Assessment of the genetic diversity of almond (*Prunus dulcis*) using microsatellite markers and morphological traits

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Abstract
The genetic diversity among 56 almond (Prunus dulcis) genotypes was analysed using 35 microsatellite markers and 14 morphological traits. Analysis of morphological traits revealed a wide range of variation among the studied genotypes. Out of 35 simple sequence repeats (SSRs) markers, 25 were polymorphic, producing 215 alleles that varied from 2 to 16 with an average of 8.76 alleles per locus. Regression analyses revealed a positive correlation between the *CPPCT03* locus and kernel yield, kernel percentage, grain weight, leaf length and tree altitude. The results of analysis of molecular variance (AMOVA) indicated that approximately 4.5% of genetic variance was observed between the collection sites. Based on SSR data, cluster analyses showed that the studied almond genotypes were classified into five main groups. The results of the present study showed that microsatellite markers could be successfully used to assay genetic diversity among Iranian almond landraces/cultivars and to identify informative markers for improving traits in breeding programs.
Keywords: *Prunus dulcis*; Genetic relationship; Microsatellite; Informative markers.

INTRODUCTION

Almond [*Prunus dulcis* (Miller) D.A. Webb, syn. *Prunus amygdalus* Batsch] occupies a very peculiar place among fruit trees (Miller *et al*., 1989). Because of almond's tolerance to cold, drought and salinity, it is considered an important tree crop and is cultivated in different climatic regions of Iran. Breeding practices in Prunus face unique challenges resulting from the narrow genetic background of commercial cultivars (Scorza *et al*., 1985). Morphological traits such as seed and kernel size, kernel yield, and blooming time are usually used for cultivar identification in almond (De iorgio and Polignano, 1999). However, morphological traits are limited because of their environmental fluctuations.

In recent years, molecular markers have been used to study genetic diversity and cultivar identification of peach and almond (Shiran *et al*., 2007; Sorkheh *et al*., 2007; Amirbakhtiar *et al*., 2006; Kadkhodaei *et al*., 2006; Sanchez-Pérez *et al*., 2006; Xie *et al*., 2006; Testolin *et al*., 2000, 2004; Aranzana *et al*., 2003). Methods based on knowledge provided by advances in molecular genetics, notably molecular markers, promise faster and more efficient approaches to cultivar improvement. In fact important tools such as molecular markers, maps, DNA sequences, and quantitative trait loci (QTLs) have been developed and made available to researchers, and applications at the breeding program level have already started (Dirlewanger *et al*., 2004). Recently, DNA microarray-based genome composition analysis has also been used in comparative genomic studies of trees (Martinez-Gomez *et al*., 2007). The objectives of the present study are to investigate the genetic diversity of major Iranian almond

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landraces/cultivars, to identify their relationship to important foreign cultivars, and to introduce informative markers for important nut traits using microsatellite markers.

MATERIALS AND METHODS

Plant materials: Fifty-one *Prunus dulcis* landraces/cultivars from different provinces of Iran along with three and two registered cultivars from Spain and USA, respectively, were used in this study (Table 1). The trees with similar ages were sown in a randomized complete block design, with four replications, at the experimental field of the Agricultural Biotechnology Research Institute of Iran (ABRII), Isfahan.

Phenotypic analysis: Fourteen independent morphological traits including leaf shape, leaf length (cm), leaf width (cm), petiole length (cm), flowering duration (day), tree altitude (cm), frostbite kernel yield (g), kernel length (cm), kernel width (cm), kernel thickness (cm), nut weight (g), kernel nut weight (g) and kernel percentage were recorded, based on food and agriculture organization (FAO).

Microsatellite analysis: Total genomic DNA was extracted according to the method described by Doyle and Doyle (1987), with minor modifications. Thirty-five simple sequence repeat (SSR) markers, isolated from peach and almond were used in this study (Testolin *et al*., 2004; Dirlewanger *et al*., 2002). Amplification reaction products were separated on a 6% (w/v) dena-

Table 1. Almond landraces/cultivars included in this study.

