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ARTICLE

Genetic screening of diploid and tetraploid cotton cultivars based on retrotransposon microsatellite amplified polymorphism markers (REMAP)

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Resumen

Cribado genético de cultivares diploides y tetraploides de algodón, basado en marcadores de polimorfismo de microsatélites en retrotransposones amplificados (REMAP)

La selección artificial continuada y el uso de variedades ha llevado a erosión genética y pérdida de loci genéticos del germoplasma disponible. El objetivo fue evaluar variedades diploides y tetraploides de algodón por REMAP. Se observó baja variabilidad genética dentro de cada variedad, pero alta dentro de cada especie (18-68%). El AMOVA reveló que el 63% de la variabilidad genética total se produjo debido a diferencia entre especies. Se detectaron alelos únicos en las especies estudiadas, que pueden ser empleados para discriminación. REMAP podrían discriminar eficazmente las especies diploides de las tetraploides y ser últiles para el cribado rápido, actuar como huella genética del gran germoplasma de algodón y para la planificación de programas de hibridadción.

Palabras clave: Algodón, Diversidad genética, REMAP, Clúster K-means.

Abstract

Continuous artificial selection and cultivation of cultivars has led to genetic erosion and loss of useful genetic loci from the available germplasm. The objective was to evaluate diploid and tetraploid cottons cultivars with REMAP. Low genetic variability within each cultivar was observed, but a high genetic variability occurred within each species (18-68%). AMOVA test revealed 63% of total genetic variability occurred due to among species genetic difference. Unique alleles were detected in the studied species, that can be used for species discrimination. REMAPs could efficiently discriminate the studied diploid from tetraploid species and may be useful for fast screening, as genetic fingerprinting of large cotton germplasm and for planning future hybridization programs.

Key words: Cotton, Genetic diversity, REMAP, K-Means clustering.

Introduction

Genetic fingerprinting and molecular screening of crop plants is an essential step in germplasm evaluation and planning proper breeding program and hybridization. This is also very important for cotton cultivars as inter-specific and inter-cultivar crossing are among the main approaches to produce genetic variability in cotton germplasm (Sheidai *et al.* 2014).

Cotton (*Gossypium* spp.) is the most important natural fiber and oil source worldwide, with an estimated production of 115 million balls (Sheidai 2008). The genus *Gossypium* L. comprises 45 diploid and 5 allotetraploid species, with their genomes allotted to 8 diploid genomes (A-G and K, Malik *et al.* 2014). Cotton has only 4 cultivated species; *Gossypium arboreum* L. (A2A2), and *Gossypium herbaceum* L. (A1A1) are diploid (2n=2x=26), while *Gossypium hirsutum* L. (AADD) and *Gossypium barbadense* L. (AADD) are tetraploid (2n=4x=52). The A-genome cottons are a potentially important genetic resource for cotton breeding program (Sheidai 2008).

Due to constant human selection for favorable agronomic characters like an annual plant habit, photoperiod insensitivity, early flowering, larger boll size, yield, and fiber qualities, as well as continuous cultivation, the most widely used cotton germplasm has a narrow genetic diversity. This has led to genetic erosion and loss of useful genetic loci from the available cotton germplasm, which in turn result in vulnerability to pests and diseases. Therefore, studying the amount of available genetic diversity in cotton germplasm and planning for intra-specific and inter-specific hybridization is important to restore adequate genetic variability in assessing the amount of genetic variability in cottons. It can be done by various approaches, such as pedigree analysis, morphological and agronomic clustering, as well as molecular studies (Noormohammadi *et al.* 2015).

Different molecular markers have been used in genetic diversity analysis of cottons, including random amplified polymorphic DNA (RAPD), allozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), and inter-simple sequence repeats (ISSRs) (Van Esbroeck *et al.* 1998, Abdalla *et al.* 2001, Sheidai *et al.* 2007, Sheidai *et al.* 2008, Kantartzi *et al.* 2009, Noormohammadi *et al.* 2013, 2015). The success of these methods seems not adequate enough as the level of polymorphism detectable is low, at both the intra- and interspecific levels. These types of markers are not efficientwhen applied to the genotyping of large germplasm collections as they are slow and time consuming (Van Esbroeck *et al.* 1998, Noormohammadi *et al.* 2015).

