGGTTAGGGGTGTAGAAAAG	AATG
StvRR_129_a	AACAAGTCGGGCTGATGAGG	TCACCAACCACAATATAAAACCCC	GAT
StvRR_130_a	TGGAATGAGGGTAAATGATGATCC	TGATTGGAAATGATCAGTGATTGG	GT
StvRR_150_a	TCACTAAAACATCACTCAAAGGCG	TTGTGTTTTCCGACTCTGTTTCTG	AG
StvRR_159_a	AGCAGTCCCTCAGTTCTAATTGC	TTCCCTTTTCTCTCTATCGTTTTG	AG
StvRR_160_b	ACGCCACTGTCAACATCTAAAACC	ATCTACAACCTACTCCGACGATGG	AC

(*continued*)

TABLE 2. Continued

SSR-Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)	Repeat Motif
StvRR_163_a	GCAGCGCTCAAGAGGTAAATG	AGGGCGTAACTGAGTGTCGTTATC	GGC
StvRR_167_a	ACTAAATGACCACTGACCTGACCG	GAGAGAATATAGGGGAGTGCGGAG	GGTCA
StvRR_167_c	TACTCCCACTTTCCTCCTCCC	AAGGATGAAAGCTGAAAGAGTAGGG	TC
StvRR_170_a	TCGGTTTGGTTTGCTCATATTACG	TAGGAGGGGAAAGACGAGACAATG	TTC
StvRR_171_b	AATTTTCAGAACCCAACAACCATC	AAAAGTTCCACAGCATTCCTCTTC	TC
StvRR_173_a	CAGAAGAAACAGCGGAAGAAGTTG	AACTATCTTTCCCCAATGTGCCAG	GA
StvRR_176_a	AGGACTACTCTAACCGTAAGCGCC	CAAGTTGAATTCGGAACGATTTTG	GA
StvRR_193_a	TCCTTCACTTCATTGTTTGTTCC	AAGACTATCTAAACTAAACAAAGGCAGG	ATCA
StvRR_199_a	ACAGGGAGGAGTAGAACAACCACC	GTTCCTCGGGAATGATTTCGTC	ACA
StvRR_204_a	AGGAGTCCACAAAGTGTCAATGG	GGCGATATATTCCTCCTTCTCCTC	GA
StvRR_207_a	TCATGCGACGTTAGATTGTATTCAC	TGTATCTTATTGGGCGCTTAACTTG	AC
StvRR_209_a	ATATTCACCTTACCCAAACCCACC	GATTCTGCTCTGCTGTGTGAATG	TC
StvRR_219_a	GTTTCTTCTCACTCACTCGCTTGC	GAGTGTGAGTGAATCGCAGGG	CT
StvRR_220_a	ATTTCGTCAATTTCCCTAACCCAC	GATCAGGGACGATCCTGTTTGAG	CT
StvRR_221_a	GTTCTCAAACGGTACCCGGAG	AATGGTTGTTTGTTTGTTTGTTGG	AACA
StvRR_223_b	GTGTGCGGTGCTATGTCGAG	TAACACCCTACACCACCACACAAC	TG
StvRR_224_a	ATCATCGGATTCAAAGAAGGACTG	ACTCTGATCGAACTTTCGTTGGAC	AG
