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QUANTITATIVE TRAIT LOCI DETECTION IN BULGARIAN COTTON MUTANT SEGREGATING POPULATION

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Identification of quantitative trait loci (QTL) linked to fiber quality is of primary interest in cotton breeding. As Bulgarian cotton varieties belong to a specific group (proles *Bulgaricum*) they are even more difficult for cross-breeding into them such quality characteristics. Therefore a segregating mutant population has been developed from the standard Bulgarian variety 'Chirpan 603' by applying seed gamma irradiation. M4 and M5 generations were used for identification of QTLs related to fiber quality characteristics. SSR markers developed in interspecific crosses and further confirmed in intraspecific crosses, together with in-house developed ISSR markers were used for association mapping of QTLs for fiber quality. Fiber strength, length, uniformity, micronaire and elongation were the main studied characteristics. QTLs with major effects on these traits identified in M4 and M5 were confirmed in M6 generation. Further ones were identified and used for map saturation and linkage group confirmation.

Keywords: Cotton, *Gossypium hirsutum*, mutation, mapping, QTL.

INTRODUCTION

Identification of quantitative trait loci (QTL) linked to fiber quality is of primary interest in cotton breeding. Upland cotton (*Gossypium hirsutum* L., 2n = 52) is the most extensively used of the four cultivated *Gossypium* species. However the level of genetic diversity in the species is low, especially among agriculturally elite types, as revealed by different means of assessment (GUTIÉRREZ *et al*., 2002; ULLOA *et al*., 2002; WENDEL *et al*., 1992). Increasing diversity is therefore essential to genetic improvement efforts.

G. barbadense (L.) is the only cultivated relative of Upland cotton (*G. hirsutum*) with the same chromosome number (2n = 4x = 52). It is valued for its fiber quality, whereas Upland

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cotton is higher valued for its yield potential. One of the challenges in interspecific introgression is to use valuable alien traits of *G. barbadense* germplasm such as fiber length, fineness, and strength to improve Upland cotton (LACAPE *et al*., 2005).

Traditional plant breeding approaches for improving fiber quality through interspecific introgression have been hindered by complex antagonistic genetic relationships that arise from the polyploidy nature of the species (MEREDITH, 2000). More recent molecular mapping studies associated *G. barbadense* chromosomes with many favorable QTLs affecting fiber and agronomic traits (LACAPE *et al*., 2005; RONG *et al*., 2004). Attempts to incorporate alleles into Upland cotton through classical breeding methods have generally not achieved stable introgression of *G. barbadense* fiber properties (BOOPATHI *et al*., 2011). Associated with these attempts at introgression have been poor agronomic qualities of the progeny, distorted segregation, sterility and limited recombination due to incompatibility between the genomes (REINISCH *et al*., 1994). As Bulgarian cotton varieties belong to a specific group (proles *Bulgaricum*) they are even more difficult to cross-breed such quality characteristics into.

One of the other main avenues for identifying and studying quality-related genes has been induced mutagenesis. This approach proved effective in many crops, including cotton (AHLOOWALIA *et al*., 2004). In the present study we chose to apply a combination of mutagenic treatment with the application of molecular markers for identifying quantitative trait loci (QTLs) in a segregating mutant population developed from commercially used Bulgarian variety.

Inter simple sequence repeat (ISSR) marker system was developed by ZIETKIEWICZ *et al*. (1994) to circumvent the requirement of SSRs for flanking sequence information. It has thus found wide applicability in a variety of plants where the most significant uses remain in genetic mapping, diagnostic fingerprinting and in the study of genetic structure within and between populations of crop species. The application of these markers to cotton was rarely attempted (MALIK *et al*., 2014; FARAHANI *et al*., 2018) and did not expand much beyond verifying the capacity of the system to reveal inter- and intraspecific variations (DONGRE *et al*., 2011; LIU and WENDEL, 2001; NOORMOHAMMADI *et al*., 2011; NOORMOHAMMADI *et al*., 2013; RANA *et al*., 2007) and an accidental use as a sub-sampling technique for producing SSR markers (ZHANG *et al*., 2009).

