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A practical method for almond cultivar identification and parental analysis using simple sequence repeat markers

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Abstract Early and accurate identification of almond [*Prunus dulcis* (Mill.) D.A. Webb] cultivars is critical to commercial growers and nurseries. Previously published simple sequence repeat loci were examined for their ability to distinguish commonly grown almond cultivars. Twelve highly polymorphic loci were selected for their ability to uniquely identify a set of 18 almond cultivars commonly grown in California, many of which are closely related. These markers also allow an accurate assessment of parent/progeny relationships among cultivars. This system can reliably identify at an early stage of development all major California almond cultivars in current production.

Keywords *Prunus dulcis* · Microsatellite markers · DNA fingerprinting · Foundation Plant Services (FPS)

Introduction

Almonds are California's largest tree nut crop and the state produces over 80% of the world's supply. Total almond production in California was a record 1.47 billion pounds in 2007/2008, a 24% increase over the previous year (ABC 2008). The consistent high demand for California almonds has been met by an increase in acreage planted over each of the past 10 years. In 2007, the estimated bearing acreage was 615,000 (United States Department of Food and Agriculture 2007). The bulk of California almonds are produced by a small number of elite cultivars; 'Nonpareil' alone produces nearly 40% of the California crop with most remaining cultivars being cross-compatible pollinizers for this self-sterile crop species. In the diploid almond, self-sterility is controlled by a single major (S-) locus, where haploid pollen containing an S-allele in common with either self or cross-pollinated pistils will result in failure of pollen growth to fertilization. Consequently, over 30% of the remaining production is from only four cultivars: 'Carmel', 'Butte', 'Monterey' and 'Padre' (ABC 2008) which are all fully cross-compatible with 'Nonpareil' and, with the exception of the intersterile 'Butte' and 'Monterey' combination, are inter-compatible with each other (Barckley et al. 2006). With substantial new plantings each year of proven inter-compatible cultivars, correct cultivar identification is critical to the continuing success of the industry. However, cultivar identification using morphological characteristics is difficult because trees are planted before distinguishing traits develop.

Simple sequence repeats (SSRs) are used widely for cultivar identification of other woody, clonally

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propagated crops such as grape and walnut (Dangl et al. 2001; Dangl et al. 2005). Foundation Plant Services (FPS) is a service department based in the College of agriculture and environmental sciences at the University of California, Davis. Its mission is to produce, test, maintain, and distribute disease-tested propagation material for use by nurseries and growers throughout the US and worldwide. FPS houses and maintains the foundation collections for the California Department of Food and Agriculture's (CDFA) registration and certification programs for grapevines, deciduous fruit and nut trees, and strawberries. FPS stock qualifies and serves as primary foundation source material for commercial increase for entire industries. Proper identification of cultivars is a critical aspect of FPS's mission.

A major benefit of using DNA markers is that trees can be identified at any developmental stage. SSR markers have been developed for *Prunus*, including almonds (Martínez-Gómez et al. 2003; Mnejja et al. 2005; Wünsch and Hormaza 2002). However, those reports did not describe streamlined protocols and markers specifically screened for efficient almond cultivar identification. In particular, they did not publish specific allelic data to facilitate development of a universal database of almond SSR marker profiles.

Here we describe an SSR marker system that distinguishes among all commercially important almond cultivars presently grown in California. The small study set contains many closely related cultivars, a particular challenge for a DNA marker-based identification system. The profiles published here uniquely identify all almond cultivars represented in the collection presently maintained by FPS. The procedures presented can be expected to distinguish among all other almond cultivars, and represents a practical system for almond cultivar identification at any developmental stage.

Materials and methods

Multiple plants of 21 almond cultivars were selected for study (Table 1). Tree leaf samples were from the almond collection at FPS with additional samples from commercial sources and the UC Davis Wolfskill Experimental Orchard included as checks. Young, non-fully expanded leaves were collected and rapidly dried at room temperature using chemical desiccant (Bautista et al. 2008). DNA extraction, PCR, fragment separation, and sizing of amplified fragments were performed according to Bautista et al. (2008) except for multiplex PCR, in which case 0.15 pmol ul^{-1} of both forward and reverse primers for each of three primer pairs was used.

