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Molecular characterization of diversity and relationship among almond [*Prunus dulcis* Miller (D.A.Webb)] cultivars and indigenous selections

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Amplification of genomic DNA from 13 almond [*Prunus dulcis* Miller (D.A.Webb)] genotypes was carried out using 15 random decamer primers. Only 13 primers generated a total of 65 scorable bands, of which 32 (49.23%) were polymorphic. Most of the genotypes under present study were not defined by a single marker but a set of several markers; however, GP-10 and GP-14 were distinctly identifiable from rest of the genotypes with unique bands obtained with OPP-10, OPP-14, OPP-15 and OPP-19. The similarity coefficient value between 13 genotypes ranged from 0.6557 to 0.9491. UPGMA based dendrogram on similarity coefficient was constructed and 13 genotypes were clustered into different sub-groups, more or less according to their origin. Seedling selections GP-10, GP-14, GP-17 and GP-19 were more divergent as they formed different groups in the dendraogram and thus, can be used in hybridization programmes with exotic introductions like Nonpariel, Drake, IXL, Promorskij, Pranyaj and Merced.

Keywords: Almond, *Prunus dulcis*, RAPD, molecular characterization, genetic relationship

Introduction

Almond [*Prunus dulcis* Miller (D.A.Webb) syn. *P amygdalus* Batsch] is an important nut fruit with widespread popularity throughout the world. Within the genus *Prunus*, almond is closely related to peach and is separated from it by evolution in the xerophytic environment of Central and South-west Asia[1]. Peach has evolved in the more humid climates of eastern Asia, separated from the almond by the uplifting of the Central Asian Massif. USA is the leading producer of almond in the world and contributed 82% to the total world production of 1.70 billion pounds in 2007[2]. India is the third largest importer of almond from USA after Spain and Germany.

The present day cultivation of almond in India is mainly based on some exotic cultivars and few seedling selections. Since the last decade or so, due to change in weather conditions, the earlier adapted cultivars are not performing well at many places. Hence, there is a need to expand the almond cultivation with the introduction of new varieties and identification of cross-compatible groups for inter-planting. In the past, the propagation of almond was done using seeds, which has resulted in the present day wide range of almond genotypes with different morphological and biological characteristics. In order to select varieties from these diverse types, characterization and evaluation of these genotypes is indispensable because of the well known fact that diversity could be created by crossing diverse genotypes. Classical methods of identification and characterization of cultivars in fruit trees are based on morphological, cytological and phytochemical traits, which present some disadvantages like high susceptibility to environmental factors and low degree of polymorphism. Thus, an accurate tool to allow an unquestionable characterization is through DNA and randomly amplified DNA is one of them.

The use of molecular markers based on DNA results in consistent and robust method to identify plant material based on their stability in different environmental conditions. Molecular analysis have been previously performed in the genus *Prunus* using different markers, such as isozymes[3], RFLPs and SNPs[4], PCR-RFLPs[5] and RAPD[6,7]. RAPD analysis has provided useful class of DNA based molecular markers[8,9] and is very often used for the study of genetic diversity and relationships both between and within plant species. Especially in the case of fruit

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trees, the molecular markers are very useful because morphological character evaluation is very time consuming and, for varied expressions, it must be evaluated in the course of several years. Markers can be used to identify polymorphisms quickly and efficiently and has proved to be a useful tool for genetic studies. Hence, the present study was undertaken based on the molecular characterization of the existing exotic cultivars cultivated both in Jammu and Kashmir and Himachal Pradesh, indigenous selections and some superior seedlings, which had been identified previously.

Materials and Methods

Molecular characterization of diversity and relationship among 13 almond genotypes, viz., four introductions (IXL, Merced, Drake & Nonpareil) from USA and two (Primorskij & Pranyaj) from Ukraine and seven indigenous selections (Shalimar, Makhdoom, Waris, GP-10, GP-14, GP-17 and GP-19) from Central Institute of Temperate Horticulture, Srinagar, Jammu and Kashmir were studied.

