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Identification of a walnut (*Juglans regia* L.) germplasm collection and evaluation of their genetic variability by microsatellite markers

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Abstract

The characterization and evaluation of walnut (*Juglans regia*) germplasm constitute important aspects of taxonomic analysis and are valuable tools for breeding programs. In this work, a collection of 57 common walnut cultivars, mainly coming from Spain and the USA, has been studied with microsatellite markers. To carry out this work, 32 primer pairs flanking simple sequence repeats previously developed in *Juglans nigra* were screened to select the loci that presented high polymorphism and that were easier to score. The 19 selected microsatellite markers allowed the discrimination of the studied cultivars, with a total of 97 alleles detected and an average of 5 alleles per locus, confirming that these markers are more suitable tools for walnut identification than other molecular markers studied previously. The genetic similarity estimated from the molecular data clearly separated the Spanish walnuts from the Californian genotypes. Allelic data are presented for use as size standards to assist in correcting laboratory-to-laboratory variation of allele size calling. Some of them are compared with previous results published and the discrepancies found are discussed.

Additional key words: cultivar discrimination; genetic relationships; molecular characterization; plant breeding; polymorphism.

Resumen

Identificación de cultivares de nogal (*Juglans regia* L.) y evaluación de su variabilidad genética mediante marcadores microsatélites

La caracterización y la evaluación de germoplasma de nogal (*Juglans regia*) constituyen aspectos importantes del análisis taxonómico y son herramientas eficaces para los programas de mejora. En este trabajo se han estudiado 57 cultivares de nogal común, procedentes principalmente de España y EEUU, mediante marcadores microsatélite. Para llevar a cabo este trabajo se examinaron 32 pares de cebadores, desarrollados previamente en *Juglans nigra*, para seleccionar los loci que presentaran un polimorfismo elevado y que fueran fácil de detectar. Los 19 marcadores microsatélite seleccionados permitieron discriminar entre los cultivares estudiados, detectando un total de 97 alelos y una media de 5 alelos por locus, confirmando que este tipo de marcadores son más adecuados para la identificación de nogal que otros marcadores moleculares previamente estudiados. La similitud genética estimada a partir de los datos moleculares, permitió separar claramente los genotipos españoles de los californianos. Se presentan los datos alélicos para su uso como tamaños estándar de utilidad en la corrección de los tamaños alélicos encontrados entre laboratorios. Algunos de ellos son comparados con los resultados previos publicados, discutiéndose las discrepancias encontradas.

Palabras clave adicionales: caracterización molecular; discriminación de cultivares; mejora de plantas; polimorfismo; relaciones genéticas.

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Abbreviations used: AF (allele frequency), AFLP (amplified fragment length polymorphism), AS (allele size), BC (Before Christ), F (fixation index), He (expected heterozygosity), Ho (observed heterozygosity), HWE (Hardy-Weinberg equilibrium), IMIDA (Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario), ISSR (inter-simple sequence repeat), Na (number of alleles per locus), Ne (effective number of alleles per locus), MEGA (molecular evolutionary genetics analysis), PD (power of discrimination), RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), SSR (simple sequence repeat), UPGMA (unweighted pair-group method with arithmetic mean).

Introduction

Juglans regia L. species (common walnut, Persian walnut, or English walnut) is widely cultivated throughout the temperate regions of the world (McGranahan and Leslie, 1990). The origin of this species seems to be a large area of the Central Asia Mountains (Nekrassowa, 1927; Berg, 1937; Browicz, 1976). During the last glaciations (Würm glaciations), walnut disappeared in Southern Europe and Southern Turkey, but survived in the warmer areas close to the Black Sea and Caspian Sea. According to available pollen data, walnut was reintroduced into former areas during the second millennium BC (Zohary and Hopf, 1988). Likewise, in Northern Spain, walnut pollen has been found in sediments from the Münsterian period, during the Low Palaeolithic time, when temperatures were expected to be low (Sánchez-Goñi, 1988). Walnut pollen has been detected also in the Carihuela Cave sediments, located in Southern Spain. This deposit of pollen has been dated to 28,000 years BC, when the Würm glaciations occurred (Carrión and Sánchez-Gómez, 1992). Walnut pollen from 11,000 to 7,000 years BC has been found in Central Italy, and has been dated to 5,000 years BC in the Southern Alps and the Balkans. In addition, Van den Brinks and Janssen (1985) reported the presence of pollen dated to 4,500 years BC in the Serra da Estrela (Portugal), which is older than the fossil pollen found in Turkey and in Greece (Bottema, 1980). Additional paleopalynologic study indicated that walnut was present also in Northern Africa during the Low and Middle Holocene (Ballouche and Damblon, 1988). Taking into consideration the previous reports and in particular the existence of walnut in both Central Asia and the Iberian Peninsula during the Würm glaciations, some questions have arisen concerning Vavilov's theory about the origin of cultivated species (Zohary, 1970). According to Frutos (2000), in the Iberian Peninsula, walnuts could have survived the rigours of the cold because the North-South oriented mountain range known as the Iberian System allowed the migration of species to the warmer areas. On the contrary, the species growing on the Northern side of the Pyrenees mountains, that are East-West oriented, did not escape from the cold because they found an insurmountable barrier when moving Southwards in the last Ice Age (Frutos, 2000). More recently, walnut has been cultivated since Greek and Roman times all around the Mediterranean basin, where this species is found as scattered individuals or as groups of trees, bordering agricultural land or rivers. In the 18th century, walnut began to be cultivated in South America by the Colonial Spaniards, who exported *J. regia* genotypes from Spain. Finally, in the 19th century, walnuts were imported into Northern California from France and into Southern California from China (Tulecke and McGranahan, 1994; Beede and Hasey, 1998). Cultivated distribution now includes North and South America (Chile, Argentine), Australia, New Zealand, South Africa and Japan. The Persian walnut is cultivated extensively for its high-quality nuts as well as base material for timber industry (McGranahan and Leslie, 2009).