Results and discussion

An initial set of 14 representative almond cultivars was used to test 53 previously published primer pairs sequenced from several *Prunus* species for their

Table 1 Almond cultivars used in this study

Accession	Source	Accession	Source
'ALDRICH'	FPS, CN1	'PADRE'	FPS, CN1
'BUTTE'	FPS, CN1	'PEERLESS'	FPS, CN1
'CARMEL'	FPS, CN1	'PRICE'	FPS
'FRITZ'	FPS, CN1	'RUBY'	FPS, CN1
'KAPAREIL'	FPS	'SOLONO'[a]	CN2, WEO
'KOCHI'	FPS	'SONORA'	FPS, CN1
'MISSION'	FPS, CN1	'SWEETHEART'	FPS
'MONTEREY'	FPS, CN1	'THOMPSON'[a]	CN2, CO2, WEO
'NE PLUS ULTRA'	FPS	'TITAN'	FPS
'NONPAREIL'	FPS, CN1	'WINTERS'	FPS
'NORMAN'[a]	CO1		

FPS Foundation Plant Services, U.C. Davis, *CN1* commercial nursery 1, *CN2* commercial nursery 2, *C01* commercial orchard 1, *CO2* commercial orchard 2, *WEO* Wolfskill Experimental Orchard, U.C. Davis

[a] Three cultivars tested at nine loci

ability to consistently amplify polymorphic fragments (Table 2). These primer pairs are described in those original publications as sequences flanking SSR loci. Here, we use the term "locus" to designate the portion of DNA amplified by a particular primer pair and refer to amplified fragments as alleles, though we did not re-sequence the amplified fragments in almond.

Based on the initial screen of 14 almond cultivars, 29 loci were eliminated from further analysis for various reasons (Table 3). Alleles could not be scored for 18 primer pairs: ten primer pairs failed to amplify fragments, six amplified apparently random fragments, and two amplified multiple loci. An additional 11 loci had alleles that could be scored, but did not provide sufficiently useful information to include in the final data set. Four of these were monomorphic for all 14 cultivars in the screen and three had extremely low polymorphism, typically resulting from the presence of one very high frequency allele. Three loci were difficult to score accurately using automated systems due to poor amplification, the presence of single base pair differences in allele lengths and stuttering of the primary fragment. At one locus, all 14 cultivars in the screen were homozygous, resulting in very low polymorphism and suggesting the presence of high frequency null alleles.

Twenty-four loci were selected for further testing: all 12 loci from Table 4 and the 12 loci marked "in data set" from Table 3. These 24 loci reproducibly amplified alleles that behaved as a single Mendelian locus: there were only one or two alleles for a given almond cultivar and these alleles were inherited in a fashion consistent with published pedigrees. The locus BPPCT 038 showed artifacts, however, these were easily distinguished by the analysis software and Mendelian alleles were scored. Reliable polymorphic data at the selected 24 loci were obtained for 18 almond cultivars, including all almond cultivars at FPS (Supplemental data). (Table Supplemental).

Each primer pair was tested under only one standard set of PCR conditions. More primers pairs might have produced useful results under different PCR conditions. However, our goal was to develop a practical forensic "DNA fingerprinting" method to uniquely characterize all almond cultivars and use this method to confirm the identity of each almond tree in the FPS foundation blocks. Adoption of a

