

IDENTIFYING RAPD MARKERS LINKED TO AN ERECT GLANDULAR HAIR TRAIT IN ALFALFA

IDENTIFICACIÓN DE MARCADORES RAPD LIGADOS AL CARÁCTER DE TRICOMAS ERECTOS EN ALFALFA

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SUMMARY

Selection for molecular markers tightly linked to an important trait may improve selection efficiency and genetic gain in hybridization. This study was conducted to identify DNA markers associated with erect glandular hairs in diploid alfalfa. One hundred and sixty nine plants of “KS94GH6”, a diploid ($2n = 2x = 16$) *Medicago sativa* var. *viscosa* (Rchb.) possessing erect glandular hairs, were crossed to cultivated alfalfa at the diploid level (CADL), a non glandular-haired alfalfa. Genotypic selection for this trait was practiced by quantifying erect glandular hair number in 10 progeny from every simple cross family. The number of erect glandular hairs was determined based on a 1 cm length of stem from the third fully elongated internode of the shoot apex from a subsample of three stems each containing 10 internodes. Two DNA pools were generated by bulking equal quantities of DNA from the original parent genotypes, whose progeny possessed the 10 highest or the 10 lowest numbers of erect glandular hairs. Both DNA pools were screened for RAPD-PCR with 100 random 10-mer oligonucleotide primers. One primer (UBC-055) gave one polymorphic DNA product of 1603 bp size between the DNA bulks. The polymorphic band, denoted UBC-055₁₆₀₃, was present in the low glandular hair number DNA bulk and absent in the high glandular hair number DNA bulk. Co-segregation of the RAPD marker with erect glandular hair phenotype was determined to be highly associated ($P \leq 0.01$).

Index words: *Medicago sativa* var. *viscosa*, DNA bulk, RAPD-PCR, glandular hairs.

RESUMEN

La selección para marcadores moleculares ligados a un carácter de interés puede mejorar la eficiencia en la selección y la ganancia genética en los programas de hibridación. El presente estudio tuvo como objetivo identificar marcadores moleculares asociados al carácter de tricomas glandulares erectos en alfalfa diploide. Para ello, se cruzaron 169 plantas diploides ($2n = 2x = 16$) “KS94GH6” de *Medicago sativa* var. *viscosa* (Rchb.) que poseen tricomas glandulares erectos, con plantas cultivadas de alfalfa a nivel diploide (CADL) que carecen de tricomas glandulares erectos. En cada una de 10 plantas por familia derivada, el número de tricomas glandulares erectos se cuantificó en un 1 cm de longitud del tallo del tercer entrenudo completamente elongado a partir del ápice, en una submuestra de tres tallos cada uno con 10 entrenudos. Con las plantas originales se formaron

dos compuestos balanceados de ADN, al mezclar cantidades iguales de ADN de las 10 plantas que mostraron los valores más altos y los 10 más bajos de tricomas glandulares erectos, respectivamente. Los dos compuestos balanceados de ADN se analizaron por RAPD-PCR con 100 iniciadores decámeros aleatorios. Sólo uno de ellos (UBC-055), arrojó un producto polimórfico entre ambos compuestos de 1603 pb. Dicho iniciador (UBC-055₁₆₀₃) está presente en el compuesto de ADN formado con el número más bajo de tricomas glandulares erectos y ausente en el compuesto de ADN constituido con el número más alto de tricomas glandulares erectos. El análisis de co-segregación indicó fuerte asociación ($P \leq 0.01$) entre el marcador RAPD y el fenotipo de tricomas glandulares erectos.

Palabras clave: *Medicago sativa* var. *viscosa*, compuesto de ADN, RAPD-PCR, tricomas glandulares.

INTRODUCTION

Insect pests cause damages valued in hundreds of millions of dollars per year to alfalfa crops (*Medicago sativa* L.) around the world. One of the most important insect pests is alfalfa weevil (*Hypera postica* Gyllenhai), which reduces forage yield. An alternative to chemical and cultural pest control is to utilize host mechanisms, such as glandular trichomes, to increase insect pest resistance. The presence of glandular trichomes in alfalfa has been shown to protect against certain stem-, leaf-, and fruit-eating insect pests (Ranger and Hower, 2001). Upon contact with an insect body the gland-tips break and the sticky exudate from erect hairs rapidly polymerizes and becomes hardened, resulting in entrapment/immobilization of insects (Elden and McCaslin, 1997).

