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A Modified-bulk Segregant Analysis for Late Blooming in Sour Cherry

Fatih Ali Canli
Department of Horticulture, Faculty of Agriculture, Central Campus,
Suleyman Demirel University, 32260 Isparta, Turkey

Abstract: A modified-bulk segregant analysis in combination with amplified fragment length polymorphism (AFLP) technique was used to identify markers associated with bloom time in a sour cherry population derived from crosses between two sour cherry (*Prunus cerasus* L., 2n=4x=32) cultivars, 'Balaton' and 'Surefire'. Screening of early and late extreme groups with 94 AFLP primer pairs resulted in the identification of two candidate bands in two different primer combinations (a 78 bp fragment in ETT/MCCG primer pair combination and a 92 bp fragment in EAA/MCGT primer pair combination). These candidate bands were present in the late bloom time group but not in the early group. Genetic markers linked to bloom time in sour cherry is very important, because utilization of markers will help the indirect selection of varieties for desirable bloom time in early generations, saving time and effort.

Key words: AFLP, molecular markers, bulk-segregant analysis, MAS, Sour Cherry, boom time, *Prunus*

INTRODUCTION

Consistent yield is one of main objectives of sour cherry breeding programs[1]. In some cherry growing regions, such as Michigan, where 72.5% of the sour cherries in US are produced, low temperature damage to flower buds and flowers is the most common factor reducing yield[2]. Therefore, cold hardiness of sour cherry flower buds is one of the most important breeding objectives for these cold production regions[1]. A delay in the spring floral bud development could decrease crop loss from a spring freeze[1]. Therefore, the development of new later blooming varieties would avoid some of the loss due to spring freeze injury.

DNA markers are essential tools in plant genetics with particular value in gene mapping and marker assisted selection. Genetic markers linked with QTL may enable indirect selection of complex traits. Molecular markers have been successfully used to map individual genetic factors or QTL controlling complex traits[3-5]. While such experiments are useful, they require large populations and are labor-intensive[6]. Construction of separate linkage maps to identify QTL for each complex trait in many different populations is frequently not feasible[7].

More efficient alternatives to the construction of saturated linkage maps for identifying QTL have been developed. Bulked segregant analysis (BSA)[8] and selective genotyping[9] have been used to identify markers linked to targeted QTL. In these approaches, polymorphic markers are evaluated across two DNA pools (BSA method) or groups of lines (selective genotype method). One DNA pool or group of lines consists of the most resistant (one-extreme) and the other the most susceptible (other extreme) lines within the population. Markers that co-segregate within groups with the trait of interest are mapped across the entire population. Thus, only a few selected markers are mapped and analyzed for association with the specific quantitative trait[7]. Chen *et al.*[10] used the selective genotyping approach with the objective of rapidly locating putative resistance loci.

Ballester *et al.*[4] studied the genetics of late bloom in almond. BSA was used in an F_1 population to identify RAPD markers linked to the Lb gene, which is located on the linkage group 4. They were able to identify three RAPD markers associated with the Lb gene. One of them ($OKP10_{1350}$) placed at 5.4 cM from Lb and possibly can be used as a selective marker for flowering time. Plants with Lb allele bloomed about two weeks later and this allele had dominant gene action. Bentolila and Hanson[20] used BSA to identify markers closely linked to the restorer of fertility (Rf) locus in petunia in a large BC1 population produced from two different parental lines carrying Rf. They were able to identify an amplified fragment length polymorphism (AFLP) marker that co-segregates with *Rf*. Decousset *et al.*[11] employed BSA in a BC2 population segregating for the Ppd-HI photoperiod response gene and were able to identify six AFLP markers closely linked to the Ppd-H1 gene. Smiech *et al.*[12] used BSA with

Corresponding Author: Dr. Fatih Ali Canli, Department of Horticulture, Faculty of Agriculture, Central Campus, Suleyman Demirel University, 32260 Isparta, Turkey
Tel: 90 533 723 4241 Fax: 90 246 2371693 E-mail: yusuf498@hotmail.com,