No.	Genotype name	Landrace/ Cultivar	Collection site	No.	Genotype name	Landrace/ Cultivar	Collection site
1	Post nazok1 (pk1)	Cultivar	Shiraz	30	H 8	Landrace	Hamadan
2	Monagha Shiraz	Cultivar	Shiraz	31	H 9	Landrace	Hamadan
3	S 8	Landrace	Shiraz	32	H 22	Landrace	Hamadan
4	S 21	Landrace	Shiraz	33	H20	Landrace	Hamadan
5	S 27	Landrace	Shiraz	34	H 6	Landrace	Hamadan
6	Post nazok 2 (pk2)	Cultivar	Shiraz	35	H 27	Landrace	Hamadan
7	S18	Landrace	Shiraz	36	H 5	Landrace	Hamadan
8	S17	Landrace	Shiraz	37	H 30	Landrace	Hamadan
9	S7	Landrace	Shiraz	38	H 7	Landrace	Hamadan
10	Sangi	Cultivar	Shiraz	39	H 4	Landrace	Hamadan
11	Mamaei1	Cultivar	Isfahan	40	H 18	Landrace	Hamadan
12	Mamaei2	Cultivar	Isfahan	55	H0	Landrace	Hamadan
13	Tadjeri	Cultivar	Isfahan	41	Ferragnes	Cultivar	France
14	Dobahre1	Cultivar	Isfahan	42	Sahand	Cultivar	Azerbaijan
15	Monagha Najafabad	Cultivar	Isfahan	43	Spain 200	Cultivar	Spain
16	Dobahre2	Cultivar	Isfahan	44	Shokofe	Cultivar	Azerbaijan
17	103	Landrace	Isfahan	45	Yalda	Cultivar	Azerbaijan
18	101-1	Landrace	Isfahan	46	Nonpareil	Cultivar	USA
19	101-2	Landrace	Isfahan	47	AR (1)	Landrace	Arak
20	Rabii	Cultivar	Isfahan	48	AR (2)	Landrace	Arak
21	H 12	Landrace	Hamadan	49	AR (3)	Landrace	Arak
22	H 15	Landrace	Hamadan	50	AR (4)	Landrace	Arak
23	H 16	Landrace	Hamadan	51	AR (5)	Landrace	Arak
24	H 17	Landrace	Hamadan	52	AR (8)	Landrace	Razan
25	H 11	Landrace	Hamadan	53	AR (6)	Landrace	Arak
26	H 21	Landrace	Hamadan	54	AR (7)	Landrace	Arak
27	H 10	Landrace	Hamadan	56	Spain 230	Cultivar	Spain
28	H 13	Landrace	Hamadan	57	Harir	Cultivar	Azerbaijan
29	H 19	Landrace	Hamadan	-	-	-	-

The names of the landraces/cultivars have been identified by their collection sites.

Table 2. Measured morphological traits.

Trait	Mean	Minimum	Maximum	Percentage of coefficient of Variation (CV%)
Leaf shape	8.95	1	14	55
Leaf length (cm)	5.26	3.5	8	19
Leaf width (cm)	1.68	1	2.2	17
Petiole length (cm)	17	10	28	24
Flowering duration (day)	8.94	4	16	31
Tree altitude (cm)	130.33	60	290	37
Frostbite	1.5	1	2	33
Kernel yield (gr)	2.3	0.03	11.4	107
Kernel length (cm)	3.05	2.5	3.91	10
Kernel width (cm)	1.99	1.67	2.51	11
Kernel thickness (cm)	1.14	1.23	1.85	12
Nut weight (gr)	34.66	14	56.7	33
Kernel nut weight (gr)	9.33	6.29	16.8	24
Kernel percentage (%)	28.72	15.91	81.11	47

turing polyacrylamide gel using a Sequi-Gen GT Sequencing Cell 30 cm gel apparatus (BioRad Laboratories Inc., Hercules, CA, USA). The amplified fragments were detected by the silver staining method as described by Bassam *et al*. (1991). The gels were visually scored by two independent observations.

