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MOLECULAR CHARACTERIZATION AND GENETIC RELATIONSHIP AMONG LOQUAT (*ERIOBOTRYA JAPONICA* LINDL.) GENOTYPES OF PAKISTAN ASSESSED BY RAPD MARKERS

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Abstract

Loquat is an important sub-tropical fruit crop of Pakistan. A number of loquat genotypes are there in the loquat growing areas of Pakistan, however, no research work has been reported regarding the description of these genotypes. As a result, there is no standard or identified loquat cultivar available to the growers in the loquat growing pockets of Pakistan. In the present study, 42 genotypes identified through the morphological and physical characters were subjected to the DNA analysis to determine the level of genetic diversity among the local loquat genotypes found in different areas of Pakistan and to assess the relationships among them. Out of 48 amplification products scored, 47 bands (97.92%) were found to be polymorphic. According to the dandrogram, two main groups of the loquat genotypes were identified. In general, all genotypes belonging to a certain location came under any one of the two groups. In this study, RAPD proved to be a reliable technique for revealing relationship among loquat genotypes.

Introduction

Loquat (*Eriobotrya japonica* Lindl.) is an evergreen fruit tree belonging to the family Rosaceae, subfamily Pomoideae. It is an important sub-tropical fruit crop having its origin in China where it is under cultivation for over 2000 years (Lin *et al*., 2007). Then it spread to Japan, Pakistan India, America, England, Italy, Spain, Brazil, Turkey, Australia and many other countries. Through thousands of years of selection a lot of cultivars have been selected with excellent quality and large sized fruit (Janick, 2007). Japan has great contribution towards the development of loquat. Japanese horticulturists selected outstanding cultivars from the progeny of seedlings introduced from China (Lin *et al*., 2007). There are a number of cultivars and selections of *E. japonica* in different provinces of China. The largest collection of germplasm, having more than 250 cultivars, is situated in Fuzhou province of China. Spain and Japan have germplasm banks with 100 cultivars and 60 cultivars respectively, while Italy has a collection of 16 cultivars (Lin, 2007).

There is no standard or identified loquat cultivar available to the growers for cultivation in the loquat growing pockets of Pakistan. As a result, most of the loquat orchards do not possess the plants with uniform fruit characteristics as they have natural chance seedling trees with variable performance owing to heterozygosity and cross-pollination. A number of loquat genotypes are being grown in the loquat growing areas of Pakistan, for which no work has been reported previously (Hussain *et al*., 2007). As a result of present study, 42 loquat genotypes have been characterized on the basis of morpho-physical characteristics in the main loquat growing areas of Pakistan.

Though the selection system of breeding material on the basis of morphological characters remains valuable, but this assessment has limitations, including the influence

of environment or management practices (Gepts, 1993). Moreover, the conventional approach to characterize the cultivars in fruit tree species on the basis of phenotypic observations is slow due to the long life cycle of plants. Therefore, there is a need to incorporate the new methods based on studies at the DNA level in order to determine the genetic relationships among different cultivars (Shiran *et al*., 2007; Hafiz *et al*., 2008).

Problems related with taxonomical classification highlight the need of complementary keys for identification and characterization of the genotypes. A standard set of RAPD primers can be established to characterize most of the common genotypes that may serve as a useful supplement to the traditional morphological information (Nandini & Chikkadevaiah, 2005).

Keeping in view the above facts, 42 genotypes identified through the morphological and physical characters were subjected to the DNA analysis to determine the level of genetic diversity among the local loquat genotypes found in different areas of Pakistan and to assess the relationships among them.

Materials and Methods

Plant material: Forty-two genotypes of loquat from nine locations in the loquat growing areas of Pakistan (Fig. 1) representing genetic diversity on the basis of morpho-physical characteristics were subjected to RAPD analysis. List of these genotypes along with respective locations is given in Table 1.