In general, European walnut production still depends largely on trees grown from seedlings. The accurate identification of walnut genotypes is a basic requirement for the management and use of germplasm (clarification of synonymy, homonymy, and misnaming) for practical breeding purposes and for protection of proprietary rights. The traditional methods for its characterization have been based on the analysis and comparison of morphological observations. However, the influence of the environment in the expression of morphological characters, as well as the long juvenile period of the walnut trees, cause difficulties for the proper classification of the plant material exclusively by morphological traits. To overcome these limitations, molecular markers have been used to differentiate, characterize, and identify walnut accessions. Such DNA-based markers are not affected by the environment and they can be detected in all tissues at all stages of development. Previous studies of walnut genetic diversity were carried out with isozymes (Arulsekar *et al*., 1986; Solar *et al*., 1994; Ninot and Aletà, 2003; Vyas *et al*., 2003), restriction fragment-length polymorphism (RFLP) (Fjellstrom *et al*., 1994), randomly amplified polymorphic DNA (RAPD) (Nicese *et al*., 1998), inter-simple sequence repeat (ISSR) (Potter *et al*., 2002), and amplified fragment length polymorphism (AFLP) markers (Andreakis *et al*., 2002; Kafkas *et al*., 2005; Bayazit *et al*., 2007). Among the DNA-based markers, microsatellites or SSRs (simple sequence repeats) allow a high level of resolution in genetic studies due to their high polymorphism, co-dominant inheritance, reproducibility, and easy detection by PCR (Gupta *et al*., 1996). There is literature related to the use of microsatellites in the study of genetic relationships in walnut (Woeste *et al*., 2002; Dangl *et al*., 2005; Foroni *et al*., 2005, 2006; Robichaud *et al*., 2006; Victory *et al*., 2006; Wang *et al*., 2008; Pollegioni *et al*., 2009; Bai *et al*., 2010; Gunn *et al*., 2010). Recently Ciarmiello *et al.* (2010) have

published the molecular characterization of *Juglans* cultivars via amplification refractory mutations system, a standard technique that allows the discrimination of alleles at a specific locus differing by as little as 1 bp (Stirling, 2003).

The main objective of this study was assessing the genetic variability of 57 Persian walnut accessions by SSR markers. The results confirmed the utility of 19 SSRs, previously developed in *J. nigra* (Woeste *et al*., 2002), for the characterization of *J. regia* varieties. This subset of SSR markers represent a powerful tool for future breeding programs involving the walnut collection included in this study and useful for programs aimed at the conservation of walnut genetic resources. Also, allelic data are presented for use as size standards, to assist in correcting laboratory-to-laboratory variation of allele size calling that may result from differences in methodologies. The differences found between the resulting data and those of previous studies are discussed.

Material and methods

Plant material

Plant material was obtained from the walnut collection at the *Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA),* in Murcia, Spain. The 57 *Juglans regia* cultivars included in this study, together with their pedigree, place of origin, and observed genetic heterozygosity (Ho, calculated as the number of heterozygous loci for a given cultivar divided by the total number of loci assayed) are listed in Table 1. Forty-four of these accessions come from Spain, 11 from the USA, 1 from France, and 1 from Chile.

DNA extraction and PCR amplification

Genomic DNA was isolated from young fresh leaves using the DNeasy Plant Mini Kit (Qiagen). The DNA extracted was quantified using a spectrophotometer, and diluted to 10 ng μL^{-1} to carry out PCR amplifications. Thirty-two primer pairs flanking microsatellites, previously developed in *J. nigra*, were assayed in 12 genetically-diverse walnut genotypes. Nineteen of these showed a high polymorphism and repeatable and clear amplification patterns, according to the detection protocol described below, and were selected for the molecular analysis of each accession (Table 2). The PCR reactions were performed in a 20-µL volume and the reaction mixture contained 1x PCR buffer (Ecogen, Barcelona, Spain), 1.9 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.25 units of *Taq* DNA polymerase (Ecogen), and 10 ng of genomic DNA. A touchdown PCR amplification protocol was programmed (Don *et al*., 1991), consisting of an initial step of 5 min at 94°C, 35 cycles of 45 sec at 94°C, 45 sec at the annealing temperature for the primer, and 45 sec at 72°C, followed by a final step of 10 min at 72°C. The annealing temperature (Ta°C) was 58 or 60°C for the first cycle and was reduced by 0.2°C per cycle for the next 14 cycles. For the last 20 cycles, the annealing temperature was 55 or 57°C (Ta-3°C), respectively (Table 2). The PCR reactions were carried out in a 96-well block Thermal cycler (Eppendorf, Barcelona, Spain). The PCR products were detected using the ABI3730 Genetic Analyzer and the GeneMapper analysis software (Applied Biosystems). For capillary electrophoresis detection, forward SSR primers were labeled with the 5'-fluorescence dyes NED (yellow), 6-FAM (blue), VIC (green), and PET (red). The size standard used in the sequencer was GS500LIZ (Applied Biosystems). Each reaction was repeated and analyzed twice for confirmation.

Data analysis

The following variability parameters were estimated from the microsatellite marker data obtained (Table 3): number of alleles per locus (Na); effective number of alleles per locus ($Ne = 1/\Sigma p_i^2$, where p_i is the frequency of the *i*th allele); observed heterozygosity (Ho, calculated as the number of heterozygous genotypes divided by the total number of genotypes); expected heterozygosity ($He = 1 - \Sigma p_i^2$, where *pi* is the frequency of the i^{th} allele); fixation index ($F = 1 - Ho/He$) (Wright, 1950); and the power of discrimination ($PD = 1 - \Sigma g_i^2$, where *gi* is the frequency of the *i*th genotype) (Kloosterman *et al*., 1993). These analyses were computed with the GeneAlEx V6 program (Peakall and Smouse, 2006). CERVUS software 3.0 (Kalinowski *et al.,* 2007) was used for computing the proportion of null alleles and the significant deviations ($p < 0.01$ and $p < 0.001$) from Hardy-Weinberg equilibrium (HWE) at individual loci. The sequential Bonferroni test was used to compute the critical significance for HWE test (Rice, 1989).

Table 1. The 57 *Juglans regia* cultivars included in this study and maintained at the IMIDA collection