StvRR_225_a	TCGCCTTTATCCTTCTCAATTCTC	TGTGCTGAGAGATAAGGCTGAGAG	TC
StvRR_225_b	TCGCCTTTATCCTTCTCAATTCTC	TGTGCTGAGAGATAAGGCTGAGAG	TC
StvRR_228_a	AAGACGTTGCCAGGCACAAC	TCTTCTCTCACCCTCACACTCTCTC	GA
StvRR_233_a	CAAATCCGTCTCACTCACTCTCAC	TGCAAAATAGCAAAGGGATAGAGC	CT
StvRR_236_a	TTGTGGAGAATATATAGGGCCTCC	CGGGATCATCATAAAATCCAACTC	TTG
StvRR_237_a	ATGAAAGTGTCCATTTGGGTGG	TTCCTTTCCTCCTTCTCTTCCATC	AGA
StvRR_241_a	AGGCTAGTTCTCATAGTAATAATCAGGC	TAAAATTCCCTCAGAAATCGGTAG	CT
StvRR_244_a	CAAGACCAGGAACAGACCGTTTAC	TTATCGGAAAATCTTCAAATTGCC	ACA
StvRR_248_a	ACTAATCACAGCCTCCAATGATGG	GCTGATCCACGTCTTCGAATTG	TTG
StvRR_249_b	GATGTTGCTTTTCCCTGTTGTAGG	ATCTCTAGCCCTTATTAGCCGTCG	TTG
StvRR_257_a	AGGACACGAGCAGATGCATTACAG	ACATTTCTCTCCCCTTCCTTGTTC	GCCA
StvRR_258_a	CGGCTCTCATGCTCTTGGTC	CCAACGGATCATTCAGGTAAATTC	ATCAATCA
StvRR_263_b	TGTTATCAGTCATCCGTTCCGTTC	CGAAAGAGGACCGGAGTTCATATC	TG
StvRR_266_a	TCTTCTTCCGCTACTTCGATCATC	GCCGATGGACATGAGCAAATAC	CTC
StvRR_272_a	CCAGAGCAACAACATGGACTAGG	CGATTGACGATACACTTCTTGTCG	ACA
StvRR_276_a	GAGTACGCCGAAAGGGAAAGG	CATTCTCTCCGAGACCATTGAAAG	AG
StvRR_281_a	TGCGTTTCTGTAGAATGCAGTAGC	CGTTCATACGAGATGTCCTTCCC	AT
StvRR_284_a	CCACCAACAACGACAATCGC	CTACTTGCGGTTTCGGTCTATGAAC	CAA
StvRR_285_a	CGCTTACTTCACAGACGAGAGTGAG	GACTCTAACCCATCTTCATCGCTC	GA
StvRR_296_a	TAGGGAGAGGATTGGAAGTGATTC	TCTTTCTTTTGATCGACTTGTCCC	GA
StvRR_311_a	CAAAACAATGGGTCCAACTTTCTC	TCAGAGACTGAAGGGATAGTGAAGG	CT
StvRR_312_a	TCGTTACTGTTTAATTGCACGCAC	AACATGACTTGCCTCGAATAATGC	ATCT
StvRR_314_a	CATTCATCTTTTAGGGAGTCCACG	GAGAGTTCAATATCGTCTCCCTGC	TGA
