

Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers

F.P. Nicese¹, J.I. Hormaza² & G.H. McGranahan

Department of Pomology, University of California, Davis CA 95616, U.S.A.; ¹ present address: Dipartimento di Ortoflorofrutticoltura, via G. Donizetti 6, 50144 Firenze, Italy; ² present address: Unidad de Fruticultura, SIA-DGA, Campa de Aula Dei, Apdo. 727, 50080 Zaragoza, Spain

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Summary

The potential use of the Randomly Amplified Polymorphic DNA (RAPD) technique for characterization and assessment of genetic relationships was investigated in nineteen walnut (*Juglans regia* L.) genotypes used as parents or released as cultivars from the breeding program of the University of California at Davis. Most of the 72 decamer primers used yielded scorable amplification patterns based on discernable bands. The results obtained produced a unique fingerprint for each of the walnut genotypes studied. Cluster analysis separated the 19 walnut genotypes into two main groups whose differences were related to their pedigree. Genotypes sharing common parents tend to group together and with at least one of the parents. Thus, RAPD markers can detect enough polymorphism to differentiate among walnut genotypes, even among closely related genotypes, and the genetic similarity based on RAPDs appears to reflect the known pedigree information. RAPD technology can be useful in current walnut breeding programs, allowing the identification of new cultivars as well as the assessment of the genetic similarity among genotypes which will help in selecting the best parents to obtain new genetic combinations.

Introduction

The family Juglandaceae consists of seven genera comprising about 60 monoecious tree species. The genus *Juglans* contains about 20 species, all producing edible nuts. Among those, the English or Persian walnut (*Juglans regia* L.) is the most widely cultivated species (McGranahan & Leslie, 1990). Persian walnuts have been grown in California since the day of early Spanish missions. In the last 50 years the genetic base of this crop has been enriched with introductions from Asia and Europe, primarily France (Forde & McGranahan, 1996). All the commercial cultivars currently grown in California can be considered descendants, at least partially, from those gene pools and have originated either as chance seedlings, or from the breeding program of the University of California (Serr, 1969). This breeding program, based on crosses between late season and laterally fruiting genotypes, has released 15 walnut cultivars (Tulecke & McGranahan, 1994). The

characterization of these genotypes is important for designing objective and repeatable criteria to protect breeders' right in newly developed cultivars.

Accurate and rapid cultivar identification is especially important in vegetatively propagated plant species such as most fruit trees both for practical breeding purposes and for proprietary rights protection. Unfortunately, the traditional methods for characterization and assessment of genetic variability in perennial fruit crop species, based on morphological, physiological and biochemical studies, are both time consuming and affected by the environment. The introduction of molecular biology techniques, such as DNA-based markers, provides an opportunity for genetic characterization that allows direct comparison of different genetic material independent of environmental influences (Weising et al., 1995).

Initial molecular studies in walnut were carried out using isozymes to assess the inheritance of some enzyme systems (Arulsekhar et al., 1986; Aleta et

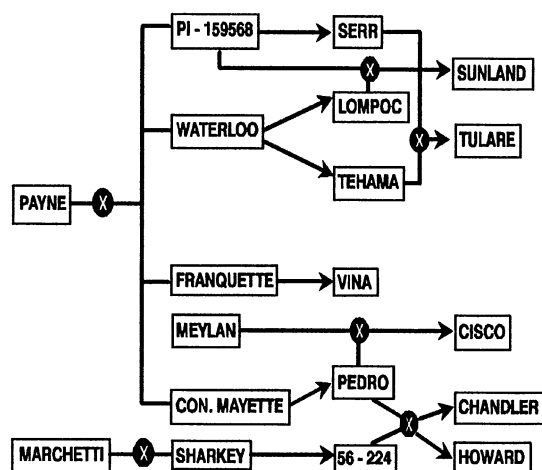


Figure 1. Pedigree diagram of the cultivars tested.