Retrotransposons (RTs) are major components of most eukaryotic genomes and are dispersed throughout the genome. Their copy-and-paste lifestyle in the genome leads to the formation of new genomic insertions without excision of the original element. The structure and copy number of retrotransposon families are strongly influenced by the evolutionary history of the host genome (Alzohairy *et al.* 2013).

All features of integration activity, persistence, dispersion, conserved structure and sequence motifs, and high copy number suggest that RTs are appropriate genomic features for building molecular marker systems. To detect polymorphisms for RTs, marker systems generally rely on the amplification of sequences between the ends of the RT, such as (long-terminal repeat)-retrotransposons and the flanking genomic DNA. The retrotransposon-microsatellite amplified polymorphism (REMAP), is commonly used PCR retrotransposon-based molecular markers (Kalendar *et al.* 1999). These markers have been used to study genetic diversity studies and to describe the profile of a population (Santana *et al.* 2012) and to discriminate between species or genotypes (Biswas *et al.* 2010). We used REMAP molecular markers to study available genetic variability in our cotton germplasm collection and discriminate between different genetic groups.

Therefore, we used inter-retrotransposon amplified polymorphism (REMAP) to study genetic diversity in different genotypes in four cotton species from our collections for the first time.

Material and methods

We used 17 genotypes of four cotton species namely *G. hirsutum*, *G. barbadense*, *G. arboreum*, and *G. herbaceum*, for genetic study (Table 1).

The cotton genotypes were cultivated in three rows of 10 m length with 20 cm interplant

distance in the experimental field of Gorgan Cotton Research Center of Iran, according to a completely randomized design (CRD) with three replications for each genotype. For each replicate, five plants were randomly selected and their fresh leaves were pooled and used for DNA extraction. Therefore, for each genotype, 15 plants were used (17x15=255 plants were used in total). Genomic DNA was extracted using the CTAB method based on Krizman *et al.* (2006). The quality and quantity of extracted DNA was tested by running on 0.8% agarose gel and Nanodrop spectrophotometer. In total six REMAP primers including combined cotton SSR and LTR primers (Teo *et al.* 2005) were used for genetic diversity analysis as they are known to produce good results in genetic diversity studies (Biswas *et al.* 2010, Santana *et al.* 2012) (Table 2).

Tetraploids	***Gossypium hirsutum***	
	Super Okra	13
	Red-leaf Okra	17
	Latif	6
	M13	7
	Golestan	1
	Gossypium barbadense	
	Giza	16
	Doctoromomi	14
	Temez14	12
	T-189	2
	T-161	15
Diploids	***Gossypium herbaceum***	
	Shahreza	5
	Redboll	10
	Kerman	9
	Garmsar	3
	Colored lentil	11
	Sorkhesemnan	8
	Gossypium arboreum	
	Arboreum	4

Tabla 1. Cultivares estudiados de algodón, poliploidia y código.
Table 1. Cotton cultivars studied, their polyploidy level and code.

Genetic diversity and population structure

REMAP bands obtained were coded as binary characters (band present=1, band absent=0). The genetic diversity parameters like, Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland *et al.* 2011, Weising*et al.* 2005), were determined for each cultivar.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall & Smouse 2006), and Nei's GST analysis of GenoDive v.2 (2013) (Meirmans & Van Tienderen 2004), were used to reveal significant genetic difference among the studied species/cultivars (Sheidai *et al.* 2014).

Nei's genetic distance was used for clustering (Freeland *et al.* 2011). Grouping of the cultivars was done by UPGMA (Unweighted Paired Group using average) clustering, PCoA (Principal coordinate analysis) plot, as well as Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000) (Sheidai *et al.* 2014). For STRUCTURE analysis, data were scored as dominant markers (Falush *et al.* 2007), and admixture model was used.

In order to identify the number of genetic groups, K-Means clustering wasperformed as

Primer name	Sequence (5'-3')	Amplicon size (bp)
SSR-BNL-0598F 3´LTR	TATCTCCTTCACGATTCCATCAT TGTTTCCCATGCGACGTTCCCCAACA	200-1000
SSR-BNL-0598R 3´LTR	AAAAGAAAACAGGGTCAAAAGAA TGTTTCCCATGCGACGTTCCCCAACA	250-1000
SSR-BNL-1162F 3´LTR	GCGCAAGCGTAGGAGTTTAC TGTTTCCCATGCGACGTTCCCCAACA	100-850
SSR-BNL-1162R 3´LTR	TTGAACGATGAAAGGGAAGG TGTTTCCCATGCGACGTTCCCCAACA	100-800
SSR-BNL-0598R NIKITA	AAAAGAAAACAGGGTCAAAAGAA CGCATTTGTTCAAGCCTAAACC	100-650
SSR-BNL-0598F NIKITA	AAAAGAAAACAGGGTCAAAAGAA CGCATTTGTTCAAGCCTAAACC	100-3000

