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Characterisation of *Juglans regia* L. with SSR markers and evaluation of genetic relationships among cultivars and the 'Sorrento' landrace

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SUMMARY
Juglans regia L. 'Sorrento' is the most important Italian walnut variety. It is cultivated for both nuts and timber in the Campania Region. 'Sorrento' is a mixture of genetic entities and is best characterized as a 'landrace', which affects the homogeneity of important commercial parameters such as fruit size and yield. To clarify the genetic variability in walnuts grown in Campania and labelled as 'Sorrento', we analyzed ten plants that originated from 'Sorrento' seeds and six grafted 'Sorrento' clones, and compared them with six other walnut cultivars using Simple Sequence Repeats (SSRs) DNA markers. Primers derived from *J. nigra* L. amplified alleles at six SSR loci in Persian walnut. A total of 33 putative alleles were detected, nine of which were unique to one genotype. Two loci, WGA5 and WGA27, were particularly useful for distinguishing walnut varieties. Cluster analysis clarified the relatively large genetic distance between most of the 'Sorrento' plants and some genotypes labelled as 'Sorrento'. Despite the genetic diversity found among seed and vegetatively propagated plants, 'Sorrento' landrace trees can be identified and distinguished from other walnut varieties. These data present a starting point for characterising the range of genetic variability among 'Sorrento' plants.

The genus *Juglans* includes about 20 species of long-lived, deciduous trees producing large, woody-shelled nuts (Manning, 1978). Most *Juglans* species are represented by native populations only, but selected breeding lines and cultivars of commercial value have been developed from *Juglans regia* L. (walnut), *J. nigra* L. (black walnut), and *J. cinerea* L. (butternut). *J. regia* cultivars, the most economically important species, are grown primarily for nut production as varietal clones (McGranahan and Leslie, 1990).

Walnut grows well in areas with a temperate climate. Production is concentrated in China (25% of world production) and the United States (California, 20%), but many other countries contribute to the total production of 1.4 million tonnes, including Turkey (10%), France (2%), India (2%) and Italy (1%) (FAO, 2004). In Italy, walnuts are produced throughout the peninsula, from the Alps to Sicily, as the species is well-adapted to the range of pedologic and climatic conditions characteristic of the country. Walnut is cultivated on approximately 65,000 ha, of which only 1,200 ha are organized into walnut plantations. In most cases, plants are scattered in orchards and in the meadows of farms. Italian nut production is based mainly on the 'Sorrento' cultivar grown in Campania, "the Italian land of walnut" and the primary location of Italian production (Piccirillo, 2000). Cultivars such as 'Malizia', Tonda di S. Martino', and 'Panella' are also grown in the same region, and the cultivars 'Feltrina', 'Bleggiana', and 'Cerreto' are grown in northern Italy. We consider 'Sorrento' to be a landrace (i.e. "a geographically or ecologically distinctive crop population, adapted but variable"), rather than a clonal cutivar. They have certain genetic integrity and are recognisable morphologically (Pingali and Smale, 2002).

In Campania, 'Sorrento' walnut plantations were established historically for both fruit and timber production. Mature rootstocks with good timber quality were traditionally grafted with genotypes having good yield and fruit quality. Furthermore, plants originating from 'Sorrento' seeds have been labelled as 'Sorrento'. The result is that 'Sorrento' is a mixture of genetic entities that is heterogeneous for important commercial traits such as fruit size and yield. Consequently, the genetic and morphological description of 'Sorrento' is extremely difficult. For this reason there is a need to develop DNA markers to monitor genetic diversity among 'Sorrento' plants. DNA markers can also be used to identify and label clones, and to characterise adapted germplasm that may be useful for breeding.