al., 1993). Isozymes have also been used in *Juglans* to identify genetic variability (Malvolti et al., 1993, 1994), to detect interspecific hybrids (Arulsekhar et al., 1985; McGranahan et al., 1986; Germain et al., 1993), to identify species and cultivars (Louskas et al., 1984; Wenheng, 1984; Arulsekhar et al., 1985; Aleta et al., 1989; Germain et al., 1993; Solar et al., 1993, 1994) and to assess mating parameters (Rink et al., 1994). RFLP markers have also been used in walnuts to determine parentage (Aly et al., 1992), to establish phylogenetic relationships in the genus *Juglans* (Fjellstrom & Parfitt, 1995), to estimate genetic diversity, and to identify cultivars (Fjellstrom et al., 1994; Fjellstrom & Parfitt, 1994a, b). Recently, RAPD markers have been used to evaluate the level of polymorphism at the interspecific level between Persian walnut (*J. regia*) and Northern California black walnut (*J. hindsii* (Jeps.) Jeps.) (Woeste et al., 1996a) and to identify a marker linked to hypersensitivity to the cherry leafroll virus (Woeste et al., 1996b).

Randomly Amplified Polymorphic DNA (RAPD) (Williams et al., 1990, 1993) can be of further use to identify closely related walnut cultivars and could complement the results previously obtained with RFLPs, mainly because of the higher level of polymorphism obtained with RAPDs. RAPD analysis has already proven to be valuable in genotype characterization as well as in population and pedigree analyses in many crop species and studies in tree crops are starting to produce interesting results (Hormaza et al., 1994; Fabri et al., 1995). RAPD markers are not only important for the characterization of the germplasm but can also be used to evaluate the effects of selection over time

Table 1. Walnut genotypes included in this study

Code	Genotype	Original source ¹
1	56-224	Univ. of California
2	Chandler	Univ. of California
3	Cisco	Univ. of California
4	Conway Mayette	France
5	Franquette	France
6	Howard	Univ. of California
7	Lompoc	Univ. of California
8	Marchetti	California
9	Meylan	France
10	Payne	California
11	Pedro	Univ. of California
12	PI-159568	Afghanistan
13	Serr	Univ. of California
14	Sharkey	China
15	Sunland	Univ. of California
16	Tehama	Univ. of California
17	Tulare	Univ. of California
18	Vina	Univ. of California
19	Waterloo	California

¹ Based on Tulecke & McGranahan, 1994.

and to aid in the development of crossing schemes in walnut improvement programs since this method allows the study of the genetic diversity of the available germplasm.

The main objective of this study was to develop RAPD markers to unequivocally characterize 19 closely related walnut genotypes from the breeding program of the University of California as well as to compare the degree of genetic relatedness obtained through RAPD analysis with that of the expected results obtained from pedigree data.

Materials and methods

Plant material

The nineteen genotypes used in this study (Table 1 and Figure 1) were obtained from the walnut collection maintained at the University of California Wolfskill Experimental Orchard in Winters, California, USA, and are part of the walnut breeding program developed at this institution. They were selected based on a pedigree assessment and include recently released cultivars as well as their parental genotypes.

Table 2. Distribution of 23 RAPD markers within the 19 walnut genotypes. Genotype numbers correspond to those in Table 1. '+' indicates presence and '-' indicates absence of the marker

Markers	Genotypes																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
K01-445	-	+	-	+	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+
K02-280	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+
K02-513	-	-	+	-	+	-	+	-	+	-	-	-	-	-	+	+	+	+	+
K03-635	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
K19-435	+	-	+	+	+	-	-	+	+	-	+	-	-	+	-	-	-	-	+
K19-880	-	-	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	+
K20-1100	+	+	-	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-
G10-560	-	+	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-
G15-690	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-
G16-620	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
G16-700	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
G18-630	+	+	-	-	-	+	-	+	-	+	-	+	+	+	-	-	+	+	-
G18-990	+	+	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-
R06-345	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	-	+	-	-
R13-430	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	-	+	-	-
R14-1130	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+
R15-490	+	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	+	-	+
R19-755	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-
S01-1550	+	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+
S10-700	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	+
T04-630	+	-	-	+	+	-	+	-	-	+	-	+	+	+	+	-	-	-	+
T04-780	-	+	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
T06-850	+	+	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-