Cultivar	Pedigree[a]	Origin	Ho[b]
Algaida	Unknown	Spain (Murcia)	0.42
Amigo	Sharkey × Marchetti[a]	USA (California)	0.58
Arriba	Unknown	Spain (Albacete, Nerpio)	0.63
As1	Unknown	Spain (Asturias, Colunga)	0.63
As12	Unknown	Spain (Asturias, Caso)	0.68
As15	Unknown	Spain (Asturias, Boal)	0.32
As16	Unknown	Spain (Asturias, Villanueva de Oscos)	0.68
As17	Unknown	Spain (Asturias, Sta. Eulalia de Oscos)	0.58
As18	Unknown	Spain (Asturias, Taramundi)	0.32
As19	Unknown	Spain (Asturias, Taramundi)	0.32
As2	Unknown	Spain (Asturias, Parres)	0.68
As22	Unknown	Spain (Asturias, Tineo)	0.74
As23	Unknown	Spain (Asturias, Tineo)	0.47
As24	Unknown	Spain (Asturias, Tineo)	0.74
As25	Unknown	Spain (Asturias, Salas)	0.68
As26	Unknown	Spain (Asturias, Llanes)	0.63
As8	Unknown	Spain (Asturias, C. Onis)	0.47
As9	Unknown	Spain (Asturias, C. Onis)	0.58
AsA	Unknown	Spain (Asturias, Llanes)	0.63
AsB	Unknown	Spain (Asturias, Sariego)	0.58
AsC	Unknown	Spain (Asturias, Gijón)	0.68
Callao	Unknown	Spain (Albacete)	0.58
Carcagente	Unknown	Spain (Valencia)	0.47
Carril	Unknown	Spain (Albacete, Nerpio)	0.47
Casa de Alfaro	Unknown	Spain (Albacete, Nerpio)	0.47
Chandler	Pedro × UC56-224[a]	USA (California)	0.53
Chico	Sharkey × Marchetti[a]	USA (California)	0.58
Eureka	Unknown	USA (California)	0.53
Franquette	Unknown	France	0.53
Garganteña seedling	Unknown	Spain (Cáceres, Garganta La Olla)	0.47
Gran Jefe	Unknown	Spain (Albacete, Nerpio)	0.47
Hartley	Franquette × Mayette[a]	USA (California)	0.42
Howard	Pedro × UC56-224[a]	USA (California)	0.47
Hoya	Unknown	Spain (Cantabria)	0.58
Isa	Unknown	Spain (Cantabria)	0.53
Isidoro	Unknown	Spain (Albacete, Nerpio)	0.42
Ladredo	Unknown	Spain (Cantabria)	0.58
Mieses	Unknown	Spain (Cantabria)	0.58
Mollarde Germán	Unknown	Spain (Albacete, Nerpio)	0.42
Payne	Unknown	USA (California)	0.37
Pedro	Conway Mayette × Payne[a]	USA (California)	0.37
Pico pájaro	Unknown	Spain (Cantabria)	0.42
Pirque	Unknown	Chile	0.47
Rubio Nerpio	Unknown	Spain (Albacete, Nerpio)	0.42
Salvador	Unknown	Spain, Málaga (Ronda)	0.32
Santa Cruz	Unknown	Spain (Cantabria)	0.11
Sendra	Unknown	Spain (Valencia)	0.47
Serr	Payne × PI-159568[a]	USA (California)	0.79
Sunland	Lompoc × PI 159568[a]	USA (California)	0.95
Taibilla	Unknown	Spain (Albacete, Nerpio)	0.32
Tobilla	Unknown	Spain (Albacete, Nerpio)	0.53
Trinta	Unknown	USA (California)	0.47
VZ-1	Unknown	Spain (Granada, Venta Zafarraya)	0.42
VZ-2	Unknown	Spain (Granada, Venta Zafarraya)	0.68
VZ-3	Unknown	Spain (Granada, Venta Zafarraya)	0.47
VZ-4	Unknown	Spain (Granada, Venta Zafarraya)	0.47
VZ-6	Unknown	Spain (Granada, Venta Zafarraya)	0.42

[a] Pedigree data obtained from Tulecke and McGranahan (1994). [b] Ho: observed genetic heterozygosity.

Table 2. Characteristics of the 19 microsatellite markers assayed

Locus	References	Ta[a] (°C)	Ta-3[b] (°C)	Size range (bp)	Flurophore[c]
WGA001	Dangl *et al.* (2005); Foroni *et al*. (2006)	58	55	180-192	6-FAM
WGA004	Woeste *et al.* (2002); Dangl *et al*. (2005); Foroni *et al*. (2006)	58	55	226-238	NED
WGA005	Foroni *et al.* (2006)	60	57	240-252	VIC
WGA009	Dangl *et al*. (2005); Foroni *et al*. (2006)	58	55	231-245	6-FAM
WGA032	Foroni *et al*. (2006); Victory *et al*. (2006)	58	55	166-198	VIC
WGA054	Woeste *et al*. (2002)	58	55	105-125	VIC
WGA069	Foroni *et al*. (2006); Victory *et al*. (2006)	58	55	160-182	VIC
WGA072	Victory *et al*. (2006)	58	55	138-146	NED
WGA079	Robichaud *et al*. (2006)	58	55	196-208	6-FAM
WGA089	Foroni *et al*. (2006); Victory *et al*. (2006)	58	55	212-222	NED
WGA118	Dangl *et al*. (2005); Foroni *et al*. (2006)	58	55	186-200	VIC
WGA202	Dangl *et al*. (2005); Foroni *et al*. (2006)	58	55	259-295	6-FAM
WGA225	Dangl *et al*. (2005)	58	55	191-203	PET
WGA276	Foroni *et al*. (2006)	58	55	168-194	NED
WGA321	Foroni *et al*. (2006)	58	55	223-245	VIC
WGA331	Dangl *et al*. (2005)	58	55	272-276	6-FAM
WGA332	Dangl *et al*. (2005)	58	55	217-228	PET
WGA349	Dangl *et al*. (2005)	58	55	262-274	NED
WGA376	Dangl *et al*. (2005)	60	57	243-253	VIC

[a] Ta: annealing temperature for the first cycle of the PCR. See Material and methods. [b] Ta-3: annealing temperature for the last 20 cycles of the PCR. See Material and methods. [c] Forward primers were modified at the 5′ end with a fluorescent label: 6-FAM (blue), NED (yellow), VIC (green) or PET (red).

Table 3. Variability parameters calculated for 19 SSR markers in 57 walnut cultivars

Locus	Na	Ne	Ho	He	F	F(Null)	HWE	PD	Genotypes
WGA001	5	2.246	0.456	0.555	0.178	0.086	NS	0.74	8
WGA004	4	2.063	0.421	0.515	0.183	0.095	NS	0.69	6
WGA005	7	3.068	0.737	0.674	–0.093	–0.059	NS	0.79	13
WGA009	4	2.958	0.561	0.662	0.152	0.084	NS	0.83	7
WGA032	10	1.891	0.421	0.471	0.106	0.056	ND	0.69	12
WGA054	7	2.445	0.526	0.591	0.109	0.062	NS	0.78	10
WGA069	6	3.602	0.544	0.722	0.247	0.149	**	0.86	11
WGA072	4	1.999	0.386	0.500	0.228	0.114	NS	0.66	6
WGA079	3	1.256	0.228	0.204	–0.118	–0.054	ND	0.36	3
WGA089	5	2.503	0.491	0.600	0.182	0.112	*	0.78	10
WGA118	3	2.493	0.561	0.599	0.062	0.033	NS	0.76	6
WGA202	6	3.850	0.614	0.740	0.170	0.096	NS	0.89	12
WGA225	3	2.427	0.526	0.588	0.105	0.062	NS	0.77	5
WGA276	9	3.730	0.649	0.732	0.113	0.066	NS	0.90	17
WGA321	6	3.241	0.719	0.691	–0.040	–0.023	NS	0.83	12
WGA331	3	2.045	0.491	0.511	0.039	0.019	NS	0.67	5
WGA332	4	2.243	0.579	0.554	–0.045	–0.033	NS	0.75	7
WGA349	5	2.849	0.439	0.649	0.324	0.196	*	0.81	9
WGA376	3	1.757	0.474	0.431	–0.100	–0.050	NS	0.58	4
Total	97								
Average/per locus	5	2.561	0.517	0.578	0.095	0.053		0.74	9

Na: allele number per locus. Ne: effective number of alleles per locus. Ho: observed genetic heterozygosity. He: expected genetic heterozygosity. F: fixation index. F(Null): frequency of alleles null. HWE: probability test for departure from Hardy-Weinberg equilibrium. ND: not done. NS: not significant. *Signicant at $p < 0.01$, ** Significant at $p < 0.001$. PD: power of discrimination.

