Molecular tagging of fiber yield genes using RI lines developed in Uzbekistan

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ABSTRACT

Fourteen fiber specific EST- SSR markers out of 85 showed polymorphic PCR-products between parental lines. These polymorphic EST-SSRs were subjected to genotype RI individuals to identify fiber QTLs. Single marker analysis revealed that four markers – EST-17, EST-47, EST-32, and EST-56 were statistically significant, and Kruskal-Wallis (KW) test for the markers were 4.8, 6.8, 13.1, and 13.5, respectively. Statistically significant two QTL regions coincided with the two EST-SSR markers that expressed the highest single marker analysis (SMA) linkage associations, EST-32 and EST-56. EST-32 marker was significantly linked to QTL and had a LOD of 4.28 explaining 48.1% of variation of lint percentage. The second marker EST-56 also was highly significant and showed a LOD of 4.34. Lint percentage QTL near the locus EST-56 explained 48.6% of variation of the trait. EST sequences linked to fiber QTLs will help in candidate gene identification and map based cloning of genes linked to these fiber QTLs. Outcomes of this research will accelerate incorporation and rapid deployment of improved agronomic fiber genes into elite cultivars through marker assisted selection programs.

Introduction

Use of reliable PCR-based DNA markers has become an important tool in molecular breeding programs. There are many types of PCR-based molecular markers such as AFLPs (Vos *et al*., 1995), CAPs and dCAPs (Konieczny and Ausubel, 1993) and microsatellites, also known as SSRs (Akkaya *et al*., 1992) or SSLPs (Bell and Ecker, 1994). Amplified fragment length polymorphism (AFLP) has also been proved powerful to identify large numbers of potentially polymorphic loci, and fingerprinting with AFLP has become widely used for characterizing diverse germplasm in cotton (Abdalla *et al*., 2001).

SSRs are the most informative marker system among available molecular markers since the number of repeats at a locus is highly variable within closely related individuals, and these can easily be visualized as AFLPs. SSR markers also have the advantage of being co-dominant providing linkage information of genomic regions segregating from both parents. SSR markers have a significantly higher expected heterozygosity than all other marker systems, thus they are the system of choice when the objective is to resolve differences between individuals, map of trait of interest, genome evolution and comparative genomics, and for the efficient utilization of wild and primitive germplasm

resources in MAS (Bell and Ecker, 1994; McCouch *et al*., 1997). A number of SSR containing loci have been identified and SSR-marker collections have been made available for cotton genome research. More than 500 microsatellite containing clones, containing mostly $(GA)_n$, repeats, have been identified at the Brookhaven National Laboratory. An additional 150 $(GA)_n$ repeat loci (designated CM) have been isolated at Texas A & M University (Connell *et al*., 1998; Reddy and Pepper, unpublished). Moreover, 309 new cotton microsatellites designated as JESPR have recently been identified and characterized by our group (Reddy *et al*., 2001). Also, EST specific microsatellite markers developed from EST data of Gene Bank have recently been identified in cotton (Qureshi *et al*., 2002). All these SSR markers can be used to tag fiber yield genes; however, we used EST-SSRs to tag fiber QTLs in this research. These EST-SSR markers have a potential in tagging fiber QTLs since many of them are specific to developing fibers of cotton.

In Uzbekistan, there are approximately 17,000 cotton germplasm collections including isogenic, inbred lines, elite AD allotetraploid varieties (*G. hirsutum* and *G. barbadense*), monosomic and translocation lines (A. Abdullaev personal communication) (Musaev *et al*.., 2000) along with wild, primitive and extant representatives of the A to G genome groups that have been developed in the Cotton Research Institutes of the Republic and collected over the world for the past 9-10 decades. In this germplasm, cotton accessions represent a very useful agronomically important traits, such as insect and pathogen resistance, tolerance to environmental stresses, fiber quality (length, strength and lint yield) and yield potential. There are recombinant inbred (RI) and isogenic lines within the collection, widely segregating for lint yield, seed fuzz formation, and seed weight, which will be very useful for molecular analysis of fiber yield genes and fiber QTL-mapping efforts using DNA marker technology.

