Identification of simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) that are associated with the nectariless trait of *Gossypium hirsutum* L

Sang-Hyuck Park · Jodi A. Scheffler · Jeffery D. Ray · Brian E. Scheffler

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Abstract Cotton (*Gossypium hirsutum* L.) has nectar containing modified stomates called nectaries that can be located on leaves, bracts or calyces. The nectar attracts some beneficial insects such as bees, but also predatory damaging insects such as heliothines and *Lygus* species. There is a naturally occurring mutation that eliminates the nectar containing nectaries and makes the cotton plants less attractive to insects. The nectariless (*ne*) trait is associated with a double recessive mutation of two genes (*ne₁* and *ne₂*) on homeologous chromosomes 12 and 26. Expression of the trait can be variable and is also affected by environmental conditions. This makes accurately selecting for the trait based on phenotype difficult. This study identified SSR and SNP markers that can be used by breeders for marker assisted selection (MAS) of the nectariless trait. DNA markers associated with the genes conditioning the trait and used for MAS, will

allow cotton cultivars to be easily developed that have decreased attractiveness to insects and reduce the need for insecticides, especially those harmful to beneficial insects such as honeybees.

Keywords Upland cotton · *Gossypium hirsutum* L. · Nectariless · Simple sequence repeat (SSR) · Single nucleotide polymorphism (SNP)

Introduction

Cotton (*Gossypium* spp.) is an economically important crop for fiber and seed oil production. The field crop was grown in 72 countries in 2019/2020 with a projected production of 123 million bales (US Department of Agriculture, Foreign Agricultural Service) and an estimated worth of \$5.67 billion (US Department of Agriculture, National Agriculture Statistics Service, www.nass.usda.gov). The United States is the third largest cotton producer (19.9 M bales) in the world after India (30.5 M bales) and China (27.2 M bales). The cotton crop is susceptible to numerous insect pests (Hou et al. 2013). In 2019, cotton yields were reduced by an estimated 4.47% by pest insects including fleahoppers (0.84%), lygus (0.83%), bollworm/budworm (0.75%), stink bugs (0.72%), thrips (0.40%), spider mites (0.28%), grasshoppers (0.15%), aphids (0.12%), and whiteflies (0.07%). Total costs and losses from pest insects were estimated at \$462

S.-H. Park · B. E. Scheffler
Genomics and Bioinformatics Research Unit, USDA-ARS, 141 Experiment Station Road, Stoneville, MS, USA

S.-H. Park
Institute of Cannabis Research, Colorado State University, 2200 Bonforte Blvd, Pueblo, CO, USA

J. A. Scheffler (✉) · J. D. Ray
Crop Genetics Research Unit, USDA ARS, 141
Experimental Station Road, Stoneville,
MS, USA
e-mail: jodi.scheffler@usda.gov

million (Cotton insect losses 2019, entomology.msstate.edu/resources/pdf/2019/table28.pdf).

The genus *Gossypium* has nectar containing glands in most of its species. These nectar containing glands are called nectaries and they attract and serve as an important food source for a variety of insects. Depending on the species, nectar is produced in floral and / or extrafloral nectaries. The floral nectaries are found between the sepals and petals of the flower bud and are often absent in modern cultivated upland cotton. Extrafloral nectaries are found in the lower part of veins on the under (abaxial) side of leaves and on many modern cotton cultivars there is a nectary only on the mid-vein. Extrafloral nectaries can also be found on the calyx or below the bracts subtending the flower (Fig. 1) (Chatt et al. 2019). These nectaries are thought to be a modified form of stomata with papillae (Paiva 2017; Hu et al. 2020). The nectar is attractive not only to mutualistic symbiotic insects such as bee pollinators and protectors such as ants, but also

herbivore predatory insects (Adjeimafo and Wilson 1983). In general, nectar possessing plants have evolved strategies to both attract and repel insects, often by producing their nectar outside of the flower in extrafloral nectaries (Wackers et al. 2001; Heil 2015; Roy et al. 2017; Llandres et al. 2019). Many of the insect predators of cotton are attracted to the nectar and research has demonstrated that extrafloral nectaries can enhance visitation behavior by insects (Stapel et al. 1997). When the nectaries are absent, the associated nectar is no longer produced and these “no nectary” cottons (nectariless) can be used as a biocontrol agent, by removing the nectar that attracts insect pests of cotton. The nectariless trait has been reported to confer resistance to lepidopterous species (Lukefahr and Rhyne 1960; Holder et al. 1968), pink bollworm, *Pectinophora gossypiella* Saunders (Wilson and Wilson 1976; Flint et al. 1991), and tarnished plant bug, *Lygus hesperus* (Benedict et al. 1981).

Fig. 1 Examples of bract and leaf extrafloral nectaries. Source <https://www.cottongen.org/find/imagesCottonGen>

Gossypium species exist in a diploid form ($2n = 2X = 26$) and a tetraploid form ($2n = 4X = 52$). The nectariless trait has been observed in diploid forms of cotton (<https://www.ars-grin.gov/>), especially *G. arboreum* (Hu et al. 2020) which has been cultivated for over a thousand years (Du et al. 2018). However, it was almost impossible to produce viable progeny by crossing a diploid and tetraploid cotton. The nectariless trait was first observed in the tetraploid cotton *Gossypium tomentosum*, a wild relative of *G. hirsutum*. Meyer and Meyer (1961) studied the trait and attributed it to mutations in each of two genes, with the nectariless condition reflecting double-recessive mutations. They transferred the trait from *G. tomentosum* to cultivated upland cotton (*G. hirsutum*) by making interspecific crosses between the two species followed by back-crossing to upland cotton for four generations until nectariless lines were produced that were comparable to upland cotton. These cotton lines did not have any floral or extrafloral nectaries. Subsequent studies indicated that the nectariless trait was controlled by two genes, designated ne_1 and ne_2 , located on chromosome 12 (A genome) and 26 (D genome) (Rhyne 1965; Holder et al. 1968). These two chromosomes are homeologous with approximately 40% overall physical similarities (Xu et al. 2008; Saski et al. 2017). An ne_2 physical and genetic map was constructed from an F_2 population derived from the interspecific crosses between *G. hirsutum* $\times$ *G. tomentosum* (Hou et al. 2013) and the genetic map supported previous findings that ne_2 was located on chromosome 26 (D12). A study by Hu et al. (2020) evaluated a diploid relative (*G. arboreum*) of tetraploid cotton and located an allele associated with nectaries on chromosome 12 which is analogous to the chromosome A12 of tetraploid cotton. While the location of the alleles is well documented, there remains a question as to whether the function of the two *ne* loci has diverged or if the genetic expression is similar. For example, is one allele more influential in the formation of leaf nectaries and the other for bract or calyx nectaries? This is the case for cotton glands, where Gl_2 has a greater effect on glanding in vegetative plant parts and Gl_3 has a greater influence on reproductive including seed (Lee Lee 1973; Romano and Scheffler 2008). There are also indications that epistatic effects may be in play, but most of the information to date is anecdotal.