StvRR_318_a	ATCACAGACGGGAGAGAATGAGAG	GACCTAATTGTAGCTCTCATCCGC	GA
StvRR_326_a	ATTGTGTTTGAATTCTTCGGGATG	CTGGTCCCCGATCACTTTCAC	TCAA
StvRR_330_a	GTCATGTGCCAATAATTTGAAAGC	ATGGAATGTCTGAGCGTCTACAAG	ATC
StvRR_334_a	ACATCACCGCTCCATCAATACC	TTCCTACGATCCATCAATTCCAAG	CGC
StvRR_338_a	TCTTCCATCTCGCCTCTTTCTTC	CAAGAGATGAAGAGAAGTTGTTGCAG	TC
StvRR_340_a	GGAGGAGTCTGATCAGTGCCG	GATGGTAAGTCCACCAAGAACACC	CAT
StvRR_349_a	TTATCCAATATCATGGATTTCGTGG	GGACCCCTAACGCTCGTTACTAC	ACAT
StvRR_351_a	ACAGAATGCCTTCACTGAGAACC	CAGTCTTCCTTTCTTATTGACGCAC	GA
StvRR_354_a	ATCCCTCTCATCCTCTTCTCCTTC	AATGGATACTGGCGGAAGTAAGG	TCT
StvRR_357_a	GTTGTGGCACTGTTCATCTTGC	AATTTCTAAACCCAACGGATTTCTG	AT
StvRR_363_b	TTGTTGTTGTTTGATGTTATCCCC	ATATCCTCAAACTTTGGATTTGGC	TC
StvRR_365_a	CTTTTCTCTTTCTCACTCGCTCAC	CAGAAACAATTTTGAGTGATTTCG	CT
StvRR_367_a	GAAGGAAACTAGGGGAGAGCAAAG	CTCCCTTCTCCTCTCATCTTTGAC	GA
StvRR_368_a	ACAGGAAGGAGTCCCACTCCAC	GATCACCGATCAAGTTGCTTCTTC	ATTG
StvRR_371_a	AGGTGCAATTTGAGAAATTTACGG	AGAGAGAACATGTGTGATGGCAAG	TG
StvRR_376_a	ACAGCCGAAGAGAAACGATTAAAG	TTATCCTCGTTGTGGATGTACTCG	TGAT
StvRR_379_b	ATTCGTCCTCTTCCTCGTCCTC	AGTCCATCGGTTGCTGTTCTG	CAT
StvRR_381_a	TCATCAGTCCATCATTCCACAATC	AGACCTCAACGACATCGTTTCC	CT
StvRR_388_b	CACCATCATCTGACATTCTTCGAC	TGACTTTTCGACAGTGAACATTGG	TC
StvRR_401_a	ACCAACACAGACGAATCGGTC	TCCTTCTCTCACTCTCGTCCTCTC	GA
StvRR_403_a	GTGTTGTCCCTGTCCCTCATCTAC	TACAATGGAGCGATGGATATGTGC	TTG
StvRR_405_a	CTAACCCCATCCATGCGTAAATC	TGGCAATCTGAGACTTTGAGTGAG	TC
StvRR_416_c	AAAACCAAGAAGAACAAGAAGGGG	AATGTTTGTGTTTCTGTGAGCACC	TGA
StvRR_429_a	AACCACCCAGTGATACCACTTCC	GTCTCTGTTCTTAAACACGCCCTC	AC