DNA extraction: Tender leaves from the subject plants were collected during April and frozen immediately in liquid nitrogen. DNA isolation was done using CTAB (hexadecyltri-methylammonium bromide) method. The leaves were ground in liquid nitrogen, and the DNA was extracted with 2% CTAB extraction buffer (2% CTAB, 1.4M NaCl, 100 mM Tris, 20mM EDTA and 1% Mercaptoethanol). Further isolation was continued according to the method described by Zidani *et al*., (2005).

Quality of the extracted DNA was checked by running on 1.5% agarose gel. Ethidium bromide solution was used to stain the gel. Stained gel was then visualized with UV trans illuminator and quality of the DNA was assessed. Appropriate dilutions of DNA were made for further amplification and RAPD analysis.

Polymerase chain reaction: Polymerase chain reaction (PCR) was performed on T-Cy Thermal cycler (Crea Con, The Netherlands) using 25 µl reaction mixture containing 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 3 mM $MgCl_2$, 200 µM of each dNTP, 1µM Primer, 50 ng of DNA and one unit of *Taq* DNA polymerase. Fourteen Ten-base pair primers were used for amplification reactions, however, only the primers showing polymorphic results were selected for the purpose of diversity and similarity analysis (Table 2).

After PCR, the amplified fragments were separated on 1.5% agarose gel in 0.5X Tris Boric EDTA (TBE) buffer, stained by Ethidium bromide, visualized and photographed with the help of Gel Documentation System (Kodak EDAS 290).

Data analysis: The photographs of gels were used to score data for RAPD markers. Each DNA fragment amplified by a given primer was considered as a unit character and the RAPD fragments were scored as present (1) or absent (0) for each of the primer-accession combinations. The molecular size of the amplification products was measured with marker DNA ladder. The presence and absence of the bands was scored in a binary data matrix. Polymorphic bands were scored and used for further analysis. RAPD analysis was carried out using the Statistica 5.5 software. Dandrogram was constructed showing the degree of relatedness / differences among all the genotypes.

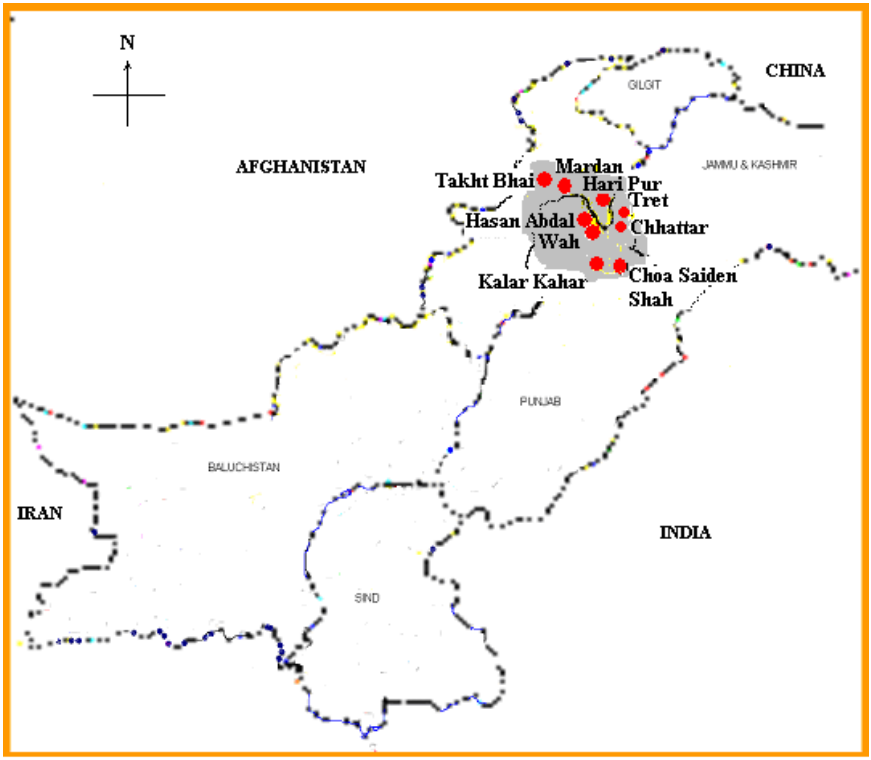

Fig. 1. Map of Pakistan showing main loquat growing area (shaded area) and circles show the sites selected for the study.

