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Genetic Relationships and Characterization of Persian Walnut (*Juglans regia* L.) Cultivars Using Restriction Fragment Length Polymorphisms (RFLPs)

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Abstract. **RFLP markers were used to investigate genetic diversity among California walnut (*Juglans regia*) cultivars and germplasm collected worldwide. Sixteen of 21 RFLP markers were polymorphic in the 48 walnut accessions tested. RFLP markers were useful for identifying walnut cultivars. All genotypes were heterozygous at ≈20% of the loci for both California and worldwide germplasm. California walnut germplasm contained 60% of the worldwide allelic diversity. Cluster analysis of genetic distance between accessions and principal component analysis of allelic genotypes showed two major groups of walnut domestication. California germplasm was associated with germplasm from France, central Europe, and Iran and had less genotypic similarity with germplasm from Nepal, China, Korea, and Japan.**

Juglans regia, the Persian or English walnut, is an important commercial crop in California where production has greatly benefited from the release of cultivars developed by the Univ. of California walnut breeding program. This breeding program was founded on crosses between late-season French selections ('Franquette' and 'Mayette') and laterally fruitful selections ('Chandler' and 'Vina'), which combined both traits (Forde, 1975; McGranahan and Forde, 1985). Lateral fruitfulness was derived from 'Payne', but was also found in 'Sharkey'. Because western Chinese walnut germplasm frequently exhibits lateral fruitfulness and French cultivars do not, it was suggested that 'Payne' and 'Sharkey' may have been derived from Chinese germplasm (Tulecke and McGranahan, 1994). Central Asian germplasm has also been used. 'Payne' was used in crosses with PI159568 from Afghanistan to produce 'Sunland' and 'Tulare'. Since a limited number of parents has been used to develop new California cultivars, an assessment of genetic variability in *J. regia* cultivars was needed to verify the availability of genetic diversity, which is required for continued crop improvement.

Little genetic information is available for this crop species despite its widespread commercial use, making genetic variability measurements difficult. Morphological variability, although readily recorded, can be an unreliable measure of genetic diversity because of the confounding effects of environment, pleiotropy, and the unknown genetic basis of most morphological attributes. Isozymes and RFLPs have advantages as genetic markers for appraising variability because 1) their codominant nature allows a plant genotype to be assayed directly and 2) they are relatively free from environmental effects. Arulsekar et al. (1985, 1986) identified isozyme loci using four enzyme systems (glucose phosphate isomerase, aspartate amino transferase, phosphoglucomutase, and esterase) in walnuts. To circumvent the potentially limiting number of isozyme loci available in walnuts, RFLP markers were developed to measure walnut genetic diversity. RFLPs have been successfully used to study genetic diversity in cross-pollinated and self-pollinated crop species (Brummer et al., 1991; Chase et al., 1991; Kesseli et al., 1991; Miller and Tanksley, 1990). In the current study, 22 walnut RFLP loci were used to determine the genetic diversity and relationships of 48 walnut accessions of diverse origins.

Materials and Methods

Germplasm. Walnut germplasm used for this study consisted of cultivars and selections from the Univ. of California, Davis, walnut breeding program; chance seedling cultivars; and plant introductions (Table 1). Trees were maintained at the Wolfskill Experimental Farm in Winters, Calif., at evaluation orchards in Davis, and as seedlings at the National Clonal Germplasm Repository (NCGR) greenhouses in Davis. Germplasm was classified into 12 groupings (Table 1) for combined cluster analysis. Carpathian germplasm is comprised of cold-hardy cultivars grown in the United States considered to have originated from eastern Europe. The germplasm classified as French germplasm are selections made in California from French lines.