Data analysis: Each polymorphic fragment was scored as either present (1) or absent (0) across all genotypes. The data were used to calculate the similarity matrix among cultivars employing simple matching coefficients. The similarity matrix was then used to construct dendrograms using the unweighted pair group method with arithmetic averages (UPGMA). This was achieved by employing the sequential, agglomerative, hierarchical, and nested clustering (SAHN) using the numerical taxonomy and multivariate analysis system (NTSYS-PC), version 2.00 (Rohlf, 1998). Observed heterozygosity (*Ho*) and expected heterozygosity (*He*) were calculated using the POPGENE version 1.32 (Yeh *et al*., 1997). The degree of polymorphism was quantified using the polymorphic information content (PIC). Probability of identity (PI) was estimated according to Paetkau *et al*. (1995). Analysis of molecular variance (AMOVA) was performed using the Arlequin version 2.00 (Schneider *et al*., 2000) to determine genetic variation (Nei, 1972). Average value of the Shannon index was also measured (Shannon and Weaver, 1949). Informative markers were determined by stepwise regression using the SPSS software version 10.0 for windows (SPSS Inc., Chicago, IL).

RESULTS

Morphological trait analysis: Mean, maximum, minimum and the percentage of coefficient of variation (CV%) of 14 morphological characters are shown in Table 2. A large diversity in the characters was observed, indicating a high level of variation in the studied plant materials.

SSR marker analysis: The results of this study showed cross amplification ability of microsatellite markers among the studied almond genotypes. Out of 35 SSR markers, Out of 35 SSR markers, 25 were polymorphic and produced 215 alleles. The number of alleles per locus ranged from 2 to 16, with an average of 8.76 (Table 3). Average value of the Shannon index was 1.79, which varied from 0.35 in *UDP96-008* to 2.6 in *CPPCT3*. Mean *He* across microsatellite loci ranged from 0.92 in *CPPCT3* to 0.17 in *UDP96-008*. The highest level of observed heterozygosity was found in *XAM18* and *CPPCT22* and the lowest in *UDP96-008*. According to PI, the most informative loci were *UDP98-412* and *CPPCT3* with values of 0.041 and 0.042, respectively. PIC for these two loci was greater (0.7) than others. The least informative locus was *XAM04* with PI of 0.98 and PIC of 0.159, followed by *XAM18* with PI and PIC values of 0.494 and 0.0018, respectively. The average of PI and PIC values for all loci were 0.258 and 0.475, respectively (Table 3). Rare polymorphic alleles (*i.e*. those with a frequency of ≤ 0.005) and their weights were determined for the pur-

Table 3. Characterized SSR markers amplified from almond *(Prunus dulcis)*.