Tabla 2. Características de los primers REMAP. Cebadores SSR y LTR de algodón
Table 2. Characteristics of REMAP primers. The cotton SSR and LTR primers.

implemented in GenoDive v.2 (2013). In this method, the optimal clustering is the one with the smallest amount of variation within clusters (Meirmans 2012). Two summary statistics of K-Means clustering, 1-pseudo-F and 2-Bayesian Information Criterion (BIC) provide information on the number of Ks. The clustering with the highest value for pseudo-F is regarded to provide the best fit, while clustering with the lowest value for BIC is regarded to provide the best fit (Meirmans 2012).

Results

REMAP primers produced common, unique and polymorphic bands in the studied cultivars; therefore, cotton cultivars can be discriminated based on these bands (Fig. 1). Genetic variability obtained for each cultivar is presented in table 3. These results revealed a low-moderate degree of genetic polymorphism for the studied cotton cultivars (0.00-18.18 %).

Since we studied few cultivars in each cotton species, we also determined the mean genetic variability present in these species. We obtained relatively a high level of genetic diversity ranging from 18-70% for REMAP molecular markers (Table 4). The highest value for percentage of genetic polymorphism (%P) was observed in *G. barbadense* tetraploid cultivar.

The four cotton species studied differed in their total number of REMAP bands due to different numbers of unique and common bands (Table 5). The highest number of REMAP bands occurred in *G. herbaceum* (2x) (35 bands), followed by *G. barbadense* (4x) (32 bands). *G. herbaceum* also had the highest number of distinct individual bands (5). The occurrence of distinct individual REMAP bands is particularly useful for identification of the cotton species.