Table 1. List of loquat genotypes included in study.

Location	Genotypes
Kalar Kahar	KK1, KK2, KK3, KK4, KK5
Choa Saiden Shah	CS1, CS2, CS3
Chhattar	CH1, CH2, CH3
Tret	TR1, TR2, TR3, TR4, TR5
Hasan Abdal and Wah	HW1, HW2, HW3, HW4, HW5
Haripur	HP1, HP2, HP3
Mardan	MN1, MN2, MN3
Takht Bhai	TB1, TB2, TB3, TB4, TB5, TB6, TB7, TB8, TB9, TB10, TB11, TB12, TB13, TB14, TB15

Results and Discussion

The local loquat genotypes selected on the basis of morpho-physical characteristics were subjected to the DNA analysis to determine the level of genetic diversity among the loquat genotypes found in different areas of Pakistan and to assess the relationships among them. For this purpose, DNA extracted from the loquat genotypes was amplified using 14 random primers and run on the agarose gel.

Table 2. Polymorphism revealed by different RAPD primers.

Primer	Sequence (5'-3')	Scored bands	Polymorphic bands	Polymorphism rate (%)
GL DecamerA-02	TGCCGAGCTG	07	07	100
GL DecamerC-02	GTGAGGCGTC	10	10	100
GL DecamerC-05	GATGACCGCC	12	12	100
GL DecamerC-07	GTCCCGACGA	09	09	100
GL DecamerC-19	GTTGCCAGCC	10	09	90
Total		**48**	**47**	**97.92**
Average		**9.6**	**9.4**	**97.92**
Range		**7-12**	**7-12**	**90-100**

Polymorphism observed: Out of 14 decamer RAPD primers used, five primers generated strong amplifications and resulted in polymorphic products (Table 2). The remaining nine primers were not considered for compiling the results because they were either not polymorphic or did not give clear amplifications.

The bands obtained through electrophoresis were photographed (Fig. 2 to Fig. 6). Out of 48 amplification products scored, 47 bands (97.92%) were found to be polymorphic. The average number of score able bands per primer was 9.6, while average number of polymorphic bands was 9.4 (having a range from 7-12 bands per primer). High frequency of polymorphism was detected with all the selected primers. The percentage of polymorphic bands was 100% with 4 primers, while GL Decamer C-19 exhibited comparatively low level of variability and the percentage of polymorphic bands was 90%.

Similar results have also been reported by Agar *et al*., (2008) who used 40 decamer primers to investigate 23 apricot cultivars. However, 28 primers did not give polymorphic bands or did not amplify clear products. Twelve primers produced good and reproducible polymorphic bands and were used for further analysis. Out of these 12 primers, 11 produced 100% polymorphic bands and overall percentage of polymorphic markers was 97.5.

Cluster analysis: Cluster analysis was performed to establish the genetic diversity among 42 loquat genotypes growing in different areas of Pakistan. The dandrogram was constructed on the basis of presence and absence of bands by using the software 'Statistica' (Fig 7). According to the dandrogram, two main groups designated as 'A' and 'B' of the loquat genotypes have been identified having a linkage distance of 33%. All the genotypes growing in Chhattar (CH), Tret (TR), Haripur (HP), Hasan Abdal and Wah garden (HW) come under the first group, while all the genotypes from Kalar Kahar (KK) and Choa Saiden Shah (CS) fall under the second group. In general, all genotypes belonging to a certain location came under any one of the two groups with the exception of those from Mardan (MN) and Takht Bhai (TB). Genotypes from Mardan and Takht Bhai were found under both the groups. Out of the three genotypes of Mardan, one (MN3), falls under the first group and the other two (MN1 and MN2) under the second group. The maximum number of genotypes (fifteen) was identified at Takht Bhai. Only two of them (TB2 and TB3) were found to belong to the first group, whereas all the remaining genotypes to the second group. One genotype from Takht Bhai (TB11) fell in group B but within the group appeared as a single solitary line, different from all other members of the group.