Within this study the main objectives set forth were to identify molecular markers linked to fiber quality characteristics and to identify QTLs with practical applicability for Bulgarian cotton breeding programs.

MATERIALS AND METHODS

Plant material

Bulgarian variety ‘Chirpan 603’ was selected for the present study as it was commercially grown over more than 80% of the cultivation area in 2005. Variety is characterized by its earliness, defined by natural cut-out in vegetative growth and transition to generative phase.

Gamma irradiation treatment and greenhouse cultivation conditions

Five hundred grams of seeds were gamma radiation treated. Based on previous experience with breeding materials from *G. hirsutum* the applied treatment was 150 Gy.

After producing the M1 generation in a greenhouse seeds from all the following generations were planted and seed cotton collected in the field.

Phenotypic observations

Fiber quality characteristics measured were: length (mm), fineness (micronaire), strength (g/tex), elongation (%), uniformity (%), and maturity (%).

The plants were phenotyped during the growing season and the fiber quality of the ones that produced enough seed cotton determined.

ISSR analysis

ISSR analysis was performed by testing a number of primers (Table 1) that demonstrated high reproducibility and polymorphism identification in our previous studies (BOJINOV and DANAILOV, 2009; IVANOVA and BOJINOV, 2009).

Table 1. List of ISSR primers used in the study

Primer	DNA sequence
E1	$(CA)_8$AA+GG
E2	$(CA)_8$AA+GC+T
E3	$(GA)_8$C+TC
E4	$(AG)_8$C+TC
E5	$(AC)_8$C+TA
E6	$(AC)_8$C+TG
E7	$(AG)_8$C+TG
E8	$(AC)_8$C+TT
E9	$(AG)_8$C
E10	$(GA)_8$T

PCR reactions were performed in 25 µl volume with the following cycling regime: denaturing at 94°C for 3 min, 40 cycles of 94°C – 1 min, AT – 45 sec, 72°C – 45 sec, followed by final extension of 72°C – 4 min, where AT is the annealing temperature for each primer calculated according to KOCHIEVA *et al*. (2002).

PCR products were analyzed through separation in 2% agarose gels and staining with ethidium bromide.

SSR analysis

Selection of the SSR primers for use in the present study (Table 2) was performed as described elsewhere (IVANOVA and BOJINOV, 2009). Sequences of SSR primers were obtained from the Cotton Genome Database (https://www.cottongen.org/find/markers), and oligonucleotides synthesized by Microsynth AG (Balgach, Switzerland).

Software used

The freely available mapping software MapDisto (LORIEUX, 2012) was used for QTL identification and mapping. The procedure for computing QTLs in Recombinant Inbred Lines

(RILs) was used as best corresponding to characterizing a population of mutation-derived sister inbred lines.