DNA Extraction, Purification and Amplification

Genomic DNA was extracted from fresh green and disease-free leaves according to the protocol described earlier[10] with some minor modifications. Young fresh and disease-free frozen leaves (2 g) were crushed in liquid nitrogen adding 20 mg of polyvinyl pyrollidone. The powder of leaves was transferred into 15 mL pre warmed (65°C) DNA extraction buffer and incubated at 65°C for 1 h in a water bath. The mixture was emulsified with 15 mL chloroform:isoamyl alcohol (24:1, v/v) and centrifuged for 15 min at 12,000 rpm at room temperature and the aqueous layer was retained. $^1/_7{}^{th}$ volume of 3 *M* sodium acetate (*p*H 4.8) followed by addition of double volume of chilled absolute ethanol was used to precipitate DNA. The DNA was spooled out with glass hook. DNA pellet was washed with 70% ethanol and centrifuged at 8,000 rpm for 10 min at 4°C. It was air dried and resuspended in 100-200 µL TE buffer (*p*H 8.0) depending upon the yield of DNA.

Purification of DNA was done by adding 10 µL of RNase (1 mg/mL) to the crude sample of DNA. The mixture was incubated at 37°C for 1 h and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was added and the contents were mixed well by gentle inversion and centrifuged at 12,000 rpm for 15 min at 4°C. To the aqueous phase so obtained, equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added and centrifuged at 12,000 rpm for 15 min at 4°C. Aqueous phase was again taken and $^1/_7{}^{th}$ volume of 3 *M* sodium acetate (*p*H 4.8) plus double volume of chilled absolute ethanol were added to precipitate the DNA. The mixture was centrifuged at 10,000 rpm for 10 min at 4°C to spin down the DNA pellet. Supernatant was discarded and the pellet was washed with 70% ethanol (100-500 µL) depending upon the pellet size. The pellet was dried for nearly 2 h using the vacuum system. Finally, the pellet was dissolved in 100 µL of TE buffer (*p*H 8.0).

DNA amplification was done with 15 random decamer primers of OPP series namely OPP-03, OPP-04, OPP-07 OPP-08, OPP-09, OPP-10, OPP-13, OPP-14, OPP-15, OPP-16, OPP-17, OPP-18 and OPP-19. For RAPD reactions, 20 ng DNA was used as tempelate in final volume of 20 µL containing 10X reaction buffer (2 µL $MgCl_2$, 2.0 µL primer, 0.5 µL dNTPs and 0.2 µL Taq polymerase). The PCR amplification was performed in 96 well microtest plates using thermal cycler (PTC-100, MJ Research Inc.). The amplification program consisted of preliminary denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C with final extension step at 72°C.

Electrophoresis of Amplified DNA

The amplified products were electrophoretically separated on 1.4% agarose gel prepared in 1X TAE buffer (40 µ*M* tris-acetate, *p*H 8.3, 0.5 µ*M* EDTA) for 1 h. Gels were stained with ethidium bromide (100 mg/mL) and then observed under UV-transilluminator. 1 Kb ladder (Fermentas SM 0313) was used as a standard. 5 µL of sample was loaded onto each well and amplified DNA was separated with 60 V constant current for $1^1/_2$ h.

For RAPD data analysis, the bands with same molecular weight and mobility were treated as identical fragments. RAPD products were scored for presence or absence of each amplicon evaluated. Only those bands that could be unequivocally scored across all the samples were included in the analysis. Pairwise similarity matrices were generated using Jaccard's coefficient of similarity[11]. Data matrices were prepared in which the presence of a band was coded as 1, whereas the absence as 0. The data matrices were analyzed by the SIMQUAL program of NTSYS-pc (Version 2.02e) and the similarities

between genotypes were estimated using Jaccard's coefficient, calculated as J=A/(N-D), where A is the number of positive matches (presence of band in both samples), D is the number of negative matches (absence of band in both samples) and N is the total sample size including both the number of matches and unmatches[12]. Dendrogram was constructed from the resultant similarity matrices using the UPGMA method[13].