The proportion of shared alleles, as described by Bowcock *et al*. (1994), was used to calculate a genetic distance between all pairwise combinations of the 57 walnut cultivars studied, using the program Populations 1.2.28 (http://www.cnrs.gif.fr/pge) (Langella, 1999). This statistic is a measure of the dissimilarity between two samples; thus, the distance between two individuals that are identical at all loci tested is equal to zero. A dendrogram, based on shared allele genetic distance was constructed using the unweighted pair group method average (UPGMA) method implemented in Molecular Evolutionary Genetics Analysis (MEGA) Program v. 2.1 (Kumar *et al*., 1994).

Results

Polymorphism and heterozygosity of SSR markers

Nineteen polymorphic SSR primer pairs, developed for *J. nigra* (Table 2), were tested in 57 accessions of *J. regia* from the IMIDA collection (Table 1). Alleles were differentiated clearly using a capillary electrophoresis sequencer and no discrepancies were found in the banding patterns of the duplicate analyses of each DNA sample. These primer pairs had different levels of amplified bands, the sizes of which ranged from 105 bp at locus WGA054 to 295 bp at locus WGA202 (Table 2). All primer pairs produced a maximum of two bands per genotype, in accordance with the diploid level of this species. Genotypes showing a single amplified fragment were considered as homozygous for that particular locus, since segregation analysis is needed to detect the presence of putative null alleles (Callen *et al*., 1993). The number of alleles observed (Na) at each locus ranged from 3 (WGA079, WGA118, WGA225, WGA331, and WGA376) to 10 (WGA032) with an average of 5 alleles per locus (Table 3), much higher than the values of 1.3 and 3.9 detected in *J. regia* with RAPDs (Nicese *et al*., 1998), and ISSRs (Potter *et al*., 2002) respectively. Altogether, 97 alleles were identified in the set of accessions. In all samples, the effective number of alleles was lower than the observed and varied from 1.256 for WGA079 to 3.850 for WGA202 (Table 3). The observed heterozygosity ranged from 0.228 for WGA079 to 0.737 for WGA005, with a mean of 0.517, and was below than the expected heterozygosity in 14 loci out of 19 (Table 3). Consequently, the fixation index (F) values, used to estimate the degree of allelic fixation, were positive and close to zero for all the loci studied except for WGA005, WGA079, WGA321, WGA332, and WGA376, with an overall mean of 0.095. WGA069, WGA072, WGA089 and WGA349 loci had the highest estimated frequency of null alleles (Table 3). Significant departure over Hardy-Weinberg equilibrium appeared only in WGA069 ($p < 0.001$), WGA089 ($p < 0.01$) and WGA349 ($p < 0.01$) loci due to a deficiency of heterozygotes (Table 3). None of these deviations remained significant after applying Bonferroni correction (data not shown).

Finally, the ability of a marker to discriminate between two random cultivars (PD) varied from 0.36 in WGA079 to 0.90 in WGA276, with an average value of 0.74. The number of genotypes detected at each locus ranged from 3 (WGA079) to 17 (WGA276), with an average of nine genotypes per locus (Table 3). The size differences detected between alleles at a locus ranged from 2 to 36 bp (Table 4). Differences between consecutive alleles ranged from 2 to 18 bp, being 2 bp in 63% of cases. Among the 97 alleles detected, there were 40 (41%) at 16 loci that showed frequencies lower than 0.05 (Table 4), whereas only nine alleles (9%) at nine loci showed frequencies higher than 0.50 (WGA001-192, WGA004-230, WGA032-196, WGA054-109, WGA072-140, WGA079-206, WGA331-276, WGA332-223, and WGA376-253) (Table 4).

Genetic diversity within the IMIDA walnut collection

The genetic heterozygosities of the walnut cultivars studied ranged between 0.11 'Santa Cruz' and 0.95 'Sunland', with an average value of 0.52 (Table 1). The allelic data for the 57 accessions studied are presented in Table 5. Fourteen private alleles were found at eight loci (WGA001-188 in 'Amigo'; WGA005-248 in 'Chico'; WGA032-184 in 'As16', WGA032-186 in 'VZ3', WGA032-188 in 'Carcagente' and WGA032-194 in 'Salvador'; WGA054-105 in 'Garganteña seedling', WGA054-119 in 'As26' and WGA054-125 in 'AsB'; WGA069-180 in 'Serr'; WGA079-196 in 'Sunland'; WGA202-277 in 'Isa' and WGA202-295 in 'VZ1'; WGA376-243 in 'Mollar de Germán') and so these loci could be very useful for genotype identification (Table 5). The 4-primers combination WGA069, WGA202, WGA276, and WGA321 allowed the unambiguous differentiation of all the cultivars studied (Table 5).

Table 4. Allele size (AS) in base pairs and allele frequencies (AF) at nuclear SSR loci

Locus	AS	AF	Locus	AS	AF	Locus	AS	AF	Locus	AS	AF
WGA001	180	0.175	WGA032	**188**	0.009	WGA089	212	0.079	WGA276	194	0.070
	182	0.009		192	0.018		216	0.377	WGA321	223	0.018
	188	0.009		**194**	0.009		218	0.026		225	0.351
	190	0.193		196	0.711		220	0.500		237	0.132
	192	0.614		198	0.053		222	0.018		241	0.404
WGA004	226	0.026	WGA054	**105**	0.009	WGA118	186	0.123		243	0.035
	228	0.342		109	0.518		198	0.465		245	0.061
	230	0.605		111	0.018		200	0.412	WGA331	272	0.377
	238	0.026		113	0.070	WGA202	259	0.132		274	0.035
WGA005	240	0.447		**119**	0.009		263	0.281		276	0.588
	242	0.333		121	0.368		265	0.263	WGA332	217	0.202
	244	0.018		**125**	0.009		275	0.307		223	0.614
	246	0.035	WGA069	160	0.035		**277**	0.009		226	0.167
	248	0.009		162	0.395		**295**	0.009		228	0.018
	250	0.061		164	0.088	WGA225	191	0.465	WGA349	262	0.316
	252	0.096		178	0.254		197	0.430		266	0.009
WGA009	231	0.018		**180**	0.009		203	0.105		270	0.158
	237	0.289		182	0.219	WGA276	168	0.009		272	0.044
	241	0.430	WGA072	138	0.316		174	0.026		274	0.474
	245	0.263		140	0.632		176	0.307	WGA376	**243**	0.009
WGA032	166	0.026		142	0.018		178	0.035		245	0.298
	168	0.018		146	0.035		180	0.132		253	0.693
	170	0.140	WGA079	**196**	0.009		182	0.018			
	184	0.009		206	0.886		190	0.018			
	186	0.009		208	0.105		192	0.386			

Private alleles found in accessions are in bold.