Utilizing nectariless cotton varieties holds promise for making the crop more profitable by reducing the number of insecticide applications. An additional advantage is that it would be safer for bee populations as fewer pesticides would be applied. Since 2001, there have been commercial releases of “nectariless” cotton cultivars (Perlak et al. 2001), but because of the difficulty in selecting for the trait visually, the cultivars were a mixture of majority nectariless, but still containing a significant number of plants with leaf and / or bract nectaries. One example is MD-15 released by Meredith (2006). In the last two years, private companies have released nectariless cultivars with a low level of nectaried plants and they most likely used DNA markers to improve the efficiency of selection; however, their methods and any DNA markers are proprietary and not freely available.

Constructing F_2 nectariless genetic linkage maps has been complicated due to cotton’s chromosomal polyploidy with the two homeologous chromosomes being very similar and many of the same DNA markers found on both homeologs (Xu et al. 2008; Saski et al. 2017). It is also difficult to accurately score the trait phenotypically. This is because the true nectariless plants need both genes to be in the recessive form (ne_1 and ne_2). Heterozygotes such as $ne_1ne_1Ne_2ne_2$ or $Ne_1ne_1ne_2ne_2$ can look nectariless to the naked eye especially under certain environmental conditions or developmental stage where reduced expression of the trait causes no visible nectary to be produced (Meyer and Meyer 1961). It is known that late in the growing season, leaf nectaries can be smaller or absent in some cotton varieties. There are also instances where other heterozygotes, for example $Ne_1ne_1Ne_2ne_2$, looks like a homozygous nectaried plant. While breeders selecting for the nectariless trait have long noted the difficulties in accurately identifying the homozygous nectariless genotypes, there are no good tools to improve their selection efficiency.

The amount of nectar in the nectary can also vary based on the time of day, environment factors such as temperature or humidity and herbivory (Wacker et al. 2001, Park et al. 2019) Unconsumed nectar can be reabsorbed with the nectar constituents recycled. In upland cotton, nectar was shown to be reabsorbed, but at a faster rate in floral nectaries than extrafloral nectaries (Cardoso-Gustavson and Davis 2015).

To facilitate nectariless trait selection, an effective selectable marker system is essential. A few attempts

have been made to develop molecular markers, however, to date, only a couple of random amplified polymorphic DNA (RAPD) markers have been developed (Amir et al. 2002; Sajid-Ur-Rahman et al. 2008) that were generally not usable for other populations.

Molecular markers called simple sequence repeats (SSRs), also known as microsatellites, have become useful in marker-assisted selection (MAS). SSRs consist of tandem repeats of short sequences (2–8 bp) that are highly polymorphic, generally abundant throughout a genome, and co-dominant (Joobeur et al. 2000; Palombi and Damiano 2002). SSR-derived genotypic polymorphism can be detected using PCR-based systems and the SSR analysis can be automated using DNA sequencers, thereby enabling simultaneous processing of multiple markers at different loci. This high-throughput analysis enables SSR marker use for genetic map construction (Liu et al. 2000). In addition, SSRs serve to fill the gap between genome sequence and genetic mapping (Schuler 1998). SSRs can also provide a framework for high-resolution marker-based map construction utilizing single nucleotide polymorphisms (SNPs). To date, a total of 78,340 SSR markers are publicly available in the CottonGen database (Yu et al. 2014) and a number of these can be localized to the cotton chromosomes (Saski et al. 2017).

Unlike SSR markers, a SNP is a genetic polymorphism caused by single nucleotide change. SNPs are the most abundant DNA markers available in *Gossypium* spp. where genetic diversity is limited (Kottapalli et al. 2016). With the development of next generation sequencing (NGS) technology, the number of identified SNP collections has increased and high-throughput data analysis has become easier. A CottonSNP63K array (SNP Chip) was developed for SNP marker development. The array contains 45,104 putative intra-specific SNPs and 17,954 putative inter-specific SNPs, enabling analysis of a mapping population derived not only from a cross within *G. hirsutum* L. but also crosses with other cotton species (Hulse-Kemp et al. 2015).

Here we report the development of a genetic map using representative sets of SSR and SNP markers to genotype 256 F_2 lines derived from crosses between nectaried and nectariless cotton lines. In this study, a total of 20 polymorphic SSR markers, selected based on their location on Chromosome 12 or 26, along with 63 K SNPs were used to construct a nectariless linkage

map to identify molecular genetic markers that can be used to improve the selection efficiency for the nectariless trait, often referred to as marker assisted selection (MAS).

