Theor Appl Genet (2009) 120:139–150
DOI 10.1007/s00122-009-1165-4

ORIGINAL PAPER

Identification and genomic location of a reniform nematode (*Rotylenchulus reniformis*) resistance locus (*Ren*^{ari}) introgressed from *Gossypium aridum* into upland cotton (*G. hirsutum*)

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Received: 9 April 2009 / Accepted: 27 September 2009 / Published online: 14 October 2009

Abstract In this association mapping study, a tri-species hybrid, [*Gossypium arboreum* × (*G. hirsutum* × *G. aridum*)^2], was crossed with MD51ne (*G. hirsutum*) and progeny from the cross were used to identify and map SSR markers associated with reniform nematode (*Rotylenchulus reniformis*) resistance. Seventy-six progeny (the 50 most resistant and 26 most susceptible) plants were genotyped with 104 markers. Twenty-five markers were associated with a resistance locus that we designated *Ren*^{ari} and two markers, BNL3279_132 and BNL2662_090, mapped within 1 cM of *Ren*^{ari}. Because the SSR fragments associated with resistance were found in *G. aridum* and the bridging line G 371, *G. aridum* is the likely source of this resistance. The resistance is simply inherited, possibly controlled by a single dominant gene. The markers identified in this project are a valuable resource to breeders and geneticists in the quest to produce cotton cultivars with a high level of resistance to reniform nematode.

Communicated by R. Waugh.

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Introduction

In recent years the reniform nematode [*Rotylenchulus reniformis* (Linford and Oliveira)] has been expanding its geographic distribution in the US as well as increasing its numbers in affected fields (Robinson 2007). Nematodes are the pathogens that cause the greatest losses in U.S. cotton, and reniform nematode is second only to root-knot nematode (*Meloidogyne* spp.), causing an estimated economic loss of approximately 2.0% nationwide. However, in the Mid South states of Alabama, Mississippi, and Louisiana, losses to reniform nematode were much higher, averaging 7.2% (Blasingame et al. 2008).

As the reniform nematode incidence increases in states east of New Mexico its economic impact is expected to increase as well. Two recent reviews summarize the present status of cotton crops in the US in relation to reniform nematode damage and strategies to manage this pest problem (Starr et al. 2007; Robinson 2007). Chemical control is somewhat successful, but it is expensive and environmentally damaging and only a temporary solution. Crop rotation, whenever possible, is a better management alternative (Brathwaite 1974; Thames and Heald 1974; Windham and Lawrence 1992; Davis et al. 2003, Stetina et al. 2007). Ultimately, however, host plant resistance is the best choice

from an environmental and human health perspective as well as for economic reasons.

Some tolerance to reniform nematode has been found in eleven lines of upland cotton (*Gossypium hirsutum* L., an $A_hA_hD_hD_h$ tetraploid) (Cook et al. 1997; Cook and Robinson 2005; Jones et al. 1988), but Robinson et al. (1999) in a survey of the 55 cultivars of upland and Pima (*G. barbadense* L.) cotton most commonly planted between 1950 and the time of the study found no resistance to reniform nematode. In a more recent survey of 52 conventional and transgenic cotton cultivars used in Alabama, Usery et al. (2005) also failed to find resistance to reniform nematode. In an extensive survey of *Gossypium* germplasm for reniform nematode resistance and tolerance, Yik and Birchfield (1984) found *G. longicalyx* J.B. Hutch. & B.J.S. Lee, to be immune to reniform nematode infection while *G. somalense* (Gürke) J.B. Hutch., and *G. stocksii* Mast. were highly resistant. Resistance was also found in *G. arboreum* L., *G. herbaceum* L. and *G. thurberi* Tod. accessions (Yik and Birchfield 1984). However, in *G. barbadense,* only 'Texas 110' showed resistance (Yik and Birchfield 1984; Zhang et al. 1998), and there was resistance in three accessions of the marie galante race of *G. hirsutum* (Yik and Birchfield 1984). In a survey of 1866 primitive accessions of *G. hirsutum* and 907 of *G. barbadense* from the U.S. Cotton Germplasm Collection, Robinson et al. (2004) found 17 moderately resistant accessions of *G. barbadense* and 6 of *G. hirsutum*; the best was GB-713, which showed 3% of the nematode reproduction in comparison to the susceptible *G. hirsutum* cultivar, DP 16. TX-1347 and TX-1348, originally identified as *G. hirsutum* but phenotypically more similar to *G. barbadense,* were found to have reniform nematode resistance in a 1997 survey of wild Mexican accessions (Robinson and Percival 1997). Previously, Carter (1981) had documented a reniform nematode resistance reaction in *G. arboreum* 'Nanking' CB 1402. Seven out of nine accessions of *G. arboreum* were found to be resistant by Stewart and Robbins (1995), confirming the potential of this species to contribute resistance genes to cultivated cotton.

Since reniform nematode resistance has been found in the diploid species *G. longicalyx*, *G. arboreum*, *G. thurberi*, and *G. herbaceum*, introgression of the resistance gene(s) into upland and Pima cultivars is a logical step, although not an easy one. The survival of the plants resulting from interspecific crosses is inescapably low due to chromosome pairing difficulties (Beasley 1940, 1942) and the probability of obtaining agronomically suitable introgressed material is even lower. Although difficult, traits of interest have been introgressed from diploid species via hexaploid bridging lines (Robinson et al. 2007; Konan et al. 2007; Mergeai et al. 2009). The most extensive group of bridging lines were developed by researchers at Gembloux Agricultural University (Maréchal 1983; Vroh Bi et al. 1999; Mergeai 2003; Ahoton et al. 2003; Benbouza et al. 2009), but other groups have also produced diploid × tetraploid lines (Beasley 1940, 1942; Brown and Menzel 1950; Muramoto 1969; Fryxell 1976; Brubaker et al. 1999).