(*continued*)

TABLE 2. Continued

SSR-Marker	Forward primer (5′→3′)	Reverse primer (5′→3′)	Repeat Motif
StvRR_431_a	AAGAGCCCAAACTCATCAGTTCAC	GATAACCCCGAAGAAATGGGTTAG	TCA
StvRR_436_a	GCCATTTTCAAATTATTTCTTCTGG	CTTATCTTCCGCTTATCCATCCC	ATGA
StvRR_437_a	ACCACGCTGACAACCATAAAATTC	CCCTCCTCTTAACCATTCTGTGC	GAAT
StvRR_438_a	TACTGTTGAGTCACCCTTTCAACG	GATTTGAAACGAAAACGGAAAGAG	TCAT
StvRR_440_a	ATGATAGCACACATCATGAGCAGC	CTCACGGAACCAACTTTACAGGTC	TTG
StvRR_441_a	TACCCCAACTACTTCATTGTTCGC	AGGAGGAGGTGTTATTGTTGTTGC	AAC
StvRR_444_a	GACTTTGTTTGTGGGATTTTGAGG	CTCCTACTTAAATTTCCCTTCCGC	AG
StvRR_447_a	GCAAATAAGTTATGACGGTAAGTGGC	TTCTGGCTTCATCAGTTCAAATCC	GA
StvRR_449_a	TACTCCTTCAGTGGTATTTTGCCC	GATCAGGTCGTTTACTCGCAAATC	TC
StvRR_451_a	GATCAAAGAATACGGAGAAGTCGG	TCCTTTCCTCTCTGTGCTATCAGG	CAAT
StvRR_453_a	CGGAACCAGGGTCCAGAGTC	GGGGAAGAAAGACAGACAGGAAGAG	TC
StvRR_454_a	GAAAATGCAAATGTACGGTCCTTC	GGCTGTTCCACTTTGCTACGAG	CT
StvRR_462_a	CACGTCATTCATTCATTCGATCTG	ATGAGGAAGAATGAAGTTGGGAGG	TC
StvRR_463_a	GATGTTCTTGACCCTGTGCATTC	AAAGGCATAGTCAATGGCTGAAAG	CT
StvRR_470_a	AGGAGGAGTCAAACAAACGGAG	TCCTATTCCTCCTCTTCTTCTCCC	GA
StvRR_471_c	TCCATTCTCCCAGGGGACATAC	AGCAGAAGCAGAAGCAGCACAT	CTG
StvRR_472_a	ACTTTCTCCTTTCACCCTCATCC	GATGATGAACTGGAACAAGCAGAG	CT
StvRR_472_d	CTCTCCCTCTTCACTTTCCTCTCC	GGCTTAACCGCCGGTAGTAGATAG	TC
StvRR_473_a	ATTCCTGTCCCGTCTCTGAATCTC	GGGAGAGAGGAGAGGATGAAGAAG	CT
StvRR_482_a	CTCCCTTTCCAAGTCCTTTTCC	TTCCTCACAGTTTCGACTGACAAG	GA
StvRR_485_a	ATTTTGTGGGAAAAGAATGGTGAG	TCCCCTGTCTTTGACTCCCTC	GA
StvRR_487_a	ATCCCAATAGGGAAGAAAACATGG	TTAGGATTTTCGGCATTATTTTCG	TCAC
StvRR_494_a	TGCTATCTTGGAATTTCAGTGCAG	AAGATCACATCTTTTCATCCCACC	GA
StvRR_506_a	GACGAGATAACTACGGAAACGACG	TTCTAACCTTGTGCCAGGACCTAC	GAA
StvRR_508_a	AAGATGAAGGGATGGAAGAGACG	CTTCTTCCTCACGGTCACTGC	GAG
StvRR_510_a	TTAATGAATTCCGAGATCAAAATCAG	TTATTTGCTTTGTTTTGTTCGTGC	ATTG
StvRR_515_a	CTCGATCCGTGCCAAAATATG	CAGTCGCAAAAGGAGATTTCG	TGTC
StvRR_530_a	GGAGCAGAATGGGGAAGATAATTC	AAATCAGAAATATTGGGGCGAAAG	GA
StvRR_542_a	GAGTATTGTTGCTTGGATGGTCAG	CCCCAACAAATAATCTTCTCTTCAAC	GTT
StvRR_544_a	AAGAGATTGTCAAGGGCGAGTG	ATTTTCCTCTCCGCTTCATTCTTC	GA
StvRR_551_a	CAGCTCTTCTTCACCCAATGTG	AGGAGGAGGAAAACGACCAAATAG	CCT
StvRR_559_a	GAGTCATAACTCATAATCTGGTGGGG	CCGCCAAAGACAAAGATATGTAATG	CGG