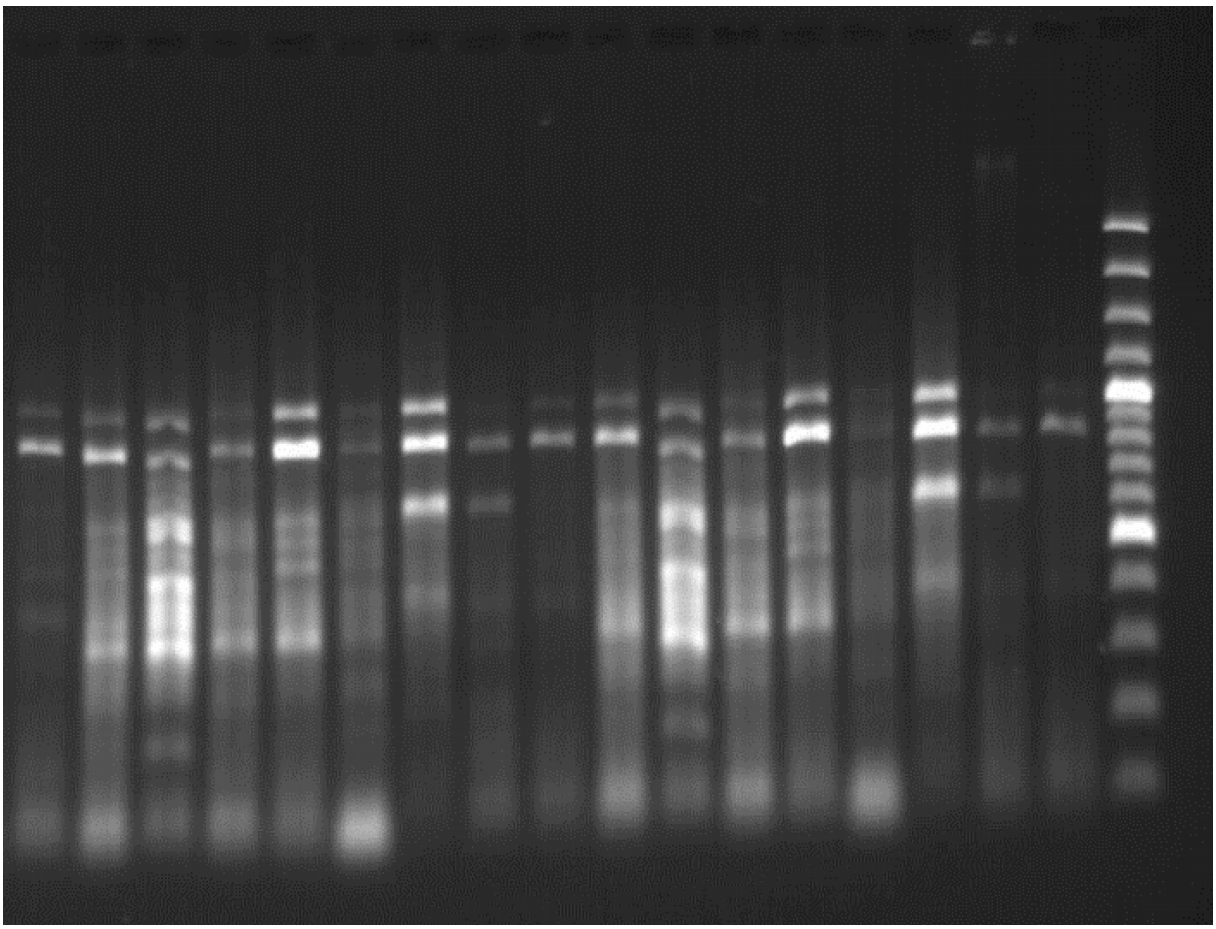

Figura 1. Patrón de bandas de los genotipos de algodón basado en los cebadores SSR-BNL0598-R3´LTR. De izquierda a derecha, variedades ordenadas según la tabla 1 y una escala de 100pb para el tamaño de los marcadores.

Figure 1. Banding profiles of cotton genotypes based SSR-BNL0598-R3´LTR primers. Left to right: cotton cultivars were arranged according to table 1 and 100bp ladder as size marker.

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	3.000	0.386	1.075	0.057	0.040	0.048	9.09
Pop2	3.000	0.591	1.102	0.096	0.063	0.076	18.18
Pop3	3.000	0.477	1.058	0.065	0.041	0.049	13.64
Pop4	3.000	0.614	1.090	0.091	0.059	0.071	18.18
Pop5	3.000	0.614	1.084	0.068	0.047	0.056	11.36
Pop6	3.000	0.500	1.051	0.048	0.032	0.038	9.09
Pop7	3.000	0.500	1.031	0.026	0.018	0.021	4.55
Pop8	3.000	0.432	1.051	0.048	0.032	0.038	9.09
Pop9	3.000	0.705	1.092	0.085	0.056	0.067	15.91
Pop10	3.000	0.545	1.073	0.063	0.043	0.051	11.36
Pop11	3.000	0.591	1.084	0.068	0.047	0.056	11.36
Pop12	3.000	0.386	1.022	0.015	0.011	0.013	2.27
Pop13	3.000	0.273	1.043	0.031	0.022	0.027	4.55
Pop14	3.000	0.455	1.092	0.085	0.056	0.067	15.91
Pop15	3.000	0.182	1.000	0.000	0.000	0.000	0.00
Pop16	3.000	0.205	1.022	0.015	0.011	0.013	2.27
Pop17	3.000	0.205	1.000	0.000	0.000	0.000	0.00

Tabla 3. Parámetros de diversidad genética en los cultivares estudiados de algodón.

Table 3. Genetic diversity parameters in cotton cultivars studied.

AMOVA produced significant genetic difference among the studied cultivars (Phi pt=0.327, P=0.01). It also revealed that 67% of total genetic variability occurred due to among population genetic difference, while, 33% was due to within population diversity (Table 6).

The grouping of the cultivars based on REMAP markers by UPGMA dendrogram (Fig. 2), separated the cultivars in distinct clusters. This means that REMAP markers can efficiently discriminate cotton cultivars from each other. The PCoA plot (Fig. 3) produced two separate main groups. The cultivars of *G. barbadense* and *G. hirsutum* (tetraploid) formed the first main group, while cultivars of *G. herbaceum* and *G. arboreum* (diploid) comprised the second major group. Therefore, REMAP markers not only differentiate the studied cultivars from each other, they can delineate diploids from tetraploids well enough. Some degree of genetic overlap was observed within diploid species as well as in tetraploid species studied. This happens due to the presence of shared ancestral alleles.