Materials and methods

Cotton plants used and genomic DNA preparation

The cotton lines used were obtained from the USDA ARS Stoneville, Mississippi cotton collection and grown at Stoneville. MD26ne was released as a cultivar (Meredith 2013). The pedigrees of the other lines are described in Romano and Scheffler (2008). Because of the difficulty in accurately phenotyping the nectariless trait, the nectariless parents used to create the F_2 progeny were tested before using. Twenty plants each of MD26ne, A1006ne and FM832ne were grown and scored for extrafloral nectaries associated with the leaves, bracts and calyces. None of the parental lines had floral nectaries. From each cotton line, five individual plants with the nectariless phenotype were self pollinated. Seed from these plants were grown out the following field season in a progeny row and scored again for the nectariless phenotype. Individual plants from progeny rows where all the plants scored as nectariless were used as the parents in crosses to develop the F_2 populations. Population size was determined by how many F_2 seed were produced from the F_1 plant.

For SSR analysis, a total of 175 F_2 individuals were used for nectariless genotyping as well as 17 parents used as controls and for validation. To be able to detect variation within the parental types not due to the nectariless trait, two plants each of MD26ne, A1006ne, JAC0gl, STVgl and FM832ne were used along with four plants of Mac7_0238 and three plants of Mac7_1238. The F_2 populations were derived from crosses between MD26ne (ne_1ne_2) and Mac7_1238 (Ne_1Ne_2), yielding 100 F_2 progeny and MD26ne (ne_1ne_2) $\times$ Mac7_0238 (Ne_1Ne_2), yielding 75 F_2 progeny. Later when the 63 K SNP Chip array became available, additional F_2 populations were developed and analyzed. A total of 259 F_2 individuals and 25 parental types were used and analyzed on a 288 SNP Chip array. To be able to detect variation within the parental types not due to the nectariless trait, three plants of MD26ne, A1006ne, JAC0gl, STVgl and

Table 1 SSR markers used for nectariless linkage map construction

	SSR markers	Chromosome	Primer sequence (5'–3')
1	BNL3482	AD_CH20, 26	BNL3482_F: ATTTGCCCCAGGTTTTTTTT BNL3482_R: GCAACACCTTTTCCTCCCTA
2	DPL0380	AD_CH12, 26	DPL0380_F: GCTAATTGCTCTCTAGGCAAAGAC DPL0380_R: CAACTGCTTCCACCTAGGATAAGT
3	CGR5793	AD_CH26	CGR5793_F: CACCAAGCACTCTAGAAACTGAAA CGR5793_R: TATGCTTCTTGACGACGAGTT
4	CGR6149	AD_CH26	CGR6149_F: CACCATAACCGAGCTACTCCAG CGR6149_R: ATCGTTCCAAGCAAACCAAC
5	CGR6471	AD_CH26	CGR6471_F: AATGGCAATCCCAGCACTAC CGR6471_R: TCTGCTTGCTCTGTTCTCTCA
6	CGR6651	AD_CH26	CGR6651_F: CCAAACCCACAATTTCTGCT CGR6651_R: GGGACTCTCTTAGGGCCAGT
7	CGR6759	AD_CH26	CGR6759_F: CGACCCATCCGATAGCAATA CGR6759_R: AAACAGAGAGGTGTGACGG
8	DC30183c	AD_CH26	DC30183_F: CTTTGGCGATCATTTTCAG DC30183_R: CATGGCGAGTTTTATCCA
9	DPL0250	AD_CH26	DPL0250_F: CATAGGCATCTGAAAGTGAATGG DPL0250_R: GGCTTAAACAAGTAAGCAATCACC
10	DPL0404b	AD_CH26	DPL0404_F: TCATCTACCATAGCCCTTGAAGTT DPL0404_R: GAAGAACCCTTCCATCAAGAATGT
11	DPL0208a	AD_CH26	DPL0208_F: CTTATTGGGTCTGCTAAGGTTGA DPL0208_R: GGATTCTTACCTCAACATGGATTC
12	CIR0167	AD_CH26	CIR167_F: CGCTCGGTTATTTAGTTT CIR167_R: AAATTCCAGCTCATGGT
13	CIR0293	AD_CH12	CIR293_F: CCGCTTTATCACATCTCT CIR293_R: GGTTGTCTTTCAGTTTGG
14	DPL0204	AD_CH12	DPL0204_F: AGTTGAGGCAGTGGTGACAGTTA DPL0204_R: TTGGAGAGGGAGAGAGACTATGAC
15	BNL2495	AD_CH26	BNL2495_F: ACCGCCATTACTGGACAAAG BNL2495_R: AATGGAATTTGAACCCATGC
16	DPL0379	AD_CH12, 26	DPL0379_F: CACCAGATGGAAGGGAAGATATAA DPL0379_R: GTTCTCCTGAGATTGTAAGTCATGC
17	DPL0565	AD_CH12, 26	DPL0565_F: CGACCATCATAATGAAAGAGAAGG DPL0565_R: GCAAGCCAAAGTATCTTGTCACAT
18	CGR6012	AD_CH12	CGR6012_F: ATGTGCCTTGTCCTCTTCCA CGR6012_R: GGGATGTGAGGGCATCATAG
19	CGR6880	AD_CH26	CGR6880_F: CGGCTGTATTTGTTTGGTGC CGR6880_R: GGGATGTGAGGGCATCATAG
20	CER0144	AD_CH12	CER0144_F: TACGCCGGGTACTTTTGAG CER0144_R: ATCGGCAACAATGGTGTITT

Markers **DPL0379** and **DPL0565** were identified as linked to the nectariless trait in this study

FM832ne were used and five plants each of Mac7_0238 and Mac7_1238. The F₂ individuals were from crosses between MD26ne × Mac7_1238 (69 F₂ progeny, POP1), MD26ne × Mac7_0238 (71 F₂

progeny, POP2), A1006ne (*ne₁ne₂*) × JACOgl (*Ne₁-Ne₂*) (69 F₂ progeny, POP3) and STVgl (*Ne₁Ne₂*)- × FM832ne (*ne₁ne₂*) (50 F₂ progeny, POP4). Leaf

tissue was collected from each plant for DNA extraction.

