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Full Length Research Paper

Genetic diversity among selected genotypes of almond *Prunus dulcis* Miller D.A. Webb assessed by random amplified polymorphic DNA (RAPD) markers

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Genetic relatedness and diversity in 32 almond genotypes were analysed using random amplified polymorphic DNA (RAPD) markers. All the genotypes maintained at Dr. Y.S. Parmar University of Horticulture and Forestry, Solan, India comprised ten exotic introductions and 22 indigenous selections. Using 16 selected 10-mer primers, 87 bands were generated and all the bands were recorded to be polymorphic. The RAPD primers namely S073 (CCAGATGCAC) and S081 (TCGCCAGCCA) gave maximum and minimum number of polymorphic bands, respectively. Cluster analysis of all the genotypes was performed based on data from polymorphic bands using Jaccard's similarity coefficient and unweighted pair group method with arithmetic mean (UPGMA) clustering method. The highest and lowest similarities detected between genotypes were 0.667 and 0.000, respectively. Average polymorphic information content (PIC) value of 16 selected primers was 0.684 and maximum and minimum PIC value was 0.8687 and 0.2551 for primers S073 and S081, respectively. Cophenetic correlation was found to be 0.89. RAPD data on genetic diversity matched classification of studied genotypes based on morphological and geographical traits.

Key words: *Prunus dulcis*, genetic diversity, accessions, DNA isolation, cetyl trimethyl ammonium bromide (CTAB), random amplified polymorphic DNA (RAPD) marker, polymorphic information content (PIC) value.

INTRODUCTION

Almond (*Prunus dulcis* (Miller) D. A Webb syn *Prunus amygdalus* Batsch) belongs to family Rosaceae subfamily Prunoideae (Martinez-Gomez et al., 2007). It is highly nourishing and of great medicinal value. Its kernels are rich source of fats, proteins, minerals and vitamins. It was spread by man in ancient times along the shores of the Mediterranean area into North Africa, South Europe and to other parts of the world, mainly to California. In India, almond is confined to hilly states such as Jammu and Kashmir, Himachal Pradesh and Uttrakhand. In spite of the fact that it is a highly valuable nut crop, its commercial cultivation is not very popular. Lack of suitable genotypes is the prime reason for its limited cultivation. Most plantation comprise chance seedling selections whose pedigree/genetic origin is not known, thereby hindering breeding efforts to develop genetically improved genotypes.

For commercial cultivation, use in breeding programmes and for Intellectual Property Rights (IPRs), genotype identification of a crop is very important. However, diversity analysis based on morphological traits is not reliable (Talhouk et al., 2000). Moreover, many genotypes are so morphologically similar, that it is very difficult or even impossible to distinguish between them (Martinez-Gomez et al., 2007). For these reasons,

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Abbreviations: CTAB, Cetyl trimethyl ammonium bromide; **NTSYS-pc,** numerical taxonomy and multivariate analysis system; **PIC,** polymorphic information content; **RAPD,** random amplified polymorphic DNA; **IPRs,** intellectual property rights.

molecular markers, particularly DNA based markers have succeeded in distinguishing accessions, clarifying synonyms, DNA fingerprinting of ambiguous genotypes, establishing genetic similarities or geographical origins and indicating the process of domestication (Wunsch and Hormaza, 2002). Among these markers, RAPD (Williams et al*.,* 1990) is one of the earliest marker systems to detect the genetic variation among numerous organisms including plants and animal species, and can be carried out in any laboratory without much infrastructure (Vidal et al., 1999; Baranek et al., 2006; Kaur et al*.,* 2010).

MirAli and Nabulsi (2003) and Shiran et al. (2007) used RAPD techniques to study the genetic relatedness among Syrian and Iranian almond cultivars, respectively. Recently Ali Al-Ghzawi et al. (2009) used RAPDs to find genetic relatedness among wild and cultivated almond genotypes in Jordan. Keeping in view the successful use of RAPDs for similar type of work elsewhere, it was planned to carry out the diversity analysis in almond germplasm being maintained at Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, India. Until now, the germplasm cannot be used as well identified genetic stock in breeding programmes, since this has not so far been characterized using any type of molecular markers. Therefore, the present investigations were undertaken with the objective to find genetic relatedness among different genotypes of almond under study using RAPD markers.