Locus name	Sequence primers (5'→3')	Average Size Base Pair (bp)	Number of alleles	Shannon Index	*Ho*	*He*	PIC	PI
XAM01	ATAAATATATACACACACACACAC CATATAGGGTCAAGGGAGTG	175	---	---	---	---	---	---
XAM02	CGTGAGGTCTCACTCTCTC ATTTAAGGGTCTGGTCA	146	9	85.1	660.0	834.0	449.0	215.0
XAM03	GCAGAACGGTTTCTTTC GATGGACCAACTCAAGC	191	---	---	---	---	---	---
XAM04	TACATTATCCCCCGGTA GAAGCTCCATTCTTGTGA	156	3	03.1	537.0	625.0	159.0	98.0
XAM05	CACACACAAACACAAATGC TTGTGCTCTTCATGGAC	172	10	96.1	518.0	873.0	676.0	083.0
XAM06	TCTCCAAGGCGATAAGCA AGGCACCTGTCCCCTACA	156	---	---	---	---	---	---
XAM07	CGCTTTGCATACATACAAGT AGGAACTGGGATTAGAGA	149	---	---	---	---	---	---
XAM08	ACATCTCTCTCCTCCATGC TCTCTGGCAGCACAAGC	220	11	22.2	673.0	888.0	658.0	054.0
XAM09	TCACATACACGTGGGTTTC TGTGATTTGTGTGTGTGC	157	9	97.1	571.0	850.0	69.0	071.0
XAM10	ATTGTTTTCCCCTGGTA GAATCTCAACTCGGAAACG	94	---	---	---	---	---	---
XAM11	CCGGGGCTCTTATAAAT TGTGATGGCCAGAGCTT	199	9	89.1	673.0	836.0	632.0	134.0
XAM12	CCTGTCACAAGATGCAA CATTTTCCAGTAGTCCA	162	2	56.0	350.0	374.0	311.0	419.0
XAM13	AATACACACGCGCACAC AAGCATCGTCACTAGCC	165	11	10.2	777.0	860.0	545.0	102.0
XAM14	CCATCGCTTGCATTT CCGTGTGTGTTTGTGTG	140	---	---	---	---	---	---
XAM15	AACTATAAAATACACACACACACA CATCATCGGCTTTATTAG	193	---	---	---	---	---	---
XAM16	GCACCAAACACAACTGA GTGTTGCCAATGTTGAT`	172	8	74.1	673.0	810.0	38.0	243.0
XAM17	CACGTACATTGTGACTGC GTGTAATGCCACAGATGC	163	---	---	---	---	---	---
XAM18	CGTCTCATTTTCCCATTA CGATGGAGGAGCACT	174	10	83.1	910.0	815.0	0018.0	494.0
XAM19	CCGTGATACACTAACAACT TGCCAAGTAAGTGCCTA	175	12	30.2	732.0	892.0	566.0	087.0

Table 3. Continued

Locus name	Sequence primers (5'→3')	Average Size Base Pair (bp)	Number of alleles	Shannon Index	*Ho*	*He*	PIC	PI
XAM20	AGAAAGCTGCACTGGTA GCTTATTCGTGTGTG	138	9	06.2	821.0	867.0	57.0	13.0
UDP98-409	TGATGGGTTTTATGGTTTTC GGACTCTTATCCTCTATCAACA	129	---	---	---	---	---	---
UDP96-015	TTGACCTATTTGTTCGTCA TAGTCAAACAATCCCCCG	174	---	---	---	---	---	---
UDP96-008	TGTACACACCCTCAGCCTG GCTGAGGTTCAGGTGAGTG	165	---	---	---	---	---	---
UDP98-412	GGGAAAGTTTCTGCTGCAC TGAAGACGACGATGATGA	129	---	---	---	---	---	---
Pchgms1	GTAAATATGCCCATTGTGCAATC ATCATTGAACTACGTCAATCCTC	194	---	---	---	---	---	---
PS7a2	GGGAAATAGATAAGATG TAATGGTGGTGTTCATT	-	---	---	---	---	---	---
CPPCT3	GTAACGAAGAAGTTACGGG AACTGTCGCTGCTGGGTT	160	16	60.2	750.0	921.0	707.0	042.0
CPPCT4	TCATTCGAAGACGACCGT GTCTAGGCACGTTGCTAG	250	---	---	---	---	---	---
CPPCT16	AATTCCCTATGGAAATTAGA CGCATATTATAGGTAGGAAA	191	13	29.2	771.0	886.0	609.0	112.0
CPPCT17	GTGACATGCATGCACTAAACA TGCAAATGCAATTTCATAAAGG	177	12	16.2	690.0	863.0	626.0	113.0
CPPCT22	CAATTAGCTAGAGAGAATTATTG GACAAGAAGCAAGTAGTTTG	240	5	31.1	771.0	690.0	053.0	535.0
CPPCT24	TTCTCCCAAAAACCAAAACC TCATTGGCTGCTAAGTGTCCT	180	6	50.1	571.0	763.0	41.0	42.0
CPPCT27	GAGCAGTTCATAAGTTGGAAC CGATAAAGATTTTGACTGCATG	114	11	12.2	842.0	866.0	513.0	155.0
CPPCT30	TGAATATTGTTCCTCAATTC CTCTAGGCAAGAGATGAGA	198	5	92.0	368.0	466.0	185.0	556.0
CPPCT33	TCAGCAAACTAGAAACAAACC TTGCAATCTGGTTGATGTT	151	9	07.2	740.0	873.0	55.0	17.0