DNA isolation. Leaves were collected from 48 *J. regia* accessions (Table 1). Leaves were ground in liquid N and stored frozen at –70C before DNA was isolated by a modification of the method of Doyle and Doyle (1987). Five grams of frozen leaves was added to 20 ml of 60C preheated 2x CTAB buffer (2% CTAB, 1% PVP, 1% b-mercaptoethanol, 0.1% sodium bisulfite, 1.4 M NaCl, 50 mM tris, 20 mM sodium EDTA, pH 8.0), and incubated at 60C for 30 min. The aqueous solution was extracted with 20 ml 24 chloroform : 1 isoamyl alcohol and centrifuged for 10 min at 1800 rpm in a bench-top centrifuge at 25C, and the aqueous layer was retained. Fifteen milliliters of isopropanol was added to precipitate the nucleic acids. The precipitate was spooled and washed in 76% ethanol with 10 mM ammonium acetate. The nucleic acid precipitate was air-dried overnight, rehydrated in 1 ml TE buffer (10 mM tris, 1 mM EDTA, pH 8.0), digested with 10 µg RNase at 37C for 1 h, and precipitated with ethanol. The DNA precipitate was washed in 75% ethanol and dried overnight before rehydration in 200 µl TE, pH 8.0. The DNA was quantified spectrophotometrically at 260 nm and visually in a 0.8% agarose gel stained with ethidium bromide containing lambda phage standards, with ≈25 $\mu g \cdot ml^{-1}$ DNA = 1.0 Å at 260 nm.

RFLP detection. Six micrograms of walnut DNA was digested

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Table 1. *Juglans regia* accessions studied for RFLP diversity.

Group	Accession	Geographic origin	Parentage[z]
Californian	Ashley	California	Unknown selection from California orchard
Californian	Chandler	California	(C. Mayette x Payne) x (Sharkey x Marchetti)
Californian	Chico	California	Sharkey x Marchetti
Californian	Cisco	California	Meylan x (Payne x C. Mayette)
Californian	Eureka	California	OP fron Persian or Kaghazi type from Iran
Californian	Hartley	California	Franquette x Mayette cross?
Californian	Howard	California	(C. Mayette x Payne) x (Sharkey x Marchetti)
Californian	Payne	California	OP seedling possibly from French x Chinese seedlings?
Californian	Sharkey	California	Selected seedling from Chinese seed?
Californian	Sunland	California	(Waterloo x Payne) x PI159568
Californian	Tulare	California	(Payne x Waterloo) x (Payne x PI159568)
Californian	Vina	California	Franquette x Payne
Californian	S61-25	California	(Hartley x Payne) x (C. Mayette x PI18256)
Californian	S77-12	California	Howard x [(Waterloo x Payne) x (Marchetti x Sharkey)]
Californian	S67-13	California	(Payne x Waterloo) x (Payne x PI159568)
French–Californian	Scharsch Franquette	California–France	Franquette seedling
Southern Californian	Early Erhardt	Southern California	Erhardt selection
Southern Californian	Placentia	Chile or China	OP seed
Not used	Concha	Chile	OP selection from Chilean orchard
Carpathian	Cascade	Washington	Russian type x Manchurian (Manregian?) type
Carpathian	Idaho	Idaho	
Carpathian	North Platte	Nebraska	
French	Conway Mayette	California–France	OP selection of Mayette
French	Meylan	France	Seedling or clone from France
French	XXX Mayette	California–France	OP selection of Mayette origin
Central European	Sibisel Precose	Romania	
Central European	Sibisel 41	Romania	
Central European	Alsoszentivani 117	Hungary	
Central European	Bulgaria 3	Bulgaria	
Central European	Geisenheim 139	Germany	
Central European	Purpurea	Germany	
Afghani–Pakistani	NCGR 122.26,.27[y]	Pakistan	
Afghani–Pakistani	NCGR 254.1,.2[y]	Pakistan	
Afghani–Pakistani	PI159568	Afghanistan seedling selection from PI127460	
Iranian	0-20-1072	Iran	
Nepal	NCGR 85.01	Nepal	
Indian	NCGR 102	India	
Russian	NCGR 155	Russia	PI264362 (Kolobok OP seedling)
Russian	NCGR 152	Russia	PI264372 (Ideal OP seedling)
Russian	NCGR 86.01	Russia	
Chinese	Beijing #5	China	
Chinese	Xinjiang #6	China	
Chinese	Xinjiang #8	China	
Chinese	Yunnan #1	China	
Chinese	PI18256	China	
Korean–Japanese	Sinensis #7	Japan	
Korean–Japanese	NCGR 63.01	Korea	
Korean–Japanese	NCGR 146	Korea	PI263512

[z]Crosses giving rise to accession or variety giving rise to open-pollinated (OP) selection.
[y]NCGR = National Clonal Germplasm Repository, U.S. Dept. of Agriculture, Davis, Calif. Two seedlings from same accession.