Seed coat of normal cottonseed is covered with lint and fuzz where lint is known as a unique textile fiber and fuzz is short fiber remaining in seed coat in ginning. There are spontaneous cotton mutants without fuzz, but linted so called "Naked seed cottons" and also cotton without fuzz and lint (Musaev and Absalov, 1972; Nadarajan and Rangasamy, 1988; Zang and Pan, 1991; Du *et al*., 2001). Many geneticists have studied the genetic inheritance of fiber formation in cottonseeds. Genetics of cottonseed fuzz and lint development have been widely studied at Institute of Genetics and Plant Experimental Biology, Uzbekistan, by academician J. Musaev and his group (Musaev *et al*., 2000), and unique RI lines collection (500 lines) on fiber formation have already been created. Musaev and Abzalov (1972) suggested that two major genes – F_{t1} and F_{t2}, control the fuzz around the micropyle whereas fuzz on chalazal and lateral parts of seed are controlled by a third gene- F_c in complementary interaction with other genes. There is additional an inhibi-

tor gene –I that in the dominant homozygous and heterozygous states of this gene block the function of all fuzz genes, resulting in dominant fuzzlessness (Musaev and Abzalov, 1972).

Cottonseed lint yield are controlled by polygenes. There might be two categories of genes for lint formation: 1) basic polymeric genes Li^A- li^A and Li^D- li^D; 2) additional polymeric genes Li_1^A- Li_2^A (came from A-genome) and Li_1^D, Li_2^D (came from D-genome) which serve as an enhancer for basic lint genes (Musaev, 1979). Basically, cotton fiber development has a very complex inheritance pattern and result of pleiotropic, polygenic and epistatic interactions of fuzz genes-F_{t1} and F_{t2}, lint genes - Li^A and Li^D, and gene-inhibitor-I (Musaev, 1979). Fuzz genes (F_{t1} and F_{t2}) contribute 35% lint yield through their pleiotropic interaction with lint genes. Sixty five percent fiber yield is a result of polygenic effect of lint genes. Gene-inhibitor in dominant state shows negative pleiotropic effect to lint genes inhibiting all major fuzz genes (Musaev, 1979).

We initiated new research project to tag fiber loci using existing RI line collection of Uzbek cotton germplasm through molecular marker technology since mapping and characterization of fiber quality genes are important to manipulate these loci effectively in cotton breeding programs. The overall objective of this research was molecular mapping of fiber yield genes from intra-specific recombinant inbred (RI) lines of *G. hirsutum* using SSR markers, in particular fiber associated EST-SSR since these microsatellites represent the coding gene sequences of developing fiber cells of cotton.

Molecular tagging of fiber yield genes will be a foundation for map-based cloning of important fiber genes and markers will be used in marker-assisted selection. As per our knowledge, there is no report on the use of any PCR based EST marker to map fiber or agronomic traits in cotton.

Experimental procedure

Plant material

For initial experiments, we selected 30 intra-specific RI lines of *G. hirsutum* derived from the cross between L-70 (fuzzless/lintless with 0% lint on cottonseed) and L-47 (fuzzy/linted with 41-42% lint on cottonseed) lines along with parental lines (L stands for and means "LINE" and the consequent number describes tag for each line). RI lines were developed through self-crossing during F_{10} generations. Selected 30 individual RI lines from the RI collection have 0 to 41% lint yield phenotype (Table 1), and represent mostly extreme phenotypic classes (0-8% to 36-41%) and some middle phenotypic classes (with 10-26% lint) with examples of segregation for fuzz type. These RI lines from Uzbek germplasm collection were re-grown at Institute of Genetics and Plant Experimental Biology field stations and detail phenotypic analysis, collecting segregation data was conducted. Tissue samples were collected from each individual of RI lines and stored under -80 °C until utilization.