The expression of glandular hair density in cultivated crops is controlled by multiple genes (Duke *et al.*, 2000). The expression of erect glandular hair in ‘KS94GH6’ in alfalfa also seems to be quantitatively inherited and influenced mainly by non-additive effects ($h^2 = 0.25$) (González *et al.*, 2000), so that this trait could be difficult to

select for. Therefore, identification of DNA markers linked to this trait should facilitate the selection process for incorporating it to cultivated tetraploid alfalfas.

Plant breeding conventional methods requires a long time to incorporate a qualitative and quantitatively controlled trait from wild germplasm into a cultivated population. This time may be significantly reduced if breeders have the ability to readily detect specific chromosomal segments linked to the desired trait (Frisch and Melchinger, 2001). In order to achieve this goal, genetic sequences (*i.e.*, DNA markers) linked to the desired character must be identified. The usefulness of a DNA marker to select for a trait depends on the degree of linkage between the trait and the marker. If the marker is tightly linked to the trait, then selection for the marker also selects for the trait, which is equivalent to genotypic selection. This process has been termed "marker-assisted selection" (MAS). Selection can take place during the seedling stage without replications, thus greatly speeding up the breeding process. In addition, MAS allows selection based upon the genotype of an individual, instead of based upon the phenotype. This reduces or eliminates the errors often associated with phenotypic selection under limited environment conditions. To be useful in MAS, a molecular marker must fulfill the following criteria: 1) The technique used to generate the marker must be reliable, relatively simple to perform, and capable of processing a large amount of samples per unit of time (Ribaut and Beltrán, 1999); 2) The marker must be tightly linked to the gene of interest if progeny testing is reduced to identify individuals with the desired genes (Johnson *et al.*, 1995).

The development of techniques based on the polymerase chain reaction (PCR; Mullis *et al.*, 1985) offers a powerful tool for identification of DNA markers linked to important agronomic traits (Zhang and Stommel, 2001). The PCR is a rapid and cyclical procedure for *in vitro* enzymatic amplification of a specific segment of DNA (Coen, 1995). DNA to be amplified is denatured by heating the sample, in the presence of DNA polymerase and excess of dNTPs, so oligonucleotides containing 10-20 bases hybridize to target sequences and serve as primers for new DNA synthesis. When using the same primer to identify different DNA templates, the presence of a PCR product in one template, but absence in another template, implies that a polymorphism has been detected.

The PCR technique can be used to identify specific chromosomal segments linked to traits of interest. Random amplified polymorphic DNAs (RAPDs; Williams *et al.*, 1990) is one PCR technique that has several advantages over other molecular marker systems. The RAPD procedure is rapid, requires only small amounts of DNA, which

needs not to be of high quality, radioactivity is not required, and polymorphisms can be detected in highly repetitive DNA. This technique uses primers with 10 nucleotides of arbitrary sequence and a GC content of approximately 50 %, to generate PCR amplification products. Since the nucleotide order within the 10-mer primers is arbitrary, no prior knowledge of DNA sequences is needed, and the primers can be universally used for eukaryotes and prokaryotes (Weising *et al.*, 1995).

No research has been conducted to identify molecular markers associated with the presence of erect glandular hairs in alfalfa. As this trait is found in wild alfalfa species, breeders might be able to use molecular markers linked to this trait to more efficiently introgress this trait from wild species into cultivated alfalfa. Based on all that information, the general objective of this study was to identify DNA markers associated with erect glandular hairs in *Medicago sativa* var. *viscosa* (Rchb.).

MATERIALS AND METHODS

Plant material and growth conditions

Plant materials used in this study were 169 'KS94GH6' glandular-haired diploid alfalfa plants ($2n=2x=16$) from *Medicago sativa* var. *viscosa* (Rchb.) and their 169 single cross families. Single cross families were obtained by manually crossing each 'KS94GH6' plant with 22 non glandular-haired testers of cultivated alfalfa at the diploid level (CADL) (Bingham and McCoy, 1979). Parents and their progenies were grown and evaluated under greenhouse conditions at Las Cruces, NM, USA, during 1995 and 1996. Seed was germinated in Jiffy-9 Peat Pellets (Jiffy Products Ltd. St. Louis, MO). When seedlings were 10 cm high, they were transplanted to 20 oz peat pots SC-10 (Stuewe and Sons, Inc. Corvallis, Oregon) containing a mixture of Metro-Mix 360 (Scotts-Sierra Horticultural Products Company, Marysville, OH) and Perlite (Thermo-o-Rock Industries, Inc., Chandler, Arizona) in a 2:1 (w/w) ratio. Greenhouse temperature was 24 ± 5 °C. Plants were grown under natural light supplemented with fluorescent light for 18 h per day. Fertilization was applied weekly using a commercial fertilizer (20N-10P-20K, Peters Professional. Grace-Sierra Horticultural Products Company. Milpitas, CA).