To date the most successful introgression of reniform nematode resistance using hexaploid bridging lines has been accomplished using *G. longicalyx* as the source of resistance [HLA-(*G. hirsutum* × *G. longicalyx*)^2 × *G. armourianum* Kearn.], (Bell and Robinson 2004; Robinson et al. 2007), and two germplasm lines LONREN-1 and LONREN-2 have been developed with this resistance source. The reniform nematode resistance has been attributed to a single dominant gene on chromosome 11, *Ren^{lon}* (Dighe et al. 2009). Another tri-species hybrid has been developed using a bridging line (*G. hirsutum*. × *G. thurberi*)^2 crossed with *G. longicalyx* (HTL, Konan et al. 2007; Mergeai et al. 2009). Screening of the BC_1 through BC_3 progeny for reniform nematode resistance and genotyping the progeny lines using simple sequence repeat (SSR) markers BNL 0836_215 and BNL3279_114 indicated that *G. longicalyx* may not be the only source of resistance (Mergeai et al. 2009); the other diploid parent in HTL, *G. thurberi*, may be an additional source. Introgression has also been reported using *G. arboreum* A_2-194 as the source of the resistance and the authors reported that one gene confers resistance (Avila et al. 2004, 2007). However, LaFoe (2005) crossed two resistant *G. arboreum* accessions (A_2-190 and A_2-019) with a susceptible accession A_2-082 and reported a distribution of susceptible and resistant F_2 plants that fit a ratio of 9 resistant to 7 susceptible individuals. He concluded that at least two partially dominant genes were responsible for the reniform nematode resistance present in *G. arboreum* A_2-190 and A_2-019.

A tri-species hybrid, HAA, obtained from a cross between *G. arboreum* A_2-190 (PI 615699) and a hexaploid bridging line G 371 (*G. hirsutum* × *G.aridum* [Rose & Standl.] Skow.) was developed by Sacks and Robinson (2007). A_2-190 was found to be resistant in reniform nematode screening assays by Stewart and Robbins (1995), and it was confirmed by independent screening tests (LaFoe 2005; Sacks and Robinson 2009). Sacks and Robinson (2009) evaluated 27 "S_2" (F_2) progeny from the bridging line G 371 (Maréchal 1983). Sacks and Robinson (2009) reported that the progeny were all resistant, and concluded that the parental line G 371 was homozygous for the resistance allele. The authors could not determine the exact source of the resistance, but postulated that it came from *G. aridum*. They argued that the *G. hirsutum* parent, NC8 was an old line that had never shown any resistance. Further, there have been no reports of any *G. hirsutum* cultivars being resistant to reniform nematode. While there have been no reports of resistance within the D genome species

G. aridum, only two accessions have been screened. Additionally, there are reports of resistance in other D genome diploids including another Mexican species, *G. thurberi*, and *G. raimondii* from Peru (Yik and Birchfield 1984).

The HAA tri-species hybrid was crossed to a *G. hirsutum* cultivar, MD51ne (Meredith 1993), and progeny from this cross was evaluated for reniform nematode resistance (Sacks and Robinson 2007, 2009). The authors found that the resistance values in the segregating progeny appeared to fit a 3:1 ratio, but the figures were obscured by variation in the nematode screening scores and possible partial dominance of the resistance gene(s) from A_2-190. Although the genetics of the resistance could not be exactly defined, it appeared that there were two sources of resistance.

Due to the difficulties in obtaining precise phenotypic data from nematode screening tests (Roberts 2002; Robinson 2002) and the multi-gene nature of the apparent reniform nematode resistance, it would be useful to identify molecular markers associated with resistance. In this association mapping study, we identify and evaluate molecular markers associated with the *G. aridum* resistance source in progeny from a cross between the tri-species hybrid [*G. arboreum* × (*G. hirsutum* × *G aridum*)^2] and MD51ne (*G. hirsutum*) and map a putative resistance locus.

Beasley (1940) introduced the designation system for cotton genomes and his cytogenetic studies demonstrated that tetraploid cottons have an AD genomic constitution (1942). Kohel (1973) designated the chromosomes by Arabic numbers and assigned chromosomes 1–13 to the A genome and 14–26 to the D genome. In this study we follow their nomenclature.

Materials and methods

Sacks and Robinson (2007, 2009) developed a line by crossing the diploid *G. arboreum* A_2-190 (PI 615699) with the hexaploid bridging line (G 371 [*G. hirsutum* × *G aridum*]^2, Maréchal 1983). A single fertile plant was produced and the chromosome number of this tri-species hybrid plant (here designated HAA, Fig. 1) was verified as tetraploid and was subsequently crossed repeatedly, both as a male and a female, to the *G. hirsutum* line MD51ne (Meredith 1993) to produce 247 plants that make up the HAAH population (Fig. 1; Sacks and Robinson 2007, 2009). The HAAH population, the parental lines A_2-190, G 371, HAA and MD51ne (also used as a susceptible check) and the susceptible check *G. hirsutum* 'DP 16' (Jones 1998; Bowman et al. 2006) were assayed for nematode resistance as described in Sacks and Robinson (2009). Briefly, these authors conducted the assays in growth chambers and the 500-ml pots containing sterilized commercial potting mix were inoculated with 8–14 vermiform reniform nematodes

