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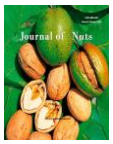

Identification of DNA Markers Linked to Blooming Time in Almond

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A B S T R A C T

In this study flowering time and other important morphologic traits were evaluated during two years in F_1 almond progenies of seventy two seedlings from cross between the intermediate flowering Italian cultivar 'Tuono' and the extra-late blooming Iranian cultivar 'Shahrood-12'. A modified-bulk segregant analysis in combination with the application of the 140 RAPD primers, 87 nuclear SSR markers spanning the whole almond genome and 5 chloroplast SSR markers, were used to identify molecular markers linked to flowering time. Results showed a quantitative inheritance of this trait in the studied progenies. The seedlings showed a wide range of flowering dates between both progenitors although some of these descendants were earlier in flowering than the early progenitor 'Tuono'. Results showed that among RAPD markers evaluated, BA-$17_{600,1000}$, BC-05_{320}, BC-06_{800}, BC-14_{1750}, BC-17_{600}, BC-20_{250}, OPC-05_{850} and OPC-$09_{700,1100}$ markers were linked to late blooming time. In addition, markers BA-04_{720}, BB-10_{630},BC-09_{2000}, BD-12_{510}andOPC-12_{350} were linked to early blooming time. Two microsatellite loci (CPPCT008 and EPPCU2584) were also found to be tightly linked to flowering time. After construction the genetic map of population, QTL analysis was performed for blooming time. QTL analysis showed that OPC-$09_{700,1100}$ and BA-$17_{600,1000}$, markers were respectively located in 2 and 4 cM distance from one of the late flowering time loci. Also the BA-04_{720}marker was located in 3 cM distances from one of the loci controlling early flowering time. These results are applicable in almond breeding programs for markers assisted strategy. The application of these results to other *Prunus* species has been also discussed.

Introduction

The almond [*P. dulcis* (Mill.) D.A. Webb; syn. *P. amygdalus* Batsch] is a species of genus *Prunus* and subgenus *Amygdalus* (Rosaceae, subfamily Prunoideae) that is commercially grown in most countries. The cultivated almond is thought to be originated in the arid mountainous regions of Central Asia (Grasselly1976a). Several wild species are also found growing in these mountainous areas from Tian Shan Mountain in western China through the mountainous areas and deserts of Turkmenistan, Afghanistan and into Iran and Iraq (Grasselly1976b; Kester

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and Gradziel 1996). Of the *Prunus* crop species, almond has the earliest blooming time, in some case along with apricot.

Flowering time in fruit trees is an important trait, especially when they require the simultaneous flowering for pollination, depending on environmental conditions during the flowering season. Rainfall during flowering, wind and also spring frosts, reduce fruit set significantly and sometimes even destroy the whole yield (Kester & Gradziel, 1996).For this reason, late flowering is among the main objectives in almond breeding programs, so that during the flowering, there be no or very low risk specially for spring frosts (Martinez-Gomez *et al.,* 2007).Flowering time is dependent to winter temperatures,chilling requirement for breaking of buds dormancy and heating requirement of the buds for opening (Campoy *et al.,* 2010).Flowering time is a quantitative trait with a high degree of heritability in many plant species, especially in *Prunus* species (Anderson & Seeley, 1993; Dicenta*et al.,* 1993; Campoy *et al.,* 2010). According to Dicenta & Garcia (1993), majority of results about the inheritance of flowering time in almond represents its quantitative inheritance with heritability range between 0.67 to 0.80.

Developing new late flowering cultivars using classical and molecular breeding techniques can help to prevent crop reduction by spring frosts.Among molecular methods markers could be used in order to find the ones associated with flowering time and applied for indirect selection (Sanches–Pereze *et al,* .2007).

Nowadays, markers based on DNA are essential tools in plant genetics with particular value in gene mapping and marker assisted selection (MAS) (Canli, 2004). Finding the molecular markers such as RAPD, AFLP and SSR linked with QTLs may enable direct or indirect selection of complex traits. Molecular markers have been successfully used to map individual genetic factors or QTLs controlling quantitative traits (Canli, 2004). The usual approach to analyse the association between marker–trait is morphological and molecular study of populations segregating for particular agronomic characters of interest (Martinez-Gomez *et al.,* 2007). The analysis of co-segregation among markers and characters allow establishment of the map position for major genes and QTLs responsible for their expression (Martinez-Gomez *et al.,* 2007; Rasouli *et al.,* 2011).

Bulked segregant analysis (BSA) and selection based on genotype are approaches that usually used to identify markers linked to quantitative trait loci (QTL). In this method, two pooled DNA samples are used that are formed from plant sources having similar genetic backgrounds but differing in one particular trait (Martinez-Gomez *et al.,* 2007). *Ballester et al.,* (2001) used BSA method in order to determine the genetic basis of late blooming in almond. They used RAPDmolecular markers to identify the late blooming gene in an F_1populationof 134 plants. They were succeededto identify three RAPD markers associated with late blooming gene that was located on the linkage group 4. One of them, $OKP10_{1350}$, placed at 5.4 cM from late blooming gene, being useful for a marker assistant selection (MAS) of late blooming time in almond. The progenies with late blooming allele bloomed 15 days later than other progenies and this allele had dominant gene action.

Canli (2004) used a modified bulk segregant analysis in combination with 94 amplified fragment length polymorphisms (AFLP) primer pairs to identify markers linked to blooming time gene in an F_1segregant population of 200 sour cherry progenies. Results showed two candidate bands present in the late bloom time group with 78 and 92 bp lengths in two different primer combinations, while, these two bands were not present in the early bloom time group.

Campoy *et al.,* (2010) studied flowering time in a BC_1 apricot progeny of 73 seedlings in combination with 46 simple sequence repeat (SSR) primers. Results showed that flowering time in apricot is also a quantitative trait and UDAP_423r and AMPA-105 microsatellite loci tightly linked to this important trait. In this study the QTL analysis for flowering time identified one significant QTL on the linkage group 5 that related to phenotypic variation.

Evaluation of flowering time in almond is a tedious process because of the long juvenile period of trees and the influence of the juvenility on the expression of the trait, as well as the climatic factors affecting on this evaluation. Marker-assisted selection (MAS) is particularly useful in these cases (Arús and Moreno-González 1993).

Since flowering time in almond is a quantitative trait (Sanches–Pereze *et al.*, 2007b), hence, identifying markers associated with blooming time can be help for selection and developing of new almond cultivars with marker assistant selection (MAS) approach. On the other hand, DNA markers of simple sequence repeats (SSR markers) which are based on the PCR technique through the specific amplification of the conserved DNA sequence flanking repetitive DNA sequences (microsatellite loci) of the genome (Tauzt, 1989), are becoming the markers of choice in mapping and genetic linkage analysis because of their high variability, multi allelic nature, relatively small size, codominant inheritance, reproducibility, relative abundance, extensive genome coverage, chromosome specific location, automation and high throughput genotyping (Sánchez-Pérez *et al.*, 2007; Boopathi *et al*., 2013). Selective genotyping involves selecting individuals from a population that represent the phenotypic extremes or tails of the trait being analyzed.