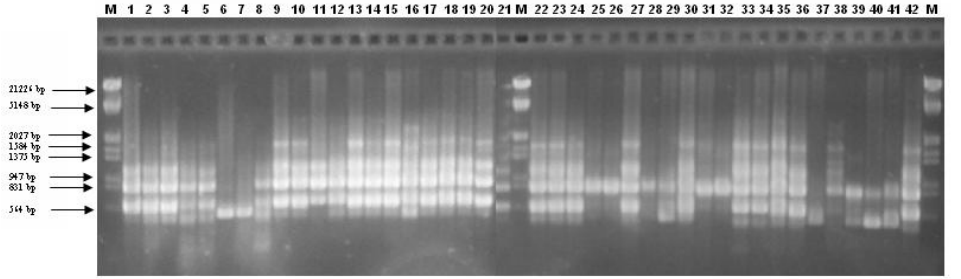

Fig. 2. RAPD pattern of 42 loquat genotypes with Primer GL DecamerA-02: M=Marker; 1=KK1; 2=KK2; 3=KK3; 4=KK4; 5=KK5; 6=CS1; 7=CS2; 8=CS3; 9=TR1; 10=TR2; 11=TR3; 12=TR4; 13=TR5; 14=CH1; 15=CH2; 16=CH3; 17=HP1; 18=HP2; 19=HP3; 20=HW1; 21=HW2; 22=HW3; 23=HW4; 24=HW5; 25=MN1; 26=MN2; 27=MN3; 28=TB1; 29=TB2; 30=TB3; 31=TB4; 32=TB5; 33=TB6; 34=TB7; 35=TB8; 36=TB9; 37=TB10; 38=TB11; 39=TB12; 40=TB13; 41=TB14; 42=TB15.

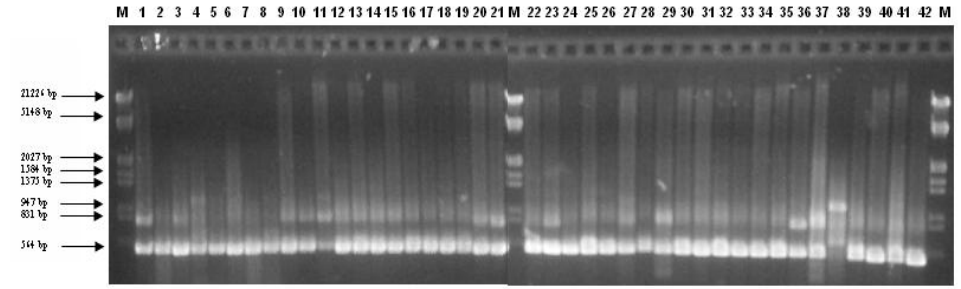

Fig. 3. RAPD pattern of 42 loquat genotypes with Primer GL Decamer C-02: M=Marker; 1=KK1; 2=KK2; 3=KK3; 4=KK4; 5=KK5; 6=CS1; 7=CS2; 8=CS3; 9=TR1; 10=TR2; 11=TR3; 12=TR4; 13=TR5; 14=CH1; 15=CH2; 16=CH3; 17=HP1; 18=HP2; 19=HP3; 20=HW1; 21=HW2; 22=HW3; 23=HW4; 24=HW5; 25=MN1; 26=MN2; 27=MN3; 28=TB1; 29=TB2; 30=TB3; 31=TB4; 32=TB5; 33=TB6; 34=TB7; 35=TB8; 36=TB9; 37=TB10; 38=TB11; 39=TB12; 40=TB13; 41=TB14; 42=TB15.