Determination of the erect glandular hair number

Erect glandular hair number was quantified in each parental plant and each single cross family, based on three stems per plant, to permit genotypic selection for this trait. In the single cross families this trait was recorded based on each of ten progeny plants. Measurements were taken on

stems with 10 internodes. The third fully elongated internode from the apex was scored, then erect glandular hairs were counted along a 1 cm transect of the stem under 75X magnification using an ocular lens containing a linear reticle scale (Edmund Scientific Company, Barrington, NJ). Within each single cross family the data were averaged over stems and progeny ($n = 30$).

DNA isolation

Total DNA was extracted from 10 immature trifoliate leaves of each 'KS94GH6' parent and each single cross family using the CTAB (cetyl-trimethyl ammonium bromide) protocol of Keim *et al.* (1988) with minimum modifications. The DNA was dissolved in 1X TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) containing 100 $\mu\text{g mL}^{-1}$ Rnase A. Purified DNA was quantified with a DyNA Quant 200 Hoefer Fluorometer (Hoefer Scientific Instrument, San Francisco, CA). The DNA solution was diluted in 1X TE buffer to a working concentration of 10 $\mu\text{g mL}^{-1}$ and stored at 4 °C until PCR amplification.

Bulked segregant analysis (BSA)

Based on the mean number of erect glandular hairs for each single cross family, two classes of the original 'KS94GH6' plants were selected. The first class (HGHN) consisted of 10 parents whose corresponding single cross progeny averaged the highest erect glandular hair number (erect glandular hair number $\geq 10.0 \text{ cm}^{-1}$). The second class (LGHN) consisted of 10 original 'KS94GH6' plants whose corresponding single cross progeny averaged the lowest erect glandular hair number (erect glandular hair number $\leq 1.25 \text{ cm}^{-1}$). Two DNA pools (LGHN = low, HGHN = high) were generated by bulking equal quantities of DNA from each of the 10 genotypes within each class. Ten plants were selected for each DNA pool in order to minimize detecting false positive markers due to non-specific annealing.

In order to avoid detecting false positive markers, a "d" value was obtained. This value represents the phenotypic effect of the gene(s) controlling erect glandular hair number in 'KS94GH6' measured in standard deviations (SD) between both populations means (high and low numbers of erect glandular hairs DNA pools). The value was calculated according to the formula:

$$d = (m_H - m_L) / \sigma$$

Where: d = effect size index (distances) expressed in units of variability; m_H and m_L = population means for high and low glandular hair number DNA pools expressed

in raw (original measurement) unit; σ = standard deviation of the whole population.

For this study, a d value ≥ 1.75 (low and high populations means differ by 1.75 SD) was considered because it means that at least 96 % of the area covered by both populations does not overlap (Cohen, 1969).

RAPD analysis

For RAPD analysis of erect glandular hair number in *Medicago sativa* var. *viscosa* (Rchb.), 100 different 10-base oligonucleotide primers were obtained from University of British Columbia (Nucleic Acid-Protein Service Unit Biotechnology Laboratory, Vancouver, BC, Canada). For each reaction a total of 30 ng of template alfalfa DNA was amplified in a 25 μL volume containing: 1x AmpliTaq Polymerase Stoffel Buffer (10 mM-Tris-HCl, pH 8.3; 50 mM KCl) (Applied Biosystems, Foster City, CA); 1 unit AmpliTaq Stoffel DNA Polymerase (Applied Biosystems, Foster City, CA); 0.2 mM final concentrations each of dATP, dCTP, dTTP, dGTP; 4 mM MgCl_2 ; and 0.2 μM of a single decamer primer. The reaction mixture was overlaid with 60 μL of light grade mineral oil (SIGMA Chemical Co. St. Louis, MO). Amplification of each reaction sample involved 45 cycles of the following thermal profile: 1 min at 94 °C for denaturing, 2 min at 37 °C for annealing, 2 min ramp from 37 °C to 72 °C, and 2 min at 72 °C for primer extension. The reaction profile was preceded by a single 94 °C soak for 5 min. At the end of 45 cycles, a single 72 °C extension was applied for 7 min. All completed reactions were held at 4 °C. The reactions were conducted on a Perkin Elmer 480 thermal cycler (Applied Biosystems, Foster City, CA).