Genotyping analysis

Genomic DNA samples were extracted from collected cotton tissues according to Iqbal *et al.* (1997) and used in genotyping analysis. Microsatellite genotyping analyses were performed as described by Reddy *et al.* (2001). Briefly, primer-pairs for EST-SSRs were amplified using a hot -start PCR protocol in genomic DNAs of the parental lines; then, if they were polymorphic between parents, the RI lines segregating with fiber yield were tested. A hot-start PCR steps were carried out as described by Brooks (2001) to improve PCR-product yield. Amplification reactions were performed in 25 μl volumes containing 2.2 μl 10 x PCR buffer with $MgCl_2$, 0.4 μl BSA, 0.2 μl 25 mM of a dATP, dGTP, dTTP, and dCTP mix, 2.0 μl 25 ng/ml of each reverse and forward primer, and 1 μl 25 ng/ml template DNA. Then, 0.2 U Taq DNA polymerase (Promega) were added to the reaction at the annealing temperature of first cycle. Amplifications were carried out with a first denaturation at 95 °C for 3 min followed by 45 cycles of 94 °C for 1 min, 50 °C for 1 min (annealing) and 72 °C for 2 min (extension). A final 5-min extension at 72 °C was then performed. Polymorphism of microsatellite amplification products was revealed using a horizontal agarose gel electrophoresis system yielding resolution of two base-pair polymorphisms, and by vertical acrylamide gel electrophoresis system capable of resolving single base-pair polymorphisms. In the agarose system, samples were electrophoresed on a 16 cm long horizontal gel (Stratagene) containing 2% agarose plus 2% Metaphor agarose (FMC) at 5.3V/cm in 0.5X TBE buffer (45 mM Tris-Borate, 1 mM EDTA, pH 8) with buffer chilling to 4 °C. Gels were stained briefly with ethidium bromide prior to photodocumentation. In the acrylamide system, samples will be electrophoresed at 20V/cm in a 10 cm high x 10 cm wide x 1 mm thick vertical gel rig (Stratagene) containing 6% polyacrylamide with 10% v/v Spreadex NAB polymer (Elchrom Scientific) in 1X TAE buffer (45 mM Tris-Acetate, 1 mM EDTA, pH 8), then visualized after staining with ethidium bromide. Gels were photodocumented using Kodak gel doc system.

QTL-mapping

Genotyping data obtained in RI lines were correlated with previously collected phenotypic data on lint percentage and the associations of polymorphic DNA markers with fiber development genes were statistically analyzed, generating QTL maps of fiber quality genes. To generate single-marker analysis (SMA) and fiber QTL maps, the Kruskal-Wallis nonparametric test and interval mapping test functions of evaluation version of MapQTL@4.0 software were used (Van Ooijen *et al.*, 2002).

Results and Discussions

Eighty-five fiber-specific EST-SSR primer pairs were screened over parental lines (L-70 and L-47) (Figures 1 and 2) to find potential polymorphic markers that will be used to identify fiber QTLs. Seventy-six EST-SSR marker primer pairs out of 85 gave visible PCR-products while 9 EST-SSRs did not give amplification product. PCR-products of sixty-three markers out of 77 were monomorphic while 14 EST-SSRs (EST-12, EST-17, EST-25, EST-32, EST-39b, EST-41, EST-47, EST-56, EST-64, EST-66a, EST-67, EST-78 EST-58, and EST-55) PCR-products showed potential polymorphisms between parents. Examples for some potential polymorphic EST markers with amplification product size differentiating the parental lines were shown in Figure 3.

To analyze associations of these EST-SSRs markers with fiber yield genes, these polymorphic markers were screened over the inter-specific RI lines of *G. hirsutum*, segregating for lint yield (Figure 4). We selected 30 individual RI lines from the RI collection having 0 to 41% lint yield phenotype (Table 1). These RI lines represent mostly extreme phenotypic classes (0-8% to 36-41%) and some middle phenotypic classes (with 10-26% lint). Genomic DNAs of these RI lines were isolated and screened with potential polymorphic EST-SSR markers. Genotypic data obtained were then analyzed using MAPQTL software and possible lint yield QTL linked EST-SSRs were identified.

To identify basic linkage information of tested EST-SSRs, single marker analysis (SMA) was conducted. Single marker analysis revealed that four EST-SSRs out of 8 polymorphic EST-SSRs showed significant association to lint percentage. These markers are EST 17, EST32, EST47 and EST56. All of these markers – EST-41, EST-32, EST-56 were significant (KW= 4.79, 6.804, 13.158, and 13.529 respectively) according to Kruskal–Wallis non-parametric test. The remaining markers tested were not significant (Table 2). This result shows that these EST-SSR markers might have close association with cotton lint yield and corresponding ESTs might determine cotton fiber development.

