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Development of a Second Generation Genetic Linkage Map for Sour Cherry Using SSR Markers

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Abstract: A second generation consensus linkage map of two tetraploid sour cherry cultivars (*Prunus cerasus* L., 2n=4x=32), 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' (EB) was constructed. Forty five SSR primer pairs from apple, peach, sour cherry and sweet cherry were screened and 10 informative SSRs yielding 17 markers were added to the sour cherry linkage map. The EB and RS consensus map consisted of 161 markers covering 442.4 cM in 19 linkage groups. The largest linkage group is group 9 covering 44.5 cM. The average distance between two markers was 2.74 cM. The longest distance between two adjacent markers was 16.9 cM in linkage group 8. SSR markers tightly linked to important quantitative traits in sour cherry, such as bloom time, fruit weight and pistil death, were obtained. These markers could be utilized as a valuable tool for trait selection. The results from current study have shown that SSR primers are co-dominant, reproducible, highly polymorphic and have high utility for cross-species amplification within *Prunus*. SSR will be the marker of choice for comparative mapping and MAS studies in *Prunus*.

Key words: Linkage mapping, SSRs, QTLs, MAS, sour cherry, boom time, *Prunus*

INTRODUCTION

Sour cherry is produced in significant quantities in about 40 countries. The majority of the world's sour cherries are produced in Russia (200,000 tons from commercial orchards and 180,000 tons from home gardens, 1986), Ukraine (2,400 tons from commercial gardens, 191,000 tons from home gardens, 1993), Yugoslavia (114,594 tons, 1990), Turkey (90,000 tons, 1990) Hungary (78,000 tons, 1990)[1] and USA (115,804 tons, 1999) (USDA/NASS 1999).

Sour cherry is an allotetraploid species (*P. cerasus* L., 2n=4x=32) with sweet cherry (*P. avium* L., 2n=2x=16) and ground cherry (*P. fruticosa* Pall, 2n=4x=32) as the presumed ancestral species[2,3]. Beaver *et al.*[2] reported that sour cherry shows disomic inheritance which is characteristic of allopolyploids.

There is an increasing interest in constructing linkage maps of crop plants so that selection of DNA markers linked to a trait of interest can be used for trait selection at early stages of cultivar development[4]. Molecular marker-based linkage maps have been useful for identifying and localizing important genes controlling both qualitatively and quantitatively inherited traits in tomato[5]. DNA based markers can be used to identify related cultivars, to assess taxonomic relationships and to indirectly select tagged loci affecting qualitative and quantitative traits. They also allow breeders to follow loci during the selection process, which helps reduce time spent in backcross programs[4]. When compared to phenotypic markers, DNA based molecular markers have some advantages: they are developmentally stable, detectable in all tissues, not affected by environmental conditions and are insensitive to epistatic effects[4].

Simple Sequence Repeats (SSRs, also known as SSR) are a class of DNA markers, consisting of tandem repeats of mono-, di-, tri-, tetra-, or penta-nucleotide units that are found throughout the genomes of most eukaryotic plants[6-8]. Due to the high rate of variation in the number of repeat units, the polymorphism level shown by SSRs is high[8]. SSR are valuable markers due to their multiallelic nature, co-dominance, abundance and extensive genome coverage. They are easy to detect by PCR and require a small amount of template DNA. Plant SSRs were first isolated and cloned from tropical species[9]. On average, there is a microsatellite in every 33 kb in plant nuclear genomes whereas they are found approximately every 6 kb in mammals[10].

The isolation and sequencing of 26 SSRs were reported from two genomic libraries of peach cultivar 'Redhaven' enriched for AC/GT and AG/CT repeats, respectively. Seventeen of these SSRs showed Mendelian

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inheritance. An assay of polymorphism in 50 peach and nectarine cultivars showed that heterozygosity ranged from 0.04-0.74 with a mean of 0.47. SSR appeared for 2-8 alleles per locus[11].