StvRR_573_a	AAAAGGCGCAATTCAGTAATGG	ATCCCCTAAAATTTTCGAGACGAC	AAAT
StvRR_618_a	TTCCTCATTCCTAGTTCAATTTGCTC	TCTCGACATGTTTCCTTATCCCAG	CA
StvRR_667_a	AGGAAGGATCCAACAATCCATTTG	CCCAGAACAAATTCCATTAGGGTC	GAT
StvRR_53_ska	TTCAATCTGTTCTGTTCCTGTTCC	TTCCTGCTTAATCTGTTCCTATTCTG	AACAG
StvRR_74_ska	GCACTCGGAGTTACCCAAAATATG	AAATTGGTGAGCGAAATCCAAC	AACC
StvRR_125_skb	GATGTAAGAGAGAAGGGGAGAGAGG	TGCCAGATCAATGGGAATTTG	AG
StvRR_169_ska	CATTAGAAACACTTCCCCGCTG	ACTACAACGCGCAATCCGAG	TGT
StvRR_188_skb	GAAGAGGAGGAGAAGGAGAAGGAG	CTTCCTCTTCATTCTTCCAGGTTG	AGGGG
StvRR_203_ska	GTGAAGGTTGTTGAGGAGGAGAGG	CCCCTTCACTCACTCCGTCTG	AGAGG
StvRR_225_ska	CACCTCTCTAATCCATCACTCAGC	TTGAGAAGGATAAAGGCGAGAGAG	ACTC
StvRR_233_ska	CAAATCCGTCTCACTCACTCTCAC	TGCAAAATAG**CA**AAGGGATAGAGC	AG
StvRR_233_skb	TTCTCTTCCAATCTCACCTTCCAC	CGTTTCAAAA**CA**CAAAGTCTTCCC	AAAG
StvRR_238_ska	GGTGGATACATCAGAATGGTCGTC	GATTGACTCAGCCACTTCTTCCTG	ACTG
StvRR_239_ska	ATTCTGAAGCCAGGGAAAATCAAC	AAGGATGAGAGTGATTTGTCGGAG	AATC
StvRR_246_ska	ATTGTGAAGAAGCGTTGAGTAGGC	TGCTTGGTCTAATGAAGTGCAATG	AG
StvRR_254_ska	CTTTCTTCCGAATCAACCCAAC	ATACCAAATAGGGAGGAATAGGGG	AACC
StvRR_309_ska	CTGACTGATGAATGTTCGCAACC	CTGCAGGATGTCAATTCATCACAG	AG
StvRR_332_skb	ATTGTTAGGCTACCGTAATCCACC	AGAGAAGCGGAACAAGAGAGATTG	AG
StvRR_354_ska	ATCCCTCTCATCCTCTTCTCCTTC	AATGGATACTGGCGGAAGTAAGG	AAG
StvRR_366_ska	GGAGTCCAATGCTTTCCAATTATG	TCGAGGTA**TTT**GCTCTGTGTTTTG	AATG
StvRR_426_ska	TGACGGAACACTACTCACTCAATTC	TTTGAAGGAAATCATTGCATATCG	ATC
StvRR_427_ska	TTTCTTCCCTTTTCAGCCTTCTTC	AAGA**GTTG**CGGAAGAGAACGG	AGG
StvRR_428_ska	GAGTACTTGTACTGGACGGGGAAG	GGCA**ACTT**TCACAACAACAATGAC	ATCG
StvRR_459_skb	TCATCCACTCCTTATTATTTCACCC	TTAA**GGAG**AATAGAGGATGAGAAAACTG	AAAG
StvRR_473_ska	ATTCCTGTCCCGTCTCTGAATCTC	GGGA**GAGA**GGAGAGGATGAAGAAG	AG
StvRR_518_ska	CAAAGAGCAAAATTGAAGAAGAAGC	TTCTG**CAG**AGGGTAAAGATTTTGG	AAC
StvRR_532_ska	CTCCACCACCAACATTCAACATC	CACAGAGAAGGAAGAGGAGGGAAG	ATCC
StvRR_553_ska	ACGAAACGTAGCAAAGGAAGAGTG	CCTTTTCCTTCCGGATAATCCTC	AAAC
StvRR_578_ska	TTGAAATTTTCTTTTGGCACAACC	CGGAAGTGACCTAACCAACCTG	ACCT
StvRR_635_ska	GCGAGTGAATGGAGATAAACAGC	ACTCGCCCTTCCTCCTTACTATTC	ACTC
StvRR_650_ska	GTCCACGACAAGGTGATCCG	TCCACCTCGTTTTGTTAAATGGTC	AGGG
StvRR_685_ska	GTCGCTGCGTTGTTCTTCTC	AAAGTGGGGAAAGGAGAGGG	AACC