RAPD's to identify markers to distinguish between resistant and susceptible forms of tomato (*Lycopersicon esculentum* Mill.). They stated that 28 out of 271 primers produced polymorphism which were tested for linkage to the resistance phenotype. They were able to identify 5 primers enabling them to distinguish between resistant and susceptible forms in a F_2 segregating progeny developed from resistant x susceptible parents for tomato spotted wilt virus. They concluded that the selection of TSWV resistant individuals can be facilitated by MAS.

Dong *et al.*[13] used BSA with the AFLP technique to identify molecular markers linked to the thermosensitive genic male sterility (TGMS) gene in a F_2 population of a cross between a TGMS indica mutant, TGMS-VN1 and a fertile indica line, CH1 of rice (*Oryza sativa*) . Out of 200 AFLP primer combinations surveyed, they identified four AFLP markers (E2/M5-600, E3/M16-400, E5/M12-600 and E5/M12-200) linked to the TGMS gene. Yu and Wise[14] identified three markers linked to the pea crown-rust resistance cluster using AFLP-based BSA in pea (*Lathyrus sativus*).

Bloom time in cherry is a quantitative trait, but has high broad sense heritability (0.91)[15] probably due to low number of genes controlling the trait. Identification of markers linked to quantitative trait loci (QTL) controlling bloom time in sour cherry could expedite the development of new cultivars or improvement of current cultivars with late blooming characteristics using marker assisted selection (MAS) and would avoid some of the loss due to spring freeze injury. The objective of this study is to search for such candidate markers associated with bloom time using BSA combined with AFLP technique.

MATERIALS AND METHODS

Plant material and bloom time: Bloom time was scored on approximately 200 progenies from a cross between two sour cherry cultivars, BalatonxSurefire located in the Clarksville Horticultural Experiment Station, for three consecutive (1999-2001) years. Bloom time was recorded as the time when approximately 50% of the flowers were open. BalatonxSurefire population was chosen because this cross displayed considerable variation in bloom time (Fig. 1, 2 and 3).

Selection of bulks: Two groups of three plants were selected from each extreme of the bloom time distribution of 'Balaton' x 'Surefire' population for selective genotyping. Three progenies, 3-24, 4-47 and 2-61, were selected as early group because they were the earliest flowering individuals over three years (Fig. 1, 2 and 3). The other three progenies, 4-22, 2-19 and 2-39, were selected as the late group since they were the latest flowering progenies for three years (Fig. 1, 2 and 3). These selected progenies carry enough flowers (about 10 flowers) to assess the bloom time accurately. Two groups were screened with a total of 94 AFLP primer pair combinations (Table 1).

Table 1: List of the AFLP primer pair combinations used to screen 'Balaton' x 'Surefire' population for candidate bands associated with bloom time

Primer combination		Primer combination		Primer combination	
ECA	MCGC	**ECC**	MCGG	**EAA**	MCCG
	MCTC		MCGC		MCCC
	MCCG		MCAA		MCAA
	MCAA		MCGG		MCTC
	MCGT		MCGA		MCAT
	MCGA		MCAT		MCGC
	MCAT		MCTC		MCTA
	MCAG		MCCG		MCCA
	MCCT		MCCC		MCAG
	MCTA		MCCT		MCCT
	MCCA		MCAG		**MCGT***
	MCCG		MCTA		MCGG
	MCCC		MCCA		MCGA
	MCAC		MCAC		MCAC
	MCTT		MCTT		MCTT
			MCGT		
ETT	MCAC	**ECT**	MCGC	**EAT**	MCTC
	MCTT		MCTC		MCCC
	MCTC		MCAA		MCTA
	MCCC		MCCC		MCTC
	MCTA		MCCG		MCAA
	MCTC		MCGG		MCGT
	MCAT		MCGA		MCGA
	MCAA		MCAT		MCGG
	MCGT		MCAG		MCCA
	MCAT		MCCT		MCAG
	MCGG		MCTA		MCCG
	MCCA		MCCA		MCGC
	MCAG		MCGT		MCAT
	MCCG*		MCAC		MCGA
	MCGC		MCTT		MCCT
	MCGA				MCAC
					MCGG

* Primer pair combinations, which amplified candidate bands that are present in late group but not in early group are shown in bold.