The objective of this work was to better understanding of the molecular basis underlying variations in blooming time in almond and the identification of markers linked to flowering time in a F1 almond progeny using a modified-bulk segregant analysis in combination with the application of SSR markers.

Materials and Methods

Plant material

This experiment was performed in Seed and Plant Improvement Institute (SPII) of Karaj, University of Tehran and Malayer University during the years 2008-2014. Plant material assayed included an F_1 almond progeny of seventy two seedlings from the cross between the intermediate flowering Italian cultivar 'Tuono' and the extra-late flowering Iranian cultivar 'Shahrood-12' (T×S).

Flowering time was evaluated in the progenitors and the F_1 (6 years old) population and expressed in a scale from extra-early, to extra late. In this population, four bulks (extra-early, early, late and extra-late) each consisting of several descendants from the almond progenies were studied using SSR markers.

Molecular Characterization Using RAPDs

Total DNA was extracted from young leaves collected in early spring, following the method described by Murray and Thompson (1980) modified by Weising *et al*,. (1995) and adapted to almond as follows: 1.5 g young leaves were ground in liquid nitrogen to fine powder and mixed with cetyltrimethyl ammonium bromide (CTAB) hot extraction buffer [100 mM Tris–HCl, pH = 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1%(w/v) PVP (polyvinyl pyrolidone) and 2% (v/v) B-mercaptoethanol]. The mixture was incubated at 60 °C for 1 h, followed by two extractions with chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids, and the pellet obtained was dissolved in Tris–EDTA (TE) buffer (10 mM Tris– HCl, pH = 8.0 and 1 mM EDTA, pH = 8.0). Co-precipitated RNA was removed by digestion with RNase A. Remaining impurities were extracted with phenol and chloroform. Total DNA was precipitated using sodium acetate and cold ethanol. The precipitate was washed twice with 10 mM ammonium acetate in 76% ethanol, and the pellet was dissolved in TE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality verified by spectrophotometry. DNA samples were stored at 4 °C. Two independent extractions were performed for each sample. Genomic DNA was PCR-amplified using 155 RAPD primers (Table 1) purchased from Tibmolbiol (Berlin, Germany) and Operon Technologies (Alameda, California, USA and Huntsville, USA). PCR reactions were performed

according to the protocol optimized by Rasouli *et al*. (2011) for RAPD primers using different annealing temperatures (from 35 ºC to 52 ºC). The amplified products were separated in 1.2% agarose gel electrophoresis using Tris–Boric acid–EDTA buffer, and stained with ethidium bromide. The stained gels were photographed under UV light with UVitec gel documentation. The molecular sizes of the amplification products were estimated using 100 bp DNA ladder plus (Fermentas). Also, some amplified PCR products were separated using regular LD-2® agarose (Conda, Madrid, Spain) and stained with GelRed™ (Biotium, Hatwad, CA, USA).

Molecular Characterization Using SSRs

Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987). Extracted almond genomic DNA was PCR-amplified using 94 primer pairs of flanking microsatellite sequences previously cloned, sequenced and reported in different *Prunus*species including almond, peach, cherry and apricot. Microsatellite amplifications and evaluation were performed as described by Sánchez-Pérez *et al.,* (2006). Amplified PCR products were separated using Metaphor® agarose (Cambrex, East Rutherford, NJ, USA) and stained with GelRed™ (Biotium, Hatwad, CA, USA). These SSR markers were well distributed across the *Prunus* genome. Fifty six of this SSR makers were previously used in the first linked map of the population 'R1000' × 'Desmayo Largueta' (Sánchez-Pérez *et al.*,2007a), as well as 15 new SSRs completing this map and mainly linked to groups 4 and 5 according to previous information of Dondini*et al*,. (2007) and Sánchez-Pérez *et al.*, (2010).

Phenotypic Data Analysis

Statistical analyses were performed using SPSS 12.0 package for Windows (Chicago, USA). The distribution of the seedlings population for each trait was represented in frequency histograms, using the mean values for 2 years of study. Bivariate correlations between different traits were calculated with row data of the 2 years, using the Pearson correlation coefficients.

Table 1. **Primers used in RAPD analysis.**

Row	Primers groups of RAPDs markers	Number of RAPDs primers used
1	BA-(01-20) (Tibmolbiol Co.Berlin, Germany)	20
2	BB-(01-20) (Tibmolbiol Co. Berlin, Germany)	20
3	BC-(01-20) (Tibmolbiol Co.Berlin, Germany)	20
4	BD-(01-20) (Tibmolbiol Co.Berlin, Germany)	20
5	BE-(01-20) (Tibmolbiol Co. Berlin, Germany)	20
6	BF-(01-20) (Tibmolbiol Co.Berlin, Germany)	7
	Sum	107
7	OPA-(01-20) (Operon Technologies Co. Alameda, USA)	5
8	OPAB-(01-20) (Operon Technologies Co. Alameda, USA)	1
9	OPB-(01-20) (Operon Technologies Co. Alameda, USA)	8
10	OPC-(01-20) (Operon Technologies Co. Alameda, USA)	5
11	OPD-(01-20) (Operon Technologies Co. Alameda, USA)	2
12	OPE-(01-20) (Operon Technologies Co. Alameda, USA)	2
13	OPG-(01-20) (Operon Technologies Co. Alameda, USA)	6
Row	Primers groups of RAPDs markers	Number of RAPDs primers used
14	OPI-(01-20) (Operon Technologies Co. Alameda, USA)	2
	Sum	31
15	OPK-(01-20) (Operon Technologies Co.Huntsville, USA)	3

Table 1. Continued

16	OPN-(01-20) (Operon Technologies Co.Huntsville, USA)	2
17	OPR-(01-20) (Operon Technologies Co.Huntsville, USA)	4
18	OPW-(01-20)(Operon Technologies Co.Huntsville, USA)	3
19	OPX-(01-20) (Operon Technologies Co.Huntsville, USA)	2
20	OPY-(01-20) (Operon Technologies Co.Huntsville, USA)	2
21	OPZ-(01-20) (Operon Technologies Co.Huntsville, USA)	1
	Sum	18
	Total	155

Genetic Linkage Analysis and QTL Identification

The genetic linkage map was constructed (Fig. 1) and QTL analysis was performed using JoinMap.4 (Van Ooijen 2006). All linkage groups were calculated for a minimum LOD of 2.50; in all cases, higher LOD values were used. Analysis of QTL was performed using the QTL Cartographer software version 1.30 (2001). The phenotypic and genotypic data were analyzed together by first performing a test of 1,000 permutations to designate a significant LOD score threshold of $\alpha = 0.05$ for each quality trait and year. Finally, data were analyzed by interval mapping with a frequency of recombination of 0.35, resulting in different QTLs in some of the linkage groups created.

Results

Blooming Time Evaluation

Fig.s 2 and 3 are showing the distribution of the seventy almond F_1 progeny seedlings of the 'Tuono' × 'Shahrood' (T×S) for flowering time according to the mean values of the first and second years. Results showed a quantitative inheritance of flowering date in the studied almond progeny. The evaluated seedlings showed a wide range of flowering dates and in most of cases a range between both progenitors. However, some of these descendants were earlier than the early progenitor 'Tuono'.