Genomic DNA extraction was conducted using a NucleoSpin Plant II kit (MN Inc., Bethlehem, PA). Briefly a young leaf, 2 cm in diameter, was ground using a FastPrep 24 homogenizer (MP Biomedical, Santa Ana, CA) for 1 min. Homogenized leaf samples were mixed with 600 µl Buffer PL2 and vortexed for at least 10 s. Then 20 µl RNase A was added and incubated at 65 °C for 30 min after gentle mixing. Afterwards 150 µl Buffer PL3 was added and mixed and incubated on ice for 5 min then centrifuged for 5 min at $11,000 \times g$. The supernatant was transferred to a NucleoSpin Filter (purple) in a collection tube and centrifuged for 2 min at $11,000 \times g$. Then 900 µl Buffer PC was added to the flow through after mixing thoroughly, then 700 µl was transferred to the NucleoSpin Plant II Column (green). The column was centrifuged for 1 min at $11,000 \times g$ and the flow through was discarded. The column was washed with 400 µl of PW1 and rinsed with PW2 buffer with 700 µl and 200 µl for 1 min and 2 min respectively. The DNA pellets were eluted with 50 µl of pre-warmed PE buffer. The DNA was quantified by a NanoDrop micro-spectrophotometer (Thermo Scientific, Wilmington, DE).

Fig. 2 Linkage analyses of SNPs that were found to be associated with the nectariless trait by Single Marker Analysis (SMA). The analyses included marker trait association (MTA) analysis, and interval mapping (IM). The graphs represent SNPs distributions on the chromosomes and location of the nectariless co-located SSR QTL on Chr. 26

Nectary/nectariless phenotypic scoring

All the nectaried parents had bract nectaries and leaf nectaries, but no floral or calyx nectaries (Fig. 1). This is common among modern cotton lines (Llandres et al. 2019). While the association of extrafloral nectaries with the *Ne₁* and *Ne₂* is well documented, floral nectary genetics is not as clear and may require additional alleles to express nectaries in the flowers (Chatt et al. 2019). While tetraploid cotton plants with the confirmed *ne₁ne₁ne₂ne₂* genotype have no floral or extrafloral nectaries, expression of bract nectaries in heterozygous plants may vary with the cotton variety, age of the plant or time of the season. Scoring leaves for nectaries at mid flower is considered the most reliable method to determine phenotype. However, because of the difficulty of seeing the nectaries with the naked eye, leaves were collected from the field and

Table 2 Descriptive statistics for F₂ phenotypic scores resulting from the cross between a nectariless parent and a nectaried parent

Population	N ^a	Genotypes			With Yates correction			
		Nectary (<i>Ne</i>)	Hetero- zygous (<i>Nene</i>)	Nectari- less (<i>ne</i>)	Chi-square <i>p</i> value 1:1	Chi-square <i>p</i> value 11:5	Chi-square <i>p</i> value 9:7	Chi-square <i>p</i> value 15:1
F2-POP1	69	13	23	33	0.81	0.01	0.57	0.00
MD26ne ^b × Mac7 1238Ne ^c	36							
F2-POP2	71	40	10	21	0.00	0.86	0.02	0.00
MD26ne ^b × Mac7 0238Ne ^c	50							
F2-POP3	69	16	23	30	0.34	0.04	0.94	0.00
A1006ne ^b × JACOglNe ^c	39							
STVglNe ^c × FM832ne ^b	50	36	12	2	0.00	0.00	0.00	0.72
F2-POP4	48							

^aNumber of individual plants

^bne, Nectariless (no nectaries)

^cNe, Cotton with leaf nectary

^dYates continuity correction for small population size

◀ **Fig. 3** Nectariless QTL linkage map on Chr. 26. The asterisk indicates SNPs that are shown consistently throughout POP1-3 and the colored line indicates SNP location. The consensus linkage group is the integrated map of POP1-3. Linkage calculations and integration of maps were calculated using JMP Genomics 8.0 (SAS Institute, Cary NC)

scored in the lab using a Keyence Digital Microscope (Keyence Corp., Itasca, IL) at 10 × magnification. The leaves were collected in early July when the plants were at mid flower and expression of nectaries was at its peak. Leaves measured 5 to 7 cm across the leaf blade and were collected from a floral branch near the top of the plant. Phenotypic scoring for nectaries was done by viewing the lower mid-vein located on the abaxial side of leaf. Two leaves were scored from each plant. The leaves were first scored using a standardized system as follows; 0- no nectaries, 1- starting to bump out from vein, 2- a ridge along two sides and sunken pad with no nectar, 3- more defined oval ridge and sunken pad with nectar, 4- well defined oval ridge with a wide open shape and sunken pad with nectar. For the final scoring used for mapping, scores 1–3 were considered some combination of *Ne* and *ne* and therefore heterozygous.

That resulted in three classes: homozygous nectaried ($Ne_1Ne_1Ne_2Ne_2$); heterozygous ($Ne_1Ne_1ne_2ne_2$ or $ne_1ne_1Ne_2Ne_2$ etc.); and homozygous nectariless ($ne_1ne_1ne_2ne_2$). Nectaries on bracts subtending the flower buds were also scored and compared to the leaf scores, but were not considered reliable enough to use for mapping. The leaves scored as nectariless also scored as nectariless for bract nectaries.

Genotyping with SSR and SNP markers

A total of 20 polymorphic SSR primers, preselected for their location on chromosome 12 or 26, were tested to determine if they were linked to the nectariless trait. The SSR primer sequences used in this study were obtained from the CottonGen (<http://www.cottongen.org/>) database and primer details are described in Table 1. For genotyping, extracted total genomic DNA was standardized to 10 ng/ul and PCR analysis conducted using a Multiplex PCR Kit (QIAGEN, Valencia, CA). Prior to DNA fragment analysis, the PCR products were mixed with formamide and size standard labeled with ROX reference dye (Life

Technologies, Grand Island, NY) using a robotic liquid handling system (Hamilton Robotics, Reno, NV). The mixed samples were then injected into an ABI 3730XL analyzer (Life Technologies, Grand Island, NY) and the genotypes were analyzed by GeneMapper5 (Life Technologies, Grand Island, NY).