Ho, *He*, PIC, and PI are observed heterozygosity, expected heterozygosity, polymorphic information content and probability of identity, respectively. The loci (*XAM01* to *XAM20*) have been determined by Testolin et al. (2004) and the others by Dirlewanger *et a*l. (2002).

Table 4. Rare polymorphic alleles and their weight for use in almond identification.

Locus name	Rare allele weight	Genotype name
XAM06	145	H 12
XAM02	190	Mamaei 1
XAM02	170	H 21
XAM05	212	AR (3)
XAM05	147	Sangi
XAM08	195	AR (5)
XAM08	307	H 13
XAM08	240	Nonpareil and S 8
XAM09	131	Shokofe
XAM16	183	Fragness
XAM16	160	S 21
XAM18	119	H 19
CPPCT16	204	H 8
CPPCT03	225	Rabii
CPPCT03	186	Spain 230 and Sahand
CPPCT17	147	AR (6)
CPPCT27	79	H 4
CPPCT30	250	Shokofe
UDP96-008	147	Spain 200

For genotype and locus names see Table 1 and 3, respectively.

pose of rapid cultivar identification (Table 4). Regression analyses revealed that there was a positive correlation between the *CPPCT03* locus and kernel yield (β = 0.424), kernel percentage (β = 0.49), grain weight (β = 0.35), leaf length (β = 0.32) and tree altitude (β = 0.327) (Table 5).

Based on sampling sites, average *He* was 0.697 and the largest heterozygosity was observed for cultivars from Hamadan (0.731). The results of AMOVA indicated that approximately 4.5% of genetic variance belonged to between collected sites (Table 6). Based on SSR data, the studied almond genotypes were classified into five main groups (Fig. 1). The first cluster included some landraces and cultivars from the Shiraz, Isfahan, Hamadan and Arak provinces. The second cluster included two sub-clusters: the first sub-cluster contained 4 landraces from the Shiraz province and the second sub-cluster contained registered cultivars from Spain, USA and Azerbaijan. Two landraces from Shiraz and Arak provinces were gathered into cluster III. One registered cultivar from USA (HO) and one registered cultivar from Azerbaijan (Harir) were located in two distinct clusters (IV and V).

DISCUSSION

The results of this study support those of Sosinski *et al*. (2000), regarding the cross amplification ability of microsatellite markers across the Prunus species. High level of heterozygosity for all loci (0.697) can be attributed to cross pollination and the self-incompatibility nature of almond. The high values of polymorphic loci (71%), average number of alleles per locus (8.76), *He* (0.775), average polymorphism information content (0.475) and PI (0.258) observed in this study indicate that SSR markers are able to identify genetic variation among the studied almond genotypes. According to PI and PIC values, *CPPCT3, UDP98-412, UDP96-409, XAM05, XAM08, XAM09, XAM15* and *XAM19* are the best loci for further studies of almond genetic diversity. The percentage of polymorphic SSR loci (71%) in this study was much higher than that estimated for RFLPs (21.9%), suggesting that SSRs can act as better systems for almond cultivar identification (Eldredge *et al*., 1992).

During this research, alleles were identified that correlated with yield-related traits. The allele belonging to the *XAM09* locus had a positive correlation with blooming duration (0.418) (Table 5). In addition, *CPPCT17* was found to be an informative marker for nut weight, average kernel thickness and leaf width (Table 5).

In this investigation, cluster analyses showed that most Iranian landraces are well separated from the Spanish and American (USA) cultivars, indicating that they may be native to Iran. However, Shiraz almond landraces are assigned to the same group as the Spanish and American cultivars. A possible explanation is that they might carry a common genetic background. According to the results of this study, SSR data failed to separate genotypes based on their sampling sites. Germplasm migration or insufficient SSR markers can explain this incomplete separation. The results show that Iranian registered cultivars including Yalda, Shokofe and Sahand are similar to the foreign cultivars.