Table 2. List of SSR markers used in the study

SSR marker	Map label	Primer sequence	Expected product size (bp)
BNL 1017	CM 1	F: AGAAAAAAACTTCCTCATGAACC R: GTTTCTCTCAGAATTTGTAGGCC	142
BNL 1122	CM2	F: TCGATAACGGCTATAGTAATCTCTC R: CAACAAATAAGCAGCCAAGAAA	174
BNL 1317	CM3	F: AAAAATCAGCCAAATTGGGA R: CGTCAACAATTGTCCCAAGA	181
BNL 1421	CM4	F: TGAAGATTTGGAGGCAATTG R: GAAATCAAGCCTCAATTCGG	228
BNL 2634	CM5	F: AACAACATTGAAAGTCGGGG R: CCCAGCTGCTTATTGGTTTC	246
BNL 2662	CM6	F: TACAACAGACCGCATCACAGTG R: TCGAGGTACTCTCTCTCTCTCTC	84
BNL 2895	CM7	F: CGATTTTACTGCTTCAGACTTG R: TACCATCTCACGGATCCACA	209
BNL 2961	CM8	F: TCGAAAGGGTGTTTCTTCTT R: GGGGATGCTTGTCACATCTT	234
BNL 3140	CM9	F: CACCATTGTGGCAACTGAGT R: GGAAAAGGGAAAGCCATTGT	103
BNL 3255	CM10	F: GACAGTCAAACAGAACAGATATGC R: TTACACGACTTGTTCCCACG	229
BNL 3279	CM11	F: CATGTCCAATGGATGTGTCA R: GGGCCACTTAAAGGCATTCT	123
BNL 3280	CM12	F: GCAGAACTGCCACTTGTTTG R: AGAAAATGGGTTGTGCTTGG	230
BNL 3383	CM13	F: GTGTTGTCATCGGCACTGAC R: TGCAATGGTTCAGTGGTGAT	190
BNL 3971	CM14	F: CACATATTTTTGCCTCACGC R: TGTGGACCCAAAAAGGAAGA	144
BNL 4108	CM15	F: TCCACCATTCCCGTAAATGT R: TGGCCAAGTCATTAGGCTTT	162
BNL3806	B1	F - GACAGGCCAGACCAGAACAT R - TCAAACAAAGCACATATATAATACACA	199

BNL1153	B2	F - CTTTATCCGGAGACGGAACA R - CTAACTTTTGCTCACCCCCA	327
BNL2884	B3	F - TCAACTCATACCAAATCAATTCC R - CCCTGTTTTGTTCAATGGGT	164
BNL2569	B4	F - CAGAGAGCCATTGTGAACGA R - ATAATGCTAGGGCATGTGGC	170
BNL3257	B5	F - CAATCTGGGATCAAAAAAACC R - GGTGAAACATAGCGTGTTGC	206
BNL2768	B6	F - TTTCTTTGTGGGGAAATTGC R - CATGGATTCTGGACACTCCC	188
JSPR58	B7	F - CCGCCCTTCTCTTGCTTAGATCTGG R - GGAGCCAATTGAGAAGTGAATCCAA	377
CIR122	B8	F - AATGTGGGCTGATACG R - CAGACACAATCCACAAAG	142
BNL2986	B9	F - TAGAGCCAAGTGGTGATCCC R - AAAGGGGGGAATGATTATGC	157
BNL3147	B10	F - ATGGCTCTCTCTGAGCGTGT R - CGGTTCAGAGGCTTTGTTGT	162
BNL4053	B11	F - TGAAGGCTTTGAAGCAAACA R - AAGCAAGCACCAAGTTAGCC	207
BNL4059	B12	F - GAGTTACGCCTGGCAATCAT R - CCATCCCCAGTGGTGTTATC	221

RESULTS

After 5 generations of selfing and reproducing the lines were considered sufficiently stable and homogenous for determining the fiber quality properties for identification of QTLs.

For molecular marker analysis a total of 15 pairs of SSR and 13 ISSR primers were screened for their capacity to reveal polymorphisms.

The two types of marker systems produced substantially different numbers of markers. While SSR primer pairs revealed 1-3 polymorphic bands per primer pair, the application of ISSR primers resulted in general in much higher number of markers per primer.

The screening of the population with the 13 ISSR primers produced varying numbers of polymorphic bands where 3 of them revealed no polymorphisms, while the 10 remaining produced between 6 and 13 polymorphic bands. Altogether ISSR primers produced 95 polymorphic bands throughout a population of 104 individuals. Together with SSR primers a total of 125 polymorphisms were identified in our population. Of these only 110 could be mapped as 15 revealed very low polymorphism.

Several markers had significant effects on more than one trait. Most notable of these were E5_305 which had significant effects on all fiber traits and marker C1_900 which affected all traits, except for fiber length (Figure 1). Further notable cases include markers that affect several

traits, such as E5_250 which significantly affects length, uniformity, elongation and micronaire. The observed polymorphisms in E5_250 could be attributed to explaining about 7% of the total variation of fiber length, 9% of the uniformity variation, 6.2% of elongation and 6.3% of the variation in micronaire.