with 30 units of *Eco*RI or *Hind*III for 6 h and electrophoresed in 0.8% agarose with 1x TAE buffer (45 mM tris acetate, 1 mM EDTA, pH 8.0) for 18 h at 0.7 V/cm. Gels were stained with ethidium bromide to visualize the DNA and transferred (Southern, 1975) to 150 × 150-mm nylon membranes (Nytran, Schleicher & Schuell, Keene, N.H.). Membranes were rinsed in 2x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), dried 2 h in a 65C oven, and stored dry at room temperature. Membranes were trimmed to 140 × 150 mm, loaded into 30 × 300-mm hybridization bottles, prehybridized for 1 h, and hybridized for 16 to 20 h at 65C according to the method of Church and Gilbert (1984). Thirty nanograms of insert DNA from 20 single-locus walnut probes (pFP01, 02, 03, 04, 06, 10, 11, 15, 17, 18, 24, 25, 26, 32, 34, 43, 45, 48, and 67) (unpublished data) and a wheat ribosomal gene (pTA71) (Gerlach and Bedbrook, 1979) were ^{32}P radiolabeled by the random priming method (Feinberg and Vogelstein, 1983),

denatured by boiling and rapid cooling, and added directly to each hybridization tube. After hybridization, membranes were washed 2 × 15 min in 2x SSC, 0.1% SDS at room temperature; 2 × 15 min in 1x SSC, 0.1% SDS at 45C; and 1 × 30 min in 0.5x SSC, 0.1% SDS at 65C; rinsed in 2x SSC; blotted on paper towels; and autoradiographed on X-OMATAR film (Kodak, Rochester, N.Y.) at –70C using Cronex (Du Pont, Wilmington, Del.) intensifying screens for 1 to 5 days. Blots were stripped by soaking in 5 mM tris, pH 8.0, 0.5 mM EDTA, pH 8.0, 0.01x Denhardts solution, and 0.05% Na-pyrophosphate at 65C for 30 to 60 min; rinsed in 2x SSC; and stored dry. Blots were reprobed 5 to 10 times.

Genetic analysis. Allelic genotypes were scored for all loci except for those identified by pFP10. Because of the complex fragment pattern displayed by hybridizations with this probe, alleles could not be assigned and fragments were scored instead. Allelic frequency analysis of 20 loci (not including pFP10) was performed using the computer program BIOSYS-1 (Swofford and Selander, 1981) to generate genetic distance matrices (Nei, 1972), and perform unweighted pair group mean (UPGM) cluster analysis (average linkage clustering). Principal component analysis (PCA) of combined fragments from hybridizations with pFP10 and allelic data from all other hybridizations were analyzed using PC-SAS version 6.03, where the presence of a fragment was scored as 1 and its absence as 0 and alleles were given a score of 1 for each haploid copy present (e.g., AB individuals scored as 11, AA as 20, and BB as 02).

Results

Polymorphisms were observed for 16 of the 21 RFLP markers. All but one RFLP, from pFP10, could be assigned conventional single-locus allelic states. Even though mapping to a single locus, pFP10 gave a complicated banding pattern (Fig. 1), which prevented allelic state designations and could not be used to estimate heterozygosity levels. Because of the highly informative nature of this probe, with 24 different fragment patterns in *J. regia*, fragment data for this probe were included in the PCA. For the other loci, 46 alleles were identified.

The relatively high degree of RFLP heterozygosity in walnuts allowed us to identify walnut genotypes and their offspring. Even though a single RFLP marker could discriminate between two genotypes, seven markers were required to identify 49 genotypes in this study. The use of the highly polymorphic pFP10 marker would reduce the number needed to five markers. 'Ashley' and 'Payne' could not be separated, supporting the hypothesis that 'Ashley' is a bud sport of 'Payne'.