Additionally, fiber yields QTLs were identified using interval mapping analysis. As expected, two QTL regions coincided with the two EST-SSR markers that expressed the highest single marker analysis (SMA) linkage associations, EST-32 and EST-56. EST-32 marker was significantly linked (Figure 4) to QTL and had a LOD of 4.28. The QTL near locus EST-32 explained 48.1% of variation of lint percentage. The second marker EST-56 (Figure 5) also was highly significant and showed a LOD of 4.34. Lint percentage QTL near the locus EST-56 explained 48.6% of variation of the trait. The QTL found with the other markers that were significant associations in SMA (EST-17 (LOD=1.81) and EST-41 (LOD=1.44)) had a low LOD scores (<2.0) and might not be significantly linked to a fiber percentage QTL (Table 2); they either might be additional fiber genes with minor effect on fiber development or might be associated with other agronomic characteristics of cotton fiber.

EST-SSRs have a particular interest since many of them were developed from ESTs of developing fiber cells. Moreover, there are a number of advantages of using expressed genes instead of 'anonymous' sequences as genetic markers. Firstly, any EST genetically linked to the trait of interest might be potential gene associated with trait and can be used to map gene directly affects the trait. Secondly, because of a high degree of sequence conservation, EST markers are more likely to be transportable across pedigree and species boundaries than markers derived from non-expressed sequences, like AFLPs, RAPD and genomic SSR markers (Cato *et al.*, 2001). Hence, EST markers linked to the trait of interest are very useful to transfer the trait through MAS approaches.

In this regard, found EST-SSR markers linked to two lint percentage QTLs, EST-32 and EST-56, particularly might be potential genes responsible for cotton fiber development or initiation, and further studies of the function of these ESTs will reveal biological role of these ESTs in lint development of cotton.

It should be noted that, our results in this study are of particular interest while genotyping of EST-32 marker over RI lines (Figure 4). The polymorphic loci that came from fuzzy/linted parent (L-47) were different in several RI lines including L-15, L-103, L-306, L-453, L-454, and L-471. In all these lines, the L-47 parent specific band (~250 bp) was in higher position (~350 bp) than expected, demonstrating either recent expansion in microsatellite repeats or rearrangements in primer binding sites. Moreover, EST-32 genotype was more complex in L-2 and L-3 (#24 and 25 in Figure 4); these lines had both L-47 specific band (250 bp) and recently expanded band (350 bp), and interestingly, the lint percentage was low in both lines although they have a band like L-47. Moreover, the lower band in these lines was also different than parents. Getting this kind of pictures we tried to analyze development history of these RI lines, and learned that these lines might have gotten exposed with radioactive treatment (x-rays) since a lot radioactive mutagenesis experiments were conducted on RI line collection during 1970s and 1980's. Hence, the repeat expansion might be due to stepwise mutations in original microsatellite sequence or chromosomal rearrangements. This expanded band was treated as L-47 specific band for mapping purposes, yet molecular cloning and sequencing of expanded band is worth to be done in the future to be sure that it is the result of expansion of the original microsatellite repeat and to understand its role in fiber development.

In this ongoing research, we presented the initial results toward tagging of fiber yield genes using in-

traspecific RI lines of *G. hirsutum* in a small number of RI individuals. To increase reliability of these results we will increase number of RI lines to strengthen the linkage associations of markers with QTLs in the future. Moreover, since QTL linked EST markers very informative, we need to put them in *G. hirsutum* x *G. hirsutum* intra-specific linkage map and identify their chromosomal locations (Lui *et al*., 2001; Ulloa *et al*., 2002) to manipulate these markers effectively in MAS programs. Since EST-SSR markers represent coding sequences, we also plan to knock out of these ESTs associated with fiber yield QTLs and study their exact physiological function using high-throughput gene knock out technologies (pHELLSGATE) in the future (Wesley *et al*., 2001).