For the SNP assay, an Illumina BeadArray™ chip with 63 K SNPs was used to explore which SNPs were associated with the nectariless trait (Hulse-Kemp et al. 2015). The dsDNA was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Scientific, Wilmington, DE). The SNP chip analyses were conducted by GeneSeek (Neogen Genomics, Lincoln, NE) or Texas A&M (College Station, TX). Quality filtering of SNP markers was performed by removing SNPs with (i) greater than 10% missing by locus (ii) allele frequency less than 1% and (iii) excess heterozygosity (a Hardy–Weinberg equilibrium p-value less than 0.0001). After filtering, approximately 8,000 SNPs remained for analysis.

Linkage analysis and genetic map construction

A series of linkage analyses including QTL single marker analysis (SMA), marker trait analysis (MTA), interval mapping (IM), and genetic map construction were performed using JMP Genomics 8.0 (SAS Institute, Cary NC). Recombination values were converted to genetic distances (cM) using the Kosambi mapping function. QTL SMA was performed to examine the regression of SNP markers and trait values. The regression is represented as the $-\log_{10}$ (probability) which is $-\log_{10}(0.01) = 2.0$ and used as a threshold of significance. Interval mapping was conducted to find putative QTLs on any genomic location flanked by two markers. IM uses the conditional probabilities of a QTL given two observed markers in the regression model to distinguish the genetic effects of a QTL from the genetic effect due to linkage (SAS Institute). The genetic map was evaluated every 2.0 cM to identify putative QTL using EM Algorithm (SAS Institute 2015a, b). A threshold LOD (Log of odd) value of 3.5 ($P < 0.05$) was used to declare presence of QTL. The nectariless QTL was considered at the position with the maximum LOD score on the linkage group. To confirm the putative QTL locus on Chromosome 26, MTA analysis was conducted on ordinal trait values. The significance of putative association is reported as the $-\log_{10}(P)$ and a

conservative $-\log_{10}$ 2.0 level was used as a threshold of significance. A linkage map for each population and its consensus linkage map were constructed using JMP Genomics 8.0 on SNPs identified using 4 mapping populations (POP1-4). The SNPs showing significant trait associations were positioned on Chr. 26 and each linkage was then merged to generate a consensus linkage map.

Results

Phenotypic evaluation of the F_2 generation

We used a total of 4 mapping populations (POP1-4) for nectariless linkage map construction and evaluated a total of 259 F_2 plants (Table 2). The leaf phenotype of each F_2 plant was scored as homozygous with nectaries, heterozygous with small or vestigial nectaries or homozygous nectariless (no nectaries). The observed scores are presented in Table 2. Previous studies have reported that the nectariless trait is controlled by two recessive alleles (ne_1 and ne_2). While it is confirmed that a plant with the $ne_1ne_1ne_2ne_2$ genotype is nectariless, there are indications that epistasis may influence expression of the trait which makes phenotypic scoring more complicated. For example, if the trait only requires one of the genes to be dominant (Ne_1 or Ne_2), the expected phenotypic segregation ratio would be 15:1. Having more nectariless phenotypes than expected has been frequently observed by the authors and others in their breeding programs. This could be because heterozygotes such as $Ne_{(1 \text{ or } 2)}ne_{(1 \text{ or } 2)}$ are phenotypically nectariless. Further complicating the scoring is the belief that environmental conditions can also affect the expression of the trait and the resulting phenotypic score. While the exact allele is not clear, all the populations exhibited some type of digenic epistasis based on Chi-square analysis.

SSR marker validation

A total of 20 polymorphic SSR primer pairs (Table 1) previously assigned to Chr. 12 and / or Chr. 26. were used to analyze 175 F_2 plants from a cross between MD26ne x Mac7 1238Ne (POP1) or MD26ne x Mac7 0238Ne (POP2). Seventeen parental lines were also used for validation and controls in the nectariless QTL

search. The 38 amplicons which were amplified by the set of SSR primers were analyzed by GeneMapper. Only two polymorphic SSR markers, DPL0379 and DPL0565 were associated with the leaf nectariless trait. The two SSRs showed an identical genotypic segregation pattern in F_2 plants. The trait association was validated through QTL SMA, MTA, interval mapping (IM) and in the integration with SNP markers. A series of QTL linkage analyses indicated that the two SSR markers were co-located in the nectariless QTL region residing on Chr. 26 for three of the mapping populations (POP1-3) (Fig. 2, 3).

SNP data processing

Because of the high degree of sequence similarity between Chr. 12 and Chr. 26 (Saski et al. 2017) and the fact that an SSR marker often amplifies fragments on both chromosomes, analysis can be difficult. The other challenge is the low number of polymorphic SSR markers available. When the CottonSNP63K array became available, we were able to partially overcome these challenges and the 63,058 SNPs were used to analyze DNA samples from a total of 259 F_2 progeny plants from 4 mapping populations (POP1-4). There were also 25 parental lines included as controls on the SNP arrays. Prior to the linkage analyses, non-informative SNPs were removed based on following criteria; 1) missing data was more than 10%, 2) parental genotypes were either identical or had missing data, 3) F_2 offspring were monomorphic. For the SNP quality processing, SNPs known to be located on Chr. 12 and 26 as well as SNPs with unknown locations were included. The final number of SNPs after data processing is listed on Table 3.