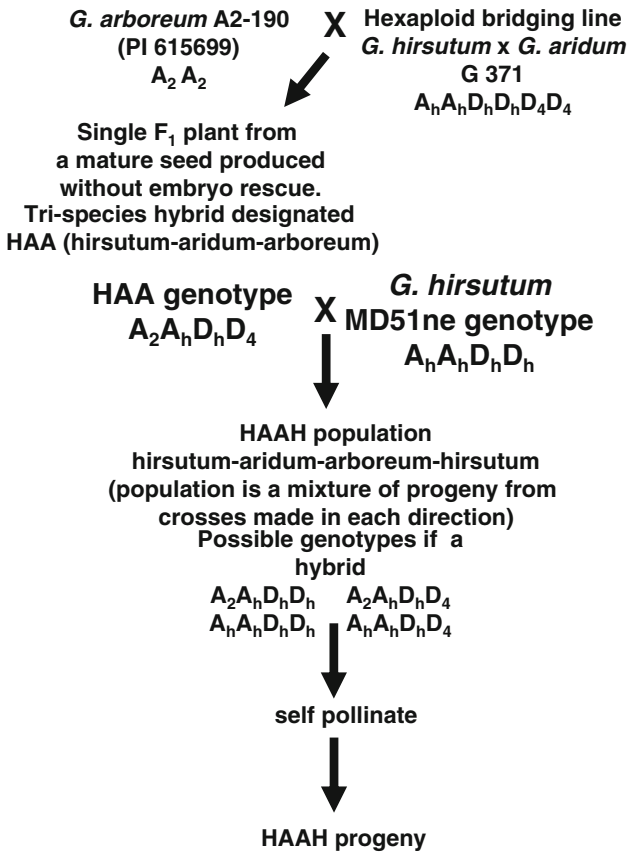

Fig. 1 Pedigree of the HAA tri-specific hybrid, HAAH population and HAAH progeny and their possible genotypes

per ml of mix within 14 days of planting. The assays were scored 8–9 weeks after inoculation and the scores were obtained by estimating the number of nematodes in the soil from each plant standardized as a percentage of the average number of nematodes present in the susceptible controls DP 16 and MD51ne. Based on the segregation of resistant and susceptible plants in the HAAH population, Sacks and Robinson (2009) concluded that at least two loci from two different sources were involved in reniform nematode resistance in these hybrids. In the present study, based on the resistance scores obtained from the previous nematode assays, a subset of the HAAH plants was selected and placed in two groups. The 'resistant group' consisted of the 50 most resistant plants, with scores from 0 to 15% of the nematode reproduction in the susceptible checks, and the 'susceptible group' contained 26 plants with the highest scores (70–175%). These 76 plants were used to identify SSR markers associated with the nematode resistance detected in the HAAH population and each sample was analyzed individually.

In an attempt to make the screening more efficient and less expensive, we selected SSR markers that had previously been located to chromosomes where root-knot nematode or reniform nematode resistance loci had been

identified in published reports (Shen et al. 2006; Wang et al. 2006; Ynturi et al. 2006; Robinson et al. 2007). Using this criterion, we targeted chromosomes 2, 3, 7, 8, 11, 14, 16, 21 (D02), and 24 (D03) instead of screening SSR markers from all 26 chromosomes. A first round of marker screening included 56 SSR markers from the nine chromosomes mentioned above, and was designed to identify associations with specific chromosomes. This screening identified a putative resistance locus (loci) associated with chromosomes 11, 21, or both. The second round of genotyping included 48 additional SSR markers and was used to more accurately locate the putative resistance allele(s) on those chromosomes.

All the SSR markers used in this study (a total of 104) are publically available and primer sequence information can be found at the Cotton Marker Database (http://www.cottonmarker.org). In addition to individual DNA samples from the 76 selected resistant and susceptible plants, the screening panel contained parental types, resistant lines GB-713 and Texas 110, LONREN-1, the susceptible cultivar DP 16, additional resistant and susceptible *G. arboreum* lines, *G. aridum* and *G. raimondii* accessions (Table 1). DNA was extracted from freeze-dried leaf tissue using a modified version of Paterson et al. (1993) where the final steps to remove residual polysaccharides were eliminated.

Primer pairs for the SSR markers were fluorescently labeled with either 6-hexachlorofluorescein (HEX) or 6-carboxyfluorescein (FAM) 5′ fluorescent label (Invitrogen, Carlsbad CA). The 5-µl PCR reaction included 5 ng DNA, 2.5 pmol each of the forward and reverse primers and 2.5 µl JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, St. Louis, MO). Amplification conditions were 95°C for a 1-min denaturation step followed by 35 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 30 s, with a final step of 72°C for 2 min. Amplified PCR products (amplicons) were separated and measured on the automated capillary electrophoresis system ABI 3730 XL (Applied Biosystems, Forest City, CA) at the Mid South Area Genomics Center (Stoneville, MS). GeneScan™-500 ROX™ (Applied Biosystems, Forest City, CA) was used as an internal DNA size standard. The output was analyzed with GeneMapper 3.7 software (Perkin Elmer, Norwalk, CT). Polymorphic SSR markers associated with reniform nematode resistance were used in further association analysis. Markers were designated by their name (e.g., BNL 3279) followed by a "_" and the fragment size associated with a resistant phenotype (BNL3279_132). The reniform nematode resistance phenotypic data were combined with the marker data and analyzed in SAS (SAS Institute, Cary NC) using a Chi-square 2×2 contingency table and an *F*-test to determine putative association. The associations and relative location of the markers on the chromosome were confirmed using JoinMap 4.0 (Van Ooijen 2006). All linkage groups were created using a minimum LOD score of 5.0.

Two of the markers found to be putatively associated with a resistance locus in the previous test were validated by using them to evaluate 198 plants from the original HAAH population (the surviving plants from the initial population of 247 plants), including the 76 used in the association analysis. These plants had nematode scores ranging from 0 to 175% of the nematode reproduction in the susceptible checks, DP 16 and MD51.