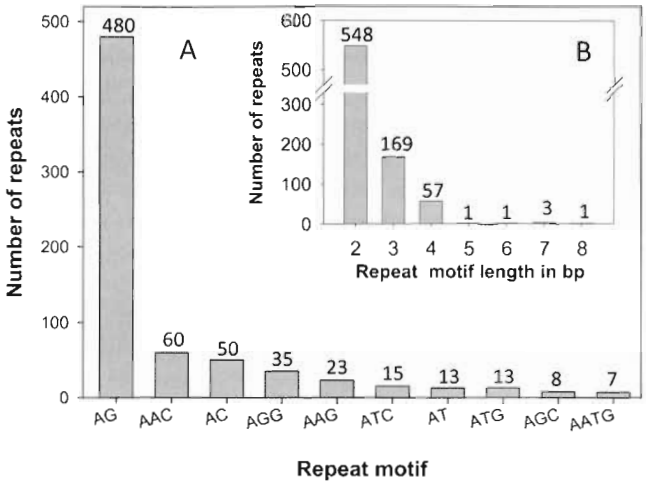

FIG. 1. Frequency of the 10 most abundant repeats detected in *Rotylenchulus reniformis* SSR-enriched libraries. A. Frequency of motif repeats detected in an SSR-enriched library after screening 694 contigs. Another 42 repeat motifs with frequencies lower than seven were not included in the plot. B. Frequency of repeats detected in *R. reniformis* for each repeat-motif length in base pairs (bp).

in Figure 2A. PIC values ranged from 0.00 to 0.82, with a general increase in PIC values as the number of alleles increased from one to five. Within this range, however, some low PIC values were observed (below 0.4) as a group of markers were monomorphic for two, three and even four alleles (Fig. 2A).

Cluster analysis

Genetic similarity coefficients based on UPGMA for the six *R. reniformis* isolates using the selected 156 markers are illustrated in the dendrogram in Figure 3. These markers allowed a clear distinction among the *R. reniformis* isolates from MS and GA vs. LA and TX as shown by the high bootstrap resampling coefficient obtained (Figure 3). A total of 62 markers distinguished the populations GA and TX, and 22 markers detected differences among the three MS populations.

Pathogenicity test of R. reniformis isolates on cotton varieties

Differences among both the cotton genotypes and the nematode populations were identified in greenhouse tests (Table 3). The resistant accessions *G. barbadense* TEX 110 and *G. arboreum* A2-190 supported significantly lower levels of reniform nematode infection than the four *G. hirsutum* lines, and they did not differ from each other. The reniform nematode population from GA infected at higher levels than did the population from TX. However, neither of these