Informative markers are most applicable for breeding purposes. These markers have previously been used in the identification of peach and nectarine varieties (Manubens *et al*., 1999). A combination of molecular and morphological data is the best choice to find informative markers. In summary, results of the present study reveal that microsatellite markers can be

Table 5. Regression between morphological and molecular data to define informative markers.

Trait	Locus name	Adjusted R^2	*P-value*	Standard β
Leaf shape	*XAM13*	0.105	0.001	-0.34
Leaf length	*CPPCT27*	0.153	0.00	0.403
Leaf length	*UDP96-412*	0.141	0.00	0.389
Leaf length	*CPPCT16*	0.093	0.003	0.322
Leaf length	*CPPCT03*	0.091	0.003	0.32
Leaf width	*CPPCT17*	0.188	0.00	0.425
Leaf width	*XAM08*	0.107	0.001	-0.343
Petiole length	*XAM02*	0.118	0.001	0.359
Petiole length	*XAM15*	0.088	0.003	-0.314
Flowering duration	*XAM09*	0.167	0.00	0.418
Flowering duration	*CPPCT27*	0.128	0.00	-0.372
Flowering duration	*Pchgm1*	0.121	0.01	0.362
Flowering duration	*XAM11*	0.074	0.007	0.292
Tree altitude	*XAM08*	0.13	0.00	-0.375
Tree altitude	*CPPCT24*	0.089	0.005	-0.305
Tree altitude	*XAM11*	0.077	0.006	-0.296
Tree altitude	*CPPCT03*	0.096	0.002	0.327
Tree altitude	*XAM13*	0.097	0.002	0.328
Frostbite	*XAM20*	0.138	0.00	0.385
Kernel yield	*CPPCT03*	0.17	0.00	0.424
Kernel yield	*XAM19*	0.122	0.001	0.364
Kernel length	*UDP96-08*	0.137	0.00	-0.384
Kernel length	*XAM15*	0.133	0.00	0.378
Kernel length	*XAM16*	0.072	0.007	0.288
Kernel width	*CPPCT03*	0.176	0.00	-0.431
Kernel width	*XAM02*	0.087	0.004	-0.313
Kernel width	*XAM13*	0.128	0.00	-0.372
Kernel thickness	*UDP98-409*	0.147	0.00	0.396
Kernel thickness	*XAM15*	0.074	0.007	-0.292
Kernel thickness	*CPPCT17*	0.129	0.00	0.373
Kernel thickness	*Pchgm1*	0.101	0.002	0.334
Kernel thickness	*XAM08*	0.083	0.004	-0.306
Nut weight	*XAM08*	0.125	0.001	-0.367
Nut weight	*CPPCT17*	0.102	0.002	0.336
Nut weight	*CPPCT17*	0.076	0.006	0.295
Nut weight	*CPPCT03*	0.112	0.001	0.35
Nut weight	*CPPCT02*	0.083	0.005	-0.305
Kernel weight	*CPPCT33*	0.149	0.00	0.398
Kernel weight	*Pchgm1*	0.093	0.003	0.323
Kernel percentage	*CPPCT03*	0.232	0.00	0.491
Kernel percentage	*XAM18*	0.039	0.039	-0.244

For locus name see Table 3.

Table 6. Analysis of molecular variance (AMOVA) and variance components for total genetic differentiation in almond based on collection sites.