Genetic relationships and clustering of genotypes

Most of the cultivars studied in this work have not been subjected previously to molecular characterization. The 57 accessions showed different two-way similarity coefficient values, ranging between 0.18 and 0.76. The highest similarity value (0.76) was observed between 'Pedro' and 'Chandler', 'Pedro' and 'Payne', 'Trinta' and 'Eureka', and 'Trinta' and 'Payne', and the lowest (0.18) was between 'Santa Cruz' and 'Chico'. Among all the genotypes tested, 'Sunland' showed a similarity value of 0.50 or more only with 3 cultivars (3%), 'Chico' with 8 cultivars (14%), and 'Amigo' with 9 cultivars (16%). On the other hand, 'As1' and 'As12' showed a similarity value of 0.50 or more with 47 cultivars (84%), 'Pirque' with 45 cultivars (80%), and 'Eureka' with 42 cultivars (75%).

The UPGMA cluster classified 57 genotypes into two main clusters that generally agree with their geographic origins and pedigree (Fig. 1). The first cluster, from 'As1' to 'Salvador', includes most of the Spanish cultivars studied and the second one, from 'Serr' to 'Trinta', consists mainly of Californian cultivars. In the first cluster, five discrete sub-clusters can be defined, and for some of these subgroups a close relationship with their geographical origin can be established. Thus, the first subgroup, from 'As1' to 'Callao', consists mostly of cultivars from Northern Spain (Asturias and Cantabria regions), but also two cultivars from Southern and Southeastern Spain ('VZ4' from Granada and 'Callao' from Albacete). This subgroup includes also a foreign cultivar from Chile, 'Pirque'. The second subgroup, from 'Garganteña seedling' to 'Tobilla', comprises cultivars from Southeastern Spain (Albacete and Valencia provinces), but also from Cáceres (1), Asturias (2), and Granada (1). The third subgroup is the smallest and includes only 'As18' and 'Santa Cruz' cultivars from Northern Spain (Asturias and Cantabria regions). The fourth subgroup, from 'VZ3' to 'Hartley', is the most diverse and includes the foreign cultivars 'Franquette' (France) and 'Hartley' (California) together

Table 5. Allelic profiles (bp) at 19 SSR loci for 57 walnut cultivars

Cultivar	WGA001		WGA004		WGA005		WGA009		WGA032		WGA054		WGA069		WGA072		WGA079	
Algaida	190	192	226	226	240	244	241	245	170	196	109	109	182	182	138	138	206	206
Amigo	**188**	192	228	230	246	250	231	241	196	196	109	109	160	182	140	140	206	208
Arriba	190	192	228	230	240	252	237	245	196	196	109	113	162	162	138	140	206	206
As1	192	192	228	230	240	242	237	241	196	196	109	109	162	164	138	140	206	206
As12	192	192	228	230	240	242	241	245	196	196	109	121	178	182	138	140	206	206
As15	192	192	228	228	242	242	241	241	196	196	109	121	178	178	140	140	206	206
As16	192	192	226	228	240	242	241	245	**184**	196	109	109	178	182	138	140	206	208
As17	190	192	228	230	242	252	237	245	196	196	121	121	162	162	138	140	206	206
As18	192	192	228	228	240	242	245	245	170	196	121	121	178	182	140	140	206	206
As19	192	192	228	228	240	242	241	241	170	196	109	109	162	178	140	140	206	206
As2	190	192	228	230	250	250	241	245	196	198	109	121	164	182	138	140	206	206
As22	180	180	228	230	240	250	241	245	170	196	113	121	162	164	140	140	206	208
As23	192	192	228	228	240	242	241	241	196	196	121	121	178	182	140	140	206	206
As24	192	192	228	230	240	240	237	245	170	196	109	121	162	178	138	140	206	206
As25	190	192	230	230	242	252	241	241	192	196	121	121	164	182	138	140	206	206
As26	180	192	230	230	240	246	241	245	170	198	**119**	121	178	182	138	138	206	206
As8	180	192	230	230	240	242	245	245	196	198	109	121	178	178	140	140	206	206
As9	190	192	228	230	240	242	245	245	196	196	109	109	162	178	138	140	206	206
AsA	180	180	230	230	240	242	237	245	196	196	109	121	162	178	138	140	206	206
AsB	180	192	228	228	240	242	241	241	196	196	109	**125**	164	182	138	140	206	206
AsC	180	180	228	230	240	252	237	241	196	198	121	121	162	178	138	138	206	206
Callao	192	192	230	230	240	242	241	245	196	196	113	121	162	178	138	140	206	206
Carcagente	190	192	230	230	242	242	237	241	**188**	196	113	121	162	162	140	140	206	208
Carril	190	192	230	230	240	242	237	237	196	196	113	121	162	162	138	138	206	206
Casa de Alfaro	190	190	230	230	240	242	237	237	196	196	109	109	162	164	140	140	206	206
Chandler	192	192	230	230	240	240	231	241	170	196	109	109	162	162	140	140	206	206
Chico	190	192	228	230	246	**248**	241	241	170	196	109	109	160	160	140	140	206	208
Eureka	192	192	228	228	240	250	241	241	170	196	109	109	162	182	140	140	206	206
Franquette	190	190	230	230	240	252	237	241	170	170	109	121	162	178	140	140	206	208
Garganteña seedling	192	192	228	230	240	240	237	245	196	198	**105**	121	178	178	138	138	206	206
Gran Jefe	190	192	230	230	240	242	237	245	196	196	109	121	162	162	140	140	206	206
Hartley	190	190	230	230	240	242	237	241	196	196	121	121	162	162	138	140	206	208
Howard	192	192	228	230	240	240	241	241	196	196	109	109	162	162	140	140	206	208
Hoya	192	192	230	230	240	250	237	241	196	196	121	121	178	182	140	140	206	208
Isa	180	192	228	228	240	240	237	241	192	196	109	121	178	178	138	140	206	206
Isidoro	192	192	230	230	240	242	237	241	170	198	109	121	162	178	140	140	206	206
Ladredo	180	180	230	238	240	242	241	245	196	196	109	109	164	182	140	146	206	206
Mieses	180	190	228	228	240	244	237	241	196	196	109	121	162	162	138	140	206	206
Mollar de Germán	192	192	230	230	240	240	237	237	196	196	109	121	162	162	138	138	206	206
Payne	192	192	228	230	240	242	241	241	196	196	109	121	182	182	140	140	206	208
Pedro	192	192	230	230	240	242	241	241	196	196	109	109	162	182	140	140	206	206
Pico Pájaro	180	192	230	230	242	252	237	245	196	196	109	109	162	162	140	140	206	206
Pirque	180	192	230	230	240	242	241	245	196	196	109	121	162	178	140	140	206	206
Rubio Nerpio	190	192	228	230	242	242	237	245	196	196	109	121	178	178	138	138	206	206
Salvador	192	192	228	230	240	240	245	245	**194**	196	109	109	164	182	138	138	206	206
Santa Cruz	192	192	230	230	242	242	237	245	168	168	121	121	162	182	140	140	206	206
Sendra	180	192	230	230	240	246	241	241	166	196	109	109	182	182	142	142	206	208
Serr	180	192	230	238	240	242	237	241	166	196	109	111	**180**	182	146	146	206	206
Sunland	182	190	230	238	242	242	237	241	166	196	109	111	160	182	140	146	**196**	206
Taibilla	192	192	230	230	252	252	237	237	196	196	113	113	162	164	138	140	206	206
Tobilla	190	192	228	230	240	252	237	237	196	196	109	121	162	162	138	140	206	206
Trinta	192	192	228	230	242	250	241	241	196	196	109	121	182	182	140	140	206	206
VZ1	190	192	228	228	240	242	245	245	170	170	109	109	162	162	138	140	206	206
VZ2	190	192	228	230	242	252	237	241	196	196	113	121	162	164	140	140	206	206
VZ3	180	192	230	230	240	252	237	237	170	**186**	109	121	162	162	138	140	206	206
VZ4	192	192	228	230	240	242	241	241	196	196	109	109	178	178	140	140	206	206
VZ6	180	180	230	230	240	240	245	245	170	196	109	121	178	178	138	140	206	208