Species	Polyploidy	Na	Ne	I	He	UHe	%P
G. hirsutum	4x	1.182	1.311	0.281	0.185	0.192	56.82
G. barbadense	4x	1.432	1.282	0.290	0.182	0.187	70.45
G. herbaceum	2x	0.614	1.090	0.091	0.059	0.071	18.18
G. arboreum	2x	1.477	1.362	0.328	0.214	0.222	68.18

Tabla 4. Parámetros de diversidad genética entre las cuatro especies estudiadas de algodón.

Table 4. Genetic diversity parameters among four cotton species studied.

Species	*G. hirsutum*	*G. barbadense*	*G. arboreum*	*G. herbaceum*
No. Bands	**27**	**32**	**19**	**35**
No. Private Bands	**2**	**1**	**0**	**5**
No. Common Bands (<=50%)	**7**	**10**	**2**	**9**

Tabla 5. Frecuencia de bandas exclusivas y comuns en especies de algodón.

Table 5. Frequency of private and common bands in cotton species.

Source	df	SS	MS	Est. Var.	V%
Among Pops	3	199.244	66.415	4.766	33%
Within Pops	47	460.089	9.789	9.789	67%
Total	50	659.333		14.555	100%
Stat	**Value**	**P**			
PhiPT	0.327	0.001			

Table 6. AMOVA basado en los dato REMAP de las muestras de las 4 especies estudiadas de algodón. df: grados de libertad, SS: Suma de cuadrados, Est. Var.: varianza estimada, V%: porcentaje de varianza.

Table 6. AMOVA test based on REMAP data for 4 cotton species samples studied. df: degree of freedom, SS: sum square, MS: mean of square, Est. Var.: estimated variance, V%: percentage of variance.

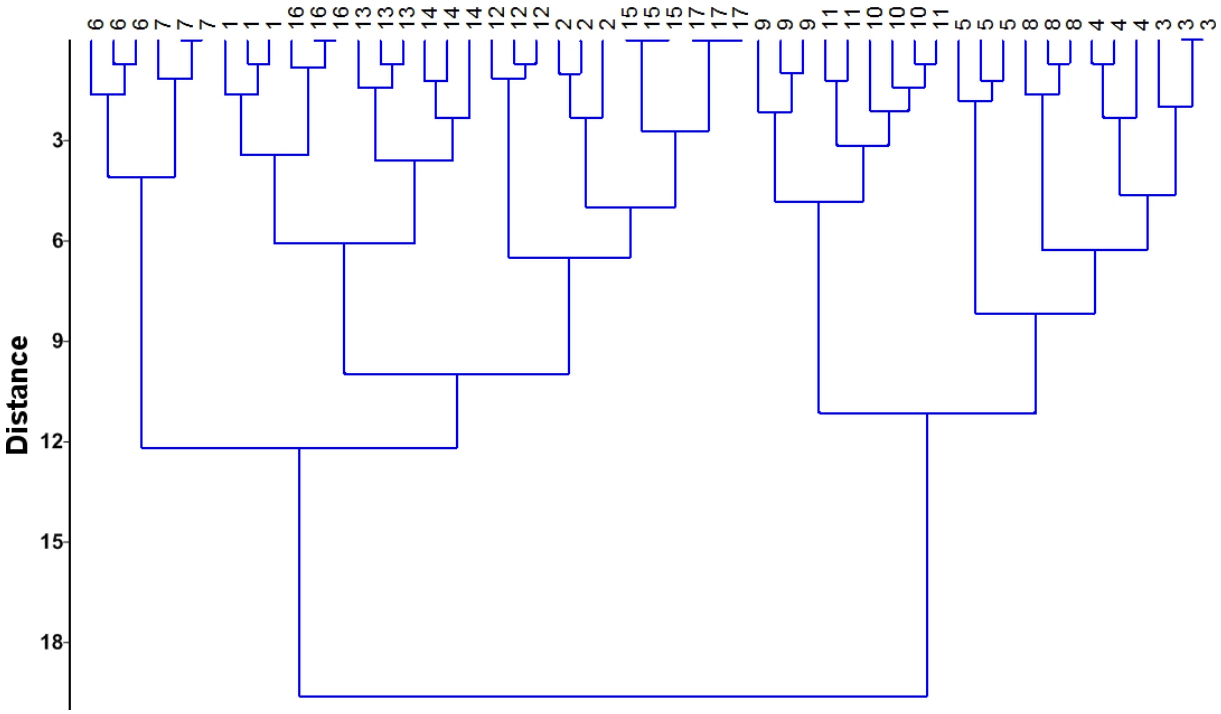

Figura 2. Dendrograma UPGMA de cultivares de algodón obtenido a partir de los datos REMAP (códigos de variedades según tabla 1).
Figure 2. UPGMA dendrogram of cotton cultivars based on REMAP data (cultivars codes as in table 1).