DNA extraction: Young unfolded leaves were obtained from trees of the 'Balaton' x 'Surefire' population located at Clarksville Horticultural Experiment Station of Michigan State University and were brought to the laboratory in a cooler and frozen at –80°C overnight and lyophilized for 2-3 days.

Modified –BSA and AFLP procedure: The DNA pooling technique proposed by Michelmore *et al.*[8] with a modification was used to find candidate markers that are present in one group but not in the other. The modification was made by not mixing the DNA of plants from the same group and keeping them separate.

AFLP markers were used because they do not have a very high development cost, genotyping cost is moderate and produces more bands (up to 100 gel^{-1}) per gel than any other markers.

Digestion, adapter ligation, preamplification and selective amplification were done as described[16,17], except with the following modifications described by Hazen *et al.*[18]; 2 μL of restriction ligation product was combined with 25 ng of MseI and EcoRI, 0.5 mM dNTPs, 1XPCR buffer (10 mM Tris-HCl, pH 7.2 50 mM KCl and 0.1% Triton X-100), 0.5 U Taq polymerase, 1.5 mM $MgCl_2$, total volume 20 μL. Preamplification was done with the following thermocycler profile [94°C 2 min - 26 cycles (94°C 1 min, 56°C 1 min, 72°C 1 min) -72°C 5 min]. The PCR product from preamlification was diluted six times with sterile water. One microliter of the dilute preamplification product was added to 19 μL of the following cocktail (25 ng EcoRI primer, 30 ng MseI primer, 0.4 mM dNTPs, 1X PCR buffer, 0.4 U Taq polymerase, 1.5 mM $MgCl_2$) and selective amplification was carried out with the following profile [94°C 2 min - 12 cycles with annealing temperatures decreasing by 0.7°C each step (94°C 30 sec, 65°C 30 sec, 72°C 1 min) - 23 cycles (94°C 30 sec, 56°C 30 sec, 72°C1min) -72°C 2 min]. The screening of early and late bulks was done by 156 AFLP primer combinations (Table 1).

Electrophoresis: The selective amplification products were separated by electrophoresis for 2.5 h at 80 W on a 6% polyacrylamide sequencing gel on a 38x50 cm Sequi-Gen GT sequencing cell (BioRad, Hercules, CA), then silver stained with sequence staining kit by Promega (# Q4132) and sizes were estimated using a 10 bp ladder (Gibco BRL #10821-015).

RESULTS AND DISCUSSION

The bloom time data for 'Balaton' x 'Surefire' population exhibited continuous variation in all three years, which is typical of quantitative inheritance. Distributions of flowering time of progenies in all three years were normal (Table 2-4). The absence of a bimodal distribution suggests that there is no major dominant gene for bloom time in 'Balaton' x 'Surefire' population. In contrary, the bloom time distribution in almond showed a bimodal distribution due to the presence of a major dominant Lb gene. Parental values were not the extremes and transgressive segregation was observed for bloom time distribution of the progenies (Table 2-4).

A modified BSA in combination with AFLP technique was used to identify candidate markers associated with bloom time. The selected individuals used for bulk analysis are indicated in Table 2-4. There were 94 AFLP (Table 1) primer pair combinations, which were used to screen early and late bulks of the population to find candidate markers present in one bulk but not in the other.

Screening of early and late bulks with 94 AFLP primer pair combinations resulted in the identification of two candidate bands in two different primer combinations that were present in late bulk but absent in early bulk (Fig. 1a-b).