Amplification products were resolved by electrophoresis (E-C360 M, Horizontal Submarine Electrophoretic Gel System, E-C Apparatus Corporation, St. Petersburg, Florida, USA) in 1.4 % (w/v) agarose gels (Agarose Wide Range/Standard 3:1, SIGMA Chemical Co. St. Louis, MO) at 100 volts for 3 h. The gels were stained in an ethidium bromide solution (0.5 $\mu\text{g mL}^{-1}$). Banding patterns were visualized and photographed on a FisherBiotech UV Transilluminator FBTI-88 (Fisher Scientific, Pittsburgh, PA). Gel images were digitized using a IS-500 Digital Imaging System (Alpha Innotech Corporation, San Bernardo, Cal.). To measure the length of PCR products, BioMarker™ Ext (Bio Ventures Incorporated, Musfreesboro, TN) (10 ng μL^{-1}) was loaded for all the gels. This size marker contains linear double stranded DNA markers of 2000, 1500, 1000, 700, 525, 500, 400, 300, 200, 100 and 50 base pairs. RAPDs were named based on the UBC primer number and the molecular weight of the polymorphic band.

For initial screening of RAPD primers for polymorphism and to ensure reproducibility of RAPDs, two reactions per primer were run for both the LGHN DNA pool and the HGHN DNA pool. RAPD fragments were scored as present (1) or absent (0). Primers that generated polymorphic fragments between the two DNA bulks were reamplified using template DNA of 30 randomly selected "KS94GH6" plants, based on the progeny test. Co-segregation of the RAPD with the erect glandular hair phenotype of the respective single cross families was then determined. Linkage between a RAPD fragment and the erect glandular hair trait was detected using a "t" test. This test compares the RAPD profile of plants of low glandular hair number (LGHN) with the RAPD profile of a high glandular hair number (HGHN) plants. Independent samples and unequal variances were assumed. The statistic used was:

$$t = (\bar{x}_1 - \bar{x}_2) / \sqrt{(S_1^2/n_1 + S_2^2/n_2)}$$

Where: t = minimum value to detect differences between two means; \bar{x}_1 and \bar{x}_2 = means of the glandular hair number for the 30 randomly selected single cross families in which the RAPD fragment was either present or absent; n_1 and n_2 = number of plants in each sample where the RAPD fragment was either present and absent; S_1^2 and S_2^2 = variances for erect glandular hair number for the samples in which the RAPD fragment was either present and absent.

The effective number of degree of freedom was estimated according to the Satterthwaite approximation (Satterthwaite, 1946).

RESULTS AND DISCUSSION

Bulk segregant analysis coupled with RAPD-PCR analysis was used to identify markers linked to the erect glandular hair trait. Then, two DNA bulks representing 10 parental genotypes whose progeny possessed a low number of erect glandular hairs (LGHN; average number of erect glandular hairs = 0.68) and 10 parental genotypes whose progeny possessed a high number of erect glandular hairs (HGHN; average number of erect glandular hairs = 11.76), were screened with 100 random 10-mer primers. Genotypes contributing to the DNA pools and their associated erect glandular hair number are shown in Table 1. A difference of 11.08 glandular hairs based on progeny tests was detected between the means of the two bulks, difference equivalent to 3.64 standard deviations. This "d" value means that the QTL has an allele phenotypic effect of 3.64 SD. The U value indicates that at least 92 % of area covered by both populations does not overlap. Thus,

there is less than an 8 % chance of bulking DNA from plants belonging to a different DNA pool. Therefore, bulk segregant analysis was effective to select the DNA bulks, as Mackay and Caligari (2000) previously discussed.

Table 1. Progeny means for erect glandular hair number per centimeter, the phenotypic effect, and power of the test for parental genotypes of 'KS94GH6', used to generate DNA pools.

LGHN [§]		HGHN ^{§§}	
Genotype	EGHN ^{§§§}	Genotype	EGHN
14	0.20	26	10.50
7	1.17	28	11.00
69	0.33	38	10.00
129	1.25	79	11.25
101	0.80	83	13.50
114	1.00	84	10.50
115	0.33	112	10.14
120	0.50	152	16.00
132	0.50	141	13.00
165	0.71	145	11.71
Mean	0.68		11.76
Standard deviation (population)	3.04		
d value	3.65		
[†] U (d = 3.6; P ≤ 0.05)	> 92 %		

^{/*} Significant at P ≤ 0.05; [§]LGHN = Low glandular hair number; ^{§§}HGHN = High glandular hair number; ^{§§§}EGHN = Erect glandular hair number; [†]U = Test power.