the primers, however, produced either no amplification or unreadable gel smears. Eighteen primers, producing 1–2 polymorphic fragments each, generated polymorphic banding patterns among the genotypes studied (Table 2). The total number of polymorphic bands obtained was 23. The apparent low level of polymorphism detected (about 25% of the primers tested) can be explained by the strict criterion adopted to score the markers. Only the conspicuous intensely stained bands between 250 bp and 1700 bp long were considered for analysis. Each RAPD analysis was repeated in separate experiments at least twice, and only highly reproducible markers were considered.

Some questions have been raised about the reliability of RAPD data due to their variable nature under different experimental conditions and by the fact that comigrating bands from different individuals do not necessarily represent homologous amplification products (Newbury & Ford-Lloyd, 1993; Bachmann, 1994). However, fragment size can be considered a reliable predictor of homology among closely related individuals, as is the case in this study, although this is

not necessarily true at higher taxonomic levels (Rieseberg, 1996). In order to maximize the reliability of the process, the reproducibility of the results obtained was tested in two different ways. First, the amplification patterns obtained with three different Taq DNA polymerases was verified using the same genotype and primer (data not shown). Second, amplification reactions with DNA obtained from different accessions of four genotypes ('Chandler', 'Cisco', 'Howard' and 'Franquette') using two different primers were also compared (data not shown). In both cases, the pattern of amplification was fully reproducible.

The results obtained, using eighteen primers that yield 23 polymorphic RAPD bands (Table 2), produced a unique fingerprint for each of the 19 walnut genotypes included in this study (Table 1) allowing a unequivocal identification of each genotype. Besides, the fingerprint of each genotype 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. This high level of polymorphism

Table 3. Table 3. Similarity matrix generated using the Nei and Li's index. Cultivar numbers correspond to those in Table 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.00																		
2	0.79	1.00																	
3	0.52	0.43	1.00																
4	0.62	0.62	0.58	1.00															
5	0.52	0.43	0.78	0.58	1.00														
6	0.73	0.73	0.35	0.43	0.23	1.00													
7	0.45	0.45	0.59	0.52	0.71	0.37	1.00												
8	0.78	0.61	0.67	0.58	0.56	0.59	0.47	1.00											
9	0.58	0.50	0.74	0.64	0.63	0.33	0.56	0.74	1.00										
10	0.64	0.54	0.47	0.43	0.59	0.50	0.75	0.59	0.44	1.00									
11	0.54	0.69	0.67	0.74	0.57	0.40	0.50	0.48	0.54	0.50	1.00								
12	0.72	0.64	0.30	0.31	0.30	0.53	0.32	0.50	0.38	0.53	0.26	1.00							
13	0.75	0.58	0.42	0.48	0.42	0.44	0.44	0.74	0.60	0.67	0.36	0.76	1.00						
14	0.69	0.54	0.09	0.52	0.29	0.50	0.20	0.38	0.27	0.40	0.33	0.61	0.54	1.00					
15	0.67	0.58	0.63	0.48	0.53	0.56	0.56	0.53	0.60	0.44	0.36	0.67	0.60	0.36	1.00				
16	0.38	0.48	0.50	0.45	0.50	0.40	0.80	0.37	0.47	0.67	0.53	0.22	0.35	0.21	0.35	1.00			
17	0.72	0.72	0.60	0.54	0.50	0.53	0.63	0.80	0.67	0.63	0.52	0.76	0.35	0.57	0.67	1.00			
18	0.45	0.54	0.59	0.35	0.59	0.37	0.75	0.47	0.44	0.75	0.60	0.32	0.44	0.20	0.33	0.80	0.74	1.00	
19	0.58	0.42	0.63	0.64	0.74	0.33	0.89	0.63	0.70	0.67	0.54	0.29	0.50	0.36	0.50	0.71	0.67	0.67	1.00

probably reflects the outcrossing nature of walnut since similar results have been obtained with RAPDs in other outcrossing fruit and nut tree species such as pistachio (Hormaza et al., 1994) or olive (Fabbri et al., 1995).