Table 1 Cotton cultivars, lines and accessions used in the mapping of the reniform resistance locus *Ren*^{ari}

Line	Species	Source information	Genome designation	Host reaction
G 371	(*G. hirsutum* × *G. aridum*)^{2a}	Maréchal (1983)	[($A_hA_hD_hD_h$)D_4D_4]	Resistant
A_2-190	*G. arboreum*	PI 615699	A_2A_2	Resistant
HAA	*G. arboreum* × (*G. hirsutum* × *G. aridum*)^2	Sacks and Robinson (2009)	$A_2A_hD_4D_4$ or $A_2A_hD_hD_4$	Resistant
MD51ne	*G. hirsutum*	Meredith (1993)	$A_hA_hD_hD_h$	Susceptible
A_2-113	*G. arboreum*	PI 529740	A_2A_2	Resistant
A_2-194	*G. arboreum*	PI 615703	A_2A_2	Resistant
D_4-1	*G. aridum*	PI 530886	D_4D_4	Unknown
D_4-2	*G. aridum*	PI 530887	D_4D_4	Unknown
D_4-3	*G. aridum*	PI 530888	D_4D_4	Unknown
D_4-4	*G. aridum*	PI 530889	D_4D_4	Unknown
D_5-8	*G. raimondii*	PI 530905	D_5D_5	Unknown
GB 713	*G. barbadense*	PI 608139	$A_bA_bD_bD_b$	Resistant
Texas 110	*G. barbadense*	PI 163608	$A_bA_bD_bD_b$	Moderately resistant
LONREN-1	[(*G. hirsutum* × *G. longicalyx*)^2 × *G. armourianum*]	Robinson et al. (2007)	$A_hA_hD_hD_h$ with F segment	Resistant
DP 16	*G. hirsutum*	Bowman et al. (2006)	$A_hA_hD_hD_h$	Susceptible

[a] The superscript 2 indicates chromosome doubling by colchicine treatment

From the SSR markers found to be putatively associated with the resistance locus, the seven markers with the closest association were tested further. The objective was to determine if these markers were able to detect resistant genotypes in progeny from the HAAH population and to confirm that resistance was still present in the next generation of plants. To this end, the 76 plants from the HAAH population (the 50 most resistant and 26 most susceptible) were self-pollinated to produce HAAH progeny (Fig. 1). As expected from a population derived from a tri-specific hybrid (HAA), the HAAH plants presented several unusual characteristics; fecundity was low, some of these plants never produced flowers while other plants produced sterile flowers. Flower morphology was abnormal in many cases (few and abnormal stamens or exserted stigmas), therefore, HAAH plants were selfed by hand. The HAAH plants shed pollen somewhat later than normal upland plants and selfings were done no earlier than 11:00 h. Selfings were done by collecting pollen from the anthers with the aid of a sterile toothpick and depositing it on the stigmatic surfaces of the same flowers. These plants were kept in pollinator-free in greenhouses. Seed production from each individual plant was also generally low and only twenty-nine of the HAAH resistant plants and 13 of the susceptible plants produced enough progeny seed for further testing. Due to the small number of seed per plant, a reniform nematode-resistance screening test was conducted using 4 progeny from each of the 29 plants along with four replications of the parental types and susceptible checks.

For the nematode screening of the HAAH progeny seedlings, individual plants were grown in a greenhouse in cone-tainers (Ray Leach SL10 Cone-tainer, Stuewe & Sons, Inc., Tangent, OR). Each cone-tainer had 120 ml of a soil mix consisting of one part steam-sterilized field soil and two parts steam-sterilized sand. Plants were divided into four sets and randomized on the greenhouse bench. A Mississippi isolate of reniform nematode maintained in greenhouse culture on tomato (*Solanum lycopersicon* 'Rutgers') was used in the assay. One week after planting, the soil in each pot was infested by pipetting 650 vermiform reniform nematodes suspended in 2 ml of tap water into an 8 cm deep depression made near the base of the plant. Plants were watered daily and fertilized every 2 weeks with general purpose 20-20-20 (N-P-K) fertilizer (Peter's Professional, The Scotts Company, Marysville, OH). Sixty days after inoculation, vermiform stages of reniform nematode were extracted from the soil in each cone-tainer using elutriation (Byrd et al. 1976) and centrifugal flotation (Jenkins 1964) and counted. The counts were standardized by expressing them as a percentage of the mean count of the susceptible check DP 16.

DNA of the HAAH progeny seedlings used for reniform nematode resistance screening was extracted using a modified version of a quick method described by Xin et al. (2003). Briefly, a 3-mm diameter hole punch was collected from each leaf and placed in a well of a 96-well plate. To each well were added 50 µl of buffer A (aqueous solution of 2% Tween® 20 and 100 mM NaOH), and the plate was incubated at 95°C for 10 min. Then 50 µl of buffer B (0.1 M Tris–HCl and 2 mM EDTA in water) were added to each well, and the plate was sealed, well contents mixed and then centrifuged briefly to pellet solids. A 1:20 dilution was made immediately, and the DNA was stored at −20 °C until ready for use. Seven SSR markers identified in the earlier analysis as the most closely associated to the putative resistance locus were used to screen these seedlings. Genotyping was done as described above.

Results

Results from the first round of screening indicated that SSR marker fragments with the greatest level of association to reniform nematode resistance in the HAAH population were also present in the bridging line G 371 and *G. aridum* accessions. Of the 56 SSR primer pairs evaluated, 24 had previously been mapped to chromosomes 11, 21 or both (Nguyen et al. 2004; Han et al. 2006; Shen et al. 2006; Wang et al. 2006; Guo et al. 2007). In the second screening round, additional SSR markers targeting those two chromosomes were added, and a total 45 SSR markers targeting chromosomes 11 and 21 were analyzed. Twenty-five of the SSR markers were significantly ($p < 0.01$ to $p < 0.0001$) associated with reniform nematode resistance and had the same fragments as *G. aridum* and G 371. Table 2 shows the frequency of each '*G. aridum*' marker fragment in each reniform nematode resistance class. BNL3279_132 and BNL2662_90 were most closely associated with a putative resistance locus named here Ren^{ari}. The order of the SSR markers associated with reniform nematode resistance was estimated using JoinMap 4 (Fig. 2).

From the original HAAH population, 198 surviving plants (nematode reproduction scores from 0 to 175%) were evaluated, using two SSR markers, BNL2662_90 and BNL 4011_133, to determine if these markers could be used to select for the Ren^{ari} locus in the entire population and not just selected extreme types (Fig. 3). The results confirmed that either marker would detect plants associated with a high level of resistance. For BNL2662_90, 65 out of the 73 plants with resistance scores from 0 to 15% had the fragment. Overall, an individual possessing either marker had approximately 70% probability of being in resistant classes 0–15%, a 28% of being in the intermediate classes 16–69% and a 2% chance of having a >70% nematode reproduction score.