Table 5 (cont.). Allelic profiles (bp) at 19 SSR loci for 57 walnut cultivars

Cultivar	WGA089		WGA118		WGA202		WGA225		WGA276		WGA321		WGA331		WGA332		WGA349		WGA376	
Algaida	216	216	200	200	259	263	197	197	178	192	241	241	272	276	223	223	262	262	245	253
Amigo	212	216	200	200	259	259	197	197	180	194	225	237	276	276	226	226	270	274	245	253
Arriba	216	220	186	198	259	275	191	197	176	178	225	241	276	276	223	223	272	272	245	245
As1	212	220	198	200	265	275	197	197	176	192	225	241	272	276	223	223	262	274	253	253
As12	212	216	198	198	263	265	191	197	176	190	225	225	272	276	217	223	262	274	253	253
As15	220	220	198	198	265	265	191	197	176	192	241	241	272	276	217	223	262	262	245	253
As16	216	220	198	200	265	265	191	197	176	182	241	241	272	276	217	223	262	262	253	253
As17	212	220	198	200	263	275	191	197	192	192	225	225	272	272	223	226	274	274	245	253
As18	220	220	200	200	263	263	191	191	176	190	225	225	272	276	223	226	262	262	253	253
As19	216	222	198	198	263	263	191	191	176	194	225	225	272	272	223	226	262	262	253	253
As2	220	220	198	200	275	275	191	203	176	176	225	237	272	276	223	223	262	270	253	253
As22	220	220	186	198	263	265	191	191	176	194	225	237	276	276	223	226	270	274	245	253
As23	216	216	198	200	263	263	191	197	174	176	225	241	272	276	223	223	270	274	245	253
As24	212	222	198	198	275	275	191	197	176	192	225	241	272	276	223	226	262	262	245	253
As25	216	220	198	200	263	275	191	197	182	192	225	225	272	276	217	223	270	274	253	253
As26	220	220	198	198	263	275	191	197	180	192	237	241	276	276	223	226	270	274	253	253
As8	220	220	198	198	265	275	191	197	176	180	241	243	276	276	217	223	262	262	253	253
As9	216	220	198	198	263	275	191	203	176	192	225	241	272	272	223	223	262	274	253	253
AsA	216	220	198	200	275	275	191	197	178	192	225	241	276	276	223	226	274	274	245	253
AsB	212	220	198	198	263	265	191	197	176	192	225	243	272	272	223	223	262	274	253	253
AsC	220	220	198	200	265	275	191	197	176	192	225	241	276	276	223	226	262	270	245	253
Callao	220	220	198	200	275	275	197	203	176	180	241	243	276	276	223	223	262	270	245	253
Carcagente	220	220	198	198	263	275	191	197	176	176	225	241	272	272	223	223	274	274	245	253
Carril	216	220	198	198	259	275	191	191	176	194	225	241	276	276	223	223	272	274	245	253
Casa de Alfaro	216	220	186	198	263	265	191	191	176	180	225	225	276	276	217	223	272	274	245	253
Chandler	216	216	186	200	259	265	197	203	192	194	241	245	272	274	223	228	274	274	245	253
Chico	216	216	186	198	259	275	197	197	192	194	241	245	274	276	226	226	274	274	245	253
Eureka	216	220	198	200	265	265	191	197	180	192	241	245	276	276	223	226	274	274	245	253
Franquette	216	220	200	200	263	265	191	203	192	192	241	241	272	276	223	223	270	270	245	253
Garganteña seedl.	220	220	198	200	265	275	197	197	176	176	241	241	272	276	217	223	274	274	245	253
Gran Jefe	220	220	186	200	259	263	191	191	176	176	225	241	276	276	217	223	274	274	245	253
Hartley	216	216	200	200	259	265	197	197	180	192	225	241	272	272	223	223	270	270	245	253
Howard	216	220	198	200	259	263	197	197	192	194	225	245	274	276	223	228	274	274	253	253
Hoya	212	220	200	200	263	263	191	197	176	192	241	243	272	276	223	226	262	274	253	253
Isa	220	220	186	198	275	**277**	191	191	176	192	241	241	276	276	217	223	270	274	253	253
Isidoro	216	216	198	200	263	275	191	191	176	176	237	241	272	272	223	223	274	274	253	253
Ladredo	218	220	198	200	259	275	197	197	192	192	237	241	272	272	217	223	262	274	253	253
Mieses	220	220	200	200	275	275	191	203	178	192	225	241	272	276	217	226	262	270	253	253
Mollar de Germán	216	220	198	200	275	275	191	197	176	180	225	225	272	276	217	217	262	274	**243**	253
Payne	216	216	200	200	265	265	197	197	192	192	237	245	276	276	223	223	270	274	245	253
Pedro	216	216	200	200	263	265	197	203	192	192	241	245	272	276	223	223	274	274	245	253
Pico Pájaro	220	220	200	200	263	265	191	203	192	192	225	241	276	276	223	223	270	274	253	253
Pirque	220	220	198	198	265	265	191	191	192	192	237	241	272	276	223	226	274	274	245	253
Rubio Nerpio	216	220	198	198	275	275	191	191	180	194	241	241	272	276	217	223	274	274	245	245
Salvador	216	216	198	200	259	275	197	197	192	192	225	241	276	276	223	223	262	262	253	253
Santa Cruz	220	220	200	200	263	263	191	191	180	180	225	225	276	276	223	223	262	262	253	253
Sendra	216	216	186	186	265	275	197	197	174	192	237	245	272	276	217	226	274	274	253	253
Serr	216	218	186	200	259	265	197	197	174	192	223	237	272	276	217	223	274	274	245	253
Sunland	212	218	186	198	259	265	197	203	168	192	223	237	274	276	217	226	266	274	245	253
Taibilla	220	220	186	198	275	275	191	191	176	176	225	241	272	276	217	223	274	274	253	253
Tobilla	216	220	186	200	263	263	191	191	176	176	225	241	272	276	223	223	272	274	253	253
Trinta	216	220	198	200	265	265	191	197	180	192	237	237	276	276	223	226	270	274	245	245
VZ1	216	220	198	200	275	**295**	191	203	180	180	225	241	276	276	223	223	262	262	253	253
VZ2	216	220	186	200	265	275	191	191	176	180	225	241	272	276	217	223	274	274	245	245
VZ3	220	220	198	200	263	265	197	197	192	192	237	241	272	272	217	217	262	262	245	253
VZ4	212	216	198	200	263	263	197	203	192	192	237	241	272	276	217	223	262	270	253	253
VZ6	216	216	198	198	259	263	191	203	192	192	225	241	276	276	217	223	262	262	253	253