The diversity of walnuts in Campania, originating either from seed or by vegetative propagation of 'Sorrento', has been studied by ALFP markers (Andreakis *et al.*, 2002). Attention focussed on plants grown by the 'Istituto Sperimentale per la Frutticoltura' (ISF-CE), the National Institute located in Campania, that grows plants for germplasm conservation and to evaluate genotypes adapted to the region. We also genotyped plants from a plantation of clonally

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propagated 'Sorrento' walnut located in Campania. The AFLP patterns showed large variability among seed-propagated plants, and clearly indicated that they had different genetic origins from vegetatively propagated plants. The latter, although not identical, were similar to one another, indicating that they originated from genetically related mother plants.

The aim of this study was to determine and compare the genetic diversity of seedling and vegetatively propagated 'Sorrento' landrace genotypes using Simple Sequence Repeat (SSR) markers that typically reveal a large number of co-dominant alleles. The SSR markers were also used to compare the 'Sorrento' landrace with six other walnut cultivars.

MATERIALS AND METHODS

Plant material

Plants originated from 'Sorrento' by grafting (G) or seed (S) and were grown for several years in the same environment as part of the 'Sorrento' landrace.

Seedling plants (S1–S9 and SO) were selected from a pool of genotypes that originated from 'Sorrento' seeds belonging to the germplasm collection of the Istituto Sperimentale per la Frutticoltura (ISF-CE). Two plants had traits associated with ancient origins: rounded fruits in S7, and elongated fruits in SO, phenotypes considered to be widespread in Campania in the past. Six plants (G1–G5 and G7) were from a walnut plantation in Campania (Nola) and produced by grafting different 'Sorrento' clones. The other six genotypes were walnut cultivars from Europe ['Blegiana'(B), 'Malizia'(M), 'Parisienne'(P), 'Franquette'(F)], or the USA ['Hartley'(H), 'Serr'(SR)].

DNA extraction.

DNA was extracted from young leaves previously ground in liquid nitrogen and stored at –80°C. The extraction protocol of Doyle and Doyle (1987) was modified as follows: 1 g of frozen, ground leaves was added to 10 ml of pre-heated (50°C) 2x CTAB buffer (2% CTAB, 50 mM DTT, 0.3% β-mercaptoethanol, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, pH 8.0) and incubated at 65°C for 30 min. The aqueous solution was extracted with 20 ml (24:1) chloroform-octanol, centrifuged at 5°C for 15 min at 13000 × g, and the aqueous layer retained. Two volumes of 100% ethanol were added (at –20°C) to precipitate the nucleic acids. The precipitate was spooled, washed with 0.2 M ammonium acetate in 75% ethanol and air-dried for 5 min. The pellet was then resuspended in 500 µl of 65°C pre-heated H_2O and treated with 125 µl (10 mg ml^{-1}) RNase at 37°C for 30 min. The DNA was precipitated, washed, dried, resuspended in 250 µl H_2O, and quantified in a 1% agarose gel stained with ethidium bromide (10 mg ml^{-1}) against Lambda DNA standards.

SSR primers and amplification

Twenty-three primer pairs with the prefix WGA, designed from the sequences of clones from an enriched (GA/CT)n library of *J. nigra* described, in part, previously (Woeste *et al.*, 2002), were used to amplify genomic DNA sequences of each plant to identify polymorphic SSR loci. Amplification products were separated initially in 2% agarose gels (not shown). Six primers combinations that produced clear, polymorphic products without artefact bands (WGA 1, 5, 9, 27, 32 and 71) were then selected for separation in higher resolution 6% polyacrylamide gels (Table I). WGA 27, 32 and 71 have been reported previously (Woeste *et al.*, 2002) while WGA 1, 5 and 9 are new.

