Variability and inheritance of peach SSR sequences in almond genotypes

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SUMMARY – Variability and inheritance of six peach [*Prunus persica* (L.) Batsch] SSR (simple-sequence repeat) markers was studied in 21 almond [*P. dulcis* (Mill.) D.A. Webb] cultivars and in a progeny of 180 descendants from the cross between the French cultivar 'R1000' and the Spanish cultivar 'Desmayo Largueta'. The results showed the molecular identification of the 21 cultivars using the six SSR polymorphic sequences. The number of alleles revealed by the SSR analysis ranged from 4 to 13, with a high level of heterozygosity. A codominant segregation of the SSRs was observed in this progeny. These results establish the value of SSR markers for distinguishing different genetic lineages and characterize the extensive gene pool available for almond breeding programs. The application of these SSR sequences in the development of markers associated with genes or QTLs involved in the inheritance of agronomic traits in almond is also discussed.

Key words: Almond, *Prunus dulcis*, molecular markers, microsatellites, SSR, breeding.

RESUME – *"Variabilité et hérédité des séquences SSR de pêcher dans les génotypes d'amandier". Au cours de ce travail nous avons étudié la variabilité et l'hérédité de six séquences SSR de pêcher [*Prunus persica *(L.) Batsch] dans 21 variétés d'amandier [*P. dulcis *(Mill.) D.A. Webb] et dans une génération de 180 descendants issus du croisement entre la variété française 'R1000' et la variété espagnole 'Desmayo Largueta'. Les résultats ont montré l'identification des 21 variétés avec les six SSRs étudiés. Le nombre d'allèles observés a varié entre 4 et 13, avec une grande hétérozygotie de ces loci. Nous avons observé une ségrégation codominante dans la génération étudiée. Les résultats ont montré la grande valeur des marqueurs moléculaires de tipe SSR pour l'identification variétale et la caractérisation de la grande variabilité génétique disponible pour les programmes d'amélioration de l'amandier. Nous avons discuté l'application de ces SSRs pour le développement de marqueurs moléculaires associés aux caractéristiques agronomiques.*

Mots-clés : *Amandier*, Prunus dulcis*, marqueurs moléculaires, micro-satellites, SSR, amélioration végétale.*

Introduction

Simple-sequence repeat sequences (i.e. microsatellites, SSR markers) (Tautz, 1989) are very good genetic markers for genetic diversity studies in a wide range of plants. Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic diversity within crop species (Gupta *et al.*, 1996). In the case of *Prunus* species, primer pairs flanking SSRs have been cloned and sequenced in peach (Cipriani *et al.*, 1999; Downey and Iezzoni, 2000; Sosinski *et al.*, 2000; Testolin *et al.*, 2000; Dirlewanger *et al.*, 2002b; Georgi *et al.*, 2002; Wang *et al.*, 2002; Aranzana *et al.*, 2002; 2003a; Yamamoto *et al.* 2002) and cherry (Downey and Iezzoni, 2000; Cantini *et al.*, 2001).

These SSR markers have been used for the molecular characterization and identification of cultivars in different species including peach (Downey and Iezzoni, 2000; Testolin *et al.*, 2000; Dirlewanger *et al.*, 2002b; Martínez-Gómez *et al.*, 2003; Aranzana *et al.*, 2002; 2003a), almond (Joobeur *et al.*, 2000; Martínez-Gómez *et al.*, 2003a), and cherry (Downey and Iezzoni, 2000; Cantini *et al.*, 2001; Dirlewanger *et al.*, 2002b; Wünsch and Hormaza, 2002). In addition, several *Prunus* rootstocks have been characterized using these SSR markers (Serrano *et al.*, 2002). SSR markers were used also for genetic mapping in peach (Sosinski *et al.*, 2000; Etienne *et al.*, 2002; Aranzana *et al.*, 2003b), almond (Joobeur *et al.*, 2000; Bliss *et al.*, 2002), and apricot (Hurtado *et al.*, 2002).

The objective of this work was to estimate the variability and inheritance of 6 peach SSR sequences in 21 almond cultivars and an almond progeny of 180 seedlings.

Materials and methods

Plant material

The plant material assayed included 21 almond cultivars from different origins, representing the genetic diversity of the species (Table 1). In addition, an almond progeny of 180 seedlings from the cross between the French cultivar 'R1000' and the Spanish cultivar 'Desmayo Largueta' was studied. This progeny was created by Dr. H. Duval (INRA, Avignon, France) in 1997 and planted in an experimental field in Santomera (Murcia, Spain) in 1998.