MATERIALS AND METHODS

Source material and isolation of DNA

Leaf material from 32 almond genotypes was collected from orchards of the University. The collections were both exotic introductions, as well as indigenous selections (Table 1). Genomic DNA was isolated from freshly collected leaves following modified CTAB method (Doyle and Doyle, 1987, 1990). Phenolics and tannins were removed by repeated purification of DNA following phenol and chloroform treatment. Also during grinding of leaves, addition of a pinch of PVP helped a lot in removing phenolics. The purity and quantity of DNA was assessed spectrophotometrically and confirmed using 0.8% agarose gel electrophoresis against known concentrations of double digest of lambda DNA.

Primer screening

Forty four 10-mer primers from Sigma-Aldrich integrated DNA technologies 'USA' were initially screened using six genotypes of almond to determine the suitability of each primer for the study. Sixteen primers were selected for further RAPD-PCR analysis (Table 2) of all the 32 accessions, based on their ability to generate clear and distinct polymorphic bands. The primers which generate smeared bands were discarded.

RAPD analysis

DNA amplification

PCR reaction was performed as described by Williams et al. (1990) with 16 selected 10-mer primers. Each PCR reaction volume of 20 µl contained 2.5 mM $MgCl_2$, 250 µM dNTPs, 1 µM of primer, 1 unit of Taq polymerase and 50 ng of genomic DNA. The chemicals used for PCR reaction were obtained from Genei, Bangalore, India, except 10-mer primers which were obtained from Sigma Aldrich USA. The PCR was performed in thermal cycler of Techne Cyclogene, Italy. Amplification conditions were: Initial denaturation at 94ºC for 2 min followed by 35 cycles each of denaturation for 1 min at 94°C, annealing for 1 min at 35°C and extension for 2 min at 72°C and finally an 8 min extension at 72°C. The reactions were repeated twice to ensure reproducibility and a few of them were repeated even thrice.

Electrophoresis of amplified product

RAPD-PCR product was detected in 1.4% agarose gel in 1× TAE buffer (pH 8.0, 242 g Tris Base, 57.1 ml glacial acetic acid, 0.5 M EDTA per litre of the buffer) and run in same buffer for at least 3 h at constant voltage at 5 V/cm. Gels were stained with 0.5 µg/ml of ethidium bromide, and DNA profiles were visualized on a UV transilluminator and photographed by using gel documentation system (Alpha imager, USA).

Data analysis

For RAPD analysis, the bands with same molecular weight and mobility were treated as identical fragments. In the data matrices, the presence of a band was coded as 1 and absence was marked as 0, and estimation of similarity among all genotypes was calculated according to the study of Nei and Li (1979). The data was analysed with SIMQUAL program of NTSYS-pc (Version 2.02), and similarities between accessions were estimated using the Jaccard's coefficient calculated as J = A / (N - D), where A is the number of positive matches (that is, presence of band in both samples), D is the number of negative matches (that is, absence of band in both samples) and N is the total sample size including both the number of matches and unmatches (Rohlf and Milligan, 1994). Dendrogram was created from the resultant similarity matrices using the UPGMA method (Sokal and Sneath, 1963) following SAHN function of NTSYS-pc (Version 2.02) (Nei and Li's, 1979). Similarity matrices were compared using Mantel test (Mantel, 1967). PIC value of various primers was analysed using formula 1-(allele/number of genotypes^2) + (allele/number of genotype^2)... or 1 - n pij^2, where p, i and j are frequency of first, second and third allele.

RESULTS

The primer screening resulted in 16 primers which detected polymorphism within 32 genotypes comprising of 10 exotic introductions and 22 indigenous selections of almond. A total of 44 random primers were used and out of these, only 16 were able to give polymorphism. The remaining primers showed either no amplification or resulted in unreadable gel smears. Some of the primers produced polymorphic bands specific to a set of genotypes. Primers S084(6), S085(5), S087(5) and S090(6) each produced single unique band of size $S084_{500}$ for 'Local Selection White', band $S085_{1475}$ for 'Nonpariel', band $S87_{1250}$ for 'Peach Almond Hybrid' and band $S090_{600}$ for 'White Quasi', respectively. Whereas primer S081 gave one unique band of size $S081_{980}$ for

Table 1. Almond genotypes included in present investigations.

