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Genetic Characterization of Walnut (*Juglans regia* L.) by Random Amplified Polymorphic DNA

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ABSTRACT

Walnut germplasm accessions representing exotic and indigenous collections, from walnut orchards of University of Horticulture and Forestry, Nauni, Himachal Pradesh, India, were used in the present investigations. A total of seven exotic and eight indigenous accessions were evaluated for randomly amplified polymorphic DNA (RAPD) variations. 21 primers out of total 36 decamerous primers generated 190 bands, out of which 157 showed polymorphism on the agarose gel, reflecting a high level of genetic diversity within the germplasms. Pairwise distances, calculated using Jaccard's coefficient of similarity, clustered indigenous and exotic accessions into separate groups except for one accession 'Blackmore' which, although exotic, was placed in a cluster of predominantly indigenous accessions. The clustering based on RAPD markers agreed to large extent with the geographical origin of the studied walnut germplasm accessions.

Keywords: DNA fingerprinting, genetic diversity, germplasm, molecular markers, RAPD

INTRODUCTION

It is well established that random amplified polymorphic DNA (RAPD) technique offers a powerful tool to detect DNA polymorphism (Williams *et al*. 1990). Because of simplicity and low input, this technique could be employed by pomologists even in less sophisticated laboratory facilities.

RAPDs can be used to distinguish different clones and cultivars and has proven extremely useful in determining genetic relationship among breeding materials, fingerprinting cultivars and constructing genetic maps in many woody and fruit crops (Hemmat *et al*. 1994). Careful handling of reaction components and standardization of reaction conditions of amplification is needed to achieve the necessary repeatability of the PCR-RAPD results (Micheli *et al*. 1994).

Walnut is an important nut tree almost all parts of which are used in one way or the other. It is commonly known as 'Akhrot' in India. The genus *Juglans* includes all walnuts and it belongs to family Juglandaceae. English or Persian walnut (*Juglans regia* L.) is the only walnut species grown in India. There are no standard walnut cultivars under cultivation in India. Exotic cultivars introduced in the past constitute merely a part of germplasm collections maintained at various research centres. Most of the walnut produce comes from trees of seedling origin grown in semi-wild state. Like elsewhere in India, walnut plantations in state of Himachal Pradesh, too, comprise of genetically diverse seedling trees of unknown origin, thereby constituting a vast gene pool. Accurate estimation of genetic distance between different genotypes can assist the breeders in crop improvement programmes. Accurate and rapid cultivar identification is especially important in vegetatively propagated plant species such as most fruit trees both for practical breeding purposes as well as for proprietary rights protection (Kaur *et al.* 2005)

In spite of extensive variation prevalent in the native walnut germplasm, there has been no systematic work on genetic characterization of indigenous and exotic germplasm of walnut in India.

So far, the characterization of walnut germplasm used in present study was based on morphological traits. These, however, are not reliable indicators of plant diversity and affected by environmental factors (Powell *et al.* 1996a, 1996b). Therefore, identification of closely related genotypes is often extremely difficult. For these reasons, RAPD is a convenient way to identify the relationship of walnut germplasm. RAPD markers have already been used by many to analyze the genetic differences and relatedness among walnut germplasm (Nicese *et al.* 1998; Shen *et al.* 2007). Here, we report the application of PCR-RAPD for correct identification of walnut accessions both of indigenous and exotic origin presently being maintained at the University of Horticulture and Forestry, Nauni, Solan (H.P). All accessions considered in the present investigation are of high commercial value.

MATERIALS AND METHODS

Plant material

The germplasm accessions of walnut analysed in this study are listed in **Table 1**.

DNA isolation

Young and healthy leaves (~1.5 g) were homogenized into fine powder in liquid N_2. The powder was transferred to 15 ml of preheated extraction buffer containing 1.4 mM NaCl, 2% (w/v) CTAB, 100 mM Tris HCl – pH 8.0, 20 mM EDTA (ethylenediamine tetraacetic acid) – pH 8.0, 1% β-mercaptoethanol and 1% PVP (Polyvinylpyrrolidone; MW-40,000) and incubated at 65°C for 60 min in a pre-heated water bath (FTS Systems, Stone Ridge, New York, USA). The mixture was extracted with an equal volume of chloroform and isoamylalcohol (24: 1 v/v). The aqueous phase was precipitated with chilled isopropanol at –20°C and DNA was spooled out with a glass hook. In some samples where spool was not formed, the mixture was centrifuged at 8000 rpm in

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Table 1 Walnut accessions included in the present investigation.