ETT/MCCG primer combination resulted in an amplified band of 78 bp, which is present in the late group,

Table 1: Bloom dates of progenies of Balaton x Surefire in 1999. Selected progenies for bulk segregant analysis are shown in bold

Apr. 26	Apr. 27	Apr. 28	Apr. 29	May 1	May 2	May 3	May 4	May 5	May 6	May 8	May 10
					4-44						
					3-37						
					2-54	4-25					
					2-44	4-14					
					2-32	3-59		4-46			
				3-29	2-29	3-42		4-35	4-54		
				2-34	2-5	2-56	4-56	3-50	2-14		
				2-31	1-66	2-33	4-2	3-44	2-7		
4-47	**2-61**	3-21	3-20	2-4	1-65	Balaton	2-45	Surefire	2-6	3-66	**4-22**

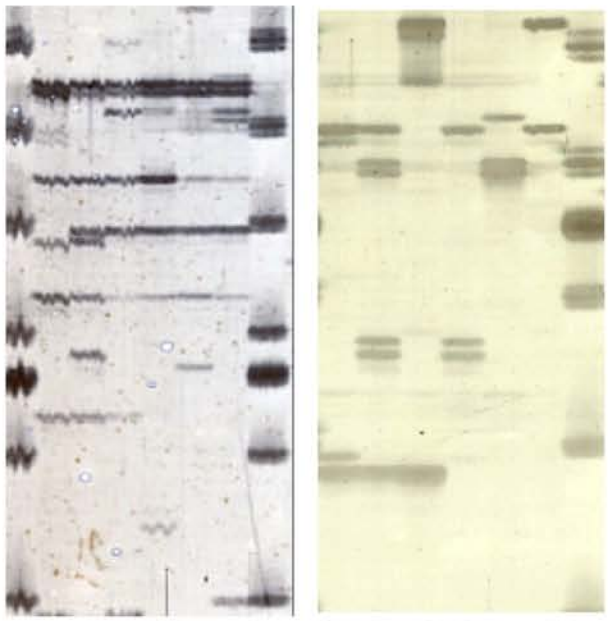
Table 2: Bloom dates of progenies of Balaton x Surefire in 2000. Selected progenies for bulk segregant analysis are shown in bold

Apr. 24	Apr. 25	Apr. 26	Apr. 27	Apr. 28	Apr. 29	Apr. 30	May 1	May 2	May 3	May 4	May 5	May 6	May 7
						4-64							
						4-44							
						4-38							
						4-26	4-61	4-7		4-54			
						4-25	4-60	4-6		4-46			
				4-31		4-15	4-43	3-62		4-39			
				4-29		4-14	4-42	3-59		4-37			
				4-21	4-2	3-63	4-11	3-50		3-44			
			3-16	3-49	3-28	3-42	4-8	3-35	4-1	3-38			
			3-2	3-27	3-18	2-56	3-5	2-58	3-17	3-37	4-66		
			2-61	2-44	2-54	2-46	2-62	2-52	3-10	2-55	4-58	4-33	
			4-56	4-34	2-37	2-42	1-66	2-36	2-45	4-55	2-14	4-45	4-24
3-24		2-6	4-28	2-31	2-34	1-65	2-32	2-43	4-23	2-7	3-22	**4-22**	3-57
4-47	3-20	2-5	3-29	4-35	2-30	Balaton	2-8	3-21	4-4	Surefire	2-9	**2-19**	**2-39**

Table 3: Bloom dates of progenies of Balaton x Surefire in 2001. Selected progenies for bulk segregant analysis are shownin bold