S/N	Genotype	Place of origin
Exotic introduction		
1	Nonpareil	USA
2	Prianyi	USSR
3	Tree No. 96	USSR
4	White brandis	Australia
5	Tree No. 104	Unknown
6	V. 31	USSR
7	V.1	USA
8	V.6	USA
9	White Quasi	Unknown
10	Star Basin	Unknown
Indigenous selection		
1	Tree No. 126	Solan (HP)
2	Tree No. 2	Kinnaur (HP)
3	V.3	Kinnaur (HP)
4	Tree No. 121	Solan (HP)
5	Tree No. 101	Kinnaur (HP)
6	Tree No. 16	Kinnaur (HP)
7	JK 184	J and K
8	Badam Jor Spillo No. 2B	Kinnaur (HP)
9	Tree No. 106	Kinnaur (HP)
10	Tree No. 125	Solan (HP)
11	Spillo No. 7	Kinnaur (HP)
12	Badam jor Spillo No. 2C	Kinnaur (HP)
13	JK 238	J and K
14	Peach Almond hybrid	Solan (HP)
15	Spillo No. 3	Kinnaur (HP)
16	Local selection white	Solan (HP)
17	Sloh	Jalandher (Pb)
18	JK 178	J and K
19	Ribba selection	Kinnaur (HP)
20	Tree No. 65	J and K
21	Spillo No. 45	Kinnaur (HP)
22	Nauni Selection	Solan HP

Pb, Punjab; HP, Himachal Pradesh; J and K, Jammu and Kashmir.

'White Brandis' and another band $S081_{1025}$ for 'Spillo No. 45'. Similarly we detected a few more primers, such as S088, S089, and S092 which gave unique bands in many more genotypes. A minimum of three fragments by each primers S081, S093, S091 and S095 and a maximum of 9 fragments by each of the primers S075, S089, S073 were recorded (Table 2). No single primer could fingerprint all the accessions. Hence, we found that a set of a few markers define each cultivar and not a single marker.

The dendrogram (Figure 1) based on polymorphism detected by 16 polymorphic primers divided the 32 genotypes of almond into three main clusters. 'Nauni Selection' was separated as a singlet at similarity value of 0.02. The rest of the accessions were divided into three main clusters namely, A, B and C. Each cluster consisted of both standard genotypes, as well as seedling selections. 'Cluster A' is the largest cluster, including a total of 26 accessions, 'Cluster B' comprised of total three genotypes which include two standard exotic genotypes 'Nonpareil' and 'V.6' and one indigenous selection namely, 'Tree No. 2', whereas 'Cluster C' has got only two genotypes, one exotic introduction 'V.31' and other indigenous selection 'Tree No. 126'.

The cophenetic correlation value was 0.89, and this high value suggested a very good fit of the dendrogram

Table 2. Amplified and polymorphic bands generated by PCR using 16 decamer random primers in 32 genotypes of almond (*Prunus dulcis*).

S/N	Primer	Nucleotide sequence 5'- 3'	Total number of amplified bands	Polymorphic bands	Unique bands
1	S075	ACGGATCCTG	9	9	0
2	S084	CAGACAAGCC	6	6	1
3	S085	CTCTGTTCGG	5	5	1
4	S081	TCGCCAGCCA	3	3	2
5	S093	CCACCGCCAG	3	3	0
6	S078	GGCTGCAGAA	5	5	0
7	S094	AGAGATGCCC	6	6	2
8	S087	GGTGCAGTCG	5	5	1
9	S088	GGTCCTCAGG	4	4	2
10	S089	CAGTTCGAGG	9	9	2
11	S090	TACCGACACC	6	6	1
12	S091	TCGGAGTGGC	3	3	0
13	S092	ACTCAGGAGC	7	7	2
14	S095	CAGTTCTGGC	3	3	0
15	S073	CCAGATGCAC	9	9	0
16	C15	TTCCGAACCC	4	4	0
Total			87	87	14

with the similarity matrix (Table 3) based on Jaccard's Coefficient. The PIC value provides an estimate of the discriminatory power of locus or loci by taking into account not only the number of alleles that are expressed, but also relative frequencies of those alleles. Referring to the PIC values recorded for all the informative primers, on an average, it was 0.6844 and varied from minimum of 0.2551 for primer S081 to maximum of 0.8676 for primer S073 (Table 4).

DISCUSSION

Estimates of genetic diversity among germplasm accessions within and between species have numerous applications in crop breeding programmes. It can be useful for organizing germplasm for identification of cultivars, assisting in identification of genetically diverse parents for hybridization. Molecular survey of parental types leads to the revelation of polymorphic level which further demonstrates the level of genetic diversity among the genotypes. Sufficiently diverse types are used in breeding programmes (Boury et al., 1992; Divaret and Thomas, 1998; Joshi et al., 1999; Couran et al., 2007). Various marker systems, including morphological and biochemical, have been employed for analysing diversity in plant germplasm.