QTL linkage analyses on Chr. 12 and 26

The association of each SNP marker with the nectariless trait was examined by a QTL single marker analyses (SMA). The SMA uses a regression model to assess marker-trait associations at individual marker locus and calculate the probability of QTL evidence for each marker. All SNPs that passed quality controls (Table 3) were used in the SMA (over 3,000 each for POP1, POP2, and POP4 and over 1700 for POP3). Results identified 72 and 138 SNPs from POP1, 122 and 137 SNPs from POP2, 74 and 68 SNPs from POP3, and 180 and 165 SNPs from POP4 that were

Table 4 SNPs that were found consistently in three of the mapping populations

SNPs	POP1: MD26ne ^a x Mac7 1238Ne ^b				POP2: MD26ne x Mac7 0238Ne				POP3: A1006ne x JACOglNe			
	Map distance ^c (cM)	SMA (-logP)	MTA (-logP)	IM (LOD)	Map distance (cM)	SMA (-logP)	MTA (-logP)	IM (LOD)	Map distance (cM)	SMA (-logP)	MTA (-logP)	IM (LOD)
00585Gh	3.67	10.7	6.7	10.2	6.1	13.3	7.6	11.9	30.5	0.4	0.2	0.1
36873Gh	5.22	10.7	6.7	10.2	6.1	13.1	7.4	13.1	30.5	0.6	0.3	0.1
08156Gh	5.22	10.6	6.6	10.2	6.1	12.7	7.3	13.1	30.5	0.5	0.3	0.1
44218Gh	5.95	10.7	6.6	10.1	2.4	17.9	8.4	17.4	32.8	0.6	0.4	0.3
51230 Gb	5.95	10.7	6.6	10.1	2.4	16.9	7.9	16.4	32.1	0.7	0.4	0.2
21834Gh	5.95	10.7	6.6	10.1	2.4	17.0	8.1	17.4	32.1	0.4	0.3	0.2
00378Gh	5.95	10.7	6.6	10.1	2.4	17.7	8.3	17.4	31.3	0.6	0.4	0.3
19456Gh	5.95	10.7	6.6	10.1	2.4	17.9	8.4	17.4	32.8	0.6	0.4	0.3

^ane, nectariless genotype

^bNe, nectary genotype

^cMapping distances based on results shown in Fig. 3

Table 5 SNP and SSR markers linked to the nectariless trait in POP1 and POP2. Highlighted markers are the most significantly associated with the nectariless trait. SNP designations ending with “NA” were previously unassigned to a specific chromosome

POP1: MD26ne x Mac7 1238Ne						POP2: MD26ne x Mac7 0238Ne					
	SNPs	Kosambi (cM)	SMA (−logP)	MTA (−logP)	IM (LOD)		SNPs	Kosambi (cM)	SMA (−logP)	MTA (−logP)	IM (LOD)
1	08157Gh_26	4.5	11.5	6.8	10.3		08184Gh_26	5.3	17.7	8.3	16.9
2	00379Gh_26	6.0	10.8	6.3	10.1		25680Gh_26	2.4	17.7	8.3	16.4
3	08162Gh_NA	5.2	10.7	6.7	10.2		20910Gh_NA	2.4	17.4	8.2	17.4
4	08161Gh_NA	5.2	10.7	6.7	10.2		50256Gb_26	5.3	17.4	8.2	16.9
5	08167Gh_26	6.0	10.7	6.6	10.1		56400Gb_26	5.3	17.4	8.2	16.9
6	16353Gh_26	6.0	10.5	6.6	10.1		16353Gh_26	2.4	17.2	8.1	16.4
7	08159Gh_NA	2.2	10.0	6.4	9.4		50696Gb_26	2.4	17.2	8.1	16.4
8	16351Gh_NA	2.2	10.0	6.4	9.4		51115Gb_26	2.4	17.2	8.1	16.4
9	36874Gh_26	2.2	10.0	6.4	9.4		SSR	2.4	17.1	8.3	16.4
10	24215Gh_26	1.5	9.9	6.3	9.3		61504Gt_26	5.3	17.0	8.1	16.9
11	42398Gh_26	1.5	9.9	6.3	9.3		21766Gh_26	2.4	16.9	8.2	16.4
12	00492Gh_NA	1.5	9.9	6.3	9.3		08185Gh_26	2.4	16.7	8.1	17.4
13	21388Gh_26	1.5	9.9	6.3	9.3		20683Gh_26	2.4	16.5	8.1	17.4
14	00592Gh_26	1.5	9.9	6.3	9.3		08136Gh_NA	9.2	15.9	7.9	15.5
15	19454Gh_26	1.5	9.9	6.3	9.3		26493Gh_26	12.6	15.7	7.8	13.8
16	36393Gh_26	1.5	9.9	6.3	9.3		16348Gh_26	12.6	15.7	7.8	15.5
17	33352Gh_26	1.5	9.9	6.3	9.3		08167Gh_26	2.4	15.7	8.0	17.4
18	32829Gh_26	1.5	9.9	6.3	9.3		22721Gh_NA	3.9	15.5	7.1	15.7
19	00667Gh_26	1.5	9.9	6.3	9.3		08138Gh_26	12.6	15.1	7.5	13.8
20	53682Gb_26	1.5	9.9	6.3	9.3		35898Gh_NA	12.6	14.9	7.6	14.3
21	49071Gh_26	1.5	9.9	6.3	9.3		20909Gh_26	2.4	14.8	7.5	16.4
22	02830Gh_26	1.5	9.9	6.3	9.3		45549Gh_NA	9.2	14.6	7.6	14.3
23	37369Gh_26	1.5	9.9	6.3	9.3		08140Gh_26	9.2	14.4	7.5	13.8
24	08149Gh_26	1.5	9.9	6.3	9.3		60525Gb_26	5.3	14.0	7.4	17.4
25	25982Gh_26	1.5	9.9	6.3	9.3		33352Gh_26	6.1	13.7	7.8	13.1
26	08152Gh_26	1.5	9.9	6.3	9.3		08155Gh_26	6.1	13.7	7.8	13.1
27	24926Gh_26	1.5	9.9	6.3	9.3		00667Gh_26	6.1	13.5	7.7	13.1
28	08155Gh_26	1.5	9.9	6.3	9.3		00592Gh_26	6.1	13.3	7.6	13.1
29	08153Gh_26	1.5	9.8	6.3	9.3		24926Gh_26	6.1	13.3	7.6	13.1
30	20910Gh_NA	6.7	9.7	6.5	9.2		08162Gh_NA	6.1	13.3	7.6	11.9
31	21766Gh_26	6.7	9.7	6.5	9.2		19454Gh_26	6.1	13.1	7.4	13.1
32	50696Gb_26	6.7	9.7	6.5	9.2		20903Gh_26	6.2	13.1	7.4	11.9
33	51115Gb_26	6.7	9.7	6.5	9.2		08161Gh_NA	6.2	13.1	7.4	11.9
34	50256Gb_26	6.7	9.7	6.5	9.2		21388Gh_26	6.1	13.1	7.6	11.9
35	25680Gh_26	6.7	9.7	6.5	9.2		37369Gh_26	6.1	13.1	7.6	13.1
36	08185Gh_26	6.7	9.7	6.5	9.2		08152Gh_26	6.1	13.1	7.6	11.9
37	SSR	6.7	9.7	6.5	9.2		08159Gh_NA	6.1	13.1	7.6	11.9
38	61504Gt_26	6.7	9.7	6.5	9.2		08149Gh_26	6.1	13.0	7.4	11.9
39	56400Gb_26	6.7	9.6	6.4	9.2		08144Gh_26	6.1	12.9	7.3	13.1
40	08184Gh_26	6.7	9.5	6.2	9.2		53682Gb_26	6.1	12.9	7.4	13.1
41	20903Gh_26	1.5	9.4	6.1	9.3		36393Gh_26	6.2	12.9	7.3	13.1
42	08140Gh_26	0.0	9.4	6.3	8.9		22395Gh_26	9.2	12.7	7.1	13.8