Private alleles found in accessions are in bold.

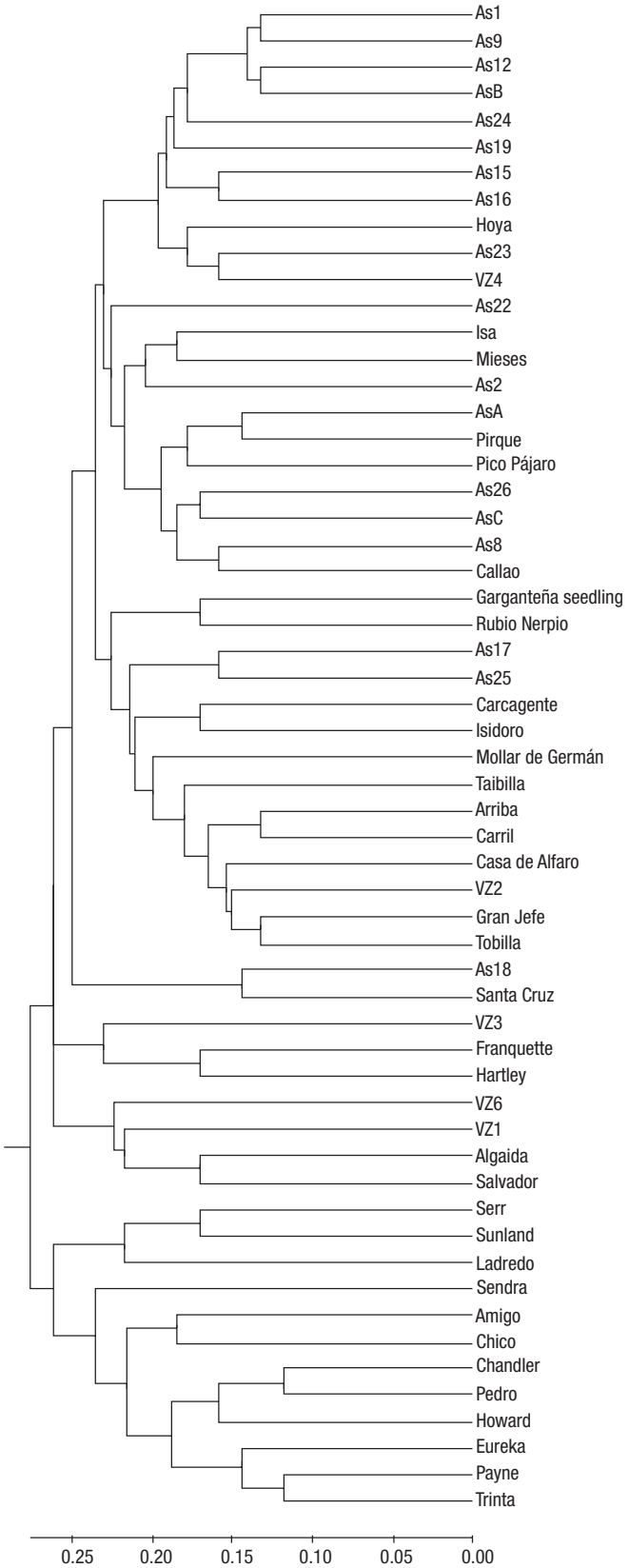

Figure 1. UPGMA dendrogram of 57 walnut cultivars based on their variation at 19 SSR loci. Drawn with MEGA program v.2.1.

with the Spanish cultivar 'VZ2' from Granada. Finally, the fifth subgroup, from 'VZ6' to 'Salvador', includes cultivars from three different provinces of Spain: Granada (2) and Málaga (1) in Southern Spain, and Murcia (1) in Southeastern Spain. The second cluster, from 'Serr' to 'Trinta', includes most of the Californian cultivars studied, but also includes two Spanish cultivars 'Ladredo' and 'Sendra'.

Discussion

In this work the genetic variability of 57 Persian walnut accessions was assessed by SSR markers. The results confirm that *J. nigra* SSR markers can be used to identify the level of genetic variability in *J. regia*, in accordance with previous studies (Dangl *et al*., 2005; Foroni *et al*., 2005, 2006; Wang *et al*., 2008; Pollegioni *et al*., 2009; Gunn *et al*., 2010). All the cultivars analyzed had a unique SSR fingerprint, which confirms the high efficiency of these markers. Furthermore, the average number of alleles detected per locus (5) and the mean power of discrimination obtained (0.74) confirm that SSRs are a more suitable tool for walnut identification than other molecular marker systems studied previously (Nicese *et al*., 1998; Potter *et al*., 2002). In all samples, the effective number of alleles was lower than the observed. These differences may be due to the presence of private alleles that exist in a few genotypes, which could be used for their identification. Observed heterozygosity was lower than expected for 14 loci out of 19 and therefore the fixation index (F) values were positive and close to zero for most of the loci studied, suggesting that the global behavior of the walnut genotypes studied was similar to that of a random mating population. In addition, the proportion of null alleles was estimated, and they were found to occur at a moderate frequency, with the mean value equaled 0.053. WGA069 and WGA349 loci had the highest estimated frequency of null alleles, 0.149 and 0.196 respectively, consistent with the high values reported by Dangl *et al.* (2005) for these loci. Significant departure over Hardy-Weinberg equilibrium appeared only in WGA069 ($p < 0.001$), WGA089 ($p < 0.01$) and WGA349 ($p < 0.01$) loci due to a deficiency of heterozygotes. However, none of these deviations remained significant after applying Bonferroni correction.