However, numerous DNA markers like restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism versus randomly amplified polymorphic DNA (RAPD AFLP), simple sequence repeats (SSR) provide an efficient means of estimating and analysing genetic relatedness among different genotypes. RAPD markers are of main interest for a laboratory without much infrastructure, and also these are simple and comparatively inexpensive. RAPDs have been used for diversity analysis of crop species including almond (Lu et al., 1996; Ortiz et al., 1997; Wooley et al., 2000; Kuden et al., 2004).

RAPD marker survey in 32 genotypes of almond indicated that 16 RAPD primers generated 89% polymorphism and amplified a total of 87 polymorphic bands. This level of polymorphism is quite high and has been reported by Shiran et al. (2007) and Ali Al-Ghzawi et al. (2009) in almond, using RAPD markers. A very high level of polymorphism has been obtained in walnut (Nicese et al., 1997; Kaur et al., 2010), strawberry (Kashyap et al., 2005) and bunium (Majeed et al., 2009). Usefulness of RAPD markers had been substantiated in almond and other nut and fruit crops (Stafne et al., 2003; Bianchi et al., 2003; Cai et al., 2006; Ali Al-Ghzawi et al., 2009). One of the most important consideration of the marker study is reproducibility of results which has been questioned in case of techniques like RAPD that is based upon arbitrary primers of small length of 10-mer (Williams et al., 1990).

Therefore, in the present study in order to avoid any ambiguous bands, the results were repeated and only clear and bright bands were retained, and ambiguous bands were not considered for analysis of results. Such ambiguity is the outcome of heteroduplex formation between the amplified products or from any other secondary artefact (Hatrys and Wohling, 2002). Sometimes, such erroneous results occur during the gel