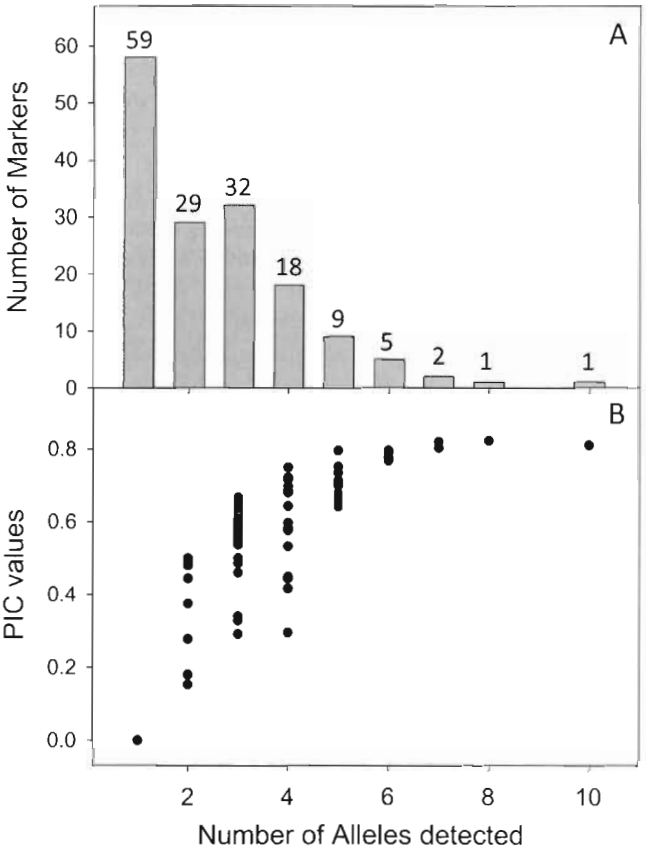

FIG. 2. A. Frequency of markers by number of alleles detected. B. Polymorphism information content (PIC) by number of alleles detected, calculated across six *Rotylenchulus reniformis* isolates.

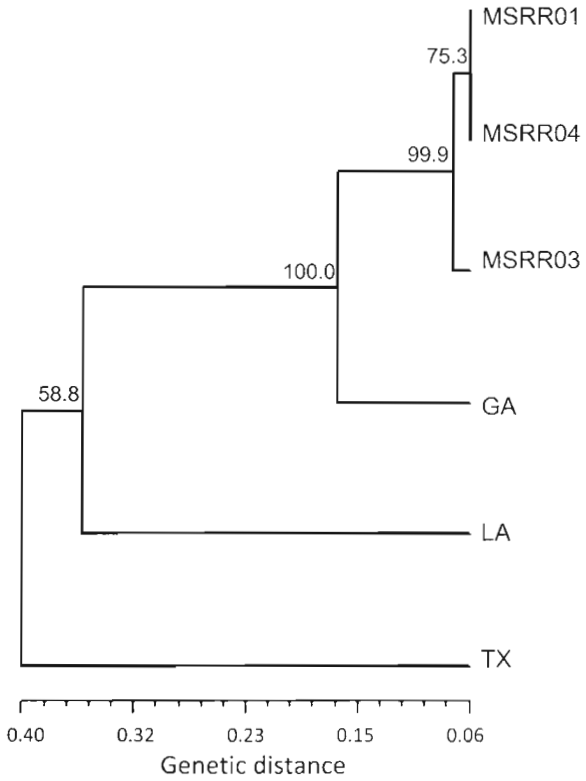

FIG. 3. Cluster analysis of six *Rotylenchulus reniformis* isolates using 156 SSR markers calculated using the unweighted paired group method using arithmetic averages (UPGMA) of NTSYSpc 2.2. Confidence levels from bootstrap analysis (5000 replicates) are indicated at the nodes.

TABLE 3. Development of female *Rotylenchulus reniformis* from four states on the roots of six cotton genotypes in greenhouse tests.

Treatment	Level	Females per g fresh root[a]	
Cotton genotype	*Gossypium hirsutum* (T 1347)	37	a
	G. hirsutum (T 19)	32	a
	G. hirsutum (FiberMax 960 BGRR)	32	a
	G. hirsutum (T 1348)	26	a
	Gossypium barbadense (TEX 110)	6	b
	Gossypium arboreum (A2-190)	5	b
	F	28.34	
	P>F	<0.0001	
Nematode population	GA	23	a
	LA	18	ab
	MSRR04	17	ab
	TX	13	b
	F	2.51	
	P>F	0.0606	
Cotton x Nematode	*F*	0.47	
	P>F	0.9523	

[a] Values are geometric (backtransformed) means of 10 replications from two combined runs of the experiment. Means followed by the same letter are not significantly different according to differences of least squares means ($\alpha = 0.05$).

populations was significantly different from the LA or MS populations tested, which caused intermediate levels of reniform nematode infection (Table 3). No interaction between cotton genotype and nematode population was detected with respect to root infection.