Accessions	Origin
Gobind	Kinnaur (IA)
Roopa Akhrot	Kinnaur (IA)
Chico	USA (EA)
Inder Akhrot	Sirmour (IA)
Tree no.12	Kullu (IA)
Netar Akhrot	Sirmour (IA)
Payne	USA (EA)
Blackmore	USA (EA)
Meylannaise	France (EA)
Kandaghat Selection	Solan (IA)
ACO-38853	USA (EA)
Tree no.101	Kullu (IA)
Ronde-de-Montignac	France (EA)
KX-Gaint	USA (EA)
Solding Selection	Kinnaur (IA)

lA: Indigenous Accession
EA: Exotic Accession

Table 2 Nucleotide sequences of random decamer primers that showed amplification in 15 accessions of walnut.

Primer	Nucleotide Sequence (5'-3')			
OPA-08	GTG	ACG	TAG	G
OPA-09	GGG	TAA	CGC	C
OPA-14	TCT	GTG	CTG	G
OPA-16	AGC	CAG	CGA	A
OPAH-20	GGA	AGG	TGA	C
OPAI-07	AGT	TCC	ACG	G
OPAI-10	TCG	GGG	CAT	C
OPAI-15	GAC	ACA	GCC	C
OPAI-17	CCT	CAC	GTC	C
OPAI-18	TCG	CGC	AAC	C
OPAJ-06	GTC	GGA	GTG	C
OPAJ-08	GTG	CTC	CCT	C
OPAJ-14	ACC	GAT	GCT	G
OPAK-04	AGG	CTC	GGT	C
OPAK-08	CCC	AAG	GGT	G
OPB-10	CTC	ACC	GTC	C
OPB-14	TCC	GCT	CTG	G
Oligo-654	ACT	CAA	AGG	C
Oligo-661	CAT	GAC	AGG	C
Oligo-664	TGG	AAG	AGG	C
Oligo-675	TTT	GCT	CGG	C

a cooling centrifuge (REMI Instruments Ltd., Mumbai, India) for 10 min at 4°C to spin down the DNA pellet. DNA was treated with 5 µg/ml of RNase (Bangalore Genei, Bangalore, India) for 60 min at 37°C. All the chemicals and reagents used for DNA isolation were of Molecular Biology ('MB') Grade and were purchased from Sisco Research Laboratories, Mumbai, India.

DNA amplification

Random primers tested in the PCR reaction had 60% G+C (**Table 2**). PCR amplification was performed in 25 µl reaction volumes, containing 1X PCR buffer (100 mM Tris HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), 2.0 mM $MgCl_2$, 200 mM each of dATP, dCTP, dGTP and dTTP, 20 pM of the primer,0.9 U of *Taq* DNA polymerase and 25 ng of walnut DNA. All the reaction chemicals were purchased from Bangalore Genei, India. Then the reaction volume was transferred to a Techne, Cyclogene Thermal Cycler programmed for initial denaturation of 2 min at 94°C. DNA amplification was performed for 39 cycles. Each cycle consisted of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. It was followed by a final extension at 72°C for 5 min.

Electrophoresis of amplified product

PCR amplified products were resolved by gel electrophoresis in a 1.5% agarose gel prepared in 1X TBE buffer (0.045 M Tris borate and 0.001 M EDTA) and run in the same buffer for at least 1 hr at 100-120 V. Gels were stained with 0.5 mg/ml of ethidium bromide and photographed under UV light of a gel documentation system (Pharmacia Biotech).

Statistical analysis

For RAPD analysis the bands with the 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. The data was analyzed with the SIMQUAL program of NTSYS-pc (ver. 2.0) and similarities between accessions were estimated using Jaccard's Coefficient calculated as J =A/(N-D), where A is the number of positive matches (i.e. presence of band in both samples), D is the number of negative matches (i.e. 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). A dendrogram was created from the resultant similarity matrices using the UPGMA method (Sokal and Sneath 1963), following the SAHN function of NTSYS-pc (version 2.0). SAHN performs the sequential, agglomerative, hierarchial and nested clustering methods as defined by Sokal and Sneath (1963).

To compare the goodness of fit of similarity coefficient (Jaccard's coefficient) and clustering obtained by UPGMA, the two matrices were subjected to the Mantel test (Mantel 1967) by using NTSYS-pc ver. 2.0.