Furthermore, to increase number of markers for further linkage mapping efforts as well as QTL analysis of agronomic traits (lint yield), microsatellite markers from the JESPR collection (Reddy *et al*., 2001) are being screened over the parental lines to find potential polymorphic SSRs. A number of potential polymorphic markers have already been determined (data is not shown). Identification of QTLs using polymorphic JESPRs is currently in progress and details are not given in this report since experiments are in a very initial stage.

Thus, our efforts to tag fiber yield genes gave promising results about genetic determinants of lint yield genes that will be useful in understanding of genetic basis of fiber development genes in cotton. The identified EST markers that are significantly associated with lint development QTL will help breeders to transfer these loci into highly adapted and resistant elite cultivars with low lint yield when linkage map information and chromosomal locations of these markers are identified.

Acknowledgments

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Table 1. *Recombinant inbred lines segregating for fuzz type and lint yield.*

#	Lines	Lint, %	Fuzz type	#	Lines	Lint, %	Fuzz type
1	L-15	41	Normal fuzz on micropyle	16	L-489	41	Fully fuzzy
2	L-16	36	Fully fuzzy	17	L-500	28	Big fuzz on micropyle
3	L-18	36	Fully fuzzy	18	L-47	40	Fully fuzzy
4	L-44	36	Fully fuzzy	19	L-100	26	Recessive naked seed
5	L-69	37	Fully fuzzy	20	L-72	0	Dominant absolutely naked
6	L-70	0	Dominant absolutely naked	21	L-102	30	Recessive naked seed
7	L-73	0	Dominant absolutely naked	22	L-462	33	Dominant absolutely naked
8	L-75	0	Dominant absolutely naked	23	L-1	0	Dominant absolutely naked
9	L-103	4	Recessive naked seed	24	L-2	0	Recessive naked seed
10	L-306	8	Little fuzz on micropyle	25	L-3	14	Little fuzz on micropyle
11	L-453	40	Fully fuzzy	26	L-4	24	Big fuzz on micropyle
12	L-454	43	Fully fuzzy	27	L-5	24	Big fuzz on micropyle
13	L-460	41	Fully fuzzy	28	L-6	12	Recessive naked seed
14	L-471	41	Fully fuzzy	29	L-7	33	Recessive naked seed
15	L-472	40	Fully fuzzy	30	L-8	14	Recessive naked seed

**Table 2.** *Single marker analysis and interval mapping of lint percentage of cotton using MapQTL @4.0.*

Locus	Nr inf.	KW* (df)	LOD	% Expl	add
EST-17	26	4.79 (1) **	1.81	27.8	-8.73
EST-32	30	13.16 (1)******	4.28	48.1	-11.703
EST-47	29	6.80 (1)**	1.44	20.0	-7.70
EST-56	30	13.52 (1)******	4.34	48.6	-11.50
EST-39(b)	28	0.81 (1)-	0.04	0.6	-1.24
EST-41	27	0.03 (1)-	0.02	0.3	-0.94
EST-64	29	1.19 (1)-	0.52	7.8	-12.31
EST-12a	26	1.97 (1)-	0.56	8.3	-4.88

*, **, **** Kruskal-Wallis non-parametric test, significant at the 0.05, 0.01 and >0.01 levels, respectively