Sosinski *et al.*[12] reported the identification of SSR loci in peach by screening a pUC8 genomic library and a λZAPII leaf cDNA library in addition to database searches. Their findings indicated that CT repeats occur every 100 kb, CA repeats every 420 kb and AGG repeats every 700kb in the peach genome[12].

CT repeats are present in at least one in every 100 kb in peach, as compared to one in every 120 kb in apple[13] and one in every 225 kb in rice[14]. CA repeats are less frequent (every 420 kb) in peach compared to apple (190 kb) and rice (480 kb).

Mapping in polyploids: Although a linkage map in sour cherry could provide broad potential advantages, linkage map construction in sour cherry is lagging compared to other *Prunus* species due to its polyploid origin. Construction of linkage maps in polyploids is difficult. There are large numbers of genotypes for each primer pair expected in a segregating population and these genotypes cannot always be identified by their banding patterns. Secondly, the genome constitution (allopolyploidy versus autopolyploidy) in many polyploids is not clearly understood[15]. To overcome the difficulty of mapping in polyploids, Wu *et al.*[15] proposed the use of Single Dose Restriction Fragments (SDRF). In the sour cherry mapping population, informative markers will be those that are Single Dose Restriction Fragments (SDRFs) in one or both parents [i.e., (+--- x ----), (---- x+---), or (+--- x +---), segregating 1:1, 1:1, or 3:1, respectively][15-17]. To identify SDRFs with a confidence level of 98 % in the four ploidy levels, a population size of at least 75 is needed[15].

Current status of mapping in *Prunus*: Linkage mapping was first initiated with diploid species due to the relative simplicity compared to polyploids. Linkage maps of peach[18,19], peach x almond[20], peach x *P. davidiana*[21], almond (*P. dulcis*)[22] and sweet cherry[23] were conducted. More recently, a map of peach rootstocks with (AFLP) markers[24], a linkage map of peach from an intraspecific F_2 population consisting of 249 markers[25], a saturated linkage map for *Prunus* using an almond x peach F_2 progeny[26], a linkage map of a BC1 progeny (*Prunus persica* x (*P. persica* x *P. ferganensis*)[27], a second-generation linkage map for almond using RAPD and SSR markers[28] and a linkage map using an interspecific F_2 population between almond and peach were published.

Linkage maps generated in *Prunus* species can be compared using common markers that have been placed on all *Prunus* linkage maps. Comparative mapping offers important benefits for genome analysis. Although an RFLP genetic linkage map of two tetraploid sour cherry cultivars, 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' (EB) was developed from the crosses of these two cultivars by Wang *et al.*[29], their map does not include any SSR markers. Moreover, the expanded *Prunus* genetic linkage map constructed from peach and almond covers 1,144 cM[30]. Sour cherry linkage map, being tetraploid, should be two times the length of the peach map. However the published map covers only one fourth of the expected length due the difficulty of having informative markers in tetraploids compared to diploids[29]. The objective of this study was to identify informative SSR markers and incorporate these markers onto the sour cherry map. Incorporation of informative SSR markers may lead to identification of homologous linkage groups in sour cherry that would be very valuable tool for comparative mapping studies in *Prunus*. Additionally, if mapped close to the QTL of important traits in sour cherry, these SSR markers would be very valuable tools in MAS for these traits.

MATERIALS AND METHODS

Mapping population, plant material and DNA extraction: The sour cherry mapping population is a 'pseudotestcross' in which informative markers are those that are homozygous recessive in one parent and heterozygous in the other parent and segregate 1:1[16]. Eighty-four progeny from crosses of 'Rheinische Schattenmorelle' (RS) x 'Erdi Botermo' (EB) were used as a mapping population. EB and RS were chosen as parents because they differ from each other for important horticultural traits such as fruit firmness, fruit color, pistil freeze susceptibility, cold hardiness, bloom date and fertility. Additionally, these parents originated from different geographical regions (Germany and Hungary, respectively)[29]. Young unfolded leaves were collected from trees of the mapping population located at the Clarksville Horticultural Experiment Station of Michigan State University. Leaves were frozen at –80°C overnight and lyophilized for 2-3 days. DNA isolation was conducted according to Stockinger *et al.*[23].