The RAPD markers were scored for the presence or absence of repeatable products. One primer (UBC-055, 5'...TCC CTC GTG C...3') detected one polymorphic DNA product of 1603 bp in size between the DNA bulks. In Figure 1A, DNA from the ten plants belonging to the LGHN DNA bulk shows the presence of the RAPD fragment. Conversely, in Figure 1B the RAPD fragment does not appear in the DNA of the ten plants that came from the HGHN DNA bulk. Thus, the polymorphic band denoted UBC-055₁₆₀₃, was present in LGHN bulk and absent in HGHN bulk. According to Figures 1A and 1B and the data in Table 1, the RAPD fragment (UBC-055₁₆₀₃) was detected in individuals possessing ≤ 1.25 erect glandular hairs cm⁻¹.

As it has been pointed out before, the advantage of molecular markers over traditional morphological markers are in their independence from environment factors, unlimited numbers, and ability to be scored at any stage of development (Simpson, 1999). DNA markers are useful for selection only if they are highly repeatable and linked to the trait of interest. A random sample of 30 plants taken from the original 'KS94GH6' population were evaluated for cosegregation of the RAPD fragment with erect glandular hair number (Figure 2), as determined based on the values of the progeny test. The t test indicated significant differences (P ≤ 0.01) in erect glandular hair number, based on marker phenotype determined by using progeny means (Table 2). No overlap was observed in the distribution of

erect glandular hair number based on marker phenotype. The data indicate that UBC-055, which was identified by bulked segregant analysis, can be used in this population to select for erect glandular hair number. Moreover, absence of the RAPD fragment amplified by primer UBC-055 in this population also could be used to identify potential parents in a breeding program for incorporating erect glandular hairs from wild alfalfa species to the cultivated alfalfa.

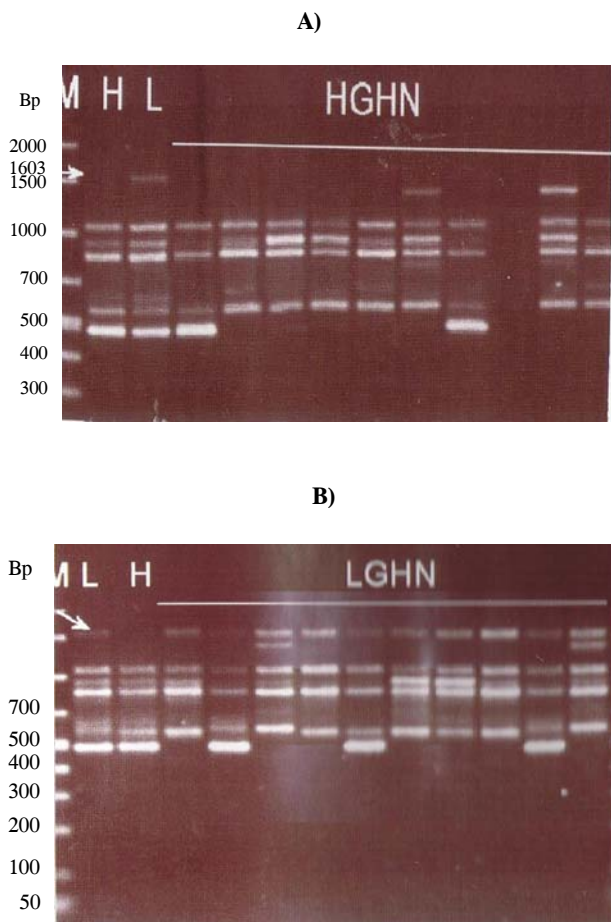


Figure 1. RAPD banding patterns from template DNA pools from: A) Ten low glandular hair original plants “KS94GH6” germplasm amplified by UBC-055 10-mer primer. B) Ten high glandular hair original plants “KS94GH6” germplasm amplified by UBC-055 10-mer primer. M = Biomarker™ Ext; L = Low glandular hair number DNA pool; H = High glandular hair number DNA pool; LGHN = Ten original plants from the low glandular hair number DNA pool; HGHN = Ten original plants from the high glandular hair number DNA pool. The polymorphic band at 1603 bp size (5'...TCC CTC GTG C...3') is indicated by arrow sign.