K	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	BIC
1	384.706	0	0	0	0	307.508
2*	384.706	112.115	272.59	0.291	**20.154**	293.87
3	384.706	146.056	238.65	0.38	14.688	291.02
4	384.706	179.853	204.853	0.468	13.755	287.164
5	384.706	204.103	180.603	0.531	12.996	284.671
6	384.706	222.75	161.956	0.579	12.378	283.044
7	384.706	238.678	146.028	0.62	11.986	281.696
8	384.706	254.011	130.694	0.66	11.939	279.971
9	384.706	266.678	118.028	0.693	11.862	278.703
10	384.706	279.095	105.611	0.725	12.039	276.966
11	384.706	291.373	93.333	0.757	12.487	274.595
12	384.706	303.373	81.333	0.789	13.225	271.508
13	384.706	312.206	72.5	0.812	13.637	269.577
14	384.706	320.539	64.167	0.833	14.218	267.281
15	384.706	328.039	56.667	0.853	14.886	264.874
16	384.706	333.539	51.167	0.867	15.21	263.599
17+	384.706	338.373	46.333	0.88	15.519	**262.47**

* Best clustering according to Calinski & Harabasz' pseudo-F: k=2

+ Best clustering according to Bayesian Information Criterion: k=17

Tabla 7. Clúster K-Means resultante. SSD: suma de los cuadrados de las distancias [T: total, AC: entre clusters; WC: dentro del clúster], BIC: criterio de infromación Bayesiana.

Table 7. REMAP K-Means clustering result. SSD: sum of squared distances [T: total, AC: among clusters, WC: within cluster], BIC: Bayesian information criterion.

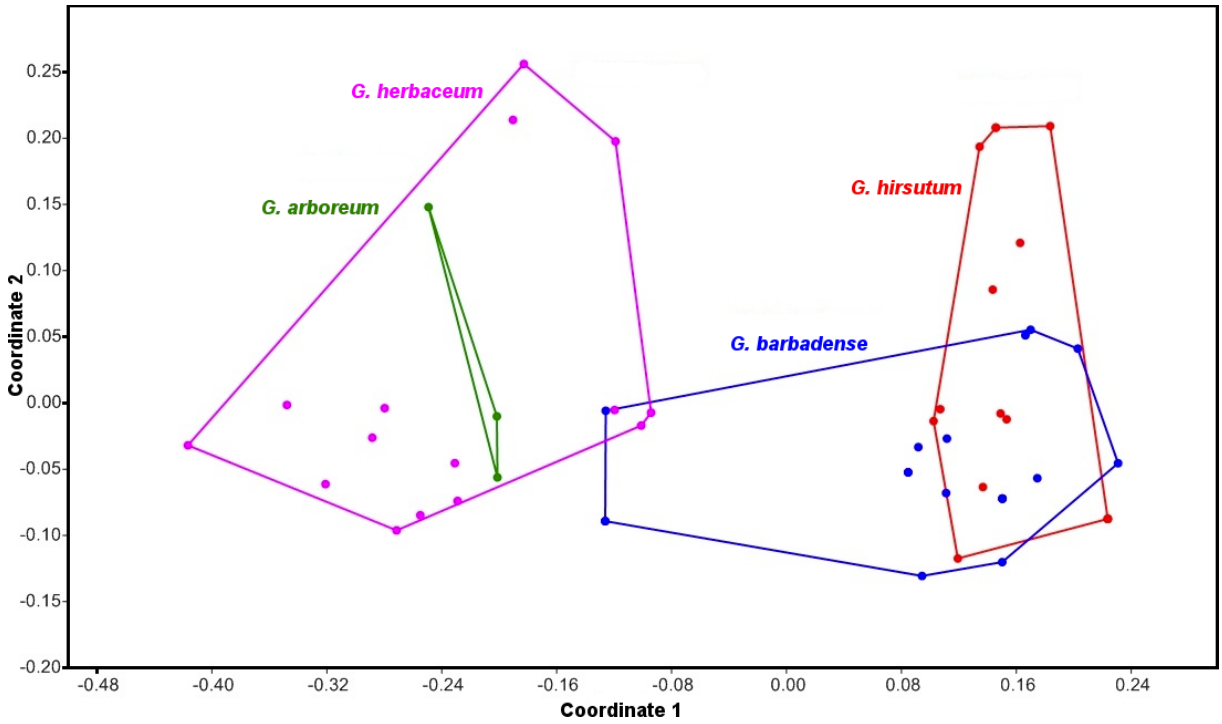

Figura 3. Análisis de coordenadas principales de cultivares de algodón, basado en los datos REMAP
Figure 3. Principal coordinates analysis of cotton cultivars based on REMAP data.