Table 2 Origin and citations for tested loci

Locus	Origin	Reference
AMPA100	Apricot	Hagen et al. 2004
AMPA105	Apricot	Hagen et al. 2004
AMPA118	Apricot	Hagen et al. 2004
ssrPaCITA10	Apricot	Lopes et al. 2002
ssrPaCITA12	Apricot	Lopes et al. 2002
ssrPaCITA14B	Apricot	Lopes et al. 2002
ssrPaCITA15	Apricot	Lopes et al. 2002
ssrPaCITA18	Apricot	Lopes et al. 2002
ssrPaCITA19	Apricot	Lopes et al. 2002
ssrPaCITA2	Apricot	Lopes et al. 2002
ssrPaCITA23	Apricot	Lopes et al. 2002
ssrPaCITA25	Apricot	Lopes et al. 2002
ssrPaCITA27	Apricot	Lopes et al. 2002
ssrPaCITA4	Apricot	Lopes et al. 2002
ssrPaCITA7	Apricot	Lopes et al. 2002
UDAp-410	Apricot	Messina et al. 2004
UDAp-411	Apricot	Messina et al. 2004
UDAp-419	Apricot	Messina et al. 2004
UDAp-420	Apricot	Messina et al. 2004
UDP96-001	Peach	Cipriani et al. 1999
UDP96-003	Peach	Cipriani et al. 1999
UDP96-005	Peach	Cipriani et al. 1999
UDP98-407	Peach	Cipriani et al. 1999
UDP98-409	Peach	Cipriani et al. 1999
BPPCT 002	Peach	Dirlewanger et al. 2002
BPPCT 004	Peach	Dirlewanger et al. 2002
BPPCT 006	Peach	Dirlewanger et al. 2002
BPPCT 014	Peach	Dirlewanger et al. 2002
BPPCT 017	Peach	Dirlewanger et al. 2002
BPPCT 034	Peach	Dirlewanger et al. 2002
BPPCT 038	Peach	Dirlewanger et al. 2002
BPPCT 039	Peach	Dirlewanger et al. 2002
BPPCT 040	Peach	Dirlewanger et al. 2002
BPPCT 042	Peach	Dirlewanger et al. 2002
pchgms1	Peach	Sosinski et al. 2000
pchgms3	Peach	Sosinski et al. 2000
MA012a	Peach	Yamamoto et al. 2002
MA015a	Peach	Yamamoto et al. 2002
MA017a	Peach	Yamamoto et al. 2002
MA023a	Peach	Yamamoto et al. 2002
MA024a	Peach	Yamamoto et al. 2002
MA027a	Peach	Yamamoto et al. 2002
MA034a	Peach	Yamamoto et al. 2002
MA035a	Peach	Yamamoto et al. 2002

Table 2 continued

Locus	Origin	Reference
MA040a	Peach	Yamamoto et al. 2002
CPSCT012	Plum	Mnejja et al. 2004
CPSCT026	Plum	Mnejja et al. 2004
CPSCT042	Plum	Mnejja et al. 2004
EMPA015	Sweet cherry	Clarke and Tobutt 2003
EMPA018	Sweet cherry	Clarke and Tobutt 2003
EMPaS06	Sweet cherry	Vaughan and Russell 2004
EMPaS10	Sweet cherry	Vaughan and Russell 2004
EMPaS12	Sweet cherry	Vaughan and Russell 2004

single protocol for DNA amplification increases productivity and reduces lab errors.

The goal of this study was to develop a method to uniquely identify all current almond cultivars using automated DNA fragment analysis, to use this method to confirm the identity of the almond cultivars at FPS and to elucidate the relationships of the commercially important cultivars grown in California. This study set represents a very narrow germplasm. Such a limited germplasm is a very good sample set for choosing highly polymorphic markers; however, the resulting data set is not the large, diverse database needed to calculate meaningful allele frequencies for probability analysis.

The twelve most informative markers were separated into four groups of three each (Table 4). These groups could be amplified and their fragments analyzed as triplexes, reducing the time and cost of analysis. The first triplex alone is sufficient to uniquely identify all 21 cultivars in the study set (Table 5). We recommend using the first nine markers as a standard profile for almond cultivar identification. Adoption of a standard set of markers for cultivar identification facilitates data sharing and helps correct for variation in data analysis among labs (This et al. 2004). As more profiles for existing almond cultivars are added to this database (Table 5), one would expect more diversity rather than less. Thus, these nine markers, selected for being highly polymorphic in a limited, closely related set of cultivars, can be expected to differentiate among all almond cultivars except those originating as bud-sports.