Table 2 Reniform nematode resistance classes for the 76 plants in the HAAH population and 25 SSR markers used for the association analysis that identified the *Ren*^{ari} locus on chromosome 21 (its relative location is shown as an empty column)

Nem res.classes (% G.h)*	Total no. of plants with any peaks	BNL1705	CIR156	CIR013	BNL3449	TMB0043	TMB1232	BNL2632	BNL2805	MUCS399	JESPR244	CM160	BNL1551	BNL0836
		Fragment size (bp)												
		162	130	218	141	193	195	211	228	189	133	109	165	230
0	7	0.14	0.12	0.12	0.08	0.06	0.09	0.11	0.11	0.11	0.08	0.09	0.10	0.12
1–5	28	0.49	0.49	0.48	0.54	0.56	0.54	0.54	0.56	0.54	0.56	0.53	0.55	0.57
6–10	9	0.12	0.12	0.12	0.16	0.18	0.17	0.16	0.17	0.16	0.17	0.18	0.18	0.17
11–15	6	0.09	0.10	0.10	0.05	0.03	0.03	0.03	0.03	0.05	0.06	0.06	0.05	0.07
70–75	6	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00
76–80	2	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
81–85	3	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.06	0.05	0.06	0.06	0.05	0.05
86–90	3	0.00	0.00	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.00
91–95	5	0.07	0.07	0.07	0.05	0.06	0.06	0.05	0.03	0.03	0.03	0.03	0.03	0.02
96–100	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
101–105	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
106–110	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
111–115	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
>115	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total no.	76	43.0	41.0	42.0	37.0	34.0	35.0	37.0	36.0	37.0	36.0	34.0	40.0	42.0
R plants w/fragment		0.84	0.83	0.81	0.84	0.82	0.83	0.84	0.86	0.86	0.86	0.85	0.88	0.93
S plants w/fragment		0.16	0.17	0.19	0.16	0.18	0.17	0.16	0.14	0.14	0.14	0.15	0.13	0.07

Nem res. classes (% G.h)[a]	Total no. of plants with any peaks	NAU984	BNL4011	BNL1066	TMB1871	*Ren*^{ari}	BNL3279	BNL2662	NAU3480	CM140	MUCS088	DPL0475	MGHES16	NAU2016
		Fragment size (bp)												
		287	133	115	219		132	90	146	110	137	165	209	233
0	7	0.12	0.16	0.16	0.15		0.15	0.15	0.14	0.15	0.15	0.16	0.13	0.13
1–5	28	0.57	0.60	0.60	0.54		0.60	0.60	0.57	0.57	0.59	0.55	0.48	0.46
6–10	9	0.14	0.16	0.16	0.15		0.17	0.17	0.18	0.17	0.15	0.18	0.14	0.15
11–15	6	0.07	0.04	0.04	0.09		0.09	0.09	0.07	0.07	0.07	0.07	0.05	0.06
70–75	6	0.02	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.06
76–80	2	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
81–85	3	0.05	0.04	0.04	0.04		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
86–90	3	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
91–95	5	0.02	0.00	0.00	0.02		0.00	0.00	0.02	0.02	0.02	0.02	0.05	0.06

Table 2 continued

Nem res. classes (% G.h)[a]	Total no. of plants with any peaks	NAU984	BNL4011	BNL1066	TMB1871	*Ren*^{ari}	BNL3279	BNL2662	NAU3480	CM140	MUCS088	DPL0475	MGHES16	NAU2016
		Fragment size (bp)												
		287	133	115	219		132	90	146	110	137	165	209	233
96–100	2	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
101–105	1	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
106–110	1	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
111–115	1	0.00	0.00	0.00	0.00		0.00	0.00	0.02	0.02	0.02	0.02	0.02	0.02
>115	2	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02
Total no.	76	42.0	45.0	45.0	46.0		47.0	47.0	44.0	46.0	46.0	44.0	56.0	54.0
R plants w/fragment		0.90	0.96	0.96	0.93		1.00	1.00	0.95	0.96	0.96	0.95	0.80	0.80
S plants w/fragment		0.10	0.04	0.04	0.07		0.00	0.00	0.05	0.04	0.04	0.05	0.20	0.20

For the analysis, the resistant class (R) contained 50 plants with a resistance score ≤15 and the susceptible class (S) contained 26 plants with a score ≥70. For each SSR marker, the frequency of plants with the marker present are indicated in each resistance class and the frequency of resistant plants with the marker and the frequency of susceptible plants without the marker are shown at the bottom of the table

[a] The average nematode reproduction of *G. hirsutum* MD51ne and DP 16 were used to calculate the nematode resistance classes (Sacks and Robinson 2009)

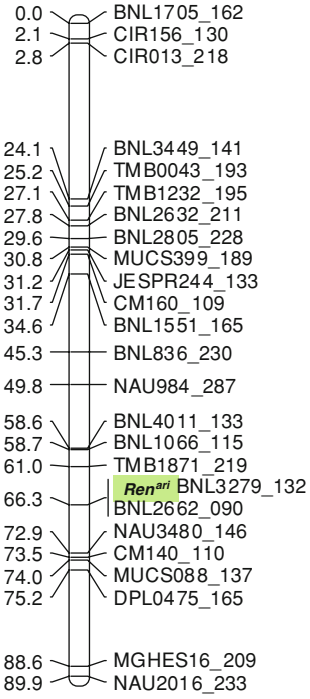

Fig. 2 Position of *Ren*^{ari} on cotton chromosome 21. On the right, markers used and location of *Ren*^{ari}. On the left, distances in cM

The HAAH population generated from the HAA tri-species hybrid crossed to MD51ne suffered high mortality, as expected, because the HAA tri-species hybrid originated from plants that have different chromosome number and genomic composition. Therefore, it is expected that the segregation distortion effect will be significant. HAAH plants presented numerous morphological anomalies (probably due to chromosomal abnormalities) and only 29 of the HAAH resistant plants and 13 of the susceptible plants produced enough progeny seed to be assayed in a reniform nematode screening test. The bolls produced by HAAH plants were always small and had few seeds; usually seeds were small but in a few cases 1 or a few large seeds were produced in one fruit. Consequently, there were generally few seed from an individual plant; therefore, the reniform nematode resistance screening was conducted using 4 progeny from each of the HAAH fertile plants along with four replications of the parental types and susceptible checks. The test confirmed that reniform nematode resistance was transferred to the progeny. Results from the screening test showed there were differences in the resistance scores among the four seeds originating from each HAAH plant (not replicates as the plants were still segregating) and between progeny from different HAAH plants. However, there was also a high level of variability among the susceptible controls indicating that observed differences may sometimes be due to environmental variation or experimental error and not genotypic differences (Fig. 4).