Table 1. Origin, pedigree, and main agronomic characteristics (shell hardness, self-compatibility, and flowering date) of the almond genotypes assayed

Cultivar	Origin	Pedigree	Shell hardness	Self-compatibility†	Flowering date
'Achaak'	Tunisia	Unknown	Semi-hard	SI	Very early
'All in one'	USA	Almond x peach	Soft	SC	Intermediate
'Antoñeta'	Spain	'Ferragnès' x 'Tuono'	Hard	SC	Late
'Ardechoise'	France	Unknown	Soft	SI	Intermediate
'Bonita'	Portugal	Unknown	Hard	SI	Intermediate
'Chellaston'	Australia	Unknown	Semi-hard	SI	Late
'Desmayo L.'	Spain	Unknown	Hard	SI	Very early
'Ferragnès'	France	'Cristomorto' x 'Aï'	Hard	SI	Late
'Garrigues'	Spain	Unknown	Hard	SI	Early
'Genco'	Italy	Unknown	Hard	SC	Late
'Glorieta'	Spain	'Primorskii' x 'Cristomorto'	Hard	SI	Late
'Guara'	Spain	Unknown	Hard	SC	Late
'Lauranne'	France	'Ferragnès' x 'Tuono'	Hard	SC	Late
'Marcona'	Spain	Unknown	Hard	SI	Intermediate
'Marta'	Spain	'Ferragnès' x 'Tuono'	Hard	SC	Late
'Masbovera'	Spain	'Primorskii' x 'Cristomorto'	Hard	SI	Late
'Mission'	USA	Unknown	Semi-hard	SI	Intermediate
'Nonpareil'	USA	Unknown	Soft	SI	Intermediate
'Primorskii'	Ukraine	'Princesse 2077' x 'Nikitski 53'	Soft	SI	Late
'R-1000'	France	'Tardy Nonpareil' x 'Tuono'	Hard	SC	Very late
'Tuono'	Italy	Unknown	Hard	SC	Late

†SC: Self-compatible; SI: Self-incompatible.

DNA isolation

Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987), with minor changes. Approximately 50 mg of young leaves were ground in a 1.5-ml Eppendorf tube with 750 µl of CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% $NaHSO_3$). Samples were incubated at 65ºC for 20 min, mixed with an equal volume of 24:1 chloroform-isoamyl alcohol, and centrifuged at 6000 g for 20 min. The upper phase was recovered and mixed with an equal volume of isopropanol at -20°C. The nucleic acid precipitated was washed in 400 µl of 10 mM NH_4Ac in 76% ethanol, dried, resuspended in 50 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and incubated with 0.5µg of RNase A at 37ºC for 30 min, to digest RNA.

PCR amplification and product electrophoresis

Extracted almond genomic DNA was PCR-amplified using six pair primers flanking SSR sequences, previously cloned and sequenced in peach (Table 2). Primers were synthesized by Invitrogen™ Life Technologies. PCR reactions were performed in a 25-µl volume and the reaction mixture contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.1 mM of each dNTP, one unit of *Taq* DNA Polymerase (Ecogen S.R.L.), and 90 ng of genomic DNA. The cycling parameters were: one cycle of 95°C for 3 min.; 35 cycles of 94°C for one min., 57°C for one minute, except for the SSR primer CPPCT 022, for which the annealing temperature was 50°C, and 72°C for two min; followed by 10 min at 72°C. The PCR reactions were carried out in a 96-well block Eppendorf Mastercycler Gradient. Amplified PCR products were analyzed by capillary electrophoresis with the automatic sequencer ABI/Prism 310 (PE/Applied Bisystems).

Table 2. Peach simple sequence repeat (SSR) sequences assayed and polymorphism obtained in the almond cultivars. H = Heterozygosity of each SSR marker

SSR marker	Reference	Linkage group†	SSR repeat motif in peach	H	No. of alleles	Size range (bp)	T annealing (ºC)
pchgms3	Sosinski *et al.,* 2000	G1	$(CT)_{14}$	0.89	10	166-195	59
BPPCT 007	Dirlewanger *et al.,* 2002b	G3	$(AG)_{22}(CG)_2(AG)_4$	0.90	4	124-158	57
CPPCT 005	Aranzana *et al.,* 2002	G4	$(CT)_{25}$	0.88	5	122-158	52
MA27a	Yamamoto *et al.,* 2002	G6	$(GA)_{28}$	0.76	13	113-146	55
MA40a	Yamamoto *et al.,* 2002	G6	$(GA)_{17}$	0.66	12	196-257	55
UDP 96005	Cipriani *et al.,* 1999	G1	$(AC)_{16}TG(CT)_2CA(CT)_{11}$	0.61	9	125-167	57

†Linkage group according to Aranzana *et al.* (2003b), or estimated by comparison with other maps containing common markers.

Data analysis

Polymorphic alleles were scored as present or absent (0/1). Band scoring was analyzed using the GeneTools gel analysis software of SYNGENE (Beacon House, Nuffield Road, Cambridge, UK). Heterozygosity of each SSR marker was calculated as the number of heterozygous genotypes divided by the total number of genotypes. In addition, heterozygosity of almond cultivars was calculated as the number of heterozygous loci for a given cultivar divided by the total number of loci assayed.