SSR primers: The sequence information on 45 SSR primer pairs from 'Redhaven' peach[11], apple[13] and sweet cherry[12] was used in this study. Sequences of peach primers (pchgms and pchcms series) and sweet cherry

primers (PS08E08, PS12A02, PS01H03 and PS07A02) were provided by Sosinski *et al.*[12]. Sour cherry SSR primers were derived from a small-insert genomic DNA library (A. Iezzoni, Horticulture Department, Michigan State University, East Lansing, Michigan). A full list of the primers and sequences used were provided by Canli[31].

Annealing temperature for each primer pairs: To find the optimum and highest annealing temperature for each primer, a Stratagene Robocycler with a temperature gradient was used. EB was used as the template DNA in the PCR mixture in optimization since the genomic library was constructed from this parent. The reaction and a 123 bp ladder was run on a 0.9% agarose gel to determine the highest optimum annealing temperature to reduce the change of mismatching and confirm the size of the amplified fragment. The gel was stained with ethidium bromide (0.5 mg μL^{-1}) for 15 min. and rinsed with double distilled water for one minute.

Screening primers for polymorphism using PCR: After determining the optimum annealing temperature for each primer pair, another DNA amplification reaction was set up with each primer pair and both parents and 12 progeny to find the primers that identify segregating fragments. Five μL of the PCR products was first run on a 0.9% agorose gel to verify amplification. To identify the presence of polymorphism, 4 μL of each remaining reaction was run on a 4% polyacrylamide gel and the bands was detected by using the DNA silver staining protocol of Promega (Promega Corporation, Madison, WI).

PCR with informative markers: After identifying SSR primers that were polymorphic, another DNA amplification reaction was conducted on the remaining progeny in the mapping population as follows; 1X PCR buffer, 0.2 mM of dNTP's, 2.5 mM of $MgCl_2$, 50 ng DNA, 0.6 unit TAQ DNA polymerase enzyme(Boehringer Mannheim Biochemicals) and ddH_2O was added to a volume of 25 μL. DNA amplification reactions were performed in a thermocycler (model 9600; Perkin Elmer Applied Biosystems, Inc., Foster City, California). The amplification products were separated by electrophoresis for 2.5 h at 80 W on a 6% polyacrylamide sequencing gel (Bio-Rad), then silver stained with sequence staining kit by Promega and sizes were estimated using a 10 bp ladder (Gibco BRL).

Scoring, X^2 analysis and map construction: The primers, which showed polymorphisms based on size in the polyacrylamide gel, were scored for the absence or presence of a band in the mapping population.

In the mapping population, informative markers are those that are SDRFs in one or both parents [i.e.,(+---x----), (----x+---), or (+---x+---), segregating 1:1, 1:1, or 3:1, respectively][15-17]. Fragments which differed between both parents were tested for fit to a 1:1 (presence:absence) ratio. Fragments which are present in both parents were tested for fit to a 3:1 (presence:absence) ratio. Those markers, which fitted the appropriate ratios at the 5% level, were used in linkage analysis. The SSR data of 84 progenies was added to the previously constructed RFLP data[29]. A linkage map was generated from the RFLP and SSR data with JOINMAP V2.0[32].

RESULTS AND DISCUSSION

Forty-five SSR primer pairs were tested to find informative markers in sour cherry. Ten (22 %) of these primer pairs were informative (Table 1), yielding 17 SDRF. This is comparable to the results in apricot where out of 45 SSR screened, 13 (28%) loci were mapped[33]. A second generation consensus linkage map of two tetraploid sour cherry cultivars, 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' was constructed (Fig. 1) by the addition of new SSR markers to a map previously constructed by Wang *et al.*[29].