Table 5 continued

POP1: MD26ne x Mac7 1238Ne						POP2: MD26ne x Mac7 0238Ne				
SNPs	Kosambi (cM)	SMA (−logP)	MTA (−logP)	IM (LOD)		SNPs	Kosambi (cM)	SMA (−logP)	MTA (−logP)	IM (LOD)
43 08144Gh_26	0.0	9.4	6.3	8.9		16351Gh_NA	6.1	12.6	7.2	13.1
44 26493Gh_26	0.0	9.4	6.3	8.9		36874Gh_26	6.1	12.6	7.2	13.1
45 45549Gh_NA	0.0	9.4	6.3	8.9		08153Gh_26	6.1	12.6	7.2	11.9
46 35898Gh_NA	0.0	9.4	6.3	8.9		32829Gh_26	6.1	12.5	7.1	13.1
47 08138Gh_26	0.0	9.4	6.3	8.9		25982Gh_26	6.1	12.5	7.1	11.9
48 16348Gh_26	0.0	9.4	6.3	8.9		24215Gh_26	6.2	12.4	7.0	11.9
49 08136Gh_NA	0.0	9.4	6.3	8.9		42398Gh_26	6.1	12.3	6.9	13.1
50 22721Gh_NA	6.7	9.4	6.3	9.2		00492Gh_NA	6.2	12.3	6.9	11.9
51 20683Gh_26	6.7	9.2	6.3	9.2		49071Gh_26	6.1	12.1	6.8	11.9
52 20909Gh_26	6.7	9.1	6.2	9.2		02830Gh_26	6.1	12.1	7.2	13.1
53 22395Gh_26	0.0	8.1	5.6	8.9		45674Gh_NA	10.9	11.1	4.6	14.0
54 45674Gh_NA	0.0	7.8	5.5	8.9		08157Gh_26	7.7	10.2	4.1	10.6
55 60525Gb_26	7.5	7.7	5.6	9.3		00379Gh_26	0.0	10.1	3.4	12.5

significantly associated with the nectariless trait on Chr. 12 and 26, respectively. Additionally, interval mapping (IM) was employed to search for QTLs on the genome location flanked by two SSR markers (Table 1) and marker trait analysis (MTA) was also used to search for QTL on each chromosome (12 and 26).

Figure 2 shows the respective nectariless QTL region located on Chr. 12 and 26. Previous research has shown that two genes (Ne_1 and Ne_2) are responsible for nectary formation with Ne_1 localized to Chr. 12 and having a major effect on presence of leaf nectaries as well as a modifying effect on bract nectaries (Meyer and Meyer 1961; Holder et al. 1968). For Chr. 12, no significant linkage was found between SNP markers and our F_2 derived nectariless phenotypic data while significant linkage was found on Chr. 26. As shown in Fig. 2, POP1-3 placed the two SSR markers on Chr. 26 whereas POP4 had no SSR markers linked on Chr. 26.

For POP1-3, eight polymorphic SNP markers that consistently appeared in the three mapping populations were identified (Table 4). POP4 was omitted because no significant linkage was found between the SNP markers and F_2 phenotypic data. In contrast, the SNP markers for POP1 and POP2 showed highly

significant linkage between the SNPs and the trait (−log P of SMA and MTA: > 10 and > 6, respectively, and LOD of IM > 10) and markers on POP3 were also significant, but to a lesser degree. The results identified similar QTL regions for the three different map populations, although the linkage on POP3 was not as significant as POP1-2.

For POP1 and POP2, 54 SNPs and 2 SSR markers (DPL0379 and DPL0565) were linked to a nectariless QTL in Chr. 26.

Figure 3 shows QTL maps constructed from individual populations (POP1-3) and a consensus map over all three populations. The three linkage maps are very closely matched to each other and eight SNP markers (marked with asterisks) were common to all three populations. The consensus map of three populations indicates that the map location at 30.5 cM is highly associated with the nectariless trait and can be used as a molecular marker for the trait selection. As shown in the consensus map, two co-located SSRs are also located in the same region, confirming a QTL region associated with the nectariless trait on Chr. 26. Notably, SNPs whose physical chromosomal location were previously unknown were also included in the analysis and some of those SNPs can now be assigned to Chr. 26 (Table 5).