Results and Discussion

Molecular characterization was investigated using 15 random primers, only 13 of which produced polymorphism (Fig. 1). Each primer generated a unique set of amplification products ranging in size from 290 bp (OPP-15) to 3750 bp (OPP-09). Number of bands for each primer ranged from 2 (OPP-03) to 8 (OPP-08, OPP-14). Total number of bands obtained was 65, of which 32 were polymorphic and 33 were monomorphic. The maximum number of bands (58) was obtained with all the primers for genotypes GP-19 and GP-10 and minimum (41) for genotype IXL. Average number of bands per primer was 5 and percentage of total polymorphic bands was 49.2%. The banding patterns obtained with 13 primers are presented in Table 1.

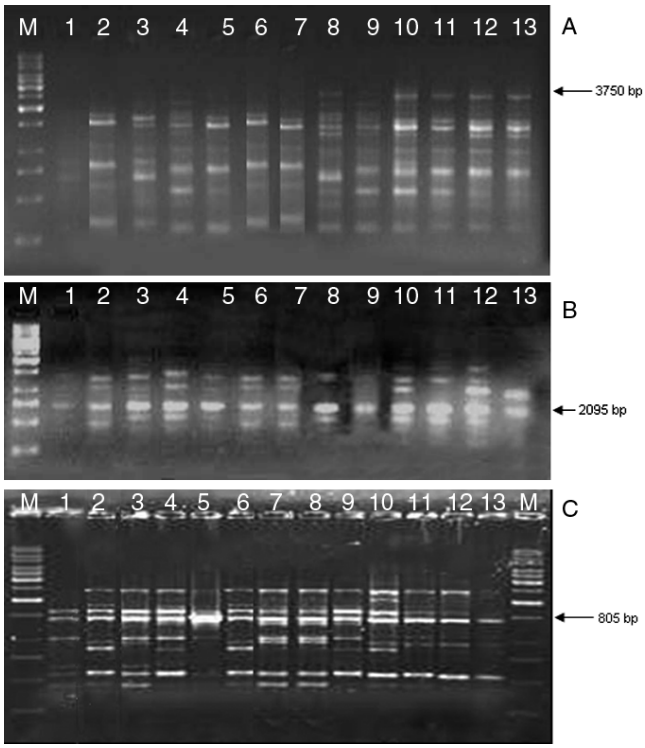

Fig. 1—RAPD pattern of 13 genotypes of *P. dulcis* generated by primers OPP-9–sequence $^{5'}$GTGGTCCGCA$^{3'}$ (A), OPP-10 – sequence $^{5'}$TCCCGCCTAC$^{3'}$(B) and OPP-14–sequence $^{5'}$CCAGCCGAAC$^{3'}$ (C). Lanes: M-Marker, 1-IXL, 2-Waris, 3-Primorskij, 4-Merced, 5-Makhdoom, 6-Shalimar, 7-Drake, 8-Pranyaj, 9-Nonpareil, 10-GP-19, 11-GP-14, 12-GP-10, & 13-GP-17.

In the present study, unique banding patterns were obtained with primers OPP-14 (2500 bp) and OPP-15 (1070 bp) for genotype GP-17, whereas for GP-19 unique bands were obtained with primers OPP-10 (3600 bp) and OPP-19 (1180 bp). OPP-04 differentiated Primorskij, Merced, Drake, Pranyaj and Nonpariel from rest of the genotypes, whereas band fragments produced by primers OPP-07 and OPP-08 were present in all the genotypes, except Waris and IXL. OPP-09 differentiated Pranyaj, GP-10, GP-14 and GP-17 from rest of the genotypes by having a band of 2450 bp, while band of 1510 bp was present in all the genotypes, except IXL. GP-10 was distinctly identifiable with a unique band of 3600 bp obtained with primer OPP-10, while a fragment of 1594 bp was present in GP-10 and GP-19 with primer OPP-13. With the same primer, OPP-13, a fragment of 2870 bp was present in all the cultivars and selections except GP-17. Similar unique bands were obtained previously[16] which distinguished Portuguese almond cultivars by RAPDs. GP-10 was distinctly identifiable from rest of the genotypes by having unique bands of 3600 bp, 2500 bp and 1180 bp obtained with OPP-10, OPP-14 and OPP-19, respectively. GP-14 could be identified with a unique band of 1070 bp obtained with OPP-15.