The revised EB and RS consensus map (Fig. 1) consisted of 161 markers covering 442.4 cM in 19 linkage groups. Forty-nine markers remained unlinked. The largest linkage group is group 9 covering 44.5 cM. The shortest linkage group is group 19 covering 5.7 cM. The average distance between two markers is 2.74 cM. The longest distance between two adjacent markers is 16.9 cM in linkage group 8 (Fig. 1). The previous EB and RS consensus map[29] consisted of 144 SDRF in 16 groups covering 443.1 cM. The incorporation of 17 new markers did not change the length of the previous map significantly. The average distance (cM/marker) between two loci decreased from 3.07 to 2.74 cM in the revised map. Marker order in the new map was mostly conserved when compared to the previous EB and RS consensus map.

The linkage groups in the previous sour cherry map[29] and in the revised map were numbered according to suspected homology to the almond x peach map[30] and the almond map[29]. Six linkage groups in sour cherry share two or more common markers with the corresponding linkage groups in the almond x peach map and in the almond map. These results suggest that these six linkage groups of sour cherry might be homologous to the corresponding linkage groups in the almond x peach map and the almond map. These conclusions about the homology relations are preliminary until more common markers are incorporated to these maps.

Table 1: Segregation ratios and product sizes of informative SSR primers in sour cherry mapping population from 'Rheinische Schattenmorelle' (RS) x 'Erdi Botermo' (EB)

Primer	Plant source	Reference	Product size	RS	EB	Ratio
GA34 (PceGA34)	Sour cherry	Downey and Iezzoni[34]	184	-	+	
			175	+	-	1:1
			170	+	-	1:1
			161	+	-	1:1
			143	+	+	
PS12A02	Sweet cherry	Sosinski *et al.*[12]	178	-	+	1:1
			167	-	+	1:1
			162	+	-	1:1
			160	+	+	
			148	-	+	1:1
PS08E08	Sweet cherry	Sosinski *et al.*[12]	188	-	+	1:1
			184	+	+	
			175	+	+	
Pchgms3	Peach	Sosinski *et al.*[12]	189	-	+	1:1
			182	+	+	
			178	+	+	3:1
			174	+	-	1:1
GA25 (PceGA25)	Sour cherry		199	-	+	1:1
			187	-	+	1:1
			174	+	+	3:1
			162	+	+	
UDP96-008	Peach	Testolin *et al.*[11]	158	+	-	
			155	+	+	
			148	-	+	
			139	-	+	1:1
			135	+	+	
			128	+	-	
UDP98-405	Peach	Testolin *et al.*[11]	112	+	-	1:1
			105	+	+	
			103	-	+	
			100	+	+	
			97	-	+	
UDP98-22	Peach	Testolin *et al.*[11]	104	+	-	1:1
			98	+	-	1:1
			90	+	+	
UDP98-410	Peach	Testolin *et al.*[11]	139	+	-	1:1
			134	+	-	2:1
			131	-	+	
UDP98-411	Peach	Testolin *et al.*[11]	164	-	+	1:2
			154	+	+	3:1
			150	+	+	
			131	+	+	3:1

- = absence of a band, + = presence of a band

Table 2: QTL detected for flower and fruit traits in sour cherry cultivars 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' (EB) by Wang *et al.*[29] and SSR markers incorporated to these QTL locations in current study

Trait	QTL	Linkage group	R^2	Nearest RFLP maker(s)	Nearest SSR marker	SSR distance to RFLP marker
Bloom time	Blm1	EB1	19.9	PS141	pchgms3-189	10.5cM
	Blm2	EB and RS2	22.3	PLG86	UDP411-154	2.3cM
					UDP411-131	4.5cM
Pistil death (%)	Pd1	EB1	12.9	EF194c	pchgms3-189	1.5cM
	Pd2	RS8	14.3	EF156b		
Pollen germination(%)	Pgr	EB1	17.0	EF146		
Ripening time	Rp1	RS4	21.5	EF158b		
	Rp2	EB and RS6	25.9	CPM20e		
Fruit weight (g)	Fw1	EB4	13.7	EF182a		
	Fw2	EB and RS 2	15.5	PLG86	UDP411-154	2.3cM
					UDP411- 131	4.5cM
Soluble solids Concentration	Ssc1	EB7	16.5	AG10b	UDP405-112	11.5cM
	Ssc2	RS6	13.1	EF159a		

R^2 = amount of phenotypic variance explained by QTL (Coefficient of determination).

