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RAPD BASED ESTIMATION OF GENETIC DIVERSITY IN WALNUT GENOTYPES GROWING IN MALAKUND DIVISION, PAKISTAN

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RAPD based estimation of genetic diversity in walnut genotypes growing in Malakund division, Pakistan, was made by collecting nut samples of 226 walnut genotypes from four walnut growing districts of Malakand division. On the basis of various qualitative traits, 18 superior walnut genotypes were selected along with two exotic varieties. Every single band was considered as a single locus/allele for all the genetic analyses. The 18 indigenous genotypes showed various levels of genetic polymorphism for the loci detected by using primers GLA-05 and GLA-10. The loci were scored as present (1) and absent (0). Bivariate 1-0 data matrix was used to estimate genetic distances (GD) and for construction of Phylogenetic tree based on cluster analysis. Maximum genetic distance (100%) was observed between Payne and Sw-58 and Payne and Serr, closely followed by 94% dissimilarity between Payne and Dr-67. Based on the data presented as dendrogram, the walnut genotypes were classified in to four major groups. The maximum numbers (13) of genotypes fall in group-I. Sw-58 was the only genotypes in group-IV and the most distinct from others. Classification of wild genotypes will help in breeding as well as vegetative propagation of walnut trees.

Keywords: Genetic polymorphism, genetic distance, genotypes, RAPD, walnut.

INTRODUCTION

Walnut belongs to the family Juglandaceae and genus *Juglans*. The family consists of about 60 species, 21 of which are placed in the genus *Juglans*. However, the most important among them are *Juglans regia* L. and *Juglans nigra* L. (Manning, 1978) that are famous for their delicious kernel and valuable wood. The Persian walnut (*Juglans regia*) is an ancient species (Fjellstorm and Parfitt, 1995) that originated in Central Asia, the West Himalayan chain and Kyrgystan (Aradhya *et al*., 2005). It has been cultivated in southern Europe since 1000 BC (Ducci *et al*., 1997). Walnut species are important sources of nuts and timbers in the temperate zones across the world (Khan and Khatoon, 2007). Walnuts are growing in the Northern regions of Pakistan since time immemorial and are one of the most important nut crops grown in Malakand division of KPJ Province. Malakand division contributes about 82% of the total walnut production of the country (Anonymous, 1999-2000). Walnut tree is a perennial, monoecious and mostly cross-pollinated. It shows high variability in both pomological and phenological traits. Most walnut genotypes are heterogamous, either protoandrous (male flowers mature first) or protogynous (female flowers mature first) and this dichogamy encourages cross pollination necessitating wind pollination and bloom overlap for good production (Pua and Davey, 2007). Walnut is extremely sensitive to site conditions and should only be planted on the most suitable frost free, fertile, well drained and deeply rootable sites (Kerr, 1993). Walnut has high nutritional value. It is rich in proteins (14-24%), fats (52-70%) and vitamins, especially vitamins B group and E, while in minerals; K and Mg are worth mentioning (Ali *et al.,* 2010, Rahman *et al*., 2012). Important amino acids are glutamic acid, arginine and leucine. Walnut contain Taurine (2-aminoethylsulfonic acid), an organic acid, that is involved in many functions such as homeostatic regulation, thermoregulation, nervous conduction and protection against oxidative stress in human beings (Cannella and Dernini, 2005). Walnut consists of mostly omega-3 and omega-6 polyunsaturated fatty acids, which are essential dietary fatty acids and helps in the prevention of coronary heart diseases (Piccirillo *et al*., 2005). Nut consumption is also associated with a protective effect against coronary heart disease, partly due to its high antioxidant content (Davis *et al*., 2006; Zhang *et al*., 2009). The walnut wood is regarded to be as one of the most valuable woods in the world for high grade furniture and joinery (Voulgaridis and Vassiliou, 2005) and the bark has been used for teeth cleaning and curing gum diseases (Ibrar *et al*., 2007).