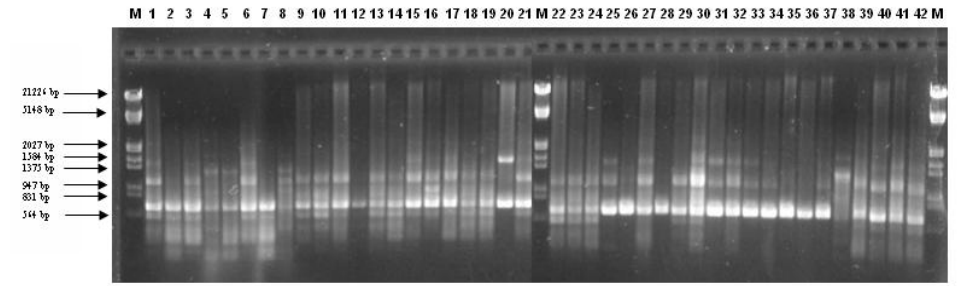

Fig. 4. RAPD pattern of 42 loquat genotypes with Primer GL Decamer C-05: M=Marker; 1=KK1; 2=KK2; 3=KK3; 4=KK4; 5=KK5; 6=CS1; 7=CS2; 8=CS3; 9=TR1; 10=TR2; 11=TR3; 12=TR4; 13=TR5; 14=CH1; 15=CH2; 16=CH3; 17=HP1; 18=HP2; 19=HP3; 20=HW1; 21=HW2; 22=HW3; 23=HW4; 24=HW5; 25=MN1; 26=MN2; 27=MN3; 28=TB1; 29=TB2; 30=TB3; 31=TB4; 32=TB5; 33=TB6; 34=TB7; 35=TB8; 36=TB9; 37=TB10; 38=TB11; 39=TB12; 40=TB13; 41=TB14; 42=TB15.

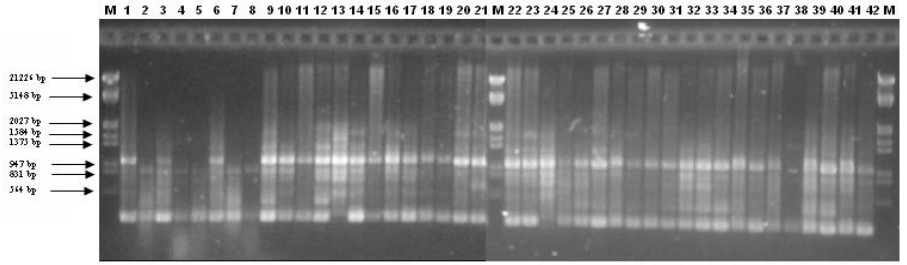

Fig. 5. RAPD pattern of 42 loquat genotypes with Primer GL DecamerC-07: M=Marker; 1=KK1; 2=KK2; 3=KK3; 4=KK4; 5=KK5; 6=CS1; 7=CS2; 8=CS3; 9=TR1; 10=TR2; 11=TR3; 12=TR4; 13=TR5; 14=CH1; 15=CH2; 16=CH3; 17=HP1; 18=HP2; 19=HP3; 20=HW1; 21=HW2; 22=HW3; 23=HW4; 24=HW5; 25=MN1; 26=MN2; 27=MN3; 28=TB1; 29=TB2; 30=TB3; 31=TB4; 32=TB5; 33=TB6; 34=TB7; 35=TB8; 36=TB9; 37=TB10; 38=TB11; 39=TB12; 40=TB13; 41=TB14; 42=TB15.