Apportioning the allelic diversity among their 13 geographic sources (Table 2) shows that, on average, each source contains 60% of the total allelic diversity found in the entire species. California germplasm contained 65% of the total allelic diversity, considerably higher than its French progenitor (with 50%), but slightly less than Chinese, Afghani–Pakistani, central European, Russian, and Carpathian groups. Since California cultivars are

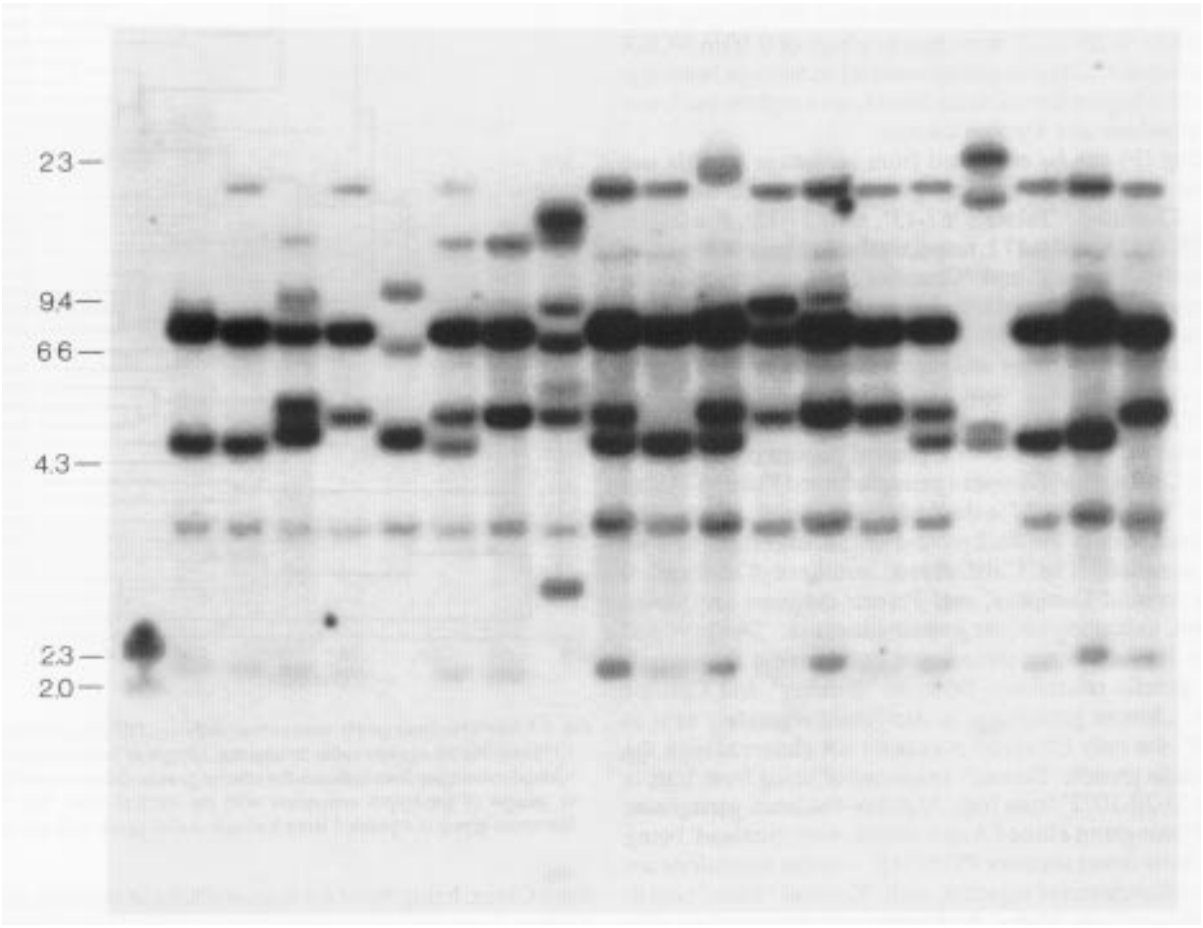

Fig. 1. Autoradiograph of 19 walnut genotypes digested with *Hind*III and hybridized with probe pFP10. Molecular length standards in kilobase pairs of DNA are presented on left.

Table 2. Summary statistics for *Juglans regia* RFLP allelic genotypes at 20 RFLP loci.