RESULTS AND DISCUSSION

Several techniques have been used to examine genetic diversity and relationships among walnut cultivars, including isozymes (Arulsekar *et al.* 1985; Solar *et al*. 1993, 1994), restriction fragment length polymorphism (RFLP) (Fjellstrom *et al.* 1994), RAPD (Malvolti *et al.* 1994; Woeste *et al.* 1996; Nicese *et al.* 1998; Shen *et al.* 2007), amplified fragment length polymorphism (AFLP) (Bayazit *et al*. 2007), inter-simple sequence repeat (ISSR) (Potter *et al*. 2002) and simple sequence repeat (SSR) (Dangl *et al.* 2005; Pollegioni *et al.* 2006). RAPD markers can detect enough polymorphism to differentiate among closely related walnut genotypes (Nicese *et al.* 1998). Besides this, RAPDs were found to be equivalent to ISSR and SSR in their characterization of walnut genotypes (Pollegioni *et al*. 2006). Thus,

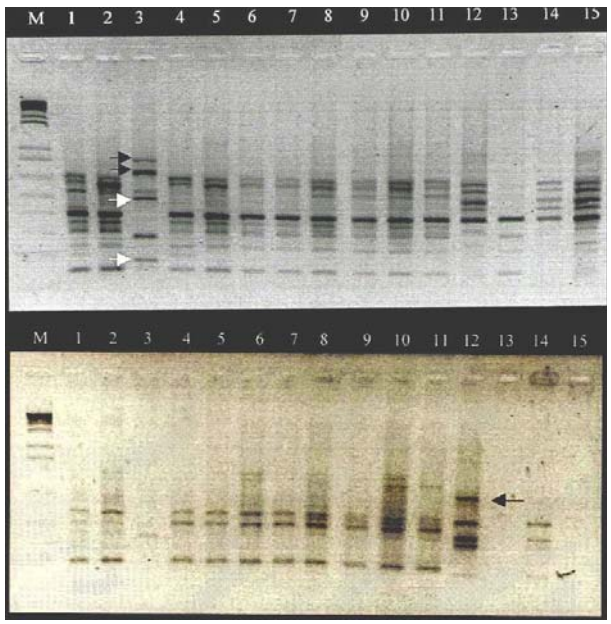

Fig. 1 (**A**) RAPD pattern with 15 indigenous and exotic accessions of walnut as generated by primer Oligo-661. M: 100 bp ladder (*Hind*III digest); Arrow(s) indicate unique bands. (**B**) RAPD pattern with 15 indigenous and exotic accessions of walnut as generated by primer OPAJ-08. M: 100 bp ladder (*Hind*III digest); Arrow(s) indicate unique bands. Lanes 1-15: 1. 'Gobind', 2. 'Roopa Akhrot', 3. 'Chico', 4. 'Inder Akhrot', 5. 'Tree No. 12', 6. 'Netar Akhrot', 7. 'Payne', 8. 'Blackmore', 9. 'Meylannaise', 10. 'Kandaghat Selection', 11. 'ACO-38853', 12. 'Tree no. 101', 13. 'Ronde-de-Montignac', 14. 'KX-Gaint', 15. 'Solding Selection'.

Table 3 Jaccard's similarity coefficient for 15 indigenous and exotic accessions of walnut.

Accessions	Gobind	Roopa Akahrot	Chico	Inder Akhrot	Tree No. 12	Netar Akhrot	Payne	Blackmore	Meylalannaise	Kandaghat Selection	ACO-38853	Tree No. 101	Ronde-de-Montignac	KX-Gaint	Solding selection
Gobind	1.00														
Roopa Akhrot	0.67	1.00													
Chico	0.03	0.04	1.00												
Inder Akhrot	0.65	0.54	0.02	1.00											
Tree No. 12	0.58	0.51	0.02	0.64	1.00										
Netar Akhrot	0.52	0.70	0.01	0.54	0.51	1.00									
Payne	0.54	0.47	0.01	0.63	0.56	0.49	1.00								
Blackmore	0.54	0.58	0.02	0.59	0.49	0.55	0.53	1.00							
Meylannaise	0.55	0.49	0.03	0.61	0.51	0.47	0.62	0.47	1.00						
Kandaghat Selection	0.62	0.74	0.03	0.64	0.57	0.69	0.50	0.65	0.52	1.00					
ACO-38853	0.52	0.48	0.01	0.57	0.52	0.43	0.61	0.52	0.60	0.51	1.00				
Tree No. 101	0.27	0.24	0.02	0.25	0.26	0.22	0.20	0.26	0.23	0.25	0.20	1.00			
Ronde-de-Montignac	0.09	0.08	0.05	0.09	0.10	0.07	0.10	0.09	0.14	0.08	0.14	0.07	1.00		
KX-Gaint	0.07	0.06	0.03	0.07	0.07	0.07	0.10	0.08	0.10	0.06	0.09	0.27	0.12	1.00	
Solding Selection	0.27	0.27	0.02	0.29	0.30	0.30	0.23	0.28	0.24	0.31	0.22	0.59	0.08	0.21	1.00

RAPDs were the markers of choice in the present investigation.