As expected, most of the fragments amplified from DNA obtained from the progenies were also present in the parents. Nevertheless, three markers (K01-445, G16-700 and R15-490) were, in some cases, present in the progeny and absent in both parents. Thus, K01-445 was present in 'Vina' and absent in 'Payne' and 'Franquette'; G16-700 was present in 'Sunland' and absent in PI-159568 and 'Lompoc'; R15-490 was present in 'Serr' and absent in 'Lompoc' and 'PI-159568'. The occurrence of non-parental bands has been reported in previous studies with RAPDs (Hunt & Page, 1992; Riedy et al., 1992; Aruna et al., 1993; Ayliffe et al., 1994; Pooler & Scorza, 1995) and different explanations have been suggested, such as formation of heteroduplex molecules between alternate RAPD alleles, mutations or recombination events within the primer binding sites or inside the amplified fragments, competition for primer binding sites or somatic rearrangements in perennial plants.

The similarity values based on 23 RAPDs (Table 3) ranged from 0.09 for 'Cisco' and 'Sharkey' to 0.89 for 'Lompoc' and 'Waterloo'. UPGMA cluster analysis of the similarity matrix (Figure 3) separated the wal-

nut genotypes included in this study into two groups whose differences were basically related to the original sources of the genotypes used as parents in the breeding program (Figure 1). The first group comprises 'Sharkey' and 'PI-159568', two genotypes originating from Asia, and some of their progeny: '56-224', 'Chandler', 'Howard', 'Serr' and 'Sunland'. The second group contains the Californian ('Marchetti', 'Payne', 'Waterloo') and French ('Conway Mayette', 'Franquette' and 'Meylan') parental cultivars and most of their progeny ('Cisco', 'Lompoc', 'Pedro', 'Tehama', 'Tulare' and 'Vina'). This is not unexpected since the three French parental cultivars probably originated in the same French region (Isere). In general, the cultivars sharing common parents tend to group together and with at least one of the parents.

Among all the genotypes tested, 'Sharkey' appears to be the most distantly related to all the others except to its progeny ('56-224') and to 'PI-159568' with a similarity value of 0.69 and 0.61 respectively. 'PI-159568' itself shows a high similarity value only with its progeny, and with 'Sharkey' and 'Sharkey's progeny ('56-224'). The markers G18-990, R19-755 and T06-400 are only present in 'PI-159568' and 'Sharkey' and some of their progeny and the marker R14-1130 is only absent in 'PI-159568' and 'Sharkey'. It is interesting to note that those two genotypes are the only ones

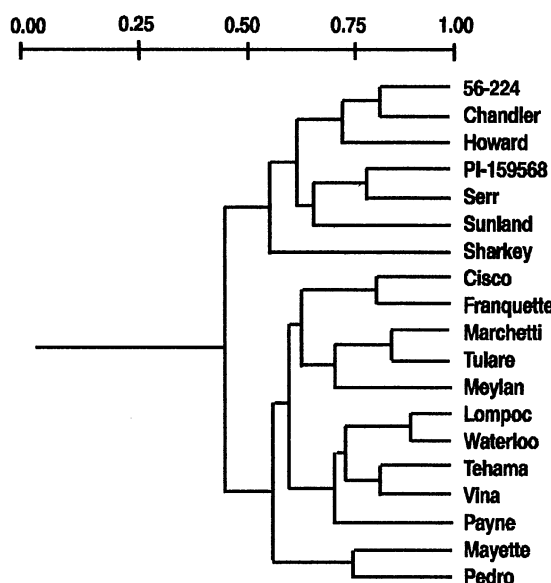


Figure 3. Dendrogram of the 19 walnut genotypes studied generated by UPGMA cluster analysis of the similarity values shown in Table 3.

in this study originating from Asia: i.e., 'PI-159568' from Afghanistan and 'Sharkey' probably from China (Tulecke & McGranahan, 1994).