Similarity Matrix and Cluster Analysis

The RAPD fragments obtained after the amplification of genomic DNA were scored for their presence (1) and absence (0) for each sample. The data matrix analyzed with NTSYS-pc software is presented in Table 2. The coefficient values between the cultivars and indigenous selections ranged form 0.6557 to 0.9491. Maximum similarity (0.9491) was found between genotypes Primorskij and Pranyaj, while IXL and GP-10 had the minimum similarity (0.6557). Dendrogram constructed using the similarity coefficient and unweighted pair group mean average (UPGMA) method to visualize genetic differentiation among various genotypes of almond and clustering pattern is shown in Fig. 2. A critical perusal of data revealed that the distribution of samples into cluster and within cluster was somewhat definite. Genotypes of same origin were more or less clustered together.

Table 1—Banding pattern of RAPD markers in *P. dulcis* with 13 random decamer primers

Geno type → / Primer ↓	IXL			Waris			Primorskij			Merced			Makhdoom			Shalimar			Drake			Pranyaj			Nonpareil			GP-19			GP-14			GP-10			GP-17		
	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB	PB	MB	TB
OPP-03	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	2
OPP-04	0	3	3	0	3	3	1	3	4	1	3	4	0	3	3	0	3	3	1	3	4	1	3	4	1	3	4	0	3	3	0	3	3	0	3	3	0	3	3
OPP-07	1	1	2	0	1	1	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4
OPP-08	0	5	5	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8	3	5	8
OPP-09	1	1	2	3	1	4	3	1	4	3	1	4	2	1	3	2	1	3	2	1	3	4	1	5	3	1	4	4	1	5	4	1	5	4	1	5	3	1	4
OPP-10	0	1	1	3	1	4	3	1	4	3	1	4	1	1	2	3	1	4	0	1	1	2	1	3	0	1	1	3	1	4	2	1	3	4	1	5	2	1	3
OPP-13	0	2	2	0	2	2	1	2	3	0	2	2	0	2	2	0	2	2	0	2	2	1	2	3	0	2	2	2	2	4	1	2	3	2	2	4	1	2	3
OPP-14	2	3	5	4	3	7	3	3	6	3	3	6	2	3	5	4	3	7	3	3	6	3	3	6	3	3	6	4	3	7	4	3	7	3	3	6	2	3	5
OPP-15	0	4	4	0	4	4	0	4	4	0	4	4	0	4	4	0	4	4	0	4	4	0	4	4	0	4	4	1	4	5	2	4	6	1	4	5	1	4	5
OPP-16	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6	0	6	6
OPP-18	0	2	2	0	2	2	1	2	3	2	2	4	0	2	2	1	2	3	2	2	4	2	2	4	0	2	2	1	2	3	1	2	3	1	2	3	1	2	3
OPP-17	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	3	1	4	0	1	1	2	1	3	2	1	3	1	1	2	1	1	2
OPP-19	1	2	3	1	2	3	2	2	4	1	2	3	0	2	3	0	2	2	1	2	3	2	2	4	1	2	3	2	2	4	1	2	3	3	2	5	1	2	3
Total	8	33	41	17	33	50	23	33	56	22	33	55	14	33	48	19	33	52	18	33	51	24	33	57	14	33	47	25	33	58	23	33	56	25	33	58	18	33	51

PB= Number of polymorphic bands
MB= Number of monomorphic bands
TB= Total number of bands scored