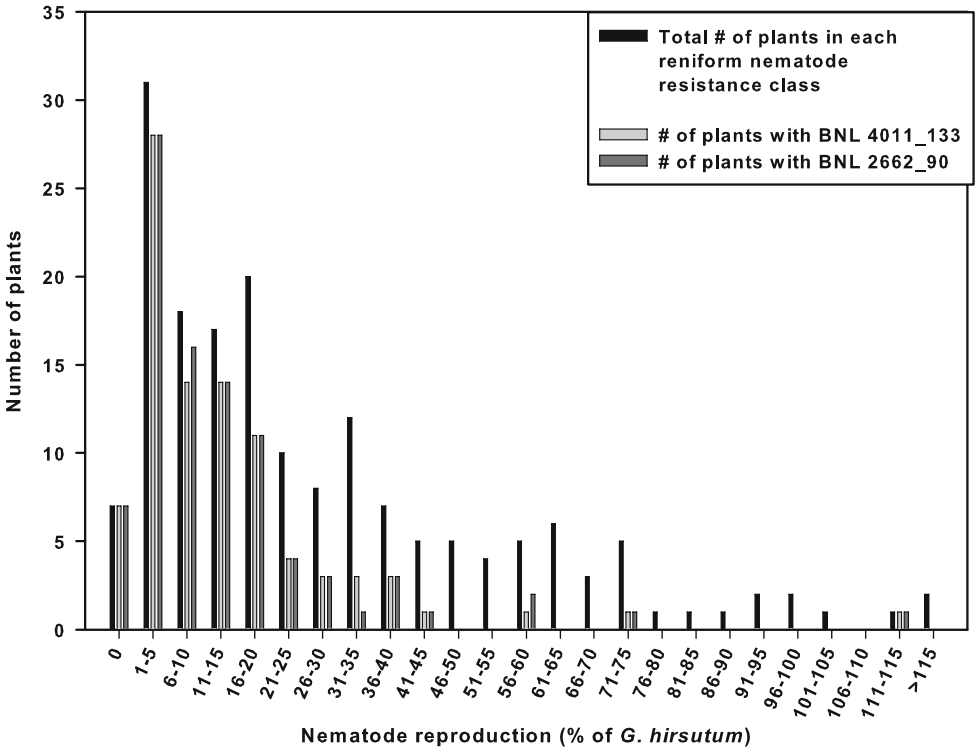

Fig. 3 Total number of plants and number of plants with the BNL 4011_133 and BNL 2662_90 markers in each reniform nematode resistance class. DNA from 198 plants of the HAAH population from the cross [*G. arboreum* × (*G. hirsutum* × *G. aridum*)^2 × MD51ne was used. The nematode reproduction (*% G. hirsutum*) is the number of nematodes present in the soil as a percentage of the nematode score of the susceptible checks MD51ne and DP 16

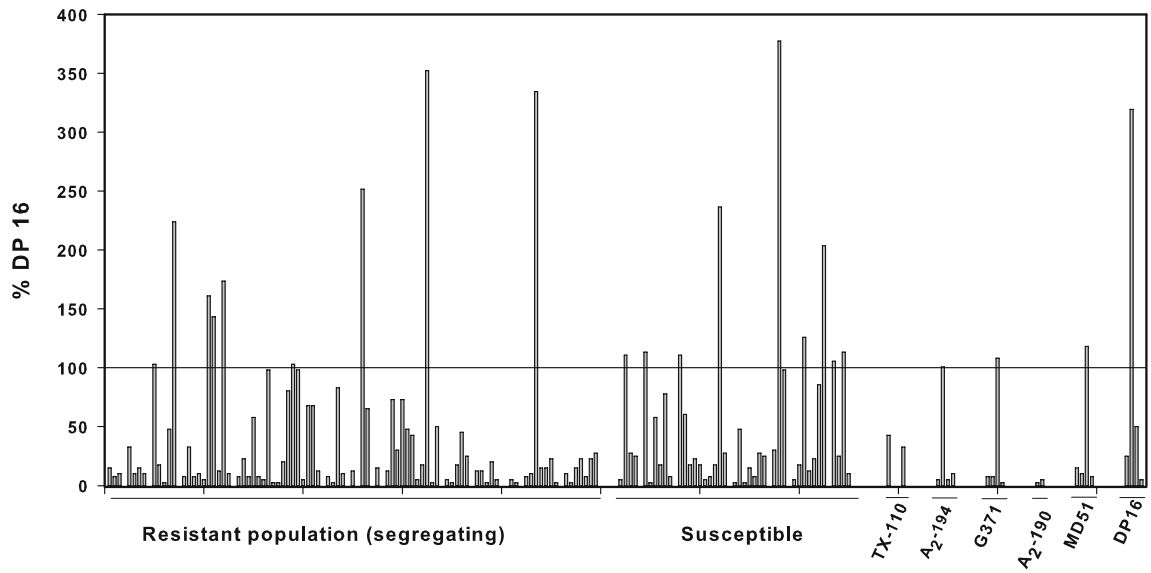

Fig. 4 Reniform nematode reproduction for 163 individual seedlings of the HAAH progeny, parental lines and susceptible (*S*) and resistant (*R*) checks; each *bar* represents an individual seedling. Nematode counts were standardized by expressing them as a percentage of the average score of the susceptible check DP 16 (reference line). The HAAH progeny seedlings have been divided into those produced by R and S plants. TX-110 and A2-194 are resistant controls, G 371 and A2-190 are resistant parents, MD51ne is the susceptible parent and DP 16 the susceptible control