Table 3 Results for failed and less polymorphic loci

Locus	Origin	Reference
AMPA100	4	In data set
AMPA105	na	No amplification
AMPA118	1	Monomorphic
ssrPaCITA10	Na	Amplified artifacts
ssrPaCITA14B	1	Monomorphic
ssrPaCITA15	2	High homoqygosity
ssrPaCITA18	Na	Amplified artifacts
ssrPaCITA19	Na	No amplification
ssrPaCITA2	Na	No amplification
ssrPaCITA23	Na	Amplified artifacts
ssrPaCITA25	2	Poor amplification
ssrPaCITA27	1	Monomorphic
ssrPaCITA7	3	Scoring difficulty
UDAp-410	Na	Amplified artifacts
UDAp-411	Na	No amplification
UDAp-419	Na	No amplification
UDAp-420	5	In data set
UDP96-001	3	In data set
UDP96-005	4	In data set
UDP98-407	Na	Amplified artifacts
UDP98-409	5	In data set
BPPCT 006	3	Low polymorphism
BPPCT 014	3	In data set
BPPCT 034	Na	Amplified 2 loci
BPPCT 038	6	In data set
BPPCT 042	3	Low polymorphism
pchgms1	4	In data set
pchgms3	5	In data set
MA012a	3	In data set
MA015a	3	Low polymorphism
MA017a	Na	Amplified 2 loci
MA023a	Na	Amplified artifacts
MA034a	3	In data set
MA035a	Na	No amplification
CPSCT026	1	Monomorphic
CPSCT042	3	In data set
EMPA015	Na	No amplification
EMPA018	Na	No amplification
EMPaS06	3	Scoring difficulty
EMPaS10	Na	No amplification
EMPaS12	Na	No amplification

[a] Number of alleles observed in 18 almond cultivars

Table 4 Suggested loci for almond cultivar identification

Locus	Alleles[a]	Size range (base pairs)	Suggested triplexes	Suggested dye
BPPCT 039	8	122–180	1	6-FAM
BPPCT 004	7	182–216	1	HEX
BPPCT 040	8	132–148	1	NED
BPPCT 002	8	199–235	2	6-FAM
UDP96-003	5	99–116	2	HEX
MA040a	7	212–259	2	NED
ssrPaCITA12	6	136–158	3	6-FAM
MA024a	7	244–250	3	HEX
ssrPaCITA4	5	129–161	3	NED
BPPCT 017	5	134–168	4	6-FAM
CPSCT012	5	143–167	4	HEX
MA027a	7	115–145	4	NED

[a] Number of alleles observed in 18 cultivars from this study set

In addition to allowing an unambiguous identification of almond cultivars, the SSR markers reported here can be used to study cultivar pedigrees. A progeny shares one allele at each locus with each of its parents. This study set of 18 almond cultivars is neither large nor diverse enough to calculate probabilities for parentage analysis. However, a consistent result for both parents and a progeny at all 24 SSR loci provides strong, if not quantifiable, evidence to support the relationship, particularly if it confirms previous reports.