Origin	No. of accessions	Mean heterozygosity	Proportion of total alleles	No. of unique alleles
California[z]	16	0.21	0.65	0
Southern California	2	0.19	0.54	0
Carpathia	3	0.27	0.67	1
France[z]	4	0.21	0.52	0
Central Europe	6	0.19	0.67	0
Russia	3	0.23	0.67	0
Afghanistan–Pakistan	5	0.16	0.70	2
China	5	0.18	0.70	2
Korea–Japan	3	0.18	0.65	1
India	1	0.10	0.48	0
Nepal	1	0.50	0.65	2
Others[y]	2	0.10	0.48	1

[z]Includes 'Scharsch Franquette'.
[y]Concha and O-20-1072.

wholly derived from introduced sources, it is not surprising that they contain no unique alleles (Table 2). Seven genotypes, primarily of Asian origin, contained unique alleles. Although the Carpathian genotypes were probably derived from eastern European germplasm, one unique allele at locus pFP43 was observed in 'North Platte' (Lindgreen and Gustafson, 1982). The small number of cultivars tested from central Europe and Russia probably accounted for the absence of this allele in the other European sources. Only 26% of the alleles was found in every source region and 37% was found in all but one source region. The average heterozygosity level among all genotypes was 0.197, ranging from a low of 0.05 in '0-20-1072' from Iran to a high of 0.50 in NCGR 85.01 from Nepal. California germplasm had an average heterozygosity of 0.21, a higher level than for most source regions but lower than for Carpathian and Russian sources.

Inbreeding (F) can be estimated from parentage records and ranges from 0 to 0.172. For most genotypes, F = 0, while for 'Howard', 'Chandler', 'Tulare', '67-13', and '77-12', F = 0.063, 0.063, 0.125, 0.125, and 0.172, respectively. In terms of retaining heterozygosity, 'Howard' and 'Chandler' had no reductions in their heterozygosity compared to their parental lines, while 'Tulare', '67-13', and '77-12' had 33.3%, 11.1%, and 30.8% reductions in their levels of heterozygosity and seemed to agree with expected inbreeding estimates.

The UPGM cluster analysis of genetic distance (Nei, 1972) between cultivars (Fig. 2) shows a general pattern of separation between the Californian–European germplasm and Russian–Asian germplasm. 'Payne' is found in the first cluster joined, as would be expected since it is in the background of the largest number of cultivars sampled. The Californian, southern Californian, Carpathian, central European, and French cultivars are joined together first, indicating similar genetic identities. 'Sharkey' and some of its derivatives are joined near two Chinese accessions, indicating genetic relatedness between 'Sharkey' and Chinese germplasm. Chinese germplasm is also found separately next to 'Bulgaria 3', the only European accession not clustered with the Euro-American groups. 'Eureka', suspected of being from Iran, is joined with '0-20-1072' from Iran. Afghani–Pakistani germplasm is scattered throughout a broad Asian cluster, with 'Sunland' being found next to its direct ancestor PI159568. Russian accessions are found somewhat clustered together, with 'Kolobok' found next to the Carpathian cultivar 'Idaho', both being cold-tolerant lines. The Sinensis types from Korea and Japan are found with the Nepal and Indian accessions, far from most other germplasm. 'Yunnan #1' from China, being fixed for unique alleles at two loci, is found the farthest from any cluster. This type of Yunnan walnut was once classified as a separate species (*J. sigilata*) (Dode, 1909) because of its unique leaf and nut morphology. When the accessions are