The observed genetic heterozygosity of the walnut collection studied was moderate, with an average value of 0.52. The presence of private alleles in some cultivars could be due to a mutation in the microsatellite sequence that would give rise to longer o shorter new alleles. 'Santa Cruz' was homozygous at 17 of the 19 loci, suggesting that this genotype could be the product of a self-pollination. The comparison of the allele sizes (in base pairs) performed by Dangl *et al*. (2005) for six varieties studied in common ('Chandler', 'Franquette', 'Howard', 'Payne', 'Serr', and 'Sunland'), with 14 of the 19 SSRs used in this study (Table 6), detected minor changes that may have resulted from differences in methodology. For five SSRs (WGA001, WGA004, WGA089, WGA202, and WGA349), the data obtained

Table 6. Comparison of the allele sizes (in base pairs) obtained in the current work and by Dangl *et al.* (2005) for 6 cultivars and 14 SSR

	WGA001		WGA004		WGA009		WGA069		WGA089		WGA118		WGA202		WGA225		WGA276		WGA231		WGA331		WGA332		WGA349		WGA376	
Current work																												
Chandler	192	192	230	230	231	241	162	162	216	216	186	200	259	265	197	203	192	194	241	245	272	274	223	228	274	274	245	253
Franquette	190	190	230	230	237	241	162	178	216	220	200	200	263	265	191	203	192	192	241	241	272	276	223	223	270	270	245	253
Howard	192	192	228	230	241	241	162	162	216	220	198	200	259	263	197	197	192	194	225	245	274	276	223	228	274	274	253	253
Payne	192	192	228	230	241	241	182	182	216	216	200	200	265	265	197	197	192	192	237	245	276	276	223	223	270	274	245	253
Serr	180	192	230	238	237	241	180	182	216	218	186	200	259	265	197	197	174	192	223	237	272	276	217	223	274	274	245	253
Sunland	182	190	230	238	237	241	160	182	212	218	186	198	259	265	197	203	168	192	223	237	274	276	217	226	266	274	245	253
Dangl *et al.* (2005)																												
Chandler	192	192	230	230	231	242	160	160	216	216	184	198	259	265	196	202	190	192	241	245	273	275	220	225	274	274	246	254
Franquette	190	190	230	230	238	242	160	176	216	220	198	198	263	265	190	202	190	190	241	241	273	277	220	220	270	270	246	254
Howard	192	192	228	230	242	242	160	160	216	220	196	198	259	265	196	196	190	192	245	245	275	277	220	225	274	274	254	254
Payne	192	192	228	230	242	242	180	180	216	216	198	198	265	265	196	196	190	190	237	245	277	277	220	220	270	274	246	254
Serr	180	192	230	238	238	242	178	180	216	218	184	198	259	265	196	196	172	190	222	237	273	277	214	220	274	274	246	254
Sunland	182	190	230	238	238	242	158	180	212	218	184	196	259	265	196	202	165	190	222	237	275	277	214	223	266	274	246	254

in the two studies were completely identical. Also, eight SSRs displayed alleles that were either smaller (*e.g.* 1-bp differences for WGA225; 2-bp for WGA069, WGA118, and WGA276; 3-bp for WGA332) or larger (*e.g.* 1-bp differences for WGA009, WGA331, and WGA376) compared with those that were detected in our laboratory. However, the differences between consecutive alleles at these SSRs were identical for the six varieties, except for 'Chandler' with WGA009 and 'Sunland' with WGA276 (Table 6). With WGA321, the data were completely identical for three cultivars ('Chandler', 'Franquette', and 'Payne') and only 1-bp discrepancies occurred for the shorter allele of 'Serr' and 'Sunland'. Most of these differences might be interpreted as stutter due to extra base additions that occur with some *Taq* polymerases (Brownstein *et al*., 1996). This result for the WGA321 locus also shows discrepancies due to misinterpretation of the homozygous (-/245 reported by Dangl *et al*. 2005) *versus* heterozygous (225/245 obtained in this work) state for cultivar 'Howard' (Table 6). Consistent with the pedigree of 'Howard' (Tulecke and McGranahan, 1994), the WGA321-225 allele observed in our experiment (Table 5) could have been inherited from the accession UC56-224 through cultivar 'Sharkey' (224/241) (Dangl *et al*., 2005), and the WGA321-245 allele could have been inherited from cultivar 'Pedro' (241/245) (Table 5). One possible explanation for the difference found between the two studies would be a mutation in the 225 bp allele to a null allele (no amplification). In this case, the mutation would affect the one or two annealing targets for the primers, and not the microsatellite sequence itself. Thus, the analysis of identical samples, and the comparison of walnut microsatellite data among laboratories working on walnut genetic resources, could help to develop a similar method of defining a common and unique reference allele set for several SSR loci, as has occurred in grape (This *et al*., 2004). This set of reference alleles could then be used in order to code the data from several laboratories and would enable a very easy comparison of data and/or genetic resources. The establishment and feeding of a uniform database with confirmed microsatellite profiles for true-to-type walnut cultivars would support better and more-rationalized management of walnut collections.

The majority of accessions examined in this work are traditional cultivars of unknown parentage. However, a close relationship between the known pedigree and the genetic similarity was observed with SSRs. Thus, the cultivars 'Payne' and 'Eureka', ancestors of most of the Californian cultivars tested, showed, as expected, a similarity value of 0.50 or more with all the cultivars related to them, and clustered together. A definite grouping of the Spanish cultivars can be established according to their geographical origin. Some cultivars, however, were placed outside of their regions. This could be due to movements of plant material from one region to another. Only one French cultivar, 'Franquette', was included in this analysis. The fact that the Californian cultivar 'Hartley' and the French cultivar 'Franquette' clustered together is consistent with previous results indicating that 'Hartley' is derived from crosses involving French cultivars (Potter *et al*., 2002; Dangl *et al*., 2005; Foroni *et al*., 2006). The level of similarity found among Californian varieties and the two Spanish varieties 'Ladredo' and 'Sendra' at the SSR level indicates that they may have a common or related ancestry.

The markers employed here will be useful for the characterization and comparison of walnut germplasm collections and for the detection of propagation errors. The evaluation of the molecular diversity of walnut genetic resources is important for the optimal development of programs aimed at conservation. These markers were able to identify uniquely all the walnut cultivars studied. In general, the cultivars sharing common parents tended to group together and with at least one of the parents. Since pedigree and passport data are often unknown or incomplete for many fruit species (Warburton and Bliss, 1996), SSRs can be a useful tool for the assessment of the degree of similarity of cultivars in this species, in order to select the best parental combinations for the production of new genetic combinations.

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