This shows the effect of genetic background on the expression of this trait.These results agree with previous report considering the mean values of each descendant for four years (2000-2003) which clearly showed the bimodal distribution of the flowering date in the population (Sánchez-Pérez *et al.*, 2007b).

Some agronomic important traits of the F_1 progeny of 'Tuono' × 'Shahrood12' (TxS) and the parents, which used in this study, were measured according to the Gulcan (1985) descriptor (Table 2). Differences were found by analysis of variance for some of the studied characteristics and the mean values for each characteristic were different (Table 2).

Modified Bulked Segregant Analysis for Flowering Time in Almond Using RAPDs Markers

Results showed that BA-$17_{600,1000}$, BC-05_{320}, BC-06_{800}, BC-14_{1750}, BC-17_{600}, BC-20_{250}, OPC-05_{850} and OPC-$09_{700,1100}$ (Figs. 4, 5, 6 and 7) markers were linked to late blooming and BA-04_{720},BB-10_{630},BC-09_{2000}, BD-12_{510}andOPC-12_{350} (Figs. 8 and 9) were linked to early blooming time. After construction of the genetic map of population, QTL analysis was performed for flowering time. Results showed that BA-$17_{600,1000}$ marker had 4 cM distance from one of the late flowering time loci (Figs. 10 and 11). Also, the OPC-$09_{700,1100}$ and BA-04_{720} markers were located at 2 and 3 cM distances from one of the genes controlling early and late flowering time, respectively (Figs. 10 and 11).

Modified Bulked Segregant Analysis for Flowering Time in Almond Using SSR Markers

In the analysis of co-segregation of SSR markers (Tables 3 and 4) and the TxS almond progeny population, two microsatellite loci (CPPCT008 and EPDCU2584) (Fig. 1) were found to be tightly linked to this important agronomic trait (Fig. 12). After construction of a genetic map of population, QTL analysis was performed for flowering time and some important morphological traits such as vegetative, nut and kernel characteristics. QTL analysis results showed that the CPPCT008 has 0cM distance from late flowering time (Table 5). Also, QTL analysis showed that UDP-97403 locus has 4 and 0cM distance from one locus controlling tree growth habit, nut width, nut thickness and kernel width respectively. Moreover, QTL analysis located the BPPCT007 locus in 2cM distance from the locus controlling kernel width. Genetic markers linked to blooming time, nut and kernel characteristics in almond are very important, because utilization of these markers will help the indirect selection of genotypes for desirable bloom time, and superior nut and kernel properties, resulting to saving time and effort. Also, by using of these molecular markers, hybrids can be separated from the flowering time in the early stages of growth.

Discussion

Flowering time showed a quantitative inheritance in the studied progeny that was in agreement with previous results in different almond progenies (Dicenta *et al*. 1993; Vargas and Romero 2001; Colic *et al*. 2012). In addition, present results were in agreement with previous results in descendants of 'Tardy Nonpareil' which also showed a bimodal distribution for this trait and explained by the presence of a late flowering major gene (Lb), that quantitatively modified by other minor genes (Sociasi Company *et al*. 1999; Sánchez-Pérez *et al*.,2007b). It seems to be a similar case in our population, because the progenitors of 'R1000' have been 'Tardy Nonpareil' and 'Tuono'.

Dicenta *et al.,* (2005) mentioned that the best strategy to obtain late-flowering descendants is to cross late-flowering progenitors. When the off springs show a bimodal distribution we must select the latest-flowering ones that are more probable of carrying the late-flowering allele (in the case of descendants from 'Tardy Nonpareil') which could be transmitted to the descendants.

Identification of genetic markers linked to blooming time in almond are very important for utilization in indirect selection of genotypes for desirable bloom time in young trees, resulting to saving time and effort. According to the obtained results, with the development of these markers, the strategy of marker assisted selection can be used in breeding programs of almond, apricot, peach and other *Prunus* species.

Present results were in line with Ballester *et al.,* (2001) that studied the genetic basis of late flowering in 61 almond progenies (F_1) by bulk segregant analysis method using RAPDs markers.They were able to identify 3 RAPDs markers associated with late flowering genes. One of them ($OKP10_{1350}$) were located at a distance of 5.4 cM of the late flowering genes. While, we found 8 RAPDs markers associated with late flowering genes and $OPC\text{-}09_{700,1100}$ was located at a distance of 2 cM of late flowering genes (Figs. 7 and 10, Table 5).

Results corroborated the suitability of the use of SSR markers for the assessment of molecular genetic variability in almond and the high degree of transportability between peach derived SSR markers in almond that previously reported by Martínez-Gómez *et al.,* (2003b). SSR detection using Metaphor® agarose gel electrophoresis was efficient and would be able to resolve most of allelic variations. In this sense, we can indicate that the use of MetaPhor® agarose and Gel Red Nucleic Acid Gel Sating® appears good indicators for molecular characterization and mapping

of population. This is due to its good resolution in comparison with the usual agarose, less toxicity in comparison with the use of ethidium bromide, and lower cost and easier routine application in comparison with the automatic capillary sequencing. In addition, results indicated the suitability of SSR markers for the analysis of QTL linked to flowering time and chilling and heat requirements as has been described before in almond (Sánchez-Pérez *et al.*,2007a) and other related species such as apricot (Olukolu *et al*. 2009), rose (Hibrand-Saint Oyant *et al.*, 2008) or peach (Fan *et al.*, 2010) as well as the future application in marker assisted selection as has been described in the case of bitterness in almond (Sánchez-Pérez *et al.*,2010).

Table 2. Data for some important traits evaluated in almond progenies of 'Tuono'(♀) × 'Shahrood-12'(♂) cross.