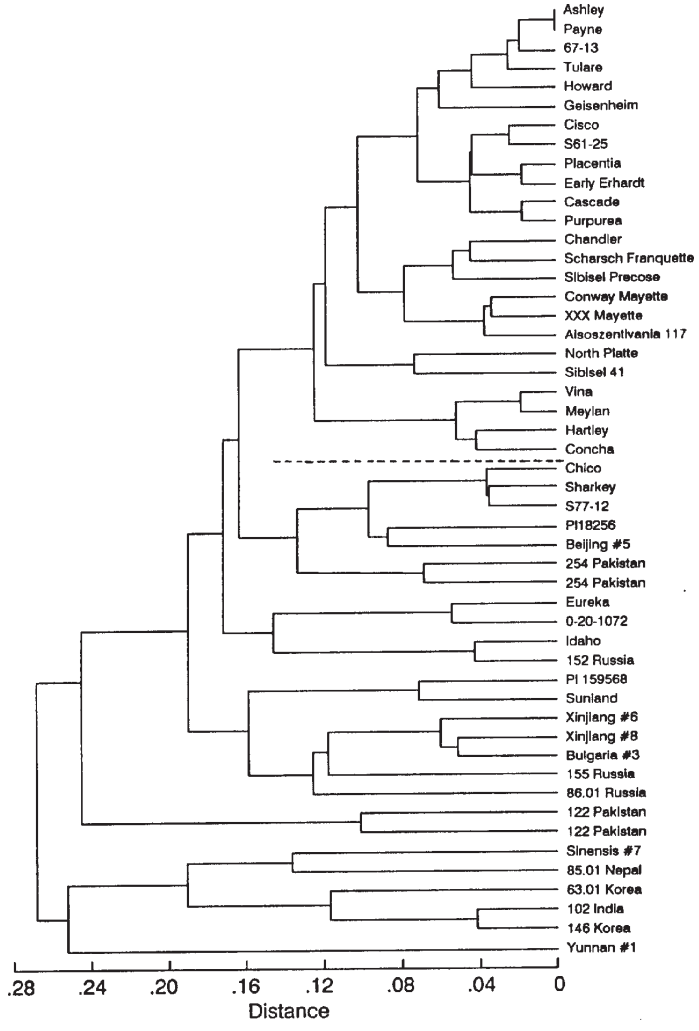

Fig. 2. Unweighted pair group mean cluster analysis of RFLP genetic distance (Nei, 1972) among 48 *Juglans regia* accessions. Length of horizontal lines joined by vertical connecting lines indicates the relative genetic differences of the genotypes or groups of genotypes connected with the vertical lines. The Californian–European group is separated from Russian–Asian group with the dotted line.

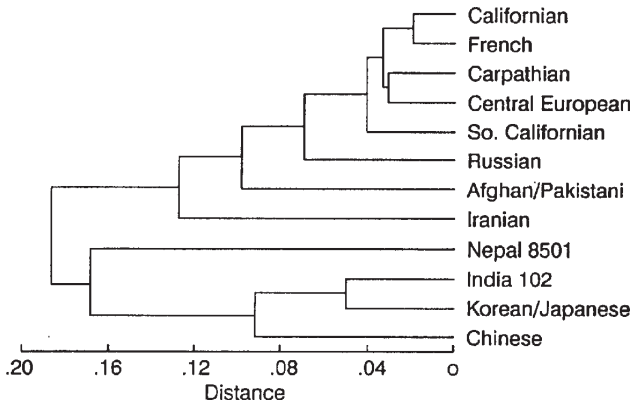

Fig. 3. Uweighted pair group mean cluster analysis of RFLP genetic distance (Nei, 1972) among 12 source groups of *Juglans regia* genotypes.

regrouped into 12 sources, placing 'Scharsh Franquette' with French lines and 'Eureka' with '0-20-1072' from Iran, and their genetic distances are recalculated and separated by cluster analysis, two major cluster groups can be identified (Fig. 3). One group extends from Europe to central Asia; the other group includes the south and east Asian region.

PCA of allelic genotypes among the cultivars showed that only 17.8% of the variation could be accounted for by the first PC, 11.2% by the second PC, 9.4% by the third PC, and only 75.0% by the first eight PCs. Although two pairs of loci, pFP03/34 and pFP24/67, map only 1.6 cM apart on linkage groups 4 and 11, respectively, of the walnut genome (unpublished data), there were no allelic correlations between any of the RFLP loci. A three-dimensional plot of the genotypes against the first three PCs (Fig. 4) divides most Asian accessions from the Euro-American accessions, but otherwise separates few members of this latter group into