Fig. 4 Theoretical genotypes from a cross of a homozygous nectaried parent and a homozygous nectariless parent with possible F₂ progeny phenotypes and genotypes

Cross				
Ne ₁ Ne ₁ Ne ₂ Ne ₂		x	ne ₁ ne ₁ ne ₂ ne ₂	
F1		Ne ₁ ne ₁ Ne ₂ ne ₂		
		Self Pollinate		
F2				
Possible F2		Genotypes		
Gametes	Ne ₁ Ne ₂	Ne ₁ ne ₂	ne ₁ Ne ₂	ne ₁ ne ₂
Ne ₁ Ne ₂	Ne ₁ Ne ₁ Ne ₂ Ne ₂	Ne ₁ Ne ₁ Ne ₂ ne ₂	Ne ₁ ne ₁ Ne ₂ Ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂
Ne ₁ ne ₂	Ne ₁ Ne ₁ Ne ₂ ne ₂	Ne ₁ Ne ₁ ne ₂ ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂	Ne ₁ ne ₁ ne ₂ ne ₂
ne ₁ Ne ₂	Ne ₁ ne ₁ Ne ₂ Ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂	ne ₁ ne ₁ Ne ₂ Ne ₂	ne ₁ ne ₁ Ne ₂ ne ₂
ne ₁ ne ₂	Ne ₁ ne ₁ Ne ₂ ne ₂	Ne ₁ ne ₁ ne ₂ ne ₂	ne ₁ ne ₁ Ne ₂ ne ₂	ne ₁ ne ₁ ne ₂ ne ₂
Possibly Scored as	nectaried	Blue	Possibly Scored as	nectariless
				Green

Discussion

The nectariless trait has proven to be a valuable agronomic trait that decreases the cotton plant's attractiveness to some insect pests. The trait has been shown to confer tolerance against pink boll worm (Percy 2001, Khan et al. 2002, flint et al. 1991), Lepidoptera (Lukefahr and Rhyne 1960; Soares et al. 1998) and tarnished plant bugs (Platt and Steward 1999). Attempts have been made to develop nectariless cultivars (Meredith 2006), but these efforts have been frustrated by the inability to accurately select for the trait phenotypically. To increase selection efficiency and allow marker assisted selection to be used, efforts have been made to develop nectariless markers including random amplified polymorphic DNA (RAPD) markers (Sajid-ur-rahman et al. 2008; Amir et al. 2002), but so far no usable markers are publicly available.

The first obstacle in developing SSR or SNP markers was to be able to accurately phenotype the progeny from F₂ mapping populations. As described in the results section, the nectariless trait is controlled by two genes with possible differential (epistatic) expression for leaf nectaries and the overall expression affected by environmental conditions. This makes traditional genetic segregation ratios difficult to determine as illustrated in Fig. 4.

To improve the chances of distinguishing between homozygous and heterozygous plants, four mapping populations with different parental combinations were used and two leaves per plant were scored using a digital microscope at 10 × magnification. Digital

images were made of the leaves and nectaries and the leaf images used for scoring. This greatly reduced the number of heterozygous plants that were scored as nectariless, but could not eliminate the heterozygous genotypes that did not produce a nectary. These possible mis-scored genotypes are highlighted in light green. While it was easier to score for heterozygous nectaried plants, there was also the possibility of mis-scores with a heterozygote being scored as homozygous nectaried which are highlighted in light blue. It is possible that Ne₁ and Ne₂ each have a different effect on a different plant part, while also having modifying effects on the other plant part (Holder et al. 1968; Waghmare et al. 2005). This is not uncommon in upland cotton where two genes, present on two homeologous chromosomes have diverged and have differential expression in different plant parts (Wilson and Smith 1977; Lee 1962, 1965).

Despite the difficulties in accurately scoring the phenotypes, analyses of the different populations, identified a consistent QTL region on Chr. 26 for POP1-3. POP1 and POP2 exhibited highly significant linkage association between the trait and Pop3 was significantly linked. The genetic markers were not always in the same order and distances varied, likely due to the small population size of each population. Markers highlighted on Table 5 will be further investigated as molecular markers to use for MAS for the nectariless trait. We expected to identify a QTL region on Chr. 12, but did not identify any statistically significant markers with these mapping populations. This may be due to a combination of small population size, the fact that the sequence in the chromosomal

Table 3 The number of SNPs used for linkage analyses after quality processing

Chr Location	POP1 MD26ne ^c x Mac7 1238Ne ^d			POP2 MD26ne x Mac7 0238Ne			POP3 A1006ne x JACOGlNe			POP4 STVglNe x FM832ne		
	12	26	n/a	12	26	n/a	12	26	n/a	12	26	n/a
Nr. SNPs Before ^a	535	542	11,777	535	542	11,777	115	125	2813	535	542	11,777
Nr. SNPs After ^b	127	101	3306	133	103	3205	77	62	1577	170	153	2855

^aNumber of SNPs before data processing^bNumber of SNPs after data processing^cne, nectariless genotype^dNe, nectaried genotype

region containing the nectariless trait is similar for the two homeologous chromosomes 12 and 26 which may confound the analysis. While it would be ideal to have DNA markers linked to both genes, because of the extreme difficulty in accurate phenotypic scoring, even markers linked to *ne*₂ will improve the efficiency and ease of selection. This is especially true in the early stages of cultivar development where large numbers of plants need to be grown and carried forward to the next generation if there is no efficient MAS. The *ne*₂ markers will help reduce the number of plants carried forward.

Further research is underway to identify SSR / SNPs for Chr. 12 by developing larger mapping populations with different parental combinations. The parents for these populations were checked for homozygosity by selecting individual plants with the nectaried or nectariless phenotype and then growing them out as a progeny row to check for segregation in the subsequent generation before using them as parents. F₃ progeny from the F₂ plants, used for mapping populations, can also be grown out to catch some of the mis-scores, but will be subject to the same phenotypic scoring challenges that are faced for the F₂ plants. Additional parental combinations are also being determined using genomic sequence information obtained from sequences in the putative chromosomal regions for the nectariless trait (Saski et al. 2017). SNPS are also being developed directly from these genomic sequences and the framework cotton genomic sequence (Saski et al. 2017). The markers and information from a recent publication that mapped the trait in diploid *G. arboreum* may also help refine

the search for markers on chromosome 12 (Hu et al. 2020).

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