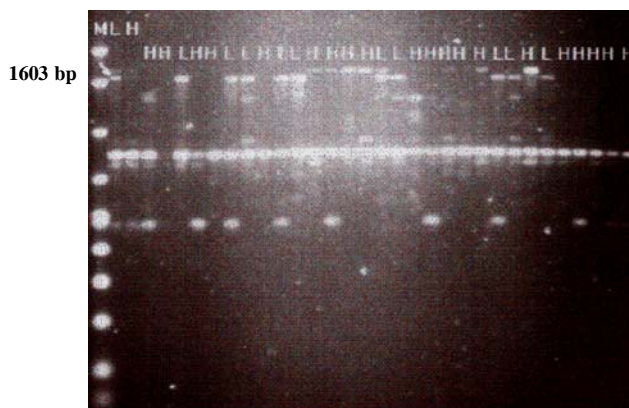


Figure 2. RAPD analysis of 30 randomly selected “KS94GH6” plants, based on the progeny test, using a segregating pattern amplified by UBC-055 10-mer primer. Lanes 1 Biomarker™ Ext (2000, 1500, 1000, 700, 500, 400, 300, 200, 100 and 50 bp); Lane 2 = Low glandular hair number DNA pool; Lane 3 = High glandular hair number DNA pool; Lanes 4-33 = Random sample of “KS94GH6” germplasm plants where H = High glandular hair number; L = Low glandular hair number. The polymorphic band at 1603 bp size is indicated by arrow.

Table 2. Progeny means for erect glandular hair number per cm and the presence or the absence of the RAPD fragment UBC-55₁₆₀₃ among single cross families from thirty randomly selected parents from 'KS94GH6'.

	Number of erect glandular hairs per cm			
	Presence		Absence	
	13.00	13.50	0.20	1.66
	10.50	10.50	1.60	0.50
	11.00	10.14	0.33	2.75
	8.67	13.00	0.75	3.80
	11.25	11.71	0.50	3.50
	16.00	9.60		
	6.25	4.80		
	5.25	6.67		
	5.80	8.00		
	7.17			
Mean	9.62		1.56	
Variance	9.77		1.83	
tc [‡]		9.66**		
t [‡] (P ≤ 0.01, 5 df)		4.03		

** Significant at P ≤ 0.01; ‡ Calculated value for the t test; † Obtained value from tables for the t test. Degrees of freedom were estimated according to the Satterthwaite approximation (Satterthwaite, 1946).

Although RAPDs provide useful markers for selection, there are inherent difficulties in the application of RAPDs in marker assisted selection (MAS), such as band reliability between runs and multiple step protocols (Johnson *et al.*, 1995). Also, it has been pointed out that RAPD markers can vary according to experimental conditions, MgCl₂ concentration (Wolf *et al.*, 1993), presence of glycerol in the reaction buffer (Hai and Négre, 1993), *Taq* polymerase quantity and quality (Schierwater and Ender, 1993), and type of thermal cyclor (Wolf *et al.*, 1993).

According to Duke *et al.* (2000), the expression of glandular hair density is controlled quantitatively, and Hill *et al.* (1999) added that it may be highly influenced by the environment. In addition, González *et al.* (2000) indicated that in *Medicago sativa* var. *viscosa* (Rchb.), erect glandular hair is a poorly heritable trait ($h^2 = 0.25$) controlled primarily by non-additive effects. Therefore, conventional selection methods will be difficult and genetic progress is likely to be slow. At this point, the UBC-055₁₆₀₃ marker may be useful for selection for erect glandular hairs in crosses between 'KS94GH6' and CADL. After that, incorporation of this trait to cultivated tetraploid alfalfa can be performed by the backcross method.

During the incorporation of the trait by backcrossing, followed by recurrent selection to increase the number of the erect glandular hair into cultivated tetraploid alfalfa, the RAPD marker identified in this study may be useful to choose individual plants possessing a high number of erect glandular hairs. If individuals in which the RAPD fragment does not appear are selected before flowering and intermated in either the backcrossing process or the recurrent selection program, genetic gain should be maximized.

CONCLUSION

One primer (UBC-055) produced one polymorphic DNA product of 1603 bp size between the DNA bulks. The polymorphic band, denoted UBC-055₁₆₀₃, was present in the low glandular hair number DNA bulk and absent in the high glandular hair number DNA bulk. At the same time, co-segregation of the RAPD marker with erect glandular hair phenotype was determined to the highly associated ($P \leq 0.01$) with the erect glandular hair trait.

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