The walnut has traditionally been propagated by seed. Thus, the resultant plants have marked diversity in nuts size, shape, shell thickness and color, quality and color of kernel and in other morphological attributes (Sharma *et al*., 2003). There are no regular orchards of walnut in the Northern areas of Pakistan. However, significant numbers of trees are grown on marginal lands in diffused plantations and thus are a source of

additional income to the farming community (Rahman *et al*., 2012). Pakistan houses a great diversity of different fruit crops (Khan *et al*., 2010; Mahmood *et al*., 2013, 2014). However, little information is available in the literature regarding studies on genetic diversity of walnut and the identity walnut germplasm (Khan *et al*., 2010). The fruit quality varies greatly due to variation in genetic makeup of different cultivars (Dogan *et al*., 2005). Since, the walnut is generally propagated by seed, there has been increasing diversity in nuts size, shape, thickness and color of shell, quality and color of kernel as well as other morphological attributes (Sharma *et al.*, 2003).

The genetic variability in perennial crop species has been investigated on the basis of morphological, physiological and biochemical characteristics but such assessments are influenced by variations due to environment (Zenelli *et al*., 2005; Vyas *et al*., 2003). The use of DNA based markers is an authentic technique of genetic characterization that allows comparison of different genetic material independent of environmental effects (Weising *et al*., 1995). Random amplified polymorphic DNA (RAPD) technique has emerged as the most extensively used techniques to develop DNA markers (Kumar and Gurusubramanian, 2011), because a single, short and arbitrary oligonucleotide primer, able to anneal and prime at multiple locations throughout the genome, can produce a spectrum of amplification products that are characteristics of the template DNA (Williams *et al*., 1990). Keeping in view the wide variations in yield and quality of walnut genotypes and aggressive removal of walnut trees for local consumption, it is crucial to explore and conserve the existing high quality walnut germplasm growing in the region. The research was, therefore, conducted to identify the desirable genotypes of walnuts using molecular characterization/ fingerprinting of promising walnut genotypes for the purpose of registration of new variety/varieties and planning future breeding programs.

MATERIALS AND METHODS

***Experiment Location and materials*:** The molecular characterization of indigenous walnut (*Juglans regia* L.) genotypes was conducted by collection, evaluation and selection of promising indigenous genotypes growing in Malakand division. The study was carried out in four Northern districts of Malakand division i.e. Swat, Dir, Chitral and Shangla.

A survey was conducted for collection of nuts of promising walnut genotypes and 226 genotypes were initially selected for having promising characters such as fruit size, kernel percentage and yield. The samples were allotted with identification number for reference and further investigations. After sun drying data on nut diameter, length, shape, shell texture and color, in-shell nut weight; kernel color, weight and percentage were recorded (Rahman *et al*., 2012). On the basis of data collected, 18 superior genotypes were selected for further studies. For comparison, two exotic varieties (Serr and Payne), available at Agricultural Research Institute Mingora, Swat, KPK, were also included in the study.

***Molecular characterization*:** For molecular characterization/ fingerprinting of selected indigenous walnut genotypes, leaves were collected from the grafted plants of each genotype at Agricultural Research Institute Mingora, early in the morning and were packed in the iceboxes. The samples were immediately taken to the Institute of Biotechnology and Genetic Engineering (IBGE), the University of Agriculture, Peshawar. Approximately 100 mg leaf tissues were collected from leaves of each genotype and immediately frozen in liquid Nitrogen. Two Randomly Amplified Polymorphic DNA (RAPD) primers (GL Decamer A-05 and GL Decamer A-10) were used to detect the level of genetic polymorphism at DNA level among the 20 walnut genotypes. The RAPD markers were selected on the basis of relative technical simplicity, the level of polymorphism detected, availability of primers and cost effectiveness.

The markers were obtained from Gene Link, USA. Only the scorable bands were included in the analyses. Every single band was considered as a single locus/allele for all the genetic analyses. The loci were scored as present/absent as described by Ahmad *et al*. (2012). Bivariate data 1-0 were used to estimate genetic distances. Unweighted Pair Group of Arithmetic Means function (Nie and Li, 1979) was used to estimate genetic distances between the genotypes as:

GD = 1-dxy / dx + dy – dxy

GD = Genetic distance between two genotypes,

dxy = total number of common loci (bands) in two genotypes

dx = number of loci (bands) in genotype 1, and

dy = number of loci (bands) in genotype 2

RESULTS

***Banding patterns with GLA-05 primer*:** The banding patterns obtained by using Gene link GLA-05 primer showed that all genotypes showed various levels of genetic polymorphism for the loci detected with GLA-05 primer (Figure 1). A total of 106 alleles (bands) were observed giving an average of 5.3 bands per genotype. Results of these analyses are presented as genetic dissimilarity matrix in Table 1. Range observed of genetic distances was 0 to 100%. Maximum genetic distances (100%) were observed among Ch20-Payne, Ch20-Sw14, Payne-Sw58, Payne- Dr67, Ch20-Serr, Payne-Serr, Sw44-Serr, Sw58-Serr, Dr62-Serr and Dr61-Serr, while 10 combinations displayed 100% homozygosity at DNA level. Average genetic distance calculated for GLA-05 was 50% (Table 1).