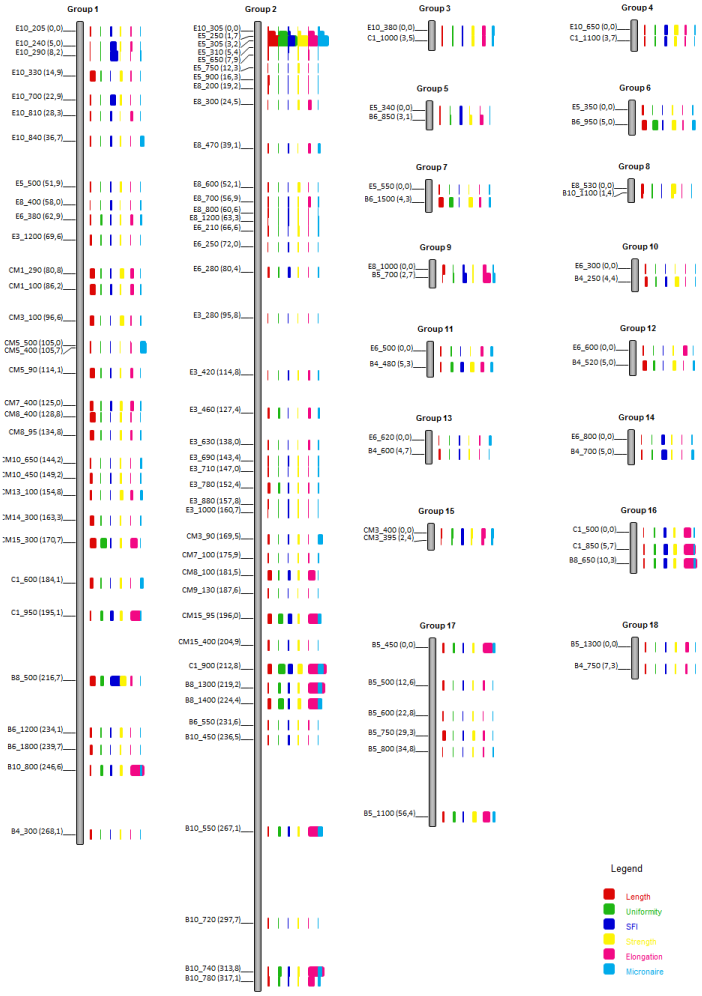

Figure 1. Combined genetic map for studied traits. The width of colored bars represents the percentage of trait variability explained by marker presence at each locus

Yet another marker (CM15_300) had significant effects simultaneously on fiber length, uniformity and elongation explaining 5.93, 6.04 and 6.67% respectively of the variability of these traits.

Marker B8_500 also had significant effects on more than one trait. It explained about 5% of the variation in fiber length, together with about 6% of fiber strength and more than 10% of the variability of short fiber index (SFI).

Several markers were identified that affected two traits simultaneously. These included B6_950, which affected length and uniformity, B10_740 and B10_550 – with effects on elongation and micronaire, B8_1400 and CM15_95 – with effects on uniformity and elongation.

Of the SSR polymorphic bands identified within the present study only two had significant effect on more than one trait (CM15_95 and CM15_400). Notably both of these are alleles of the same marker, therefore further supporting its association with important fiber traits, initially identified in an interspecific cross (REDDY ET AL., 2001).

Phenotyping for the fiber properties revealed significant differences in fiber quality within the population (Table 3).

Table 3. Range of quality characteristics observed in tagged M6 plants

Range	Mic.*	Str. (g/tex)	Length (mm)	Unif. (%)	Elng. (%)	SFI
Minimum	3.53	25.6	21.97	82.4	5.2	5.50
Maximum	5.90	39.0	30.78	90.9	9.0	10.80
Mean	4.72	32.3	26.37	86.7	7.1	8.15
'Chirpan 603' untreated	5.20	35.6	27.62	88.5	7.6	8.40

* Mic. – micronaire; Str. – fiber strength; Unif. – fiber uniformity; Elng. – fiber elongation; SFI – short fiber index

The results showed that the variations generated by mutagenic treatment were sufficient for identification of lines with improved fiber quality characteristics. For example, several progenies had high micronaire readings with one progeny reaching 5.90. At the other extreme of this trait there was one progeny with micronaire reading of 3.53 and two other progenies with micronaire values of 3.6-3.7.