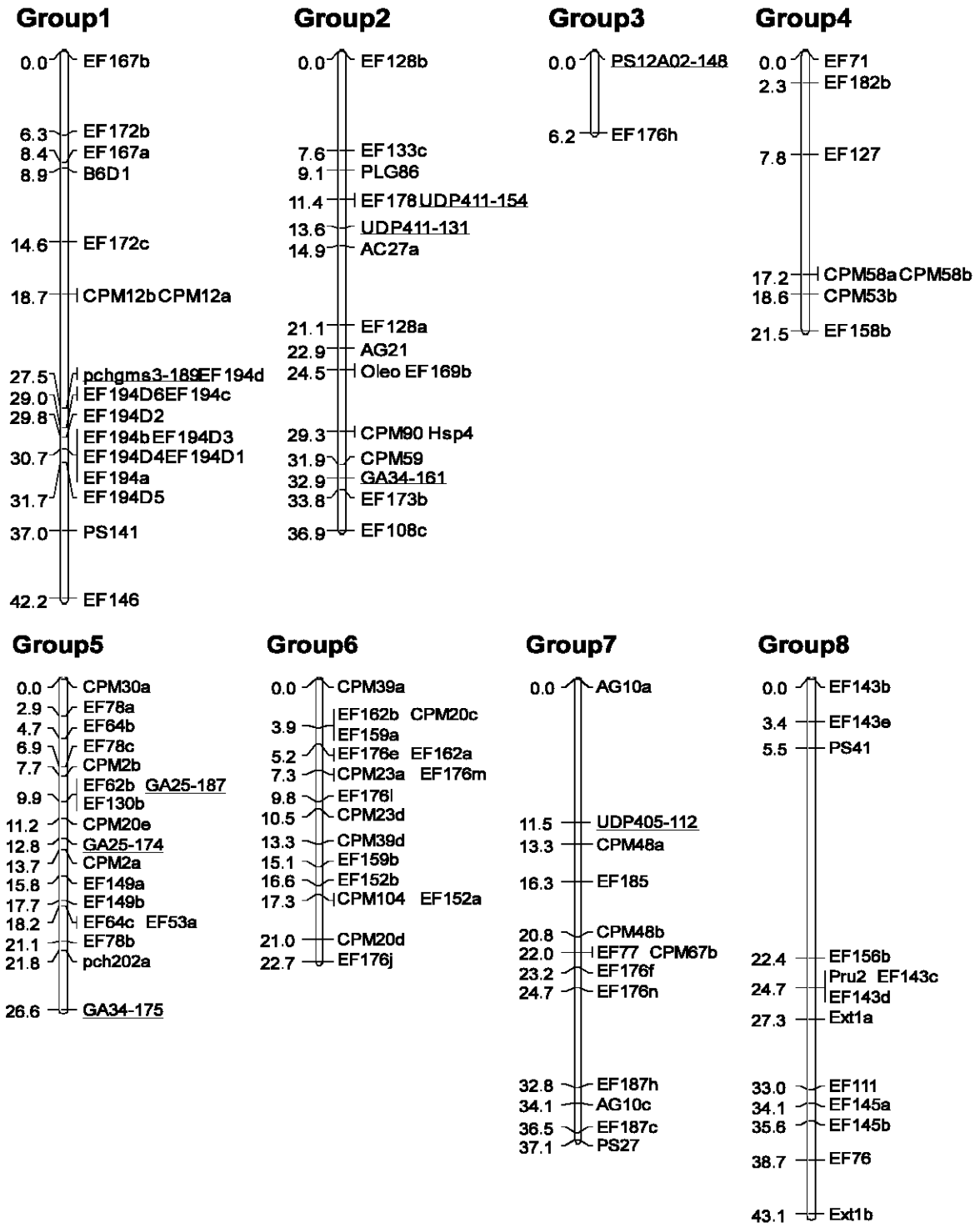

Fig. 1: The consensus map of two sour cherry cultivars, 'Rheinische Schattenmorelle' (RS) and 'Erdi Botermo' (EB), constructed from combined data of RFLP and SSR markers using JoinMap with a minimum LOD of 3.0 and a maximum recombination frequency of 0.35. SSR markers are underlined