Despite the variation observed in the nematode screening test, there was still a lower but detectable association between presence of the fragment using the seven markers most closely associated to *Ren*^{ari} and reniform nematode resistance scores in the progeny generation. An individual possessing the either the BNL2662_90 or the BNL3279_132 fragment had a 67% probability of being in resistant classes 0–15% (% of the nematode reproduction in the *G. hirsutum* DP 16 susceptible control); the probability was 63% for BNL4011_133 (Table 3). Although the association between the reniform nematode resistance score and the SSR markers decreased, the association among markers

Table 3 Reniform nematode resistance scores for the 144 HAAH progeny plants and presence of the fragment for 7 SSR markers

Nematode resistance classes (% *G. hirsutum* DP 16)	Total no. of plants in each class	BNL4011	BNL1066	*Ren*^{ari}	BNL3279	BNL2662	CM140	MUCS088	DPL0475
		Fragment size (bp)							
		133	115		132	90	110	137	165
0	20	0.16	0.13		0.17	0.17	0.18	0.18	0.12
1–5	23	0.19	0.18		0.22	0.22	0.21	0.20	0.14
6–10	20	0.19	0.20		0.17	0.17	0.15	0.16	0.16
11–15	14	0.08	0.08		0.10	0.10	0.08	0.08	0.11
16–70	40	0.23	0.22		0.19	0.21	0.25	0.25	0.30
>70	27	0.15	0.18		0.14	0.13	0.13	0.13	0.16
Total no. of plants	144	62	60		63	63	61	61	98

The relative location of the *Ren*^{ari} locus is shown as an empty column. Plants with a resistance score $\leq$15% were classified as resistant. For each SSR marker, the frequencies of plants with the marker present are indicated in each resistance class

remained consistent. When one marker exhibited the '*G. aridum*' fragment, the other markers did as well (Table 3).

Discussion

Plants derived from a hybrid between *G. arboreum* A_2-190 and G 371 (designated HAA) were found to be resistant to reniform nematode infection (i.e., to support only low levels of nematode reproduction). Because several *G. arboreum* accessions have been found to be resistant (Yik and Birchfield 1984) and A_2-190 was chosen for its resistance (Sacks and Robinson 2009); it was expected that it would be the major contributor of reniform nematode resistance in the tri-species hybrid plants. However, based on nematode screens performed using HAAH individuals as well as the G 371 "S_2" and HAA F_2 plants (Sacks and Robinson 2009) it became clear that the bridging line G 371 was also a contributor to resistance. In the first round of the SSR marker analysis, a close association was found between the plants scored as resistant and unique SSR fragments found in G 371 and *G. aridum*; this pattern was observed in multiple markers. This discovery was fortuitous as it indicated that markers associated with the *G. aridum* resistance source had been identified and that this source of resistance was possibly one locus. It further indicated that the *G. aridum* resistance source was not the same as that functioning in *G. arboreum*.

The map generated for chromosome 21 (Fig. 2) was not identical to any existing cotton linkage map. Direct comparison of cotton maps is difficult as maps have been based on populations originating from crosses of a variety of different species or cultivars and the markers used are also variable among studies. Additionally, it is known that the genetic distance and order of markers can be affected by segregation distortion (Lorieux et al. 1995a, b), which probably had a significant effect in this HAAH population. Also as the HAAH plants are newly synthesized polyploids, they may have a different marker order than in typical *G. hirsutum*. The map of chromosome 21 presented here shows some similarities with previously published maps; the order of markers BNL1705, CIR156, CIR013, BNL2632, BNL2805, BNL1551 and BNL3279 coincide with those on D02 (chromosome 21) in Nguyen et al. (2004) for a cross between *G. hirsutum* cv. Guazuncho 2 and *G. barbadense* cv. VH8-4602. Three of the markers used in this work, NAU2016, BNL1551, and BNL2632, present the same order as in LGD02 (chromosome 21) in a map constructed with EST-derived SSRs in *G. hirsutum* (Han et al. 2006) and two, NAU2016 and BNL1066 are similarly located to those in LGA03 (chromosome 11) in the same map. Six of the markers associated with reniform nematode resistance in the present map, NAU2016, BNL3279 (one of the two closest to *Ren*^{ari}), BNL1551, BNL3449, CIR156, and BNL1705 are in the same order as in the chromosome 21 map based on [(TM-1 × Hai7124) × TM-1] by Guo et al. (2007); and two of the markers in the present map, NAU2016 and BNL1066 were also found on Guo et al. (2007) chromosome 11. NAU2016, BNL1066 and BNL2632 have been mapped to a region on chromosome 11 of a *G. hirsutum* × *G. barbadense* cv. Pima S-6 map (Shen et al. 2006).

The map generated in this study revealed a region on chromosome 21 (Fig. 2) that is duplicated on chromosome 11. The region on chromosome 11 that has been identified as the location of root-knot nematode resistant genes *rkn1* (Wang et al. 2006) and Mi_2 (Niu et al. 2007), an RKN resistance QTL (Shen et al. 2006), and another putative RKN resistance QTL (Ynturi et al. 2006). It is also the location of the LONREN-1 resistance source (Dighe et al. 2009) and possibly resistance QTLs for Fusarium wilt (*Fusarium oxysporum*) and Verticillium wilt (*Verticillium dahliae*) (Roberts et al. 2009).