A more detailed information on genetic variability within each cultivar and each species as well as genetic admixture was revealed by STRUCTURE plot (Fig. 4). For example, cultivars 1,2, and 13-17 showed a high degree of genetic affinity due to genetic admixture and shared common alleles. Similarly, cultivars 6 and 7, and 9-11 showed high degree of genetic similarity. In general, high degree of genetic admixture was observed in all the studied cultivars.

K-MEAN clustering produced optimum number of genetic groups k=2 and 17 (Table 7). K=17 supports the AMOVA result and indicates that the cultivars are genetically differentiated. K=2 indicted the presence of two genetic groups among the studied cultivars and is in agreement with PCoA result. Cultivar numbers 1, 2, 6 and 7, as well as 12-17 (all tetraploid) were placed in the first genetic group. The cultivars 3-5, and 8-10 (all diploid), were placed in the second genetic group. Therefore tetraploid cultivars studied were also genetically discriminated.

Discussion

Available germplasm diversity is important for cotton breeders, as they use the existing genetic variability to plan inter-specific and inter-cultivar crossings. Breeders rely ongenetic variation between parents to create unique gene combinations necessary for new superior cultivars. Having an efficient and rapid genetic screening approach is of importance while dealing with a large cotton germplasm. Efficient molecular and genetic screening of the cotton cultivars increases the probability of identifying diverse genotypes and therefore producing unique genotypes via different crossing plans (Van Esbroeck *et al.* 1998, Sheidai *et al.* 2007, Sheidai *et al.* 2008, Tyagi *et al.* 2014, Noormohammadi *et al.* 2015).

Genetic finger printing characterizes the individuals and accessions for the choice of parental genotypes in breeding programs. For any meaningful plant-breeding program, accurate determination of genetic diversity and portioning within and between gene pools is an essential step for effectively utilizing germplasm resources (Ulloa *et al.* 2007, Zhang *et al.* 2011). Therefore, using proper statistical and bioinformatics approaches are essential for efficient grouping of the genotypes and to illustrate detailed genetic differences of the parental genotypes.

The present study revealed that REMAP molecular markers are suitable molecular markers