Similar variation distributions were observed for all other quality characteristics (Table 3) with uniformity being the least affected and short fiber index (SFI) – the most.

DISCUSSION

The first linkage group (LG1) contained several loci that had statistically significant effects over numerous traits' variation (Figure 1). Constructing graphical genetic map helped to quickly identify broader regions encompassing two or more markers and affecting two or more traits. One such region is near the top of LG1 and included markers E10_240, E10_290, E10_330 and E10_700 (encompassing 17.9 cM and explaining 19.07% of the total variation in SFI with a second significant effect – on fiber length). Another region was tagged by markers CM1_290 and CM1-100 and encompassed 11.2 cM. It was identified as responsible for 8.87% of the variation in fiber length.

Even more interesting regions could be identified on the longest linkage group of our study – LG2. The region at the top of this group is relatively small – it encompasses 1.6 cM but affects all fiber traits. This region is defined by the markers E5_250 and E5_305 the latter being the only one that affects all fiber traits. This important finding is corroborated by the identification of the closely situated marker E5_250 which also significantly affects numerous fiber traits. Therefore this relatively small region tagged by two markers appears as a very good candidate for inclusion in marker assisted selection (MAS). Not only is this region tagged by two markers with significant effects on several traits, but it is well defined, too, as the other two markers on both sides of the region that are tightly spaced (E10_305 at 1.7 cM and E5_310 at 2.2 cM) have very little effect on all the traits.

There is one more region at the other end of LG2 that significantly affects more than one trait. That is the region tagged by markers CM15_95, CM15_400, C1_900, B8_1300 and B8_1400, which covers about 28 cM. This region affects significantly 5 of the 6 studied fiber traits, explaining 17.27% of the variation in uniformity, 4.46% in SFI, 4.53% in fiber strength, 58.84% in elongation and 5.27% in micronaire. Apparently the region covers several loci with significant effects on different traits and it therefore could become a good candidate for MAS if these loci could be further characterized and better tagged (with more markers in a denser map). Simultaneous improvement of multiple traits related to both fiber quality and yield were also found in studies of cropping systems (ABBAS *et al*., 2016) – an observation that supports our findings.

Identification in our study of regions that had simultaneous statistically significant effect on fiber strength, micronaire and elongation is in line with the observations that micronaire (a measurement affected by both fiber fineness and maturity) is affected by the buildup of cellulosic fibrils. The process results in improved strength, but reduced elasticity. Therefore, the mutagenic treatment applied at the beginning of present study may have affected some basic processes of cellulose synthesis and/or deposition that are defined by the locus/loci tagged with the abovementioned markers. Further studies would be needed to identify which of the many possible mechanisms are affected, but the simultaneous effect on the modification of all three traits by the genetic constitution of these regions strongly supports such theory.

The fiber of the progeny with the lowest micronaire has apparently reached maturity as both its strength (31.8 g/tex) and length (27.86 mm) were similar to those of the parent variety. The fact that this and one of the other two genotypes with low micronaire had the trait affected by the mutagenic treatment is confirmed by their strength (31.8 and 33.0 g/tex), length (27.86 mm and 30.3 mm) and elongation (5.8% and 6.9%) indicating that their fiber has completed its development. The low micronaire (3.71) of the other plant apparently resulted from incomplete development as its fiber was quite short (at 24.38 mm), with high strength (37.4 g/tex) and elongation (8.1%) and low uniformity (85.6%).