The almond cultivars 'Aldrich', 'Butte', 'Carmel', 'Monterey' 'Norman', 'Price' and 'Thompson' have previously been reported to be chance seedling selections probably originating from 'Nonpareil' × 'Mission' crosses (Asai et al. 1996; Brooks and Olmo 1997). This preliminary characterization was based on early cross-compatibility studies (Kester et al. 1994) where it was shown that most chance-selection cultivars could be grouped into four cross-incompatibility groups (S_1S_7, S_1S_8, S_5S_7, S_5S_8). These cross-incompatibility genotypes were presumed to be the result from natural crosses between the dominant cultivar 'Nonpareil' (S_7S_8) and the cultivar 'Mission' (S_1S_5) which was the major pollenizer for 'Nonpareil' during the early to mid 20th century (Asai et al. 1996; Wood 1925). However, other potential donors of the S_1, S_5 or S_7 allele have now been identified, including 'Languedoc' (S_1S_5), 'Ne Plus Ultra' (S_1S_7), and 'Peerless' (S_1S_6), (Barckley et al. 2006; Lopez et al. 2006) all of which have been reported to be widely planted in California from the late 19th to mid 20th century (Asai et al. 1996; Wood 1925).

The SSR markers used in this study fully support a 'Nonpareil' by 'Mission' parentage for these chance seedlings. In 'Carmel,' we assumed that a null allele for the MA034a locus is inherited from 'Mission' (Table 6, Bautista et al. 2008). There is no evidence of contributions by either 'Ne Plus Ultra' or 'Peerless' ('Languedoc' unavailable for analysis). In fact, no evidence of genetic contributions from 'Ne Plus Ultra' can be observed in any of the evaluated cultivars despite 'Ne Plus Ultra' being a widely planted cultivar originating from the same seedling block as the original 'Nonpareil' (Wood 1925).

The SSR data does, however, support both 'Fritz' and 'Ruby' as having the cultivar 'Peerless' in their lineage since both have the unique alleles 142 at BPPCT039 and 156 at ssrPaCITA12 markers (Table 5) as well as the unique S_6 incompatibility allele. Molecular marker data also support 'Mission' as the other parent (Table 5) as does the presence of the S_1 incompatibility allele (Barckley et al. 2006). It is assumed the same null allele at MA034a inherited by 'Carmel' is also inherited from 'Mission' by both 'Fritz' and 'Ruby' (Table 6).

Similarly, while the recently released cultivar 'Kochi' was discovered as a volunteer seedling near a 'Drake' almond orchard (Kochi 2004), the SSR data show that it most likely results from a 'Peerless' × 'Nonpareil' cross. 'Kochi' shares one allele at each locus with both 'Nonpareil' and 'Peerless', including the unique 'Peerless' alleles 142 at

Table 5 Allele sizes (in base pairs) for almond cultivar addressed in this study

Cultivar	BPPCT 004	BPPCT 039	BPPCT 040	BPPCT 002	UDP96 003	MA 040a	MA 024a	ssrPaCI TA 012	ssrPaCI TA 004	MA 027a	CPSCT 012	BPPCT 017
Aldrich	182 196	122 130	130 142	199 233	110 114	212 227	236 236	148 148	147 155	212 145	143 167	134 168
Butte	182 196	122 130	136 146	199 233	99 114	227 259	224 236	148 158	147 155	115 145	143 151	152 166
Carmen	182 196	122 148	130 142	199 233	99 144	212 227	234 236	136 158	148 155	121 145	143 167	134 168
Fritz	194 196	142 146	130 146	199 211	110 114	221 227	224 236	148 156	129 155	131 145	143 165	134 168
Kapareil	182 205	130 130	130 146	233 235	99 110	221 255	236 250	138 158	155 155	121 121	149 167	134 134
Kochi	182 194	142 148	142 146	211 233	99 110	225 225	224 224	156 158	155 155	115 121	151 167	134 152
Mission	196 216	122 146	130 136	199 203	108 114	227 227	234 236	136 148	129 147	141 145	143 167	166 168
Monterey	182 216	146 148	130 142	203 233	99 114	227 259	224 234	148 158	129 155	115 141	167 167	134 168
Ne Plus Ultra	182 184	130 148	130 142	231 233	99 114	212 227	236 238	148 158	129 155	121 145	143 167	134 166
Nonpareil	182 194	130 148	142 146	211 233	99 110	212 259	224 236	148 158	155 155	115 121	151 167	134 152
Norman	194 196	122 130	130 146	199 211	110 114	212 227	224 236	148 158	147 155			
Padre	182 196	122 180	136 142	199 209	99 108	227 227	236 244	148 148	129 147	141 145	143 149	146 168
Peerless	182 194	136 142	138 146	231 233	99 110	225 227	224 236	156 158	155 155	121 145	143 167	134 152
Price	194 196	146 148	130 146	199 211	110 114	212 227	224 236	148 148	129 155	121 141	167 167	134 166
Ruby	194 196	122 142	136 146	199 211	108 110	227 227	224 236	148 156	147 155	141 145	143 167	134 168
Solano	182 194	130 130	130 142	211 233	99 99	212 255	224 236	138 158	155 155			
Sonora	182 194	148 148	130 142	211 233	99 99	255 259	224 236	138 158	145 155	115 121	149 151	134 152
Sweetheart	194 194	130 148	134 146	211 215	110 114	229 255	244 224	138 148	155 161	121 121	149 167	134 152
Thompson	194 216	122 130	130 142	203 211	99 114	212 227	224 236	136 148	147 155			
Titan	194 196	146 148	136 142	199 211	99 108	229 259	224 236	146 158	155 155	115 137	151 168	134 152
Winters	182 200	130 136	132 132	233 233	116 116	227 229	236 242	146 158	155 155	121 145	143 167	134 134