Apr. 27	Apr. 28	Apr. 29	Apr. 30	May 1	May 1	May 2	May 2	May 3	May 3	May 4	May 5	May 6
						4-61	3-47	3-52				
						4-59	3-43	3-52				
						4-55	3-42	3-50				
						4-52	3-36	3-45				
						4-46	3-33	3-44				
						4-41	3-27	3-38				
				3-63	3-54	4-40	3-19	3-34				
			4-21	3-55	3-51	4-32	3-12	3-22	4--65			
			4-8	3-59	3-41	4-30	3-11	3-21	4-64			
			3-62	3-56	3-37	4-28	3-8	3-10	4-62			
			3-49	4-60	3-33	4-26	2-64	3-5	4-57			
			3-35	4-56	3-31	4-25	2-62	2-63	4-54			
			3-29	4-49	2-60	4-23	2-55	2-59	4-45	4-66		
			3-18	4-44	2-57	4-12	2-54	2-58	4-39	4-58		
		4-47	1-65	4-42	2-56	4-7	2-51	2-53	4-38	4-53		
		4-43	1-66	4-29	2-48	4-6	2-45	2-46	4-37	**4-22**		
	4-36	3-46	2-43	4-19	2-44	4-4	2-35	2-40	4-35	4-20		
	4-34	3-28	2-37	4-18	2-42	54-1	2-17	2-38	4-33	3-51		
	4-31	3-16	2-34	4-15	2-33	3-64	2-15	2-28	4-27	3-17		
	3-20	2-24	2-29	4-14	2-32	3-61	2-14	2-11	4-16	2-52		
	3-2	2-22	2-25	4-10	2-31	3-58	2-13	2-10	4-11	2-47		
	3-1	2-16	2-23	4-3	2-30	3-48	2-12	2-9	3-65	2-36		
3-24	**2-61**	2-6	2-5	4-2	2-20	2-4	2-8	2-7	3-57	**2-19**	4-24	**2-39**

a. EAA/MCGT combination b. ETT/MCCG combination



L [1 2 3] [4 5 6] L [1 2 3] [4 5 6] L

Fig. 1 a-b: Two candidate AFLP bands that are present in one group, but not in another; a 94 bp band pointed by an arrow in EAA/MCGT primer combination is present in late group, but not in early group. A 78 bp band pointed by an arrow in ETT/MCCG primer combination is present in late group, but not in early group. Lane L is 10 bp ladder; lanes 1, 2 and 3 are late group (2-19, 4-22 and 2-39, respectively); lanes 4, 5 and 6 are early group (4-47, 2-61 and 3-24, respectively)

but not in the early group (Fig. 1b). The parent, 'Balaton', also had the band as the late group; 'Surefire' did not have this band.

EAA/MCGT primer combination amplified a band at 94 bp, which is present in the late group, but not in the early group (Fig. 1a).

The significant relationship between these AFLP markers and the bloom time could be confirmed by genotyping the whole 'Balaton' and 'Surefire' with these markers and testing for a statistically significant relationship. Then, informative AFLP markers could be converted into STS and be utilized in MAS for late blooming.

For a better understanding of the genetics of bloom time, the AFLP markers obtained here could be incorporated into the existing sour cherry linkage map in EB and RS population. The incorporation of these markers into the map may provide useful linkage information between these AFLP markers and bloom time.

If these markers are not closely linked and if each one maps independent of each other or into different linkage groups, then this information may allow us to have better understanding of the number of genes controlling bloom time. The amount of variation explained by the locations of the AFLP markers could also be calculated using QTL CARTOGRAPHER[19]. If these candidate markers are closely linked or map in to the same location, this might indicate that they are part of the same QTL.

From a breeding standpoint, availability of informative markers associated with bloom time has high value, because cold damage to flower buds and flowers is the most common factor reducing yield in sour cherry. Selection of late blooming varieties could decrease crop loss due to freezing temperatures in late spring. Genetic

markers obtained in this study are very important, because utilization of markers will help the indirect selection of varieties for desirable bloom time in early generations, saving time and effort. These candidate markers may also be incorporated in to existing *Prunus* maps and lead to isolation of genes for blomm time.

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