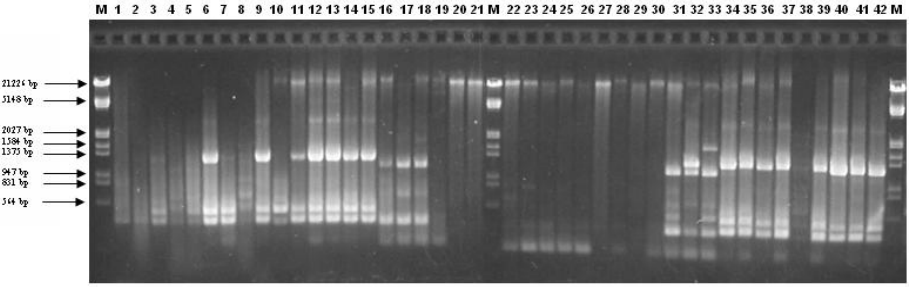

Fig. 6. RAPD pattern of 42 loquat genotypes with Primer GL Decamer C-19: M=Marker; 1=KK1; 2=KK2; 3=KK3; 4=KK4; 5=KK5; 6=CS1; 7=CS2; 8=CS3; 9=TR1; 10=TR2; 11=TR3; 12=TR4; 13=TR5; 14=CH1; 15=CH2; 16=CH3; 17=HP1; 18=HP2; 19=HP3; 20=HW1; 21=HW2; 22=HW3; 23=HW4; 24=HW5; 25=MN1; 26=MN2; 27=MN3; 28=TB1; 29=TB2; 30=TB3; 31=TB4; 32=TB5; 33=TB6; 34=TB7; 35=TB8; 36=TB9; 37=TB10; 38=TB11; 39=TB12; 40=TB13; 41=TB14; 42=TB15.

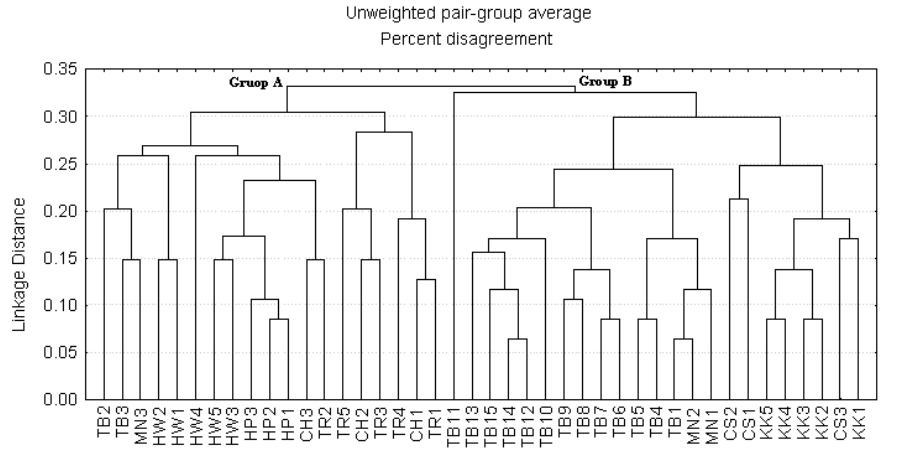

Fig. 7. Clustering pattern of 42 loquat genotypes based on RAPD markers.

Grouping together of the genotypes of Chhattar, Tret, Haripur, Hasan Abdal and Wah garden is understandable because many families of local people from these areas are linked to one another with social ties and blood relations, and have a continuous interaction among them. So exchange of material among these locations is not surprising. Similarly, genotypes of Kalar Kahar and Choa Saiden Shah formed a distinct cluster under the group B. It is also due to continuous exchange of material between the two locations, as both are located in the same district and have strong social bonds.

Grouping of genotypes of Choa Saiden Shah and Kalar Kahar together with those of Mardan and Takht Bhai was unexpected as they are located very distantly in two different provinces of the country, which are not connected directly through any rail or road link. But when the socio economic set up of Choa Saiden Shah area was taken into consideration, the situation became clear. Choa Saiden Shah is an old town of the Punjab province where coal mines are abundantly found. Majority of the mine workers or labourers in these mines belong to the North Western Frontier Province (NWFP), to which Mardan and Takht Bhai belong. They have their settlements in Choa Saiden Shah and its periphery for almost three generations, while they frequently travel between the two provinces. Moreover, Khewra, a town adjacent to Choa Saiden Shah, is rich in salt mines and same is the situation of workers over there. These mineworkers might have been a source of transfer of material at such a long distance.