Phenolics posed a great problem during DNA isolation. Therefore, the first step of our study was to optimize the DNA extraction procedure. The addition of 1% (w/v) PVP to the extraction buffer was essential to eliminate compounds such as phenolics and tannins. We verified the reproducibility of the RAPD bands by performing DNA amplification on serially diluted DNA over a sufficiently wide range.

The PCR protocol was as described by Williams *et al.* (1990) with small modifications. Different components of PCR including *Taq* polymerase, primers, duration and temperature of annealing, dNTPs were optimized using only one cultivar i.e. 'Gobind'. 25 ul reaction volume consisted of 1X PCR buffer, 200 uM dNTPs, 2.0 mM $MgCl_2$, 20 pmol of primer, 0.9 U *Taq* polymerase and 25 ng template DNA and MilliQ water to make up the volume to 25 µl.

A total of 36 random primers were used but only 21 were able to amplify the genomic DNA of walnut accessions. The remaining primers showed either no amplification or resulted in an unreadable gel smear. Therefore, each PCR reaction was repeated at least twice.

Some of the primers produced polymorphic bands specific to a set of varieties, e.g. OPAI-18 produced two unique bands, one each, in 'Gobind' and 'Blackmore', whereas primer OPA-08 produced two unique bands only in 'Solding selection'. 'Tree No. 101' could be clearly distinguished by OPAJ-08 and Oligo-664, while OPAJ-14 gave a single specific band in 'Roopa Akhrot'. Primer Oligo-661 generated four specific bands (Oligo-661_{1500}, Oligo-661_{1400}, Oligo-661_{1200} and Oligo-661_{1600}) in a single accession i.e. 'Chico' (**Fig. 1A**) and primer OPAJ-08 produced an informative marker of size OPAJ-08_{1500} for 'Tree No. 101' (**Fig. 1B**), which is an indigenous accession.

A minimum of three (OPAI-17) and a maximum of 16 (Oligo-675 and OPAJ-14) DNA fragments were observed (**Table 4**). No single primer could fingerprint all the accessions. Besides, the fingerprint of many of the genotypes is defined by multiple RAPD bands, presumably at multiple genetic loci. This is important for cultivar characterization, since each cultivar is not defined by a single marker but by a set of several markers. A polymorphism level as high as 82.63% reflects high genetic diversity within walnut germplasm available in Himachal Pradesh. This high level of polymorphism also reflects the outcrossing nature of walnut. Another reason being that germplasm accessions are comprised of both indigenous as well as exotic genotypes. Similar results have been obtained with RAPDs for characterizing genotypes of different origins, in other outcrossing nut and fruit tree species such as pistachio (Hormaza *et al.* 1994) and olive (Fabbri *et al.* 1995).

Table 4 Amplified and polymorphic bands as generated by 21 random decamer primer in 15 accessions of walnut.

Primers	Total number of bands	Total number of polymorphic bands
OPA-08	6	6
OPA-09	5	4
OPA-14	6	5
OPA-16	4	2
OPAH-20	9	4
OPAI-07	7	7
OPAI-10	11	11
OPAI-15	10	10
OPAI-17	3	3
OPAI-18	11	11
OPAJ-06	9	9
OPAJ-08	13	11
OPAJ-14	16	13
OPAK-04	11	7
OPAK-08	9	8
OPB-10	7	7
OPB-14	7	4
Oligo-654	8	4
Oligo-661	15	8
Oligo-664	7	7
Oligo-675	16	16

Cluster analysis based on the result of all 21 polymorphic primers was used to generate a dendrogram (**Fig. 2**).

The dendrogram from UPGMA divided all accessions into four main clusters based on combined scores of all 21 primers. The first cluster includes six indigenous accessions namely 'Gobind', 'Inder Akhrot', 'Tree No.12', 'Roopa Akhrot', 'Kandaghat Selection', 'Netar Akhrot' and only one exotic accession i.e. 'Blackmore', which is separated from rest of the genotypes in this cluster at an average genetic distance of 0.48. The placement of most of the indigenous accessions in one cluster obviously indicates that their genetic base is narrow. Average genetic distance is only 44% in first cluster. Second and third clusters include exotic accessions namely Payne, 'Meylannaise', 'ACO-38853', and 'Tree No.101' and 'Solding Selection' (both indige-