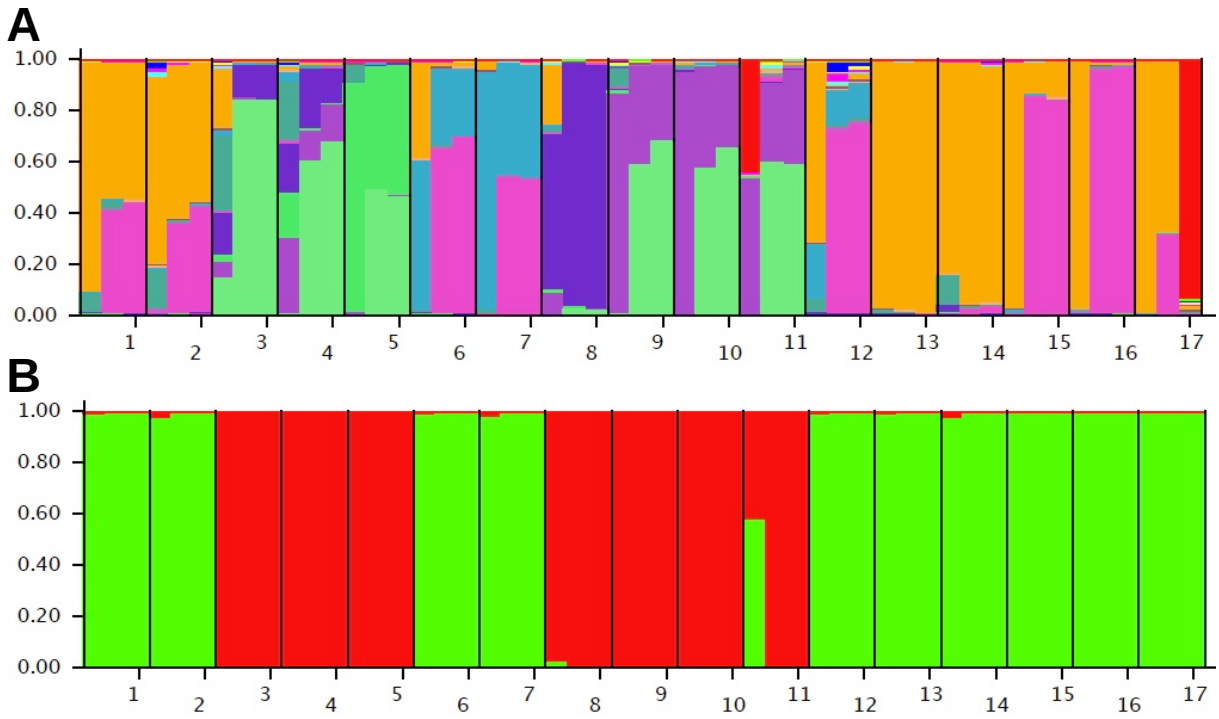

Figura 4. Gráfico STRUCTURE de variedades de algodón, basado en los datos REMAP. **A:** k=17; **B:** k=2. Códigos de variedades según tabla 1.

Figure 4. STRUCTURE plot of cotton cultivars based on REMAP data. **A:** k=17; **B:** k=2. Cultivars codes as in table 1.

to investigate genetic variability in cotton cultivars markers as they are easy to use and fast and also produce large number of bands including distinct individual bands. Therefore, these markers are efficient enough to discriminate cotton cultivars and cotton species. Moreover, clustering and PCoA grouping based on REMAP markers, not only discriminated each cultivar from the others but also revealed genetic distinctness of diploid versus tetraploid cultivars. The genetic distance obtained among these cultivars can be regarded as a suitable guide for choosing distant parental plants for future hybridization. Usually, plants that show a higher degree of genetic distance have different genetic content and are thought to produce new combination of genes in their offspring (Van Esbroeck *et al.* 1998, Sheidai *et al.* 2007, Sheidai *et al.* 2008). The STRUCTURE plot also suggests that we can use plants from two different genetic groups identified as the parental plants for hybridization.

The earlier studies in cotton reported low degree of genetic variability in these plants (Liu *et al.* 2006, Murtaza 2006, Wang *et al.* 2007, Tyagi *et al.* 2014). This is mainly due to continuous artificial selection for higher productivity in cotton farming led (Iqbal *et al.* 1997). This in turn is hindering breeding programs worldwide. The present investigation revealed low to moderate genetic variability within each cotton cultivar studied but also revealed relatively high genetic diversity within each species. Therefore, we suggest first to perform multiple crossing between cultivars in each species to combine different genes in resulting offspring, and then cross diploid/tetraploid species together. In this way we can combine maximum genetic variability available to produce new cultivars.

We also identified some cultivars with good genetic variability, viz. cultivar No. 2 (T-189 of *G. barbadense*, and cultivar No. 4 (Arboreum of *G. arboretum*). These are good candidates for hybridization with other cultivars with suitable agronomic characteristics.

We obtained a good number of unique REMAP bands in the individual cotton species. Identification of bands is very important for both genetic fingerprinting and cultivar discrimination in cotton as well as for developing association mapping populations in cotton (Tyagi *et al.* 2014). In a similar study performed by using SSR molecular markers in accessions of *G. hirsutum*

(Upland cotton) and *G. barbadense* (Tyagi *et al.* 2014), several alleles were unique in the studied accessions that is in agreement with the present investigation by REMAP markers. Moreover, population structure analysis revealed extensive admixture in the studied cultivars and identified five subgroups corresponding to Southeastern, Midsouth, Southwest, and Western zones of cotton growing areas in the United States, with the accessions of *G. barbadense* forming a separate cluster. These results are in agreement with our findings based on REMAP molecular markers.

The present study revealed almost a high degree of genetic variability in diploid cotton species (68.18% in *G. arboretum* and 18.18% in *G. herbaceum*). The Upland cotton (*G. hirsutum*) is known to have relatively low levels of genetic diversity. The genetic variability available especially in the diploid species, which are the putative donors of the A and D genomes for the commercially important allotetraploid cottons (AADD), could be used as the genetic resources for cotton improvement. In conclusion we suggest using REMAP molecular markers as efficient molecular marker for genetic fingerprinting of cotton germplasms.

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