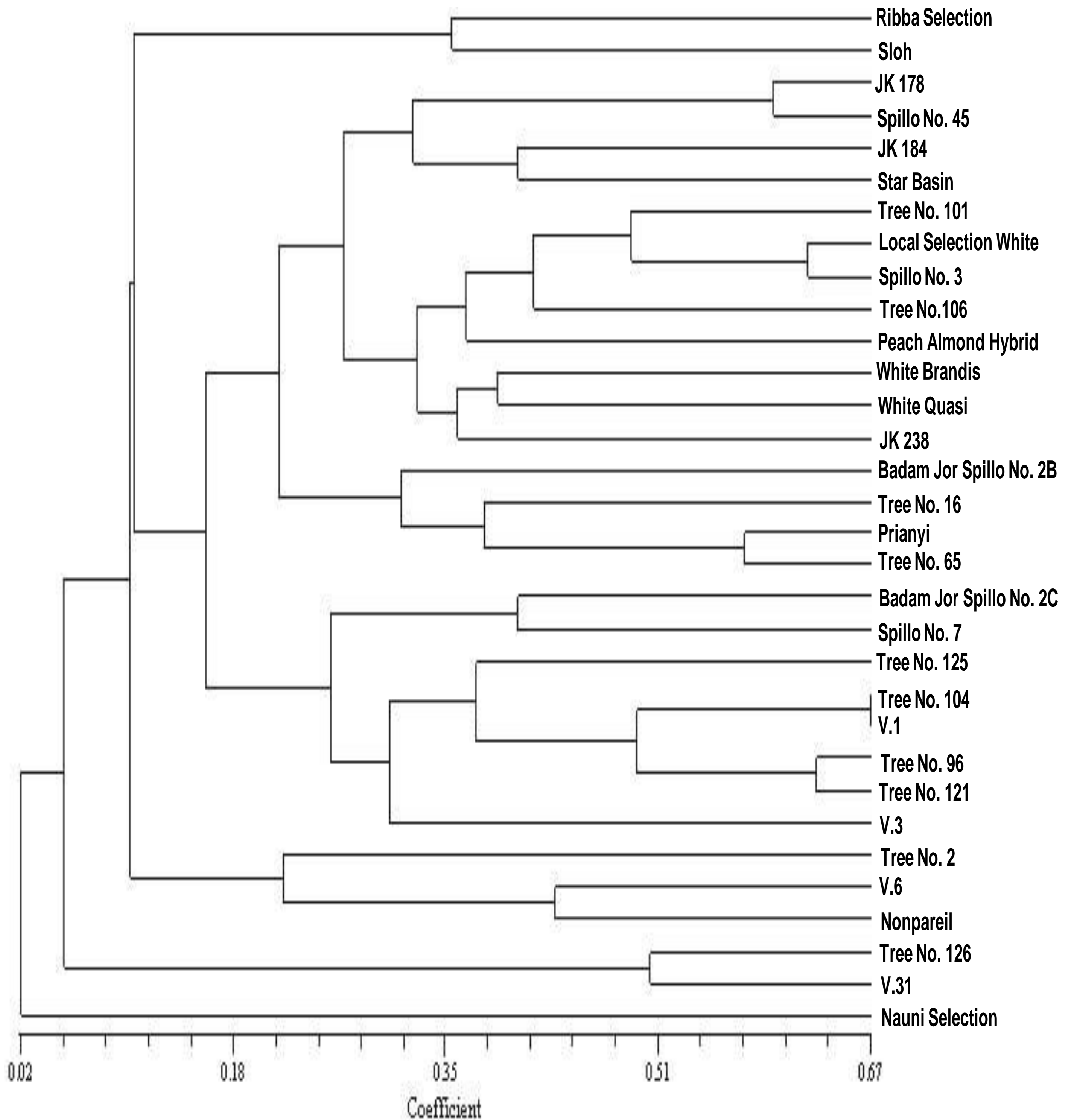

Figure 1. Dendrogram of 32 accessions of almond based on RAPD analysis.

run. Perez et al. (1998) found a moderate to high reproducibility for major bands when faint bands and rare profiles were rejected.

The range of similarity was found to be between zero and 0.667 among 32 genotypes of present investigation. The zero value ranges between a number of genotypes

Table 3. Jaccard's similarity coefficient for 32 accessions of almond.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	1.000																															
2	0.350	1.000																														
3	0.154	0.321	1.000																													
4	0.100	0.212	0.536	1.000																												
5	0.154	0.276	0.333	0.593	1.000																											
6	0.000	0.207	0.226	0.323	0.407	1.000																										
7	0.083	0.320	0.241	0.345	0.565	0.619	1.000																									
8	0.000	0.103	0.207	0.310	0.296	0.375	0.409	1.000																								
9	0.050	0.077	0.033	0.172	0.292	0.381	0.421	0.300	1.000																							
10	0.069	0.267	0.242	0.333	0.281	0.300	0.423	0.385	0.280	1.000																						
11	0.030	0.139	0.257	0.306	0.294	0.313	0.333	0.345	0.207	0.364	1.000																					
12	0.095	0.071	0.065	0.161	0.222	0.292	0.381	0.167	0.263	0.214	0.276	1.000																				
13	0.190	0.143	0.129	0.226	0.250	0.296	0.292	0.154	0.300	0.241	0.182	0.400	1.000																			
14	0.000	0.038	0.034	0.138	0.200	0.273	0.238	0.250	0.313	0.148	0.214	0.278	0.190	1.000																		
15	0.105	0.272	0.292	0.214	0.192	0.160	0.227	0.181	0.048	0.231	0.296	0.043	0.130	0.050	1.000																	
16	0.000	0.160	0.143	0.250	0.333	0.429	0.474	0.350	0.353	0.320	0.385	0.316	0.227	0.375	0.211	1.000																
17	0.000	0.185	0.296	0.267	0.250	0.296	0.240	0.200	0.130	0.200	0.345	0.077	0.034	0.190	0.300	0.286	1.000															
18	0.050	0.272	0.192	0.172	0.240	0.450	0.500	0.238	0.222	0.280	0.207	0.143	0.181	0.105	0.294	0.278	0.238	1.000														
19	0.077	0.050	0.043	0.174	0.200	0.158	0.250	0.188	0.250	0.190	0.167	0.308	0.267	0.273	0.071	0.231	0.000	0.071	1.000													
20	0.000	0.156	0.333	0.387	0.333	0.310	0.286	0.296	0.192	0.281	0.333	0.138	0.129	0.154	0.148	0.391	0.400	0.148	0.143	1.000												
21	0.000	0.222	0.190	0.167	0.190	0.211	0.235	0.176	0.067	0.182	0.160	0.000	0.053	0.000	0.333	0.308	0.333	0.333	0.000	0.250	1.000											
22	0.000	0.211	0.182	0.160	0.182	0.263	0.294	0.235	0.063	0.174	0.154	0.056	0.050	0.000	0.308	0.286	0.235	0.417	0.000	0.182	0.571	1.000										
23	0.000	0.000	0.000	0.115	0.131	0.263	0.291	0.313	0.417	0.227	0.154	0.267	0.235	0.333	0.000	0.385	0.000	0.133	0.429	0.182	0.000	0.000	1.000									
24	0.000	0.091	0.038	0.034	0.039	0.000	0.000	0.000	0.000	0.037	0.033	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.125	0.000	0.000	0.083	1.000								
25	0.000	0.130	0.074	0.143	0.115	0.174	0.190	0.263	0.250	0.250	0.222	0.222	0.200	0.267	0.053	0.235	0.043	0.053	0.300	0.160	0.000	0.000	0.364	0.143	1.000							
26	0.063	0.043	0.080	0.200	0.227	0.250	0.278	0.296	0.286	0.217	0.240	0.333	0.294	0.417	0.059	0.357	0.048	0.125	0.571	0.227	0.000	0.000	0.625	0.077	0.333	1.000						
27	0.077	0.105	0.091	0.080	0.091	0.158	0.250	0.118	0.250	0.190	0.120	0.133	0.188	0.077	0.154	0.143	0.056	0.364	0.143	0.043	0.125	0.111	0.250	0.000	0.083	0.222	1.000					
28	0.077	0.167	0.091	0.080	0.143	0.100	0.176	0.000	0.154	0.087	0.077	0.063	0.118	0.167	0.250	0.066	0.118	0.250	0.143	0.043	0.125	0.111	0.000	0.000	0.000	0.100	0.333	1.000				
29	0.067	0.095	0.040	0.036	0.083	0.043	0.100	0.000	0.063	0.038	0.034	0.056	0.105	0.143	0.133	0.000	0.050	0.133	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.111	0.428	1.000			
30	0.067	0.095	0.083	0.208	0.300	0.263	0.294	0.235	0.308	0.174	0.200	0.267	0.235	0.455	0.063	0.200	0.105	0.133	0.667	0.182	0.000	0.000	0.333	0.000	0.250	0.625	0.111	0.250	0.200	1.000		
31	0.000	0.000	0.150	0.130	0.045	0.050	0.000	0.125	0.000	0.043	0.080	0.000	0.000	0.083	0.077	0.000	0.200	0.000	0.000	0.095	0.000	0.000	0.000	0.000	0.091	0.111	0.000	0.000	0.000	0.125	1.000	
32	0.000	0.000	0.095	0.083	0.000	0.050	0.056	0.125	0.077	0.091	0.080	0.067	0.059	0.083	0.077	0.071	0.125	0.077	0.000	0.045	0.000	0.000	0.125	0.000	0.200	0.111	0.167	0.000	0.000	0.000	0.500	1.000