Table 2—Jaccards similarity coefficient for 13 almond cultivars

Genotypes	IXL	Waris	Primorskij	Merced	Makhdoom	Shalimar	Drake	Pranyaj	Nonpareil	GP-19	GP-14	GP-10	GP-17
IXL	1.0000												
Waris	0.7884	1.0000											
Primorskij	0.7368	0.8620	1.0000										
Merced	0.7017	0.8596	0.8983	1.0000									
Makhdoom	0.7843	0.8518	0.8596	0.8571	1.0000								
Shalmimar	0.7272	0.8909	0.8965	0.8947	0.8889	1.0000							
Drake	0.7735	0.8070	0.8793	0.8771	0.9056	0.8750	1.0000						
Pranyaj	0.7241	0.8166	0.9491	0.8833	0.8448	0.8500	0.8965	1.0000					
Nonpareil	0.7307	0.8000	0.8103	0.8392	0.8653	0.8035	0.8518	0.7966	1.0000				
GP-19	0.6833	0.8333	0.9016	0.8387	0.8000	0.8666	0.7903	0.8870	0.7833	1.0000			
GP-14	0.7068	0.8000	0.8688	0.8064	0.8275	0.8333	0.8166	0.8852	0.7796	0.9016	1.0000		
GP-10	0.6557	0.7741	0.8709	0.7812	0.7704	0.8064	0.7619	0.8571	0.7540	0.9344	0.8709	1.0000	
GP-17	0.6785	0.7758	0.8474	0.7833	0.8035	0.8421	0.8245	0.8333	0.7857	0.8814	0.8793	0.8813	1.0000

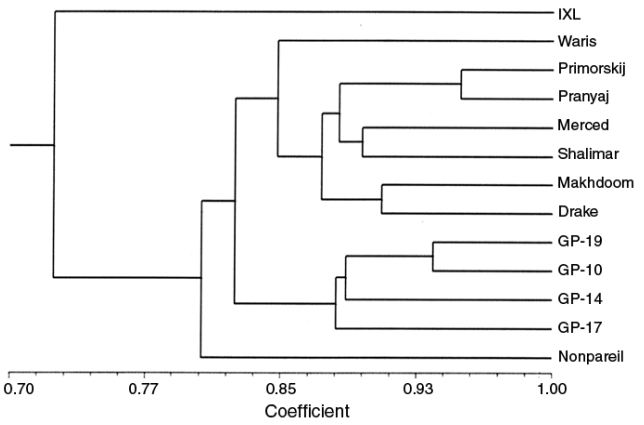

Fig. 2—Dendrogram of the genetic relatedness of 13 almond genotypes based on RAPD primers.

The first major bifurcation in the dendrogram divided the 13 almond genotypes into two groups. First group consisted of only one cultivar IXL, whereas all other genotypes constituted the second group. Further bifurcation of second group demarcated the genotypes of Ukrainian/Russian origin (Primorskij and Pranyaj) and seedling selections of India (GP-19, GP-10, GP-14 and GP-17) into separate groups.

Out of 15 decamer primers, 13 produced polymorphism and the extent of polymorphism in the studied gentoypes was 49.2%, which is close to 57.7% reported in 17 Apulian cultivars[15] and 56.4% in *P. dulcis* and *P. webbii*[16].

Most of the genotypes under present study could not be defined by a single marker but by a set of several markers as reported earlier in walnut[17]. This probably reflects the out crossing nature of almond owing to self incompatibility which prevents self pollination. Similar results with RAPDs have been obtained earlier in almond[18] and other out crossing fruit and nut species such as olive[19] and walnut[20]. The similarity index calculated during the present study showed values ranging from 0.6557 to 0.9491, the lowest value being observed between Primorskij and Pranyaj. The similarity values are in accordance with the origin of the genotypes, wherein genotypes of same origin show higher values. Previously the similarity values between 19 almond cultivars have been found to range between 0.70 and 0.96[21] which is almost similar to the present findings. A UPGMA based dendrogram based on similarity coefficient was constructed and accordingly 13 genotypes of almond could be clustered into different sub-groups, more or less according to their origin, whereas, the indigenous seedling selections formed a separate group. Earlier, based on the similarity values, various workers constructed dendrograms for different cultivars and genotypes of genus *Prunus* and clustered them according to their geographic origin[21-24].

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