Source of variation	Degree of freedom	Mean squared	Variance component	Percentage of variation	*P value*
Among collection sites	4	4.98	0.116	4.41	0.001
Within collection sites	109	2.26	2.51	95.43	-
Total	113	7.25	2.63	100	-

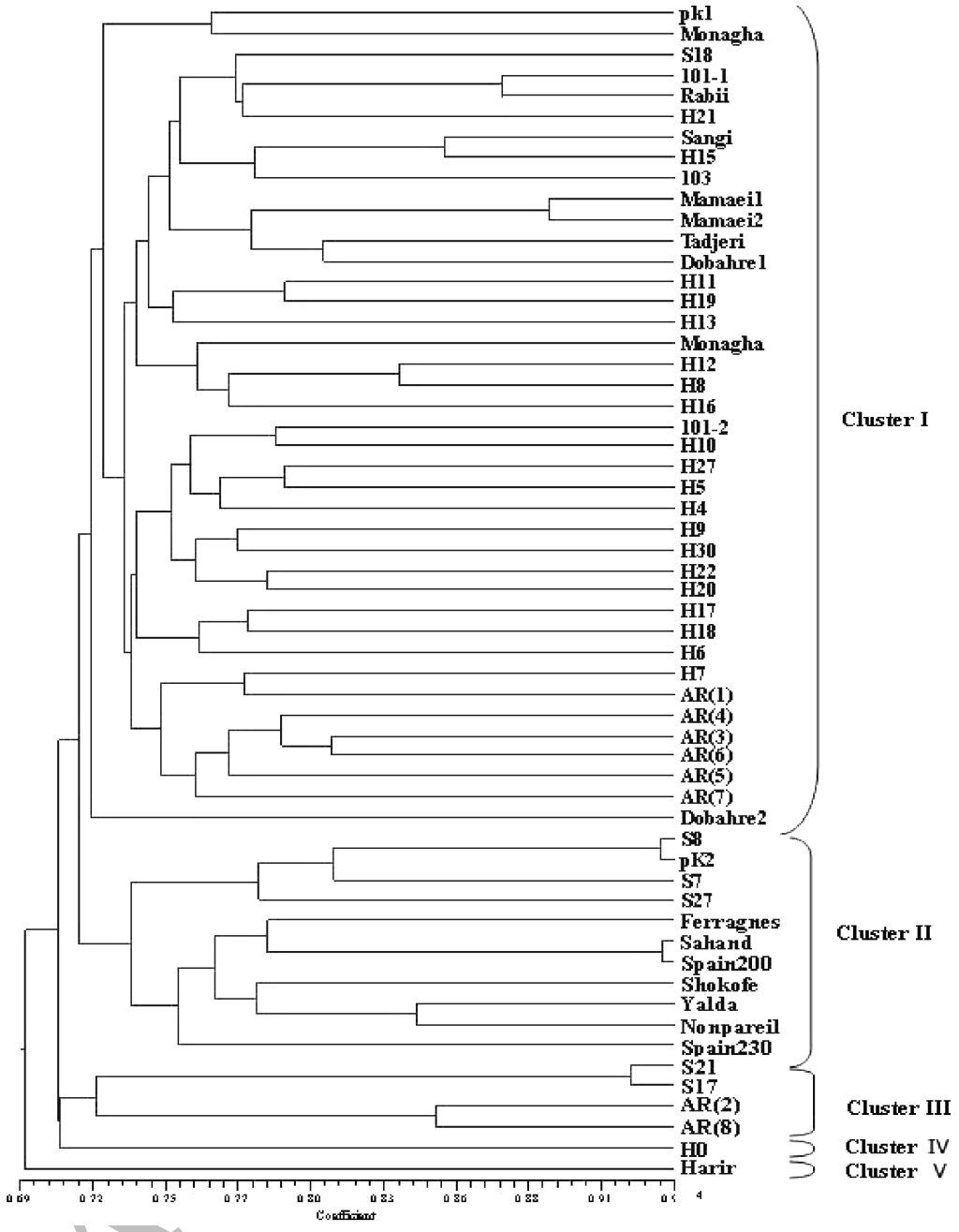

Figure 1. Dendrogram showing the relationships between 57 almond accessions using simple matching index and unweighted pair group method whit arithmetic mean (UPGMA).

successfully used to assay genetic diversity among Iranian almond landraces/cultivars and to identify informative markers for breeding of important traits.

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