which include pairs between exotic and selected genotypes. Ali Al-Ghzawi et al. (2009) recorded similarity values ranging from 0.000 to 0.500 among sixteen Jordanian almond genotypes using RAPD markers. This research group used forty RAPD primers, and only six primers showed reproducible fragments. Even our research group recorded sixteen RAPD primers as polymorphic primers out of a total of 44 primers. This is because of high genetic variation which is further due to heterozygous nature of almond. The clustering based on RAPD analysis placed the accessions in appropriate groups. 'Nauni Selection' is extremely thin shelled, early maturing and late flowering and it formed a singlet. The Cluster B comprised of all the three accessions namely 'Tree No. 2', 'V.6' and 'Nonpareil' which are thin shelled, early to mid season in maturity than those in cluster A which

are late flowering, late maturing and hard shelled.

From these results, we infer that each seed selection like any other genotype is a unique genotype in itself, due to the fact that almond is a typical example of strong gametophytic and self-

Table 4. PIC value of sixteen informative random decamer primers.

Primer with code	PIC value	Alleles
S075	0.7655	44
S084	0.8047	16
S085	0.7449	14
S081	0.2551	14
S093	0.6666	54
S078	0.7219	53
S094	0.8047	13
S087	0.7040	28
S088	0.6172	9
S089	0.8587	10
S090	0.7911	15
S091	0.5207	26
S092	0.7654	27
S095	0.3506	24
C 15	0.7109	16
S073	0.8676	40

incompatible nature. This suggests the inherent differences between the indigenous selections and exotic introductions studied.

Conclusion

Discriminating power of RAPD markers have been demonstrated in a variety of fruit and other crops. RAPD markers employed in the present investigation characterized the almond genotypes which will assist a breeder to use them as well as identified genetic stocks. The grouping of genotypes had been found to be in congruence with few of the morphological traits which further paves way for their use in breeding programmes.

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