LOD – the LOD score; add-the estimated additive effects

% Expl – the percentage of the variation explained for by the QTL **.**

Figure 1.
Parental lines: L-70 –fuzzless/lintless; L-47- fuzzy/linted (from J. Musaev's collection).
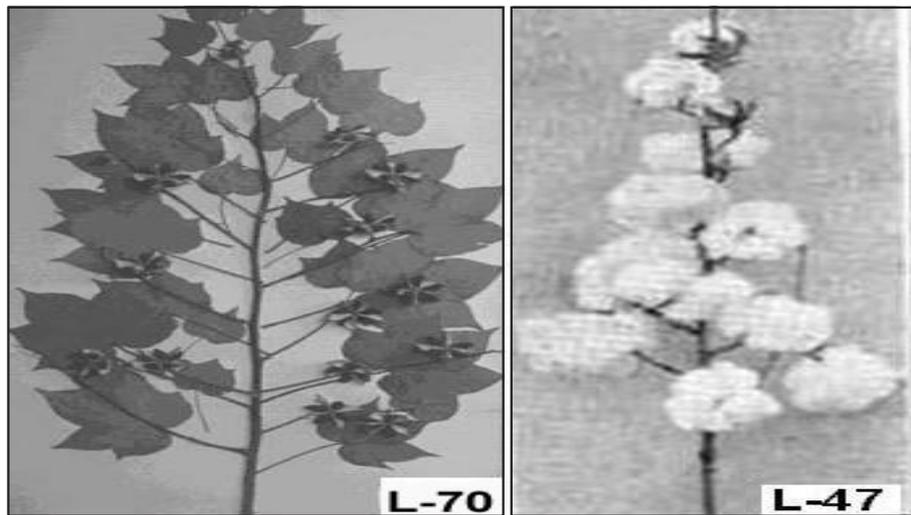
Figure 2.
Examples for fiber yield and seed fuzz segregation. A. Lint yield of naked seed lines showing variation of 0-32%. B. Lint yield of fuzzy seed lines showing 1-45% variation. C. Types of seed fuzz. D. Fiber yield per boll (1-10g) (from J. Musaev's collection).
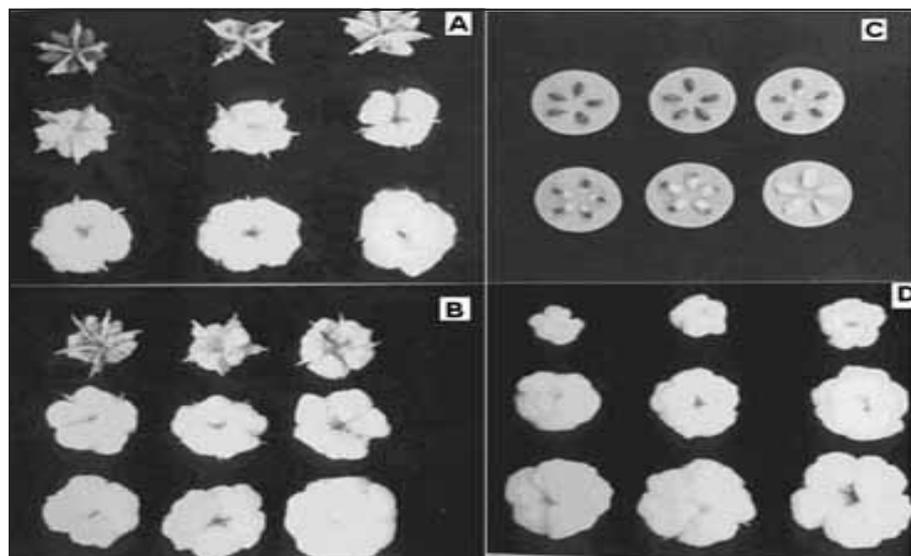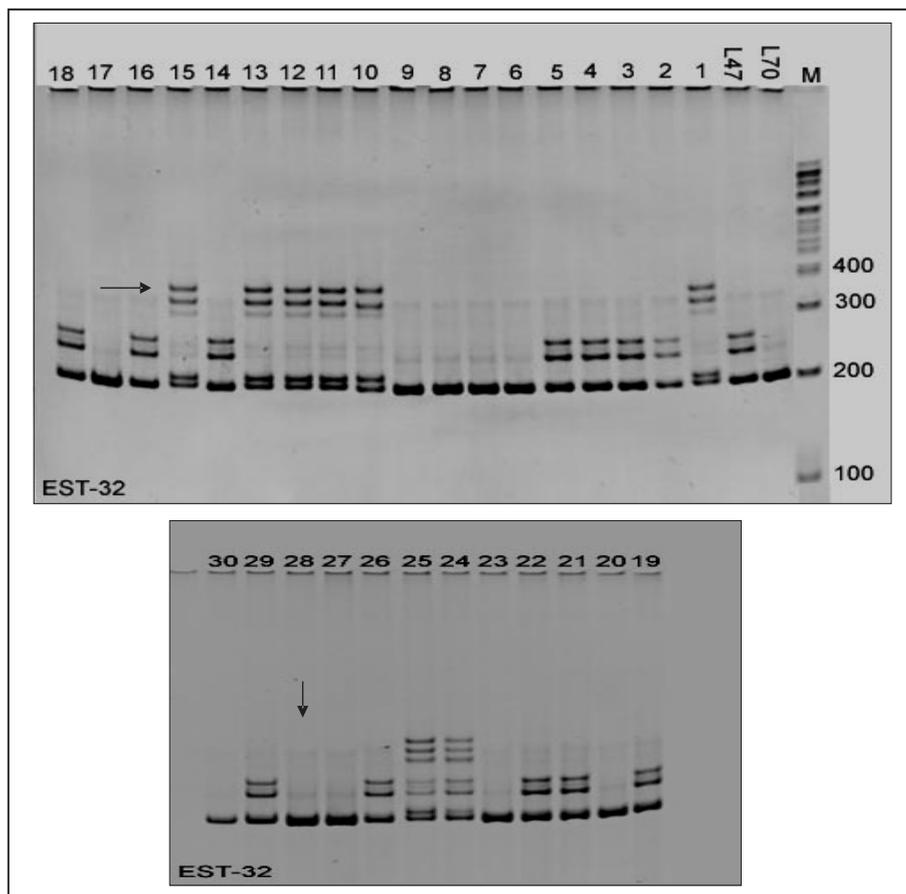