The cultivar Payne, ancestor of most of the cultivars tested, showed, as expected, a similarity value of 0.50 or more with all 10 cultivars related to it except 'Cisco' (similarity value of 0.47) and 'Sunland' (similarity value of 0.44), two cultivars that are two generations away from 'Payne' in the pedigree.

In our research, as detected with other crop species (Aruna et al., 1993; Dunemann et al., 1994; Dweikat et al., 1993; Hallden et al., 1994), we observe a fairly close relationship between the known pedigree and the genetic similarity obtained with RAPDs. This is of great interest in breeding tree crop species since very often the pedigree of the cultivars is unknown. However, it was not possible to compare the genetic similarity values estimated with the Nei & Li index with the coefficients of coancestry (Falconer, 1989) due to three main reasons. One, that the available pedigree consists of a maximum of just two generations. Second, the French and Californian cultivars are probably related since the genetic base of the Californian cultivars has been enriched with introductions from France. Third, although the markers we have used allow us to unequivocally distinguish all the cultivars studied, a higher number of markers will probably be

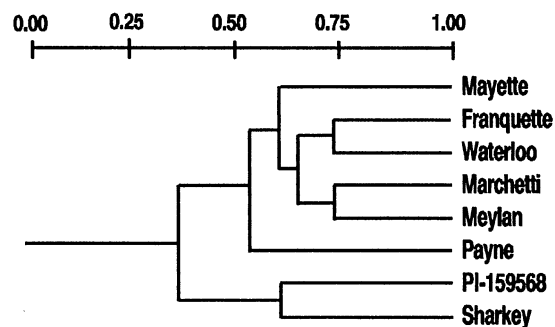


Figure 4. Dendrogram of the parental cultivars generated by UPGMA cluster analysis of the similarity values shown in Table 3.

needed to obtain a dendrogram that accurately reflects the similarity matrix; in our case the correlation coefficient between the cophenetic matrix developed from the dendrogram and the similarity matrix was 0.65. However, if only the eight initial parental genotypes are analyzed, the dendrogram obtained (Figure 4) is a very good representation of the similarity matrix since the correlation coefficient between the two matrices is 0.86. Moreover, in this case, two clear clusters are obtained: one with the genotypes of Asian origin, 'PI-159568' and 'Sharkey', and the other with the French and Californian cultivars. This is the expected result based on the fact that the gene pool of the Californian cultivars has been enriched with French germplasm. Comparisons of genetic distances obtained with molecular markers and theoretical data based on pedigree information have been already made in different herbaceous species and generally molecular marker-based measures of genetic distance agree with pedigree information (Dudley, 1994). Since pedigree and passport data are often unknown or incomplete for many fruit and nut tree species (Warburton & Bliss, 1996) RAPDs can be a useful tool to assess the degree of similarity of accessions or cultivars in these woody species in order to select the best parents to obtain new genetic combinations; this is especially important if we consider the long generation times of most fruit and nut tree species and, consequently, the length of the breeding process.

The results obtained show first that the RAPD technique can detect enough polymorphism to differentiate among walnut genotypes, even among cultivars closely related because of their common parents (for example 'Lompoc'-'Tehama' and 'Chandler'-'Howard'). Second, a general pattern of separation between Californian-European and Asian genotypes was obtained in this study confirming the results previ-

ously reported with RFLPs by Fjellstrom et al. (1994). Third, the RAPD method is a relatively simple technique to study genetic relationships in walnut thus allowing the study of the influence of genetic drift and selection which cannot be predicted using pedigree information alone. From the data obtained in this study we can conclude that RAPD technology can be useful in current walnut breeding programs, allowing the identification of new cultivars as well as the assessment of the genetic similarity among different genotypes.

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