The 12 loci designated in the top row are listed according to the ability to distinguish between almond cultivars. The first nine loci are recommended for routine almond cultivar identification

Table 6 Null allele inherited by three 'Mission' progeny

Cultivar	MA 034a initial score		MA 034a correct score	
Misson	178	178	178	Null
Carmel	222	222	222	Null
Fritz	226	226	226	Null
Ruby	226	226	226	Null

Single null alleles will appear homozygous at the given locus. Individuals inheriting the null allele will appear homozygous at that locus

BPPCT039 and 156 at ssrPaCITA12. 'Kochi' also possesses the unique 'Peerless' S_6-allele (Barckley et al. 2006).

Molecular markers can also support published parentage by analyzing only parent/progeny pairs, which will share one SSR allele at each locus. Though this analysis does not show the direction of descent (which is the parent and which the progeny), it can be used in conjunction with other information to support reported pedigrees. In this study marker data for 'Padre' support earlier reports of 'Mission' being the seed parent. The data are also consistent with 'Nonpareil' being one parent of 'Kanpareil', 'Solano', 'Sonora' and 'Titan' (Brooks and Olmo 1997). 'Titan's' seed parent is actually known to be 'Tardy nonpareil', a late blooming somatic mutant or "bud-sport" of nonpareil [only rarely are differences between somatic mutants observed with SSR data (Riaz et al. 2002)]. S-allele data are also consistent for the reported parentage of 'Sonora', 'Solano' and 'Kapareil'. There are no S-allele data for 'Titan' which is used primarily as an almond parent in generating almond x peach hybrid rootstocks. Unique molecular marker patterns were also observed for the recent cultivars 'Sweetheart' and 'Winters', supporting the reported use of novel germplasm to incorporate improved productivity and pest resistance to these cultivars (Gradziel et al. 2007; Martínez-Gómez et al. 2004).

Conclusion

The goal of this study was to develop a "DNA fingerprinting" method to uniquely identify all almond cultivars and to use this system to confirm the identity of each almond tree in the FPS foundation blocks. Previously published loci were screened with the objective to reduce time and cost of testing. The system developed has streamlined protocols compatible with automated high through-put DNA fragment analysis.

The twelve recommended markers form the basis for a practical method to uniquely identify almond cultivars. The loci show Mendelian inheritance and the profiles are consistent with known parentage and have proven informative in evaluating possible parentage for the many chance seedling selections. The limited database of profiles published here contains all important almond cultivars grown in California. Since these cultivars are readily available worldwide, they provide good reference profiles to facilitate data sharing among different labs.

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