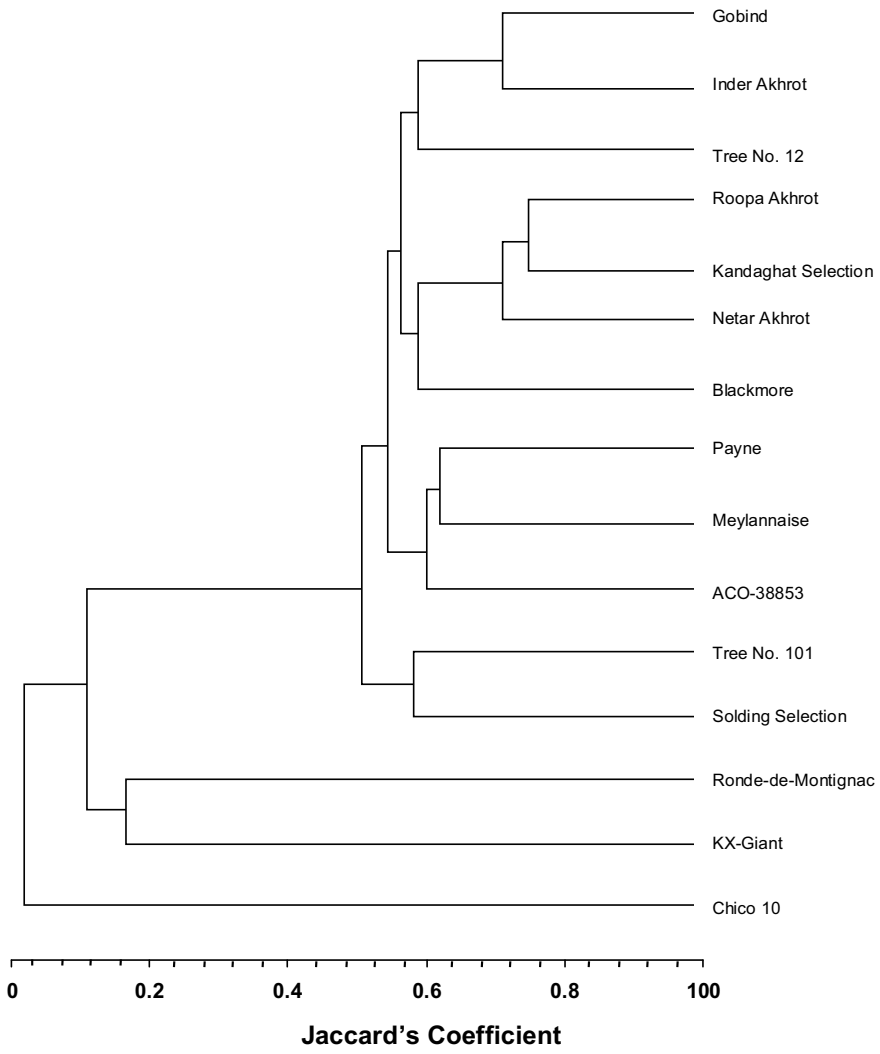

Fig. 2 Dendrogram of 15 indigenous and exotic accessions of walnut based on RAPD analysis.

nous), respectively. 'Ronde-de-Montignac', 'KX-Gaint', fall in cluster fourth. Based on UPGMA clustering, Nicese *et al.* (1998) could separate 19 walnut genotypes into two main groups using RAPDs.

Among all the genotypes studied, 'Chico' appears to be the most distantly related to all others with an average similarity value of 0.02. Similar results are obtained from Jaccard's Similarity Cofficient (**Table 3**). It is interesting to note that this genotype is lateral bearing (those walnut trees which bears fruits on lateral as well as terminal parts of the shoot) and obviously making an entirely different group and rest being terminal bearers. In 'Gobind' (indigenous) and 'Blackmore' (exotic) OPAI-18 generated a specific marker in each, though both belong to different groups. The closeness shared between 'Blackmore' and 'Gobind', though both belong to separate groups, is perhaps, due to common parentage at some stage. Among the indigenous accessions, 'Kandaghat Selection' is having maximum similarity with 'Roopa Akhrot'. Similarly RAPDs analysis supports the achievement of objectives by Emmarold *et al.* (2001) in cashew wherein they could cluster cashew accessions in different groups obtained from India, Tanzania, Mozambique, Brazil and Cook Island.

The cophenetic correlation, based on Mantel test is used to measure the goodness of fit of a cluster analysis to the similarity or disimilarity matrix on which it was based. The value of cophenetic correlation as 0.93 being very high and statistically significant at 0.01 probability level shows its goodness of fit for studying genetic diversity in walnut accessions.

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