Genotypes	Blooming time (Code)	Growth vigor (Code)	Foliage density (Code)	Leaf area (mm^2)	leaf Chlorophyll (CCI)	Nut weight (gr)	Nut length (mm)	Nut width (mm)	Kernel Weight (gr)	kernel width (mm)	Kernel length (mm)
TS-1	early medium	intermediate	dense	1114.72	13.20	2.48	31.40	17.70	0.75	10.85	23.00
TS-2	medium	weak	low	670.12	12.37	2.50	31.80	19.30	0.76	11.05	22.80
TS-3	medium late	strong	dense	1418.31	14.20	2.48	31.84	19.40	0.75	11.08	22.80
TS-4	medium late	strong	dense	1278.89	12.87	1.95	24.78	19.78	0.65	12.00	18.82
TS-5	medium	strong	intermediate	1271.56	12.73	2.28	26.12	19.96	0.78	12.12	20.16
TS-6	medium late	strong	intermediate	1187.03	10.33	2.14	28.90	18.38	0.68	11.06	21.42
TS-7	extra late	strong	dense	1497.99	12.77	1.60	24.62	17.86	0.64	10.52	18.28
TS-8	medium late	weak	dense	765.21	12.23	2.00	24.90	20.70	0.66	12.00	18.50
TS-9	medium late	weak	intermediate	665.40	11.83	2.05	24.20	20.00	0.67	12.00	18.30
TS-10	medium	strong	dense	1130.93	13.07	3.25	28.84	22.36	0.72	12.87	19.64
TS-11	medium	strong	dense	1579.77	12.07	3.30	28.72	23.00	0.75	12.96	19.72
TS-12	medium late	strong	dense	1157.94	17.03	2.28	26.50	20.00	0.78	11.70	20.60
TS-13	Late	strong	intermediate	1318.43	10.57	2.30	26.20	19.50	0.80	11.30	19.70
TS-14	Late	strong	dense	1241.99	12.20	2.50	31.10	21.10	0.70	12.50	23.05
TS-16	medium	weak	low	1169.37	13.90	2.13	28.50	19.20	0.68	10.90	21.90
TS-17	Late	strong	dense	1158.41	12.70	2.12	29.00	18.20	0.67	11.50	21.50
TS-18	early medium	strong	dense	958.58	9.50	2.16	30.00	17.50	0.68	10.90	21.50
TS-19	early medium	intermediate	low	1326.79	13.63	3.94	32.38	26.44	0.95	12.71	22.44
TS-20	medium	strong	dense	1344.81	12.33	1.15	24.40	18.74	0.57	11.78	18.56
TS-21	medium	strong	dense	1269.51	14.57	1.1	25.50	19.00	0.48	10.86	20.2
TS-22	medium	strong	intermediate	1911.06	15.63	2.18	29.00	18.44	0.71	11.08	21.46
TS-23	early medium	strong	dense	1051.13	15.30	3.36	30.94	23.88	1.06	13.36	21.84
TS-26	early medium	strong	intermediate	1126.47	11.73	2.26	25.40	20.00	0.76	12.5	19.1
TS-27	extra late	strong	dense	1431.74	17.73	1.85	24.78	17.26	0.62	10.52	18.4
TS-28	Late	weak	dense	575.15	13.60	3.26	27.90	23.00	0.75	13.3	19.8
TS-29	medium late	strong	intermediate	1089.91	18.03	3.45	31.52	23.94	1.1	13.62	21.9
TS-30	early medium	strong	dense	1290.66	14.43	3.42	31.50	23.90	1.08	13.59	21.87
TS-31	early medium	strong	dense	1264.76	15.17	3.42	31.50	23.90	1.08	13.59	21.87
TS-32	medium	strong	dense	1152.39	13.07	3.75	31.26	23.54	0.94	13.4	22.25
TS-33	medium	strong	dense	873.42	15.57	4.06	33.10	24.16	1.16	13.84	23.98
TS-34	medium late	strong	intermediate	1285.72	15.97	1.27	24.96	15.10	0.43	9.58	19.28
TS-35	Late	weak	low	273.95	12.10	2.50	31.10	21.10	0.70	12.50	23.05
TS-36	medium late	strong	low	1192.93	14.47	2.60	31.00	21.20	0.70	12.50	23.05
TS-37	Late	strong	low	195.87	11.20	2.70	30.90	21.30	0.70	12.50	23.05
TS-39	medium late	strong	low	1297.26	11.13	2.76	32.50	20.75	0.99	11.35	23.00
TS-40	medium late	strong	dense	1089.29	8.23	2.59	27.28	18.26	0.63	11.00	19.94
TS-41	very early	strong	dense	726.32	15.60	3.45	33.00	22.60	1.10	13.25	21.00
TS-42	very early	strong	dense	777.96	12.83	3.45	30.90	24.30	1.10	13.75	23.50
TS-43	extra early	strong	dense	865.44	17.53	3.45	32.40	25.70	1.10	13.30	21.00

Table 2. Continued

Genotypes	Bloomingtime (Code)	Growth vigor (Code)	Foliagedensity (Code)	Leaf area (mm^2)	leaf Chlorophyll (CCI)	Nut weight (gr)	Nut length (mm)	Nut width (mm)	Kernel Weight (gr)	Kernelwidth (mm)	Kernel length (mm)
TS-45	early medium	strong	dense	791.04	11.63	2.50	31.10	21.10	0.70	12.50	23.05
TS-46	medium	strong	dense	650.54	13.03	2.60	31.00	21.20	0.70	12.50	23.05
TS-47	extra late	strong	dense	4158.94	12.00	2.70	30.90	21.30	0.70	12.50	23.05
TS-48	late	strong	dense	966.42	12.23	2.40	31.20	21.00	0.69	12.40	23.00
TS-49	medium late	intermediate	dense	689.26	16.67	1.15	24.00	18.80	0.57	11.80	19.10
TS-50	early medium	strong	dense	795.41	8.20	1.15	25.20	19.10	0.57	11.90	18.90
TS-51	late	strong	dense	786.75	18.60	1.16	25.20	19.40	0.55	11.80	18.30
TS-52	medium late	strong	dense	892.18	6.47	3.32	31.30	24.00	1.06	14.10	21.40
TS-53	late	strong	intermediate	650.46	12.30	1.10	25.50	19.00	0.48	11.10	20.60
TS-54	late	strong	intermediate	967.78	10.80	1.10	25.30	19.00	0.50	10.60	19.80
TS-55	medium late	strong	dense	773.83	18.07	1.10	25.10	19.00	0.51	11.10	20.60
TS-56	early	strong	dense	984.68	12.77	1.10	25.70	19.20	0.45	10.60	19.80
TS-60	early	strong	dense	841.98	19.57	2.16	30.00	19.30	0.68	10.90	21.60
TS-61	early	weak	low	748.30	10.23	1.14	23.80	18.40	0.59	12.00	18.00
TS-62	early	weak	low	1087.32	7.73	3.40	29.90	23.10	1.06	13.40	22.30
TS-63	early	weak	intermediate	608.81	9.03	3.30	29.90	22.80	0.75	13.00	20.50
TS-64	early medium	strong	dense	780.88	14.23	3.30	29.70	22.40	0.75	13.30	19.50
TS-65	very early	strong	dense	1020.16	11.70	3.26	27.90	23.00	0.75	13.30	19.80
TS-66	very early	strong	dense	941.47	26.57	3.34	28.30	23.10	0.77	12.30	19.80
TS-67	early medium	intermediate	intermediate	875.43	17.63	2.80	32.00	20.50	0.99	10.70	22.60
TS-68	early medium	intermediate	low	1012.94	9.60	2.70	33.00	21.00	0.99	12.00	23.40
TS-69	medium late	strong	dense	654.07	9.80	2.80	32.00	20.50	1.01	10.70	22.60
TS-70	very late	weak	intermediate	670.38	11.70	2.70	33.00	21.00	0.98	12.00	23.40
TS-71	very late	intermediate	dense	479.69	17.37	2.59	26.40	18.00	0.62	11.30	19.90
TS-72	early medium	intermediate	dense	908.94	15.10	2.59	26.90	18.50	0.63	11.00	20.90
TS-73	early medium	weak	low	486.43	7.63	2.59	27.70	18.00	0.64	10.70	19.80
TS-74	very late	weak	dense	1197.48	11.97	2.58	28.20	18.40	0.65	10.80	19.70
TS-75	extra early	strong	dense	886.55	10.23	1.27	25.50	15.40	0.43	9.40	18.70
TS-76	early medium	intermediate	intermediate	778.07	11.43	1.26	24.30	15.00	0.44	9.90	19.50
TS-77	medium late	strong	dense	565.45	13.37	1.28	24.70	14.40	0.42	10.00	19.60
TS-78	extra early	strong	dense	1178.61	10.47	1.27	25.50	15.40	0.43	9.40	18.70
TS-79	early medium	strong	dense	1203.81	18.87	1.26	24.30	15.00	0.44	9.90	19.50
TS-80	medium	strong	dense	1299.47	8.97	1.28	24.70	14.40	0.42	10.00	19.60
Tuono	medium	strong	dense	1424.21	14.60	3.96	24.08	22.70	0.77	13.80	22.00
Shahroo12	extra late	intermediate	dense	1077.70	12.73	6.21	32.70	25.40	0.81	12.70	23.00

Table 3. Specifications of the simple sequence repeat (SSR) markers tested in present study.