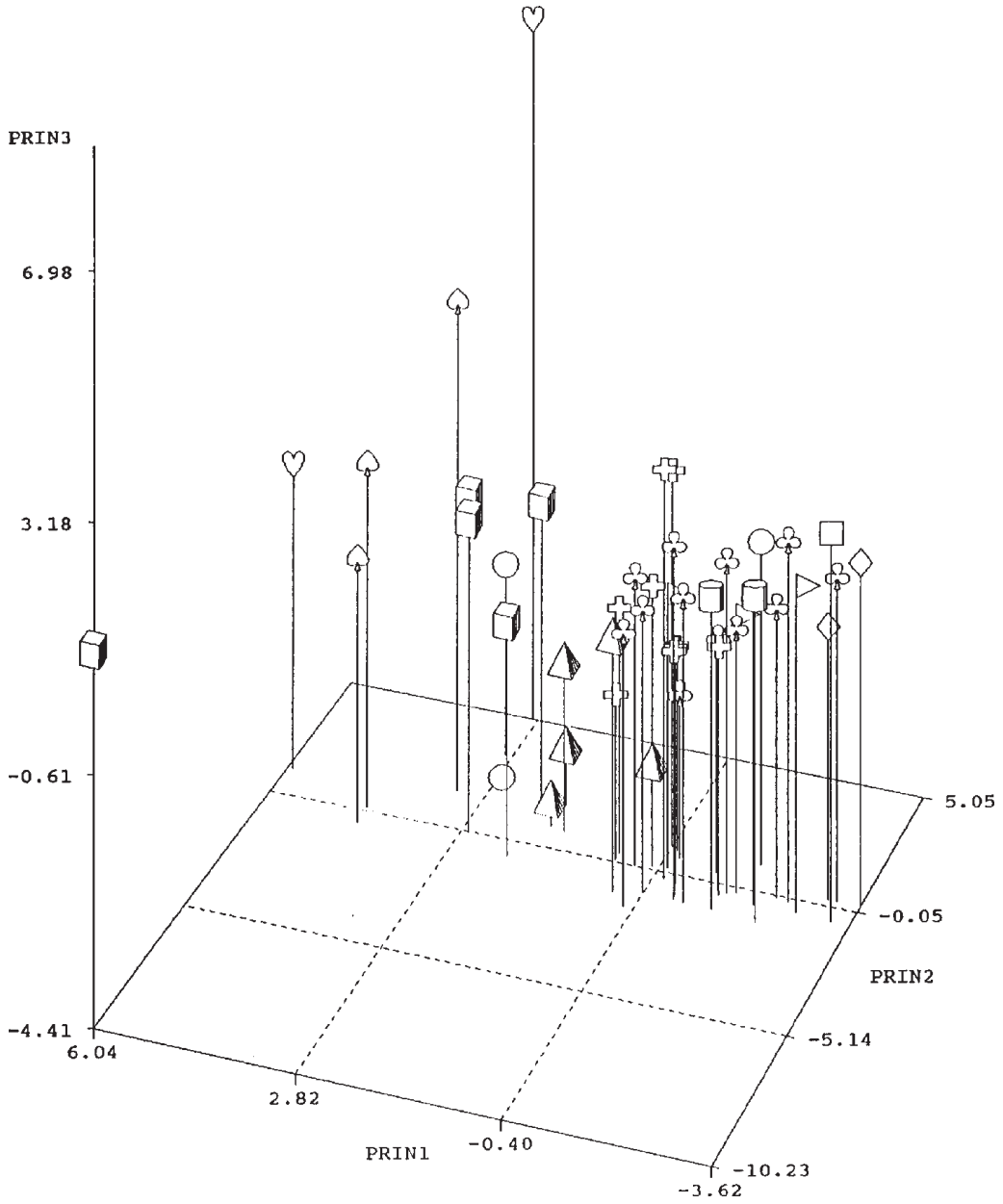

Fig. 4. Plot of *Juglans regia* accessions coded by geographic origin against the first three principal components of RFLP allelic genotypes. Clubs = California, flags = France, crosses = central Europe and Carpathia, diamonds = Iran, squares = Concha, cylinders = southern California, pyramids = Afghanistan–Pakistan, circles = Russia, cubes = China, spades = Korea–Japan, hearts = India and Nepal.

any obvious pattern. Groupings can be seen for germplasm from Korea–Japan (sometimes referred to as *J. sinensis*), China, Afghanistan–Pakistan, France, and Iran. Carpathian–central European and California germplasm overlapped each other and the French and Afghani–Pakistani germplasm, which could be expected because of their shared backgrounds. Like the cluster analysis, it can also be seen that two of the Russian accessions group with east Asian germplasm and one accession groups with more western germplasm. Inspection of additional PCs did not appreciably increase the differentiation of germplasm groups, but only changed the relative spread of the same groups previously identified. Two of the Russian accessions are found in the east Asian group and one in the Euro-American group. Two Pakistani accessions are found in the east Asian group and one is found in the more western group.