***Banding pattern with GLA-10 primer*:** The banding pattern using primer GLA-10 is shown in Figure 2. Walnut genotypes exhibited various levels of genetic polymorphism for the loci detected by using primer GLA-10. A total of 132 alleles

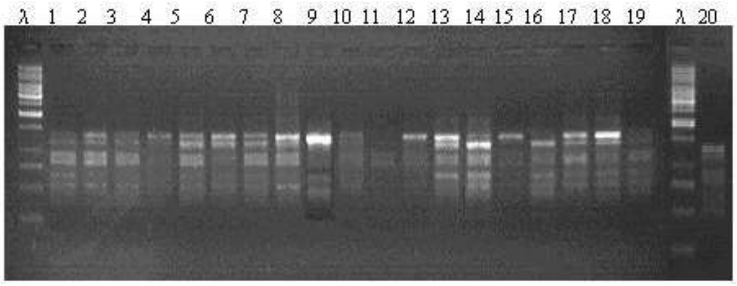

Figure 1. PCR profile of 20 genotypes of walnut using RAPD primer GL A-05, λ=marker, 1. Ch-20, 2. Ch-6, 3. Dr-45, 4. Payne, 5. Ch-1, 6. Sw-14, 7. Ch-7, 8.Ch-22, 9. Sw-110, 10. Sw-44, 11. Sw-58, 12. Dr-62, 13. Dr-37, 14. Dr-66, 15. Dr-61, 16. Dr-67, 17. Ch-14, 18. Sw-8, 19. Serr and 20. Sw-46

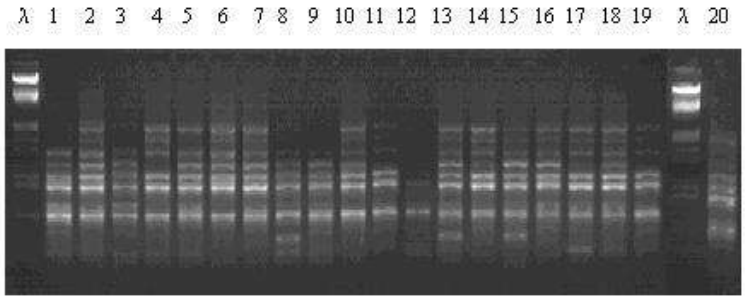

Figure 2. PCR profile of 20 genotypes of walnut using RAPD primer GLA-10. λ=marker, 1. Ch-20, 2. Ch-6, 3. Dr-45, 4. Payne, 5. Ch-1, 6. Sw-14, 7. Ch-7, 8. Ch-22, 9. Sw-110, 10. Sw-44, 11. Sw-58, 12. Dr-62, 13. Dr-37, 14. Dr-66, 15. Dr-61, 16. Dr-67, 17. Ch-14, 18. Sw-8, 19. Serr and 20. Sw-46

Table 1. Genetic distances (in percentage) among different walnut genotypes using GLA-05 primer.

	Ch-20	Ch-6	Dr-45	Payne	Ch-1	Sw-14	Ch-7	Ch-22	Sw-110	Sw-44	Sw-58	Dr-62	Dr-37	Dr-66	Dr-61	Dr-67	Ch-14	Sw-8	Serr
Ch-6	50																		
Dr-45	50	0																	
Payne	100	80	80																
Ch-1	66	20	20	75															
Sw-14	100	60	60	50	50														
Ch-7	66	20	20	75	0	50													
Ch-22	50	0	0	80	20	60	20												
Sw-110	50	0	0	80	20	60	20	0											
Sw-44	60	40	40	66	25	75	25	40	40										
Sw-58	50	60	60	100	50	100	50	60	60	33									
Dr-62	50	60	60	50	50	66	50	60	60	33	66								
Dr-37	71	33	33	80	20	60	20	33	33	40	60	60							
Dr-66	33	16	16	83	33	66	33	16	16	50	66	66	16						
Dr-61	80	60	60	50	50	66	50	60	60	33	66	66	60	66					
Dr-67	20	33	33	100	50	83	50	33	33	66	60	83	33	16	83				
Ch-14	33	16	16	83	33	66	33	16	16	50	66	66	16	0	66	16			
Sw-8	83	40	40	66	25	33	25	40	40	50	75	33	40	50	75	66	50		
Serr	100	80	80	100	75	50	75	80	80	100	100	100	80	83	100	80	83	66	
Sw-46	66	20	20	75	0	50	0	20	20	25	50	50	20	33	50	50	33	25	75