SSR marker group	Species	Origin	No of SSRs amplified	No of SSRs polymorphic	Reference
BPPCT	Peach	Genomic	22	12	Dirlewanger *et al*. 2002
CPDCT	Almond	cDNA	4	3	Mnejja *et al*. 2005
CPPCT	Peach	Genomic	13	8	Aranzana *et al*. 2002
EPDCU	Almond	cDNA	3	3	GDR[1]
EPPCU	Peach	cDNA	2	2	GDR[1]
MA	Peach	Genomic	1	1	Yamamoto *et al*. 2002
PceGA	Cherry	Genomic	1	1	Downey and Iezzoni 2000
pchcms	Peach	Genomic	1	1	Sosinski *et al*. 2000
pchgms	Peach	cDNA	1	1	Sosinski *et al*. 2000
PMS	Cherry	Genomic	1	1	Cantini *et al*. 2001
PS	Cherry	Genomic	1	1	Sosinski *et al*. 2000
Tpscp	Plum	Chloroplastic	5	0	Ohta *et al*. 2005
UDA	Almond	Genomic	2	2	Testolin *et al*. 2004
UDP	Peach	Genomic	14	10	Cipriani *et al*. 1999
UDP	Peach	Genomic	6	5	Testolin *et al*. 2000
Total	--	--	77	51	--

[1] GDR, Genome data base for Rosaceae (http://www.rosaceae.org/peach/genome)

Table 4. Polymorphism obtained in the application of SSR in the modified bulk segregant assay in the almond F1 progeny 'Tuono' x 'Shahrood-12'.

SSR marker	Polymorphism[1]	SSR marker	Polymorphism[1]	SSR marker	Polymorphism[1]
BPPCT002	PPD	CPPCT002	NP	Tpscp07	NP
BPPCT004	NP	CPPCT006	NP	Tpscp08	NP
BPPCT006	NP	**CPPCT008**	**PEL**	Tpscp011	NP
BPPCT007	PPD	CPPCT013	PPD	UDA002	PPD
BPPCT008	NP	CPPCT022	NP	UDA045	PPD
BPPCT010	PPD	CPPCT026	PPD	UDP96001	PPD
BPPCT011	NP	CPPCT027	PPD	UDP96003	PPD
BPPCT012	NP	CPPCT028	NP	UDP96005	PPD
BPPCT014	PPD	CPPCT030	PPD	UDP96008	PPD
BPPCT017	PPD	CPPCT033	PPD	UDP96013	PPD
BPPCT020	PPD	CPPCT034	PPD	UDP96019	NP
BPPCT021	PPD	CPPCT035	NP	UDP97401	PPD
BPPCT025	NP	CPPCT047	PPD	UDP97402	PPD
BPPCT024	NP	**EPDCU2584**	**PEL**	UDP97403	PPD
BPPCT026	PPD	EPDCU4658	PPD	UDP98021	PPD
BPPCT028	PPD	EPDCU5183	PPD	UDP98024	NP
BPPCT029	PPD	EPPCU3392	PPD	UDP98025	PPD
BPPCT033	PPD	EPPCU9830	PPD	UDP98405	PPD
BPPCT035	NP	MA027	PPD	UDP98406	NP
BPPCT037	NP	PceGA025	PPD	UDP98407	PPD
BPPCT038	PPD	pchcms3	PPD	UDP98408	PPD
BPPCT039	NP	pchgms2	PPD	UDP98409	PPD
CPDCT016	PPD	PMS67	PPD	UDP98410	NP
CPDCT027	PPD	PS01H03	PPD	UDP98411	PPD
CPDCT028	NP	Tpscp01	NP	UDP98412	NP
CPDCT047	PPD	Tpscp03	NP		

P- no polymorphism
PPD - polymorphism in the parents and descendants
PEL – polymorphism between extra-late blooming parent and descendants

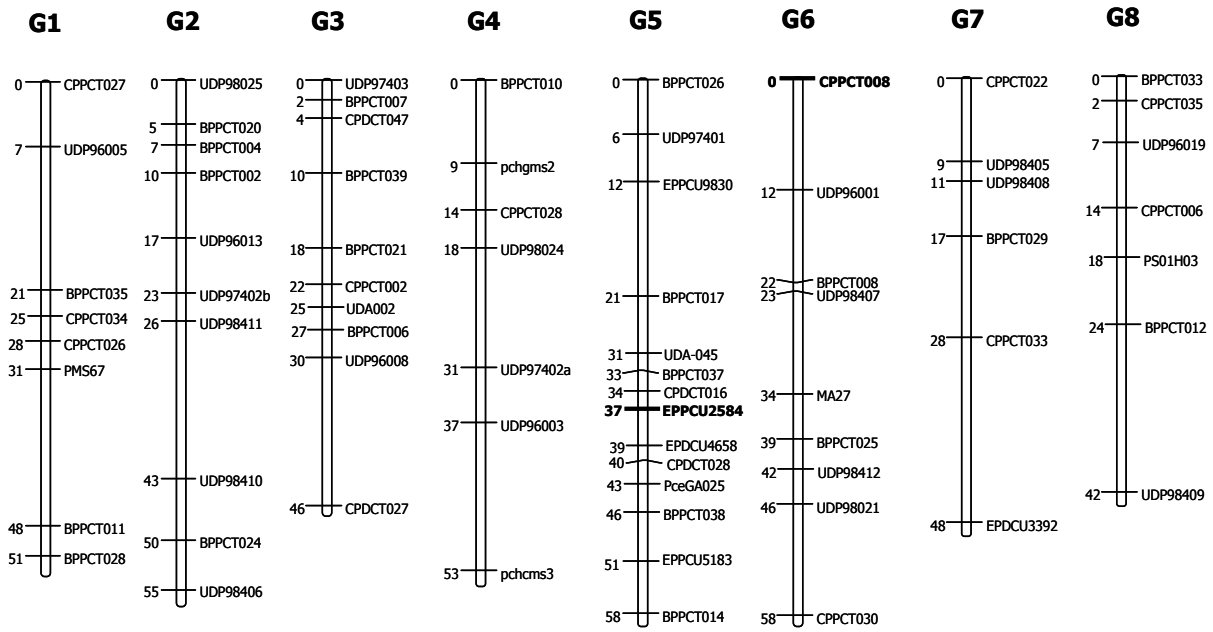


Fig. 1. Approximate location of SSR markers assayed in the eight linkage groups described in almond according to Sánchez-Pérez *et al.*, (2007); Dondini*et al*,. (2008) and Sánchez-Pérez *et al.*, (2010).