The maximum number of genotypes (fifteen) as well as the maximum level of genetic diversity was observed at Takht Bahi in a single orchard of a progressive grower. Genotypes from Mardan were mainly found in group B, however, some members also fell in group A, which predominantly includes genotypes from Chhattar, Tret, Hari Pur, Hasan Abdal and Wah. This exchange is, however, not unexpected as these areas are socially linked among one another. Moreover, Mardan as well as the last three of the sites mentioned above are physically linked through the old Grand Trunk Road (GT Road).

While observing the clustering pattern, it is evident that in most of the cases the genetic diversity level among the genotypes of the areas in close proximity is very narrow, forming small sub-clusters with very little linkage distances. Kalar Kahar and Choa Saiden Shah fall under one cluster. Genotypes from Haripur come under a small sub-cluster with very small linkage distance. Low genetic diversity within certain locations may be due to single introduction and/or shorter history of cultivation. Genetic diversity within these locations may therefore be attributed to the cross pollination and heterozygosity as most of the plants may be siblings of the seedling parents.

On the other hand, a wide range of genetic diversity exists at Mardan and Takht Bhai. It is due to the fact that there are many progressive growers of loquat in this area who have been involved in the commercial cultivation of loquat for the past many decades. They have made a number of loquat introductions and selections over the years. The results of study indicate that there is good level of genetic diversity among loquat genotypes growing in different areas of Pakistan. Since it is the first study on loquat ever conducted in Pakistan, it can provide a basis for the documentation of loquat cultivars and for establishment of loquat germplasm unit. It would also be helpful for the planning of breeding strategies and future programs.

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References

Agar, G., S. Ercisli, N. Yildirim, A. Esitken and E. Orhan. 2008. Identification of apricot (*Prunus armeniaca* L.) cultivars in Turkey using RAPD markers. *Turk J. Biol*., 32: 1-7.

Gepts P. 1993. The use of molecular and biochemical markers in crop evaluation studies. In: Hecht MK (ed), *Evolutionary Biology*, Vol. 27. Plenum Press, New York, p 51-94.

Hafiz, I.A., N.A. Abbasi., T. Ahmad., A. Hussain. 2008. DNA methylation profiles differ between juvenile and adult phase of crab apple (*Malus micromalus*). *Pak. J. Bot*., 40 (3), 1025-1032.

Hussain, A., N.A. Abbasi and A. Akhtar. 2007. Fruit characteristics of different loquat genotypes cultivated in Pakistan. 2nd Int. Sympo on loquat. *Acta Hort*., 750: 287-291.

Janick, J. 2007. Genetic alteration associated with fruit domestication. 2nd Int. Sympo on loquat. *Acta Hort*., 750: 27-35.

Lin, S. 2007. World loquat production and research with special reference to China. 2nd Int. sympo on loquat. *Acta Hort*., 750: 37-43.

Lin, S., X. Huang, J. Cuevas and J. Janick. 2007. Loquat: An ancient fruit crop with a promising future. *Chronica Hort*., 47 (2): 12-15.

Nandini, R. and Chikkadevaiah. 2005. DNA fingerprinting of sunflower genotypes (*Helianthus annuus* L.). *HELIA*., 28 (42)**:** 9-18.

Shiran, B., N. Amirbakhtiar, S. Kiani, Sh. Mohammadi, B.E. Sayed-Tabatabaei and H. Moradi. 2007. Molecular characterization and genetic relationship among almond cultivars assessed by RAPD and SSR markers. *Scientia Horticulturae*, 111:280-292.

Zidani, S., A. Ferchichi and M. Chaieb. 2005. Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves. *African J. Biotech*., 4(8): 862-866.

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