(bands) were observed in 20 genotypes giving an average of 6.6 bands per genotype. Thus, the primer GLA-10 on an average basis, yielded higher numbers of bands per genotype (6.6) as compared to primer GLA-05 (5.3). Genetic distances were observed in a range of 0 to 100% (Table 2). The maximum genetic distances (100%) were observed among genotypes Payne-Ch1, Payne- Ch7, Payne-Sw110, Payne-Sw58, Payne-Dr37, Payne-Dr61, Payne-Ch14 and Payne-Serr, while 86 combinations showed no polymorphism at molecular level. Average genetic distance calculated for

Table 2. Genetic distances (in percentage) among different walnut genotypes using GLA-10 primer.

	Ch-20	**Ch-6**	**Dr-45**	**Pay-ne**	**Ch-1**	**Sw-14**	**Ch-7**	**Ch-22**	**Sw-110**	**Sw-44**	**Sw-58**	**Dr-62**	**Dr-37**	**Dr-66**	**Dr-61**	**Dr-67**	**Ch-14**	**Sw-8**	**Serr**
Ch-6	25																		
Dr-45	0	12																	
Payne	50	0	37																
Ch-1	0	25	0	100															
Sw-14	37	0	25	75	14														
Ch-7	0	12	0	100	0	0													
Ch-22	44	0	33	67	25	0	33												
Sw-110	0	37	0	100	0	28	0	37											
Sw-44	37	0	25	75	14	0	0	0	28										
Sw-58	0	25	0	100	0	14	0	44	0	14									
Dr-62	66	0	71	29	66	0	71	0	60	0	66								
Dr-37	0	22	0	100	0	12	0	22	0	12	0	75							
Dr-66	37	0	25	75	14	0	0	0	28	0	14	0	12						
Dr-61	0	22	0	100	0	12	0	22	0	12	0	83	0	12					
Dr-67	25	0	12	88	25	0	12	0	37	0	25	0	22	0	22				
Ch-14	0	11	0	100	0	22	0	45	0	22	0	84	0	22	0	11			
Sw-8	25	0	12	88	25	0	12	0	37	0	25	0	22	0	22	0	11		
Serr	0	25	0	100	0	14	0	44	0	14	0	80	0	14	0	25	0	25	
Sw-46	37	0	25	75	14	0	0	0	28	0	14	0	12	0	12	0	22	0	14

Table 3. Genetic distances (in percentage) among different walnut genotypes using RAPD primer.

	Ch-20	**Ch-6**	**Dr-45**	**Pay-ne**	**Ch-1**	**Sw-14**	**Ch-7**	**Ch-22**	**Sw-110**	**Sw-44**	**Sw-58**	**Dr-62**	**Dr-37**	**Dr-66**	**Dr-61**	**Dr-67**	**Ch-14**	**Sw-8**	**Serr**
Ch-6	38																		
Dr-45	25	6																	
Payne	75	40	59																
Ch-1	33	23	10	88															
Sw-14	69	30	43	83	32														
Ch-7	33	16	10	88	0	25													
Ch-22	47	0	17	74	23	30	27												
Sw-110	25	19	0	90	10	44	10	19											
Sw-44	49	20	33	71	20	38	13	20	34										
Sw-58	25	43	30	100	25	57	25	52	30	24									
Dr-62	58	30	66	40	58	33	61	30	60	17	66								
Dr-37	36	28	17	90	10	36	10	28	17	26	30	68							
Dr-66	35	8	21	79	24	33	17	8	22	25	40	33	14						
Dr-61	40	41	30	75	25	39	25	41	30	23	33	75	30	39					
Dr-67	23	17	23	94	38	42	31	17	35	33	43	42	28	8	53				
Ch-14	17	14	8	92	17	44	17	31	8	36	33	75	8	11	33	14			
Sw-8	54	20	26	77	25	17	19	20	39	25	50	17	31	25	49	33	31		
Serr	50	53	40	100	38	32	38	62	40	57	50	90	40	49	50	53	42	46	
Sw-46	52	10	23	75	7	25	0	10	24	13	32	25	16	17	31	25	28	13	45

GLA-10 was 21.54%, which was the lowest between the two RAPD markers used (Table 2).