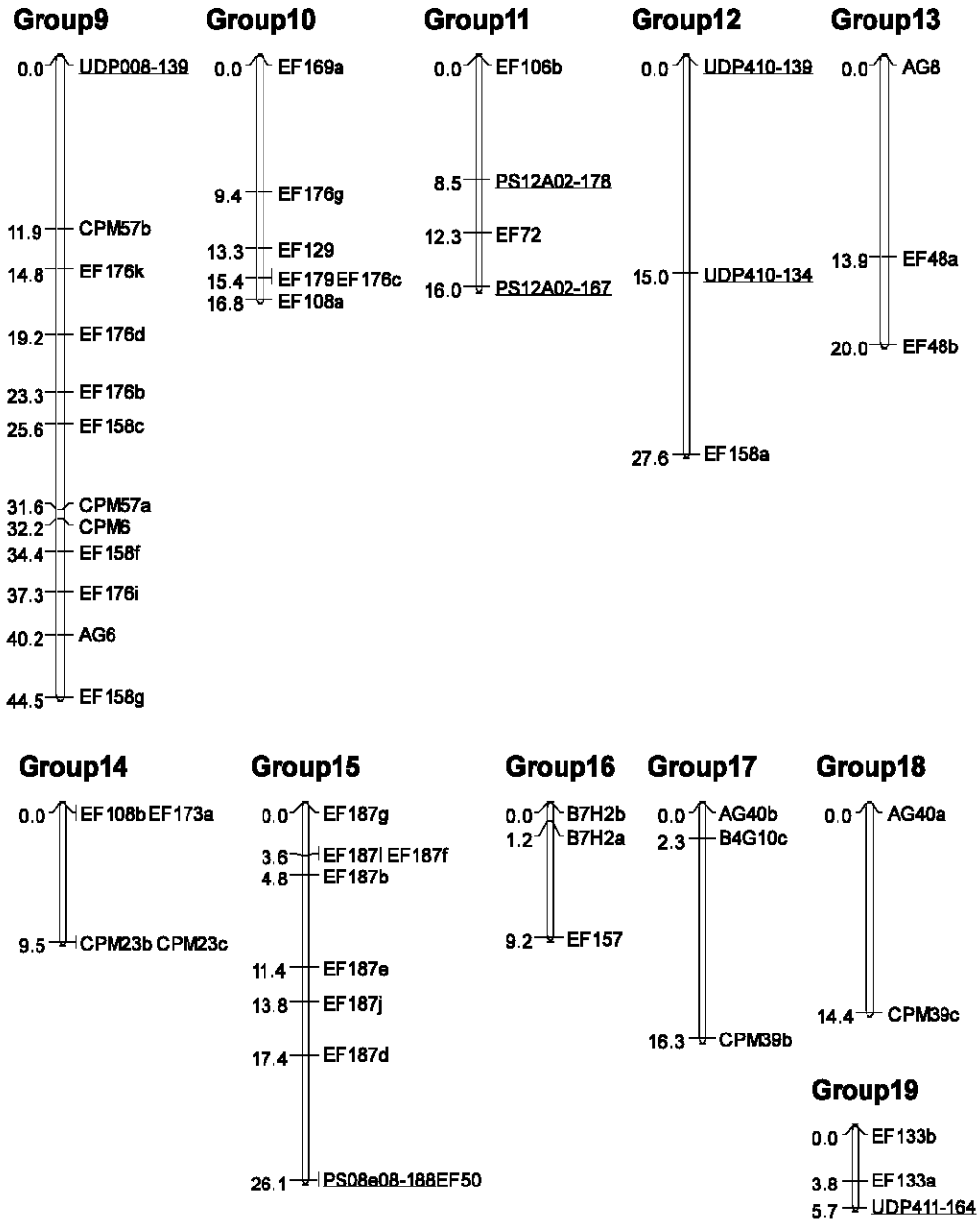

Fig. 1: Continue

QTL locations for six fruit and flower traits were detected by Wang *et al.*[29]. In the current study, SSR markers were mapped to the locations of QTL detected earlier (Table 2). Two peach SSR markers UDP411-154 and UDP411-131 (Fig. 2) were linked to the bloom time (Blm_2) location, at the distances of 2.3 and 4.5 cM, respectively (Table 2). The same peach markers are also tightly linked to fruit weight QTL (Fw2), at the distances of 2.3 and 4.5 cM, respectively (Table 2). The pchgms3-189 marker was mapped to 10.5 cM of the PS141 which located in bloom time (blm1) area. The same marker is also tightly linked to EF194c marker at a distance of 1.5 cM which is located in pistil death (Pd_1) area (Table 2). UDP405-112 marker mapped 11.5 cM distance of the AG10b marker which is the closest marker to soluble solids concentration (Ssc_2) QTL location (Table 2). SSR markers obtained are horticulturally very important. Being tightly linked to important traits and highly polymorphic, these SSR markers will be utilized for breeding for these traits saving considerable time and resources. A negative

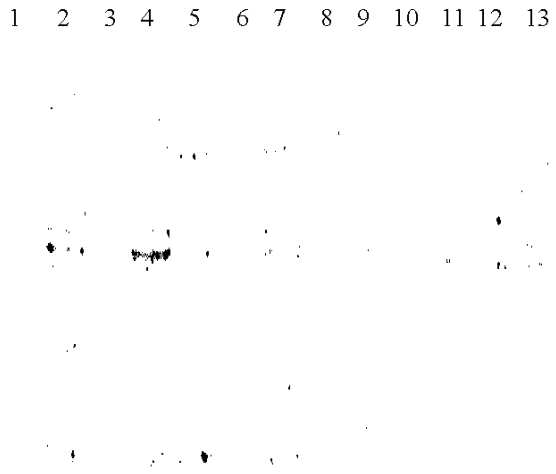

Fig. 2: DNA fragment patterns of the SSR primer UDP411. Arrows indicates two segregating fragments which were mapped to bloom time 2 (blm2) location. The upper arrow indicates a 154 bp fragment named UDP411-154 and the lower arrow indicates a 131 bp fragment named UDP411-131. Lanes 1-13 are progenies from 'Rheinische Schattenmorelle' (RS) x 'Erdi Botermo' (EB) population