DISCUSSION

To develop effective management practices for plant pathogens and long lasting resistance in crop varieties, the genetic variability of pathogen and host need to be known (Werlemark et al., 2006; Silva et al., 2008), and this is particularly true in the case of nematodes (Cook, 2004). Large morphological differences have been reported for *R. reniformis* around the globe (Germani 1978; Nakasono 2004; Soares et al., 2004; Agudelo et al., 2005). However, for the most part, the presence of genetic variability in *R. reniformis* has been inconsistent, as analysis of ITS1 sequences showed no differences among populations from various southern states of USA (Agudelo et al., 2005), whereas also ITS1 and 18S rDNA showed large differences among isolates of a narrow area in Alabama (Tilahun et al., 2003, 2008).

We have developed 156 molecular markers based on SSR-enriched libraries of *R. reniformis* that will enable detection of genetic variability in this species. We found that 88 of these markers were polymorphic among six populations from four southern states (TX, LA, MS, GA). However, not only these 88 markers are important, as all 156 could show polymorphism if testing isolates from more extensive areas. It was surprising that among three populations collected from a same location in Mississippi (MSRR01, MSRR03 and MSRR04) there was enough genetic diversity to show polymorphism in 22 of the markers. At the same time this shows once more the need to measure the genetic variability of this nematode for proper evaluation in plant breeding programs, as indicated by Agudelo et al. (2001, 2005).

Regarding the repeats found in the SSR-enriched libraries for *R. reniformis* the general trend was similar to reports for other species. We found a significant reduction in the number of repeats detected as the length of the motifs increased from 2 to 4 bp, this also was observed in complete genome screening of other eukaryotes (Katti et al., 2001). Dinucleotide repeats AC and AT have been the most frequently found throughout five complete eukaryote genomes, where either one of these motifs was predominant (Katti et al., 2001; Anwar and Khan 2005). In our *R. reniformis* SSR-enriched libraries the most abundant repeat was AG, present in a frequency eight times higher than any other repeat motif. Though in low frequency, the repeat motifs CG, CCG and AGGGG found in *R. reniformis* were rather curious, as they are very rare or absent in other eukaryote genomes (Katti et al., 2001; Anwar and Khan 2005). We also report here for the first time the presence of at least three minisatellites, with motif lengths of 18, 20 and 40 bp in *R. reniformis*. Minisatellites in yeasts are usually related to cell wall proteins or cell wall metabolism (Richard and Dujon, 2006), however it would be interesting to find the role for minisatellites in nematodes.

The SSR markers developed here detected between one and ten alleles with an average of 1.7 alleles per marker and a maximum PIC value 0.8. The relatively high polymorphism observed across the 156 loci allowed a preliminary cluster analysis of the populations of the four southern states and the large values of Bootstrap resampling supported the discrimination. The percentage of multiallelic loci occurring within each population was similar (40-45%) for the six *R. reniformis* isolates independently of them being originated from a single egg mass or from many individuals.

Though correlations between heterozygosity and environmental fitness is still not completely understood (Hansson and Westerberg, 2002), there is evidence that the presence of dissimilar length alleles at microsatellite loci (multiallelic locus) are more likely to mutate (Amos et al., 2008) and this could have a potential effect on the environmental fitness of the nematode.

As expected, lower levels of reniform nematode infection were associated with the known resistant genotypes *G. barbadense* TEX 110 (Yik and Birchfield, 1984; Robinson, 2007; Starr et al., 2007) and *G. arboreum* A2-190 (Robinson, 2007; Starr et al., 2007; Sacks and Robinson, 2009). The GA population of reniform nematode caused higher levels of infection than did the TX population. Our cluster analysis based on 156 markers showed a genetic distance of 0.35 between these two populations with 62 SSR markers that distinguished GA and TX populations from each other. While the results of the present work do not identify markers associated with differences in infection, those that showed population differences could be used as the starting point for future studies. Differences in the level of infection by reniform nematode have been reported on various host plants (Dasgupta and Seshadri, 1971; McGawley and Overstreet, 1995; Nakasono, 2004; Agudelo et al., 2005). However, a previous study (Agudelo et al., 2005) did not identify differences between reniform nematode populations at the molecular level even though differences in reproductive indices were documented.

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