***Average genetic distance*:** Average genetic distances among 20 walnut genotypes using the two RAPD primers are presented in Table 3. Maximum genetic distance (100%) was observed between Payne and Sw-58 and Payne and Serr, closely followed by 94% genetic distance between Payne and Dr-67. Minimum genetic distances (0%) were observed between Ch1-Ch7, Ch6 - Ch22, Dr45-Sw110 and Ch7- Sw46. About 90% similarities were noted among the genotypes Dr 45-Ch1, Dr45-Ch7, Ch1-Sw110, Ch1-Dr37, Ch6-Sw46 and Ch22-Sw46 (Table 3).

Genetic relatedness: The walnut genotypes under study could be classified into four distinct groups at 60% dissimilarity level (Fig. 3). Thirteen out of 20 walnut genotypes were classified in Group I, which can further be classified into three sub-groups, IA, IB and IC. Group II contained four and group III two genotypes. Sw-58 was the only genotype in group IV and was the most distinct from all the other genotypes. Among the genotypes studied, this single plant (Sw-58) is altogether different at genetic level.

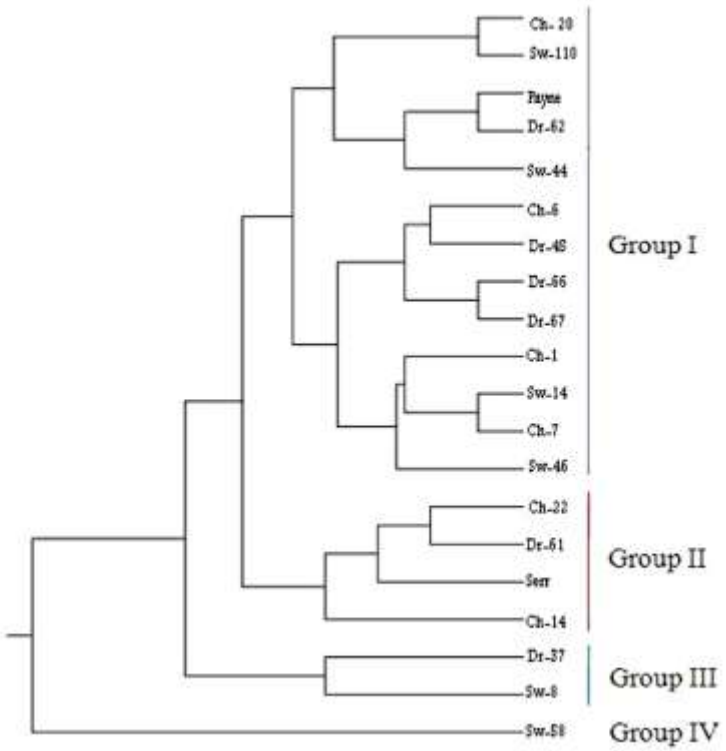

Figure 3. Dendrogram of 20 walnut genotypes generated by UPGMA cluster analyses of the dissimilarity values shown in Table 3.

DISCUSSION

Morphological, physiological and biochemical attributes have been used to investigate the genetic variability in perennial fruit crops but growth and environment can interfere with such assessments (Zenelli *et al*., 2005; Vyas *et al*., 2003). The use of DNA based markers is an authentic technique of genetic characterization that allows comparison of different genetic material independent of environmental effects (Weising *et al*., 1995). Random amplified polymorphic DNA (RAPD) technique has emerged as the most extensively to develop DNA markers that helps to detect genetic polymorphism between genotypes at molecular level (Kumar and Gurusubramanian, 2011).

Genotypes Dr45-Ch1, Dr45-Ch-7, Ch1-Sw110, Ch1-Dr37, Ch6-Sw46 and Ch22-Sw46 showed 90% similarities (Table 3). Since, the genotypes sharing common parents tend to group together (Nicese *et al*., 1997), the 90% similarities among these genotypes indicate that these genotypes have similar genetic base. It has been reported that foreign pollens through natural crosses may result in about 10% dissimilarities (Karimi *et al*., 2010). Thus, the 10% dissimilarity could be due to out crossing in walnut genotypes.