Results and discussion

Polymorphism and heterozygosity of SSR markers

Amplification was successful with the six peach SSR sequences assayed, with a total of 53 polymorphic bands scored. The number of presumed alleles revealed by the SSR analysis ranged from 4 to 13. The mean number of alleles per locus was 8.8. A larger size range of amplified bands was observed commonly in the almond cultivars and breeding lines assayed, between 113 bp and 257 bp. Direct-count heterozygosities ranged from 0.61, for the SSR marker UDP 96005, to 0.90, for BPPCT 007, with an average value of 0.78 for all the loci studied (Table 2).

Amplification was successful in almond for all markers initially developed for peach. Overall, the results showed a high degree of homology for the SSR loci between peach and almond, and the transportability of these markers. These results agree with reports by Cipriani *et al.* (1999), Sosinski *et al.* (2000), Testolin *et al.* (2000), Cantini *et al.* (2001), Decroocq *et al.* (2003), and Martínez-Gómez *et al.* (2003) of the successful utilization of these markers in different *Prunus* species. The similar order of SSR markers observed in different *Prunus* maps suggests a high level of synteny within this genus (Dirlewanger *et al.* 2002a; Aranzana *et al.* 2003b). This homology among *Prunus* species partly

explains the low level of breeding barriers to interspecific gene introgression and highlights the opportunity for successful gene transfer between closely-related species.

Heterozygosity of almond cultivars

Heterozygosities of almond genotypes ranged between 0.33, for 'Bonita', 'Garrigues' and 'Glorieta', and 1, for 'Achaak', 'All in one', 'Antoñeta', 'Genco', 'Guara', and 'Primorskii', with an average value of 0.72 for all the genotypes studied (Table 3).

Table 3. Heterozygosity level (H) of the almond cultivars

Cultivar	H	Cultivar	H
'Achaak'	1.00	'Guara'	1.00
'All in one'	1.00	'Lauranne'	0.83
'Antoñeta'	1.00	'Marcona'	0.80
'Ardechoise'	0.50	'Marta'	0.67
'Bonita'	0.33	'Masbovera'	0.50
'Chellastone'	0.83	'Mission'	0.50
'Desmayo L.'	0.80	'Nonpareil'	0.80
'Ferragnès'	0.50	'Primorskii'	1.00
'Garrigues'	0.33	'R-1000'	0.80
'Genco'	1.00	'Tuono'	0.67
'Glorieta'	0.33		

The results showed the higher level of heterozygosity in almond cultivars in comparison with the results obtained in peach cultivars (0.22) by Martínez-Gómez *et al.* (2003). The level of heterozygosity described by Martínez-Gómez *et al.* (2003) in a wide range of almond cultivars was 0.56, on average, for all the genotypes studied. The higher heterozygosity for almond relative to peach can be attributed to mating-system differences within each of these two species, with peach being self-compatible in the majority of cultivars, and almond normally being self-incompatible and thus outcrossing. These results confirm the narrow genetic base observed in peach when compared to the great diversity observed in the almond cultivars. Higher levels of heterozygosity for almond relative to peach had been observed previously when isozyme variability was evaluated by Arulsekar *et al.* (1986).

Inheritance of peach SSR markers in almond

The results showed the codominant segregation of the SSR markers assayed in the progeny from the cross between 'R1000' and 'Desmayo Largueta'. MA27 SSR marker segregation ('R1000' *ab*; 'Desmayo Largueta' *cd*) is close to 25% of each different expected genotype (*ac*, *ad*, *bc*, *bd*). In addition, UDP 96005 SSR marker segregation ('Desmayo Largueta' *ab*; 'R1000' *cc*) is close to 50% of each different expected genotype (*ac*, *bc*) (Table 4). These results confirm the codominant inheritance of these markers indicated by Tautz (1989) and described by Gupta *et al.* (1996) in a wide rage of plant species.

Utilization of peach SSR markers in almond breeding

These results establish the value of SSR markers for distinguishing different genetic lineages and characterize an extensive gene pool available for almond breeding programs, because of their high polymorphism, abundance, and codominant inheritance.

Table 4. Inheritance of SSR peach sequences in an almond progeny

SSR marker	Parents	SSR allele sizes	SSR segregation†			
			a c	*a d*	*b c*	*b d*
MA27a	'R-1000'	*a* = 119 bp, *b* = 133 bp				
	'Desmayo L.'	*c* = 137 bp, *d* = 146 bp	19	28	22	31
UDP96005	'Desmayo L.'	*a* = 125 bp, *b* = 141 bp				
	'R-1000'	*c* = 155 bp, *c* = 155 bp	53	----	47	----

†Percentage of seedlings with each SSR genotype.

Another application of these SSR sequences could be the application in the development of markers associated to agronomic traits in almond. The application of SSRs for Marker Assisted Selection was described by Testolin (2002), who indicated the possibility of find SSR near the encoding genome sequences. Further studies of SSR markers in progenies segregating for agronomic traits could be very interesting in the development of markers associated with genes or QTLs involved in the inheritance of agronomic traits.

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