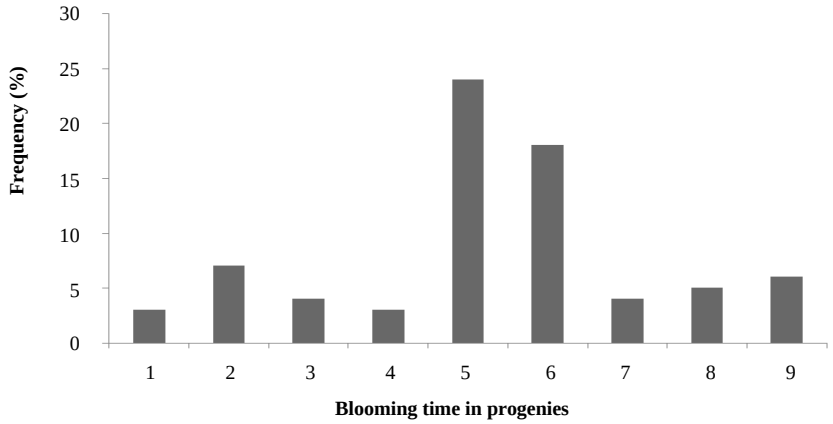


Fig. 2. Frequency of flowering time in almond seedlings from the cross between 'Tuono'(♀)× 'Shahrood-12'(♂) in the first evaluation year (1= Extremely early, 2= Very Early, 3= Early, 4= Early / intermediate, 5= Intermediate, 6= Intermediate / late, 7= Late, 8= Very late, 9= Extremely late).

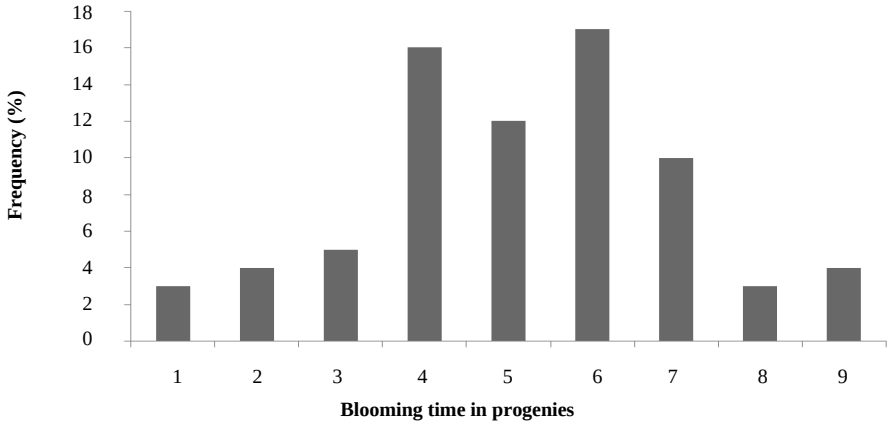
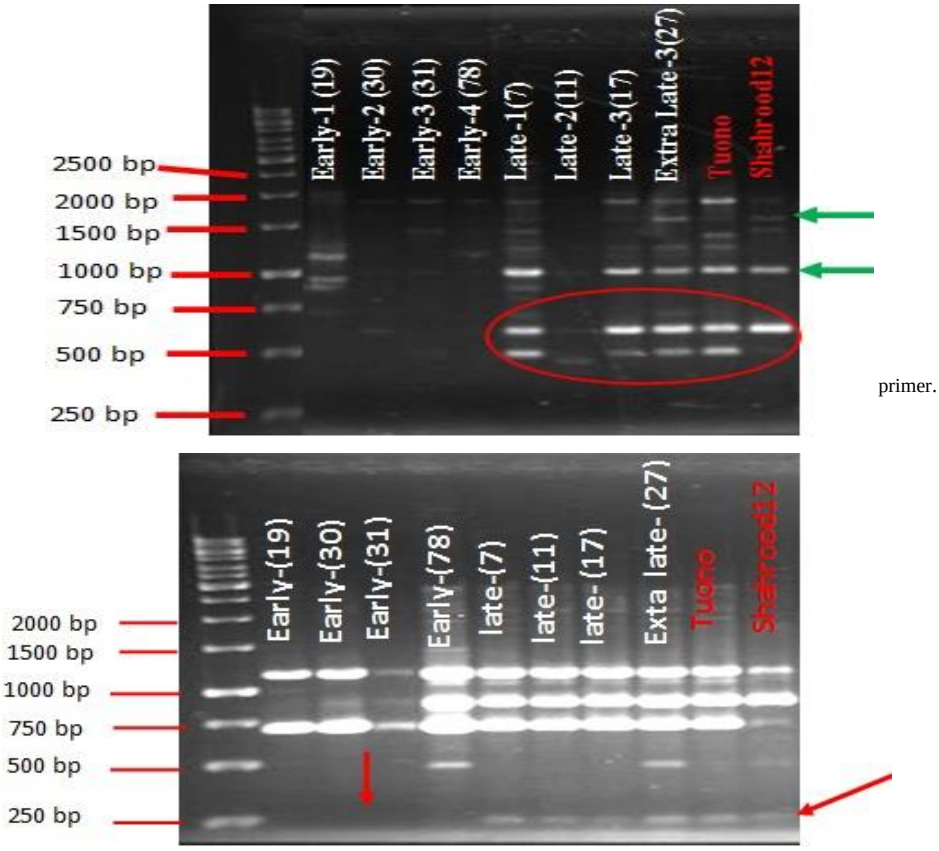


Fig. 3. Frequency of flowering time in almond seedlings from the cross between 'Tuono'(♀)× 'Shahrood-12'(♂) in the second evaluation year (1= Extremely early, 2= Very Early, 3= Early, 4= Early / intermediate, 5=Intermediate, 6= Intermediate / late, 7= Late, 8= Very late, 9= Extremely late).



primer.

Fig. 5. Candidate band presumably linked to late flowering time in almond using$BC\text{-}20_{250}$ RAPD primer.

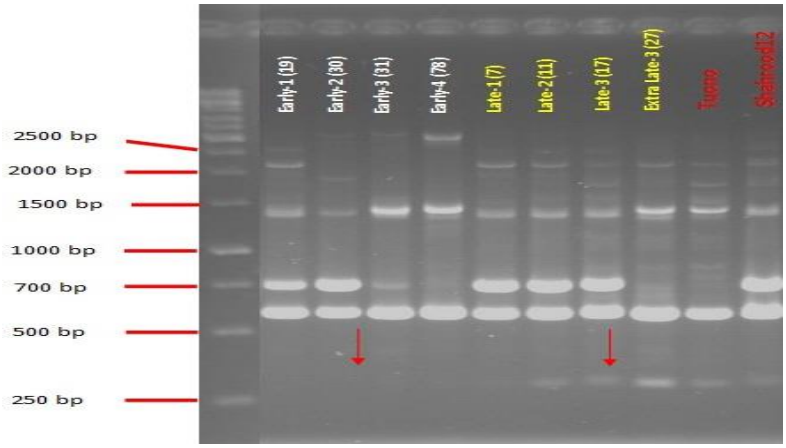


Fig. 6. Candidate band presumablylinked to late flowering time in almond using$BC\text{-}05_{320}$ RAPD primer.