Genotypes Ch1-Ch7, Ch6-Ch22, Dr45-Sw110 and Ch7-Sw46 expressed zero percent dissimilarity (100% similarity). Thus, it is clear that Ch1 and Ch7 are genetically identical. Similarly, Ch6-Ch22, Dr45- Sw110 and Ch7-Sw46 are also genetically identical. Their genetic identity is also reflected in chemical analyses as reported by Rahman *et al*. (2012), where Ch-1 and Ch-7 were high in carbohydrates and low in crude fats and nearly equal in protein contents (Rahman *et al.*, 2012). On other hand, Ch-6 and Ch-22 were exactly similar in protein content and almost at par in crude fats and carbohydrates. Furthermore, these two genotypes are comparatively low in carbohydrates and crude fats as compared to Ch-1 and Ch-7 (Rahman *et al*., 2012), which suggest that Ch-6 and Ch-22 are genetically identical but dissimilar to Ch-1 and Ch-7. However, Dr-45 and Sw-110 are identical at molecular level but their biochemical analyses do not support this fact, as was the case in genotypes growing in Chitral. It can be argued that since these genotypes are grown in two dissimilar climates (Swat and Chitral) therefore, the variations in protein, carbohydrates and fats could be due to different agro-climatic conditions (Sharma *et al*., 2003; Rahman *et al*., 2012). The 100% similarity of Dr-45 and Sw-110, and Ch-7 and Sw-46 growing in different regions indicate that the same genotype moved from one region to the other back in history. Similar arguments can be given for molecular similarity between as well. In an earlier study,

Khan *et al*. (2010) investigated 20 walnut germplasm of Northern Pakistan using protein markers and reported modest genetic diversity (ranging from 0-60%). In a similar study, Sharma and Sharma (2001) investigated the genetic diversity in Indian walnut using kernel characters. They grouped 229 accessions in 16 clusters and reported that the clustering pattern of the genotypes from the same location revealed their distribution in more than one cluster indicating non-parallelism between geographic and genetic diversity. Dogan *et al*. (2005) evaluated genetic diversity in walnut from Turkey on the basis of fruit characters and reported high genetic diversity in walnut genotypes. However, non-significant genetic differences, based on isozyme analysis, in Italian walnut genotypes are also reported by Malvolti *et al*. (1993). The study of leaf isozyme patterns to identify various cultivars of walnut *Juglans nigra* and *Juglans regia* revealed that *Juglans nigra* was more heterozygous than *Juglans regia* (Vyas *et al*., 2002).

The dendrogram (Fig. 3) of various genotypes reveals that RAPD markers revealed diversity of *Juglans* genotypic (Francesca *et al*., 2010) and the genotypes under study can be classified into four distinct groups at dissimilarity level of about 60%. Among the genotypes studied, this single plant (Sw-58) is altogether different at genetic level. It has been historically famous for its nuts quality, with a medium thick nutshell, high kernel percentage with extra light color and excellent yield. Beside the visual attributes, its chemical composition reveals that it has the lowest carbohydrates (13.10%) and the highest (14.0%) in protein and crude fats (Rahman *et al*., 2012).

The information revealed during this study establishes the identity and genetic distances among promising walnut genotypes in Pakistan and can be helpful in establishing a gene bank of walnut genetic resources, breeding and propagation of walnut in Kyber-Pakhtunkhwa, province of Pakistan.

***Conclusion*:** It can be concluded that walnut genotypes revealed various levels of genetic polymorphism for the loci detected by using RAPD primers GLA-05 and GLA-10. GLA-05 detected 106 alleles as compared to 132 by GLA-10. Thus, primer GLA-10 on an average yielded higher number of bands per genotype compared with primer GLA-05. Average maximum genetic distance (100%) was observed between Payne and Sw-58 and Payne and Serr closely followed by Payne and Dr-67 (94%). Based on the data from the dendrogram, the walnut genotypes were classified into four major groups. Thirteen out of 20 genotypes were placed in group-I. Sw-58 was the only genotype in group-IV which was genetically the most distinct from other genotypes. The study also revealed that among 20 genotypes under study, Ch1-Ch7, Ch6-Ch22, Dr45-Sw110 and Ch7-Sw46 were genetically identical, though designated as different genotypes during survey.

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