correlation was found between bloom time and percent pistil death (r = - 0.25) and also a negative correlations exits between bloom time and fruit weight (r = - 0.45)[29]. The existence of correlation between these traits further increases the value of these markers enabling breeder to select more than one trait at the same time.

The expanded *Prunus* genetic linkage map constructed from peach and almond covers 1144 cM[30]. The sour cherry linkage map, being tetraploid (2n=4x =32), should have 16 linkage groups covering two times of the length of the peach map. Mapping has a drawback in sour cherry due to the requirement for SDRF in a tetraploid state, which limits the availability of informative markers. However, new SSR primer pairs were recently published by Aranzana *et al.*[35], Dirlewanger *et al.*[36] and Wang *et al.*[37]. Incorporation of new markers should extend the current sour cherry map and bring the linkage group number down to 16 groups.

The results in this study clearly indicate that SSR developed in other *Prunus* species have good utility in sour cherry and are transportable into *Prunus* species. SSR are distributed throughout the sour cherry genome. Having cross-species amplification, they are highly useful for comparative mapping analysis. Further incorporation of currently unavailable SSR loci into sour cherry map will likely provide an excellent source for identification of homologous linkage groups in sour cherry[29]. With the availability of more SSR markers and an increased number of common SSR loci mapped in *Prunus* species, it should be possible to identify homologous areas and regions of translocations, insertions, or deletions. Such data would provide information on gene order conservation in *Prunus* and the family Rosaceae.

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