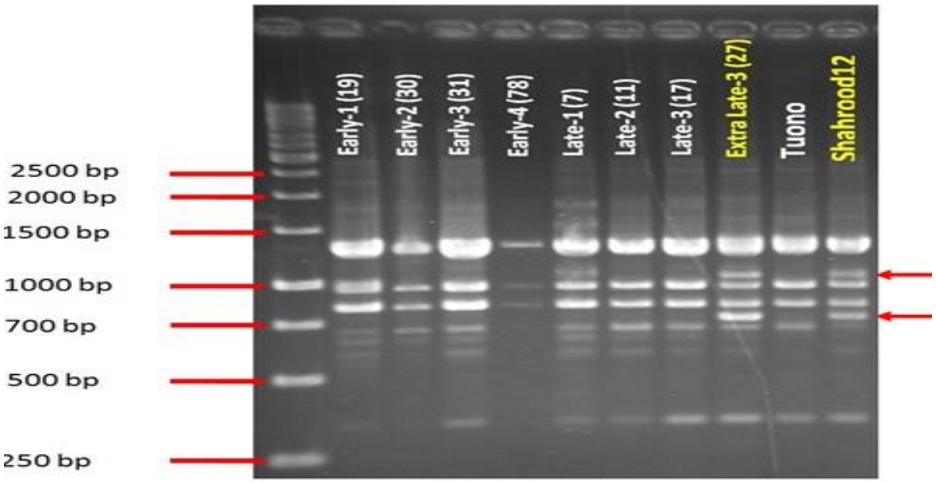


Fig. 7. Candidate bands presumably linked to late flowering time in almond using OPC-$09_{700,1100}$ RAPD primer.

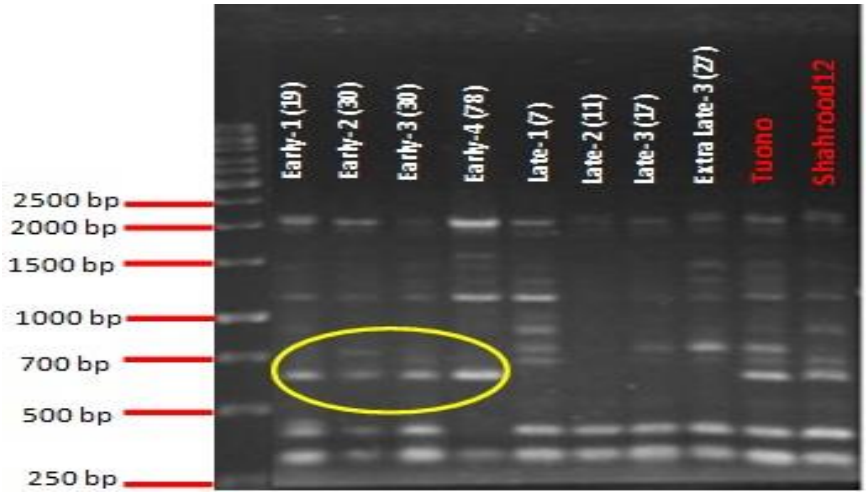


Fig. 8. Candidate band presumablylinked to early flowering time in almond using BB-10_{630} RAPD primer.

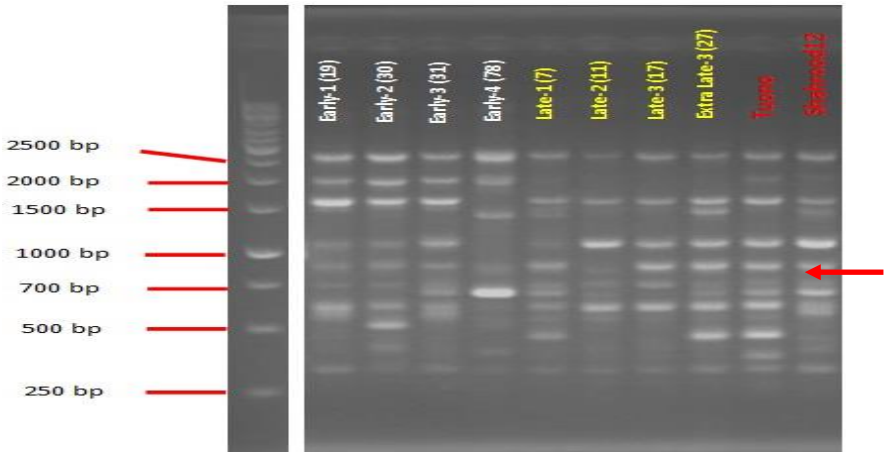


Fig. 9. Candidate band presumablylinked to early flowering time in almond using BC-09_{2000}RAPD primer

Table 5. Name of linked primers with flowering time in RAPD and SSR analysis with their sequences.

Row	marker	Candidate band	Marker sequences	Linked with flowering time	Marker distance from QTL of flowering time
1	RAPD	BA-04_{720}	5′-TCCTAGGCTC-3′	Early flowering	3 cM
2	RAPD	BB-10_{630}	5′-ACTTGCCTGG -3′	Early flowering	-
3	RAPD	BC-09_{2000}	5′-GTCATGCGAC -3′	Early flowering	
4	RAPD	OPC-12_{350}	5′-TGTCATCCCC -3′	Early flowering	-
5	RAPD	BD-12_{510}	5′-GGGAACCGTC -3′	Early flowering	-
6	RAPD	BA-$17_{600,1000}$	5′-TGTACCCCTG-3′	Late flowering	4 cM
7	RAPD	BC-06_{800}	5′-GAAGGCGAGA -3′	Late flowering	-
8	RAPD	BC-14_{1750}	5′-GGTCCGACGA -3′	Late flowering	11 cM
9	RAPD	BC-05_{320}	5′-GAGGCGATTG-3′	Late flowering	-
10	RAPD	BC-17_{600}	5′-CCGTTAGTCC -3′	Late flowering	-
11	RAPD	BC-20_{250}	5′-AGCACTGGGG -3′	Late flowering	-
12	RAPD	OPC-05_{850}	5′-GATGACCGCC -3′	Late flowering	-
13	RAPD	OPC-$09_{700,1100}$	5′-CTCACCGTCC -3′	Late flowering	2 cM
14	SSR	CPPCT008_{200}	F:GAGCTCTCACGCATTAGTTT R:TTTGACTGCATAACAAAACG	Late flowering	0 cM
15	SSR	EPPCU$2584_{125,150}$	F:TTCAGCTCATCTAGTTTCATCACC R:CACGGTTCGAACAACATCTG	Late flowering	37cM