Figure 3.
The potential polymorphic EST-SSR markers (polymorphic bands were shown with arrows), differentiating parental lines: L-70 -fuzzless/ lintless parent; L-47 - fuzzy/linted parent; M-100 base pair marker (without 6% PAAG gel).
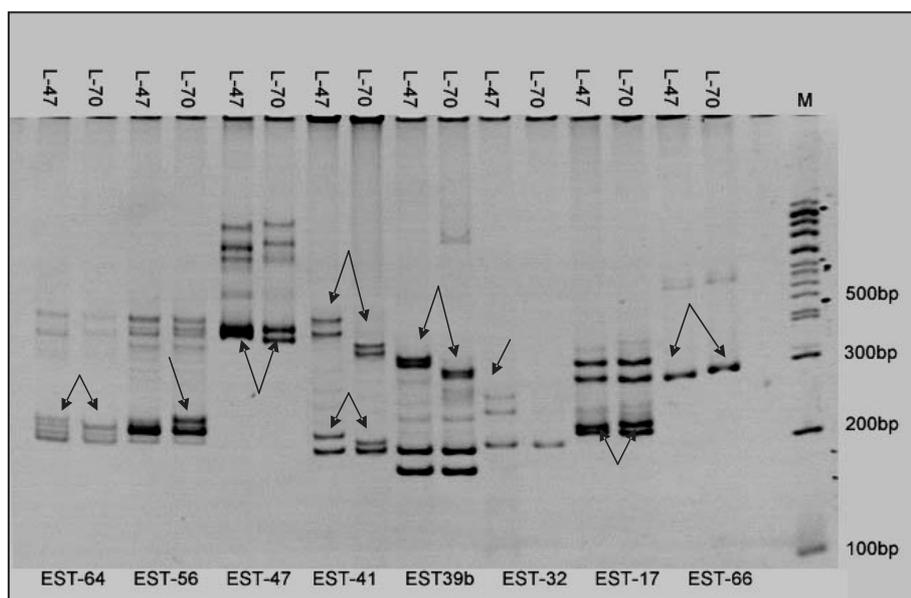
Figure 4.
Genotyping of RI lines with EST-32 marker in 6% polyacrylamide gel: M- 100 base pair marker; L70- fuzzlees/lintless line; L-47 fuzzy/ linted line; band specific to L-70 parent was scored as " a" while band specific toL-47 parent was considered as "b"; arrows show possible expansion of SSR sequence.

Figure 5.
Genotyping of RI lines with EST-56 marker in 6% polyacrylamide gel. L70- fuzzlees/ lintless line; L-47 fuzzy/linted line; band specific to L-70 parent was scored as " a" while band specific to L-47 parent was considered as "b".