Discussion

This RFLP study has provided comparative information on California walnut germplasm with respect to foreign germplasm and has illuminated previously untested hypotheses. The California germplasm pool seems to be as diverse as most other walnut germplasm pools and does not seem to have significantly reduced genetic diversity levels. However, by examining the considerable amount of allelic diversity available in other germplasm sources, untapped genetic diversity may be found, particularly from eastern Asia. There is no general loss of heterozygosity in California walnut germplasm compared to foreign germplasm, except in the few cases where heterozygosity loss was expected from increased inbreeding. Selection '77-12', which has the highest F value (based on ancestry), but not the lowest amount of RFLP heterozygosity, is a relatively weak tree, which could be expected from inbreeding in this normally outcrossed species. Trees that have the lowest amount of RFLP heterozygosity are not weak trees, suggesting that these loci are not linked to deleterious recessive alleles.

The RFLP variation identified in this study is also useful for identifying cultivars. The minimum number of probes needed to tell 49 genotypes apart is seven, the same number predicted by the equation $P = 3^k!/3^{kn}(3^k - n)!$, where P = probability of discriminating n cultivars and k = the number of loci with two alleles per locus (Parfitt, 1989; Soller and Beckman, 1983). Variations on the number of probes needed will depend on the allelic variability in the populations surveyed. More probes would be needed to separate many closely related genotypes. Fewer probes would be needed where more than two alleles per locus are present. Because of the time involved in Southern hybridization analyses (Southern, 1975), it could be more efficient to use isozyme or randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) marker analysis instead of RFLP analysis for routinely fingerprinting cultivars. Nevertheless, these RFLP probes have effectively identified parentage of walnut offspring (Aly et al., 1992) and can overcome the problems of low polymorphism levels in isozyme loci and the potential unreliability of RAPD markers.

Cluster analysis of the genetic distance among accessions grouped most accessions within the same source together, but also identified some additional groups of interest. 'Sharkey' was grouped with Chinese accessions, affirming its Chinese origins, while 'Payne' seems to be of western origin. These results also suggest that the lateral-bearing trait found in 'Payne' and 'Sharkey' had unrelated origins from Europe and Asia. 'Bulgaria #3' is apparently derived from Chinese germplasm. The proposed Iranian origin of 'Eureka' (Tulecke and McGranahan, 1994) is supported by its grouping with '0-20-1072' from Iran.

Cluster analysis of the 12 germplasm sources placed the California germplasm near the French and placed the Carpathian germplasm near the central European. The two main clusters identified indicate that most European germplasm originated from Iran and the Asian germplasm originated near the Himalaya mountain range. These hypotheses are supported from historical accounts (McGranahan and Leslie, 1991) that the European germplasm was initially brought via Greek and Roman trade from Persia and that Chinese germplasm came from areas to the west of China. PCA also divided the 48 accessions into these two main groups, with Iranian germplasm found on one extreme of the first PC and east Asian germplasm on the other margin. A small number of PCs could not explain most allelic variation seen, as would be expected if there is an absence of linkage disequilibrium among the RFLP loci. Even though a few of the RFLP loci are closely linked, the lack of allelic correlations between these loci implies that there has been no selection over time to retain allelic linkage disequilibrium. A similar lack of allelic correlation between closely linked RFLP loci was reported in *Triticum* (Lubbers et al., 1991).

The concept of core collections, for which the number of accessions within germplasm collections is condensed but still represents the total species diversity (Frankel and Brown, 1984), can be empirically evaluated with the results from this experiment. The characters in this study had minimal genetic relatedness and were assumed to be unlinked to selected characteristics. Thus, these markers can be considered to represent the actual variation for these accessions. Selecting 10% of accessions by randomly sampling each of five RFLP geographic clustering groups (California–France, central Europe–Carpathia, China, Korea–Japan, and other Asia) will on average retain 78.1% of the species allelic diversity, which is well within the 70% objective goal (Brown, 1989) of core collections. However, there is a considerable amount of phenotypic variability within these clustering groups, and a single accession (≈10%) from each of these groups does not sufficiently reflect the expressed genetic variability found in that group. Also, because many *J. regia* plant introductions, especially wild germplasm accessions, were not included in this study (as it focused primarily on California germplasm), there is a high probability that there is considerably more allelic diversity within this species than was detected in this study.

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