The two progenies with lowest fiber strength (25.6 - 26.2 g/tex) had high micronaire as well (5.90 – 5.63), thus the trait variation in this case could be attributed to the very coarse fiber, probably resulting from mutagenic treatment. At the other extreme there were three progenies with fiber strength varying only slightly (38.3-39.0 g/tex). Fiber length of these progenies varied little, too (28.70-30.78 mm), while micronaire was more variable (3.88-4.82). Interestingly the

elongation of the progenies within this group varied little (6.2-6.9%) thus indicating that within these progenies the only affected trait was fiber strength.

In line with the preliminary expectations fiber length was affected both positively and negatively with extremes evenly spaced from the parent. All of the four progenies with shortest fiber (21.97-23.80 mm) had high to very high micronaire (5.05-5.91) and equal or lower fiber strength to the parent genotype (30.8-34.0 g/tex). Combined this indicates that respective progenies have reached full maturity and their fiber length is indeed negatively affected by the mutagenic treatment.

The analysis of the progenies with longest fiber did not result in similarly clear-cut conclusions. The progeny with the longest fiber (30.78 mm) had its uniformity (88.5%) and elongation (6.9%) essentially equal to that of the parent, while its strength (39.0 g/tex) was significantly increased. The next two progenies with longest fiber (30.51-30.71 mm) had their strength unaffected (35.6-37.7 g/tex), while micronaire was significantly decreased (4.38-4.43).

The complex nature of the inheritance and the requirement to combine several different (and often negatively correlated) traits makes breeding for the varieties with good agronomic performance, and carrying at the same time multiple improved fiber characteristics a daunting problem that every cotton breeder strives to resolve. Marker assisted selection (MAS) has risen in the recent years as a tool of choice as it significantly facilitates the selection of genotypes for intercrossing in each generation. As more and more loci having positive effect on the traits of interest are identified and molecularly tagged, this tool becomes more and more accessible to the applied breeding programs. By using one of the most commercially important varieties in the country this study attempts to provide knowledge and tools needed to keep Bulgarian breeding programs competitive.

The possibilities for using SSR markers, identified in inter- and intraspecific crosses as linked to fiber quality characteristics in cotton, for identification of loci affected by a mutagenic treatment of seeds from the standard Bulgarian variety (‘Chirpan 603’) were demonstrated in the present study. Furthermore, the potential for using ISSR markers in MAS mutation breeding was demonstrated as well. Genetic linkage map has been constructed which consisted of 18 linkage groups. Markers linked to all studied fiber characteristics were found with loci having statistically significant effect on corresponding traits explaining between 4 and 19% of the total trait variation. Combining maps for individual traits allowed for identification of several regions, affecting multiple quality traits at once. The markers tagging these regions may prove of particular interest for future breeding efforts that would aim at applying marker assisted selection (MAS) for pyramiding multiple positive alleles.

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QTL DETEKCIJA KOD BUGARSKE SEGREGIRAJUĆE POPULACIJE MUTANTA PAMUKA

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Izvod

Identifikacija lokusa kvantitativnih svojstava (QTL) vezanih za kvalitet vlakana je od primarnog interesa za oplemenjivanje pamuka. Bugarske sorte pamuka pripadaju određenoj grupi (proles *Bulgaricum*), i u njih je veoma teško ubaciti kvalitativne karakteristike ukrštanjem. Stoga je segregirajuća mutantna populacija razvijena iz standardne bugarske sorte "Chirpan 603" primenom gama zračenja semena. Generacije M4 i M5 su korišćene za identifikaciju QTL-ova vezanih za karakteristike kvaliteta vlakana. SSR markeri razvijeni u interspecifičnim ukrštanjima i dalje potvrđeni u intraspecifičnim ukrštanjima, zajedno sa *in-house* razvijenim ISSR markerima, korišćeni su za mapiranje QTL-ova za kvalitet vlakana. Snaga vlakana, dužina, ujednačenost, mikronaura i izduženje bili su glavne proučavane karakteristike. QTL-ovi sa velikim efektima na ove osobine identifikovane u M4 i M5 generaciji, potvrđeni su i generaciji M6. Ostali su identifikovani i korišćeni za dopunu mape i potvrdu grupnih veza.

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