For the moment, it is not possible to say with certainty where the *Ren*^{ari} locus is located, as a number of the SSR markers have been located to both chromosomes 11 and 21. However, we propose that the *Ren*^{ari} locus is located on chromosome 21. The first indication that the *Ren*^{ari} locus is located on chromosome 21 is that *G. aridum* is a D genome diploid, and therefore its genetic contribution would be most closely related to chromosome 21. The second line of evidence is that, to date, the markers DPL0209, and BNL3592 have been mapped only to chromosome 11 (Nguyen et al 2004; Han et al. 2006; Shen et al. 2006; Wang et al. 2006; Yu et al. 2007; Guo et al 2007; Ma et al. 2008). In this study, these two markers amplified in *G. arboreum* and *G. hirsutum* samples but did not show the characteristic '*G. aridum*' fragment pattern. It appeared their amplicons mapped to chromosome 11 and were not associated to *Ren*^{ari}. Another way to assess the location of the *Ren*^{ari} locus will be to genotype interspecific hypoaneuploids lines using the markers (Stelly 1993; Gutierrez et al. 2009). This work is currently in progress.

The reniform nematode resistance screening (Fig. 4) and subsequent genotyping of the HAAH progeny seedlings indicated that nematode screening on a single plant basis was not very effective. The nematode screen data of even the susceptible controls DP 16 and MD51ne showed much variation. A similar degree of variation has been documented in previous nematode screening assays (Roberts 2002; Robinson 2002; LaFoe 2005; Robinson et al. 2007; Sacks and Robinson 2009), suggesting that replicated testing or assays under more tightly controlled conditions, such as in a growth chamber, may be needed to get more reliable phenotypic resistance scores required for mapping. A second potentially complicating factor may have been the composition of the group of progeny tested. If the single HAA plant had the genotype $A_2A_hD_hD_4$ then the HAAH population plants could have four possible genotypes, and the selfed progeny could have up to nine different genotypes (Fig. 1). It is possible that a combination of either a homozygous D_4D_4 or A_2A_2, or a heterozygous $A_2A\_D_4D\_$ could confer resistance. In this case, some individuals would be resistant, but not contain the '*G. aridum*' (D_4) fragment.

Another consideration is that the HAAH progeny population analyzed in this study probably is not a "random sample" of genotypes because a preponderance of abnormal morphology and fertility was observed among the HAAH plants. In this population, bolls on the surviving plants were always small, there were few seeds per boll and many plants were sterile. Such features were also reported for other tri-species hybrids (Beasley 1940, 1942; Brown and Menzel 1950; Muramoto 1969; Fryxell 1976; Brubaker et al. 1999; Robinson et al. 2007). As only 29 of the resistant plants and 13 of the susceptible plants produced viable progeny seed, it is likely that certain genotypes were more fertile and produced more selfed progeny, thus biasing the composition of the group of progeny evaluated in the nematode screening assay.

Despite the challenges confronted in obtaining phenotypic data (Fig. 4), the presence or absence of the '*G. aridum*' fragment in the seven markers remained consistent (Table 3). Based on the nematode resistance assay data, in these progeny, the presence of the '*G. aridum*' fragment using either BNL3279_132 or BNL2662_90 would select for resistant plants (0–15% nematode reproduction of the susceptible check) at least 67% of the time and select a susceptible type (>70%) 14% of the time. Thus, the availability of SSR markers identified in this study should allow researchers to select in early generations when reliable reniform nematode screening assays are not practical.

We have mapped the location of the *Ren*^{ari} reniform nematode resistance locus, which appears to have originated from the D genome diploid (*G. aridum*) of the G 371 hexaploid bridging line. The next step will be to resolve the *G. arboreum* contribution to resistance in the HAA line. To achieve this objective, F_2 progeny of *G. arboreum* resistant × *G. arboreum* susceptible accessions need to be evaluated. Development of *G. arboreum* resistant × *G. arboreum* susceptible F_2 populations have already been reported (LaFoe 2005; Avila et al. 2007). Any markers identified as associated with a *G. arboreum* resistance locus (loci) can be tested using the HAAH population and its progeny to see if a combination of SSR markers associated with the *G. aridum* and *G. arboreum* resistance sources could more accurately identify resistant lines. Identification of markers and mapping of the source of resistance from *G. arboreum* is currently underway. Based on reniform nematode reproduction assays, it appears that the resistance is at least as strong as that from *G. aridum*, but it may be multi-genic and therefore more difficult to assess. There is a precedent for this finding in the previous observation of multigenic root-knot nematode resistance and identification of QTLs for it (Ynturi et al. 2006).

In the course of our research, we evaluated not only the HAAH progeny, but also a set of lines from a LONREN-1 introgression program (Wallace et al. 2009). The results showed that the *Ren*^{ari} was associated with BNL3279_132 while the LONREN-1 (*Ren*^{lon}) introgressed segment from *G. longicalyx* was associated with BNL3279_114, indicating that one could select for both resistance sources with one SSR marker. Other markers, including BNL2662_73, BNL4011_177, CM140_99, DPL0209_198, and DPL0475_157, were associated with the *Ren*^{lon} locus, and may be useful as flanking markers. These two independent sources of reniform nematode resistance will be combined in future crosses.

In the present study, we identified and evaluated molecular markers associated with the *G. aridum* resistance source in the G 371 bridging line (*G. hirsutum* × *G aridum*)^2 and mapped the putative resistance locus *Ren*^{ari}. The markers identified here provide valuable tools for plant geneticists developing new lines and cultivars, especially in light of the difficulty assessing nematode resistance using reniform nematode screens.

Acknowledgments The authors are indebted to Sheron Simpson and the MSA Genomics Center for the fragment analysis, Hans Hinrichsen for germplasm maintenance, Jennifer Tonos for assisting with some of the pollinations, Pameka Johnson for tissue and primer preparation; Robin Jordan for DNA extractions; Kristi Jordan, and Mike Gafford for assistance with reniform nematode resistance screening, and Peggy Thaxton (DREC, Stoneville, MS) for providing the LONREN-1 breeding material. Cotton Inc. (Cary NC) provided funding for primers used in this project. We also thank Drs. Jack McCarty and Jeff Ray and two anonymous reviewers for helpful suggestions on how to improve this manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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