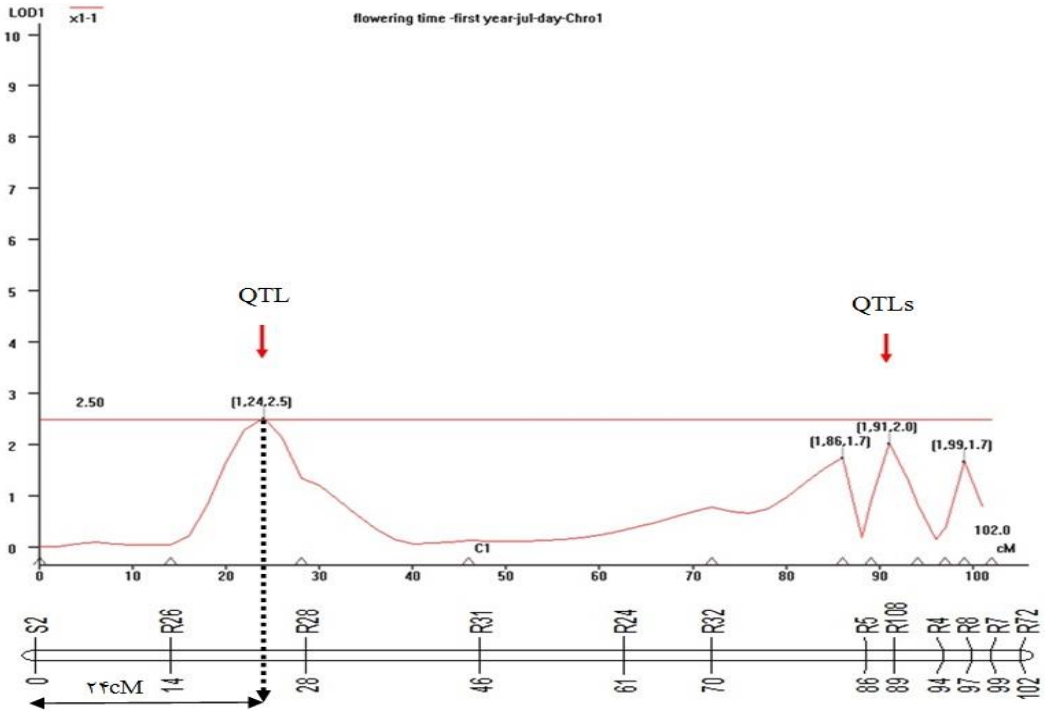


Fig. 10. LOD Position, distance and of flowering time QTL that have been identified in Linkage groups on a map prepared on almond population from the cross between 'Tuono'(♀) × 'Shahrood-12' in the first year .

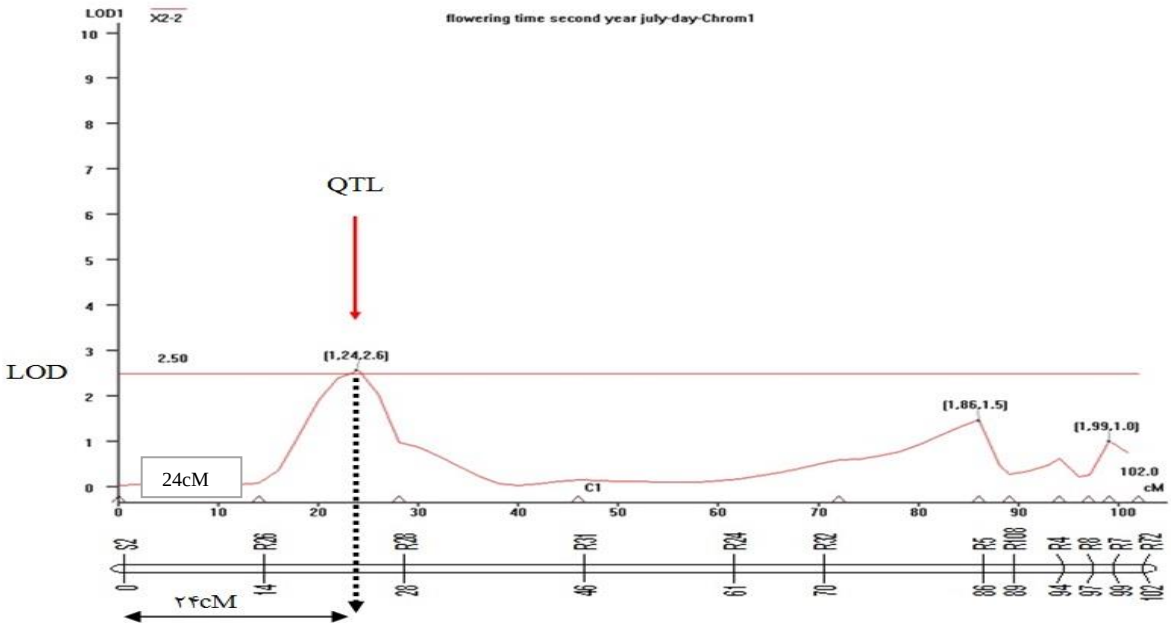
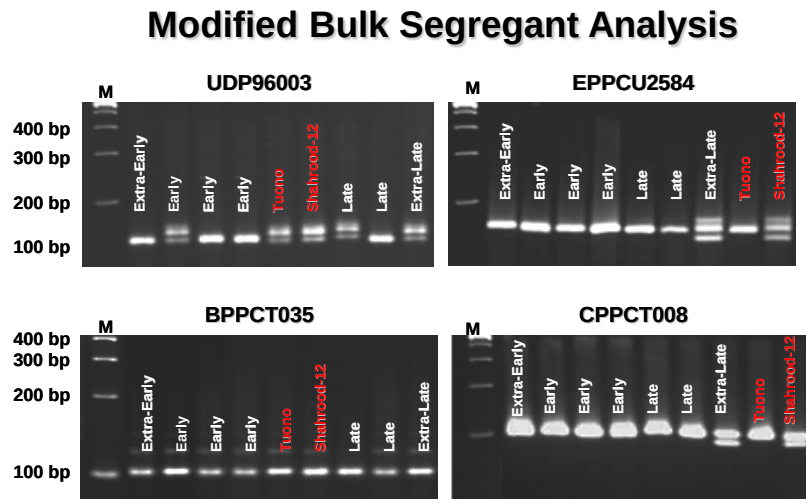


Fig. 11. Position, distance and LOD of flowering time QTL that have been identified in Linkage groups on a map prepared on almond population from the cross between 'Tuono'(♀)× 'Shahrood-12' in the second year.



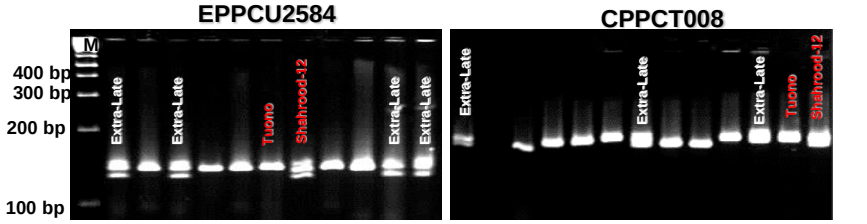


Fig. 12. Metaphor® agarose gels showing polymorphism observed in the application of SSR markers UDP96003, EPPCU2584, BPPCT035 and CPPCT008 to the modified bulk segregantanalayis of the F1 almond population 'Tuono' × 'Shahrood-12'.

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