PCR reactions contained 3.3 mM $MgCl_2$, 0.25 mM dNTPs, 0.3 unit AmpliTaq Gold (Perkin-Elmer), 0.2 µM unlabelled primer (reverse), 0.2 µM fluorescent primer

TABLE I

Properties of the microsatellite loci used to characterize 22 walnut genotypes

SSR Locus	Tm** (°C)	Primer pair sequences (both 5'-3')	Size range (bp)	'Sorrento' seedlings		'Sorrento' clones		Other cultivars		All alleles	Overall H_o	Unique Allele
				H_o[1]	Na[2]	H_o[3]	Na[4]	H_o[5]	Na[6]			
WGA1	48	ATTGGAAGGGAAGGGAAATG CGCGCACATACGTAAATCAC	181 - 195	0.53	3	0.35	2	0.63	3	3	0.58	
WGA5	47	CAGTTTGTCCCACACCTCCT AACCCATGGTGAGAGTGAGC	240 - 266	0.60	4	0.59	3	0.79	6	7	0.70	248 bp:M 252 bp:S1 255 bp:F 265 bp:B
WGA9	48	CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	236 - 248	0.71	4	0.65	4	0.76	4	6	0.76	236 bp:G4
WGA27	51	AACCCTACAACGCCTTGATG* TGCTCAGGCTCCACTTCC	192 - 210	0.58	3	0.48	2	0.77	5	6	0.69	192 bp:F 207 bp:P
WGA32	47	CTCGGTAAGCCACACCAATT* ACGGGCAGTGTATGCATGTA	120 - 196	0.43	3	0	1	0.59	3	4	0.65	120 bp:S1
WGA71	45	ACCCGAGAGATTTCTGGGAT* GGACCCAGCTCCTCTTCTCT	136 - 210	0.74	6	0.75	5	0.74	4	7	0.81	136 bp:S1
Total					23		17		25	33		
Mean				0.60 ± 0.11		0.47 ± 0.26		0.71 ± 0.08				
Allelic richness[7]					3.8		2.8		4.2	5.5		

[1]Number of alleles (Na) and observed heterozygosity (Ho) calculated for 'Sorrento' seedling samples only.
[2]Number of alleles (Na) and observed heterozygosity (Ho) calculated for 'Sorrento' clone samples only.
[3]Number of alleles (Na) and observed heterozygosity (Ho) calculated for other cultivar samples only.
[4]Size of specific alleles that are present in only one genotype or landrace.
[5]Allelic richness = (number of alleles per locus / number of individuals tested).
*Sequences previously published (Woeste *et al.*, 2002).
**Tm = DNA melting temperature.

(forward), and 60 ng DNA template in a final volume of 15 µl. Thermal cycling conditions were as follows: denaturation for 5 min at 95°C; then 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; with a final extension 10 min at 72°C. Each amplification included a negative control reaction without DNA template. To prepare samples for loading, 1.0 µl PCR product was mixed with 2 µl 6x loading buffer (20 mg blue dextran in 1 ml formamide) and 0.5 µl Tamra 500-labelled molecular size standard. The samples were denatured at 94°C for 1 min before loading onto an ABI 373 sequencer (Applied Biosystems) and electrophoresed in the 6% polyacrylamide gels under denaturing conditions [7 M urea, 1 × TBE buffer (90 mM Tris-borate, pH 8.3, 2 mM EDTA)]. Data were analyzed with GENESCAN (Applied Biosystems) software. Two replicate experiments were carried out for each SSR primer pair.

Evaluation of polymorphisms and data analysis

The SSR allele composition of each plant was determined and the observed heterozygosity (H_o) value of each microsatellite was calculated according to the formula (Nei, 1987):

$$H_o = 1 - \sum_{j=1}^{n} x_{ij}^2$$

where x_{ij} is the frequency of the *j*th allele for each SSR marker *i* and summation extends over *n* alleles.

The CONVERT (Glaubitz, 2003), MICROSAT (Minch *et al.*, 1997) and PHYLIP (Felsenstein, 1989) programs were used to analyse the SSR data and to construct a Neighbor-joining dendrogram based on allele frequency data calculated by Nei's genetic distance (Nei, 1972).

RESULTS

The SSRs were highly polymorphic in the 22 walnut genotypes or landraces analysed. Each sample had just 1 or 2 alleles, as expected. The number of alleles per primer pair ranged from 3 to 7, with a mean of 5.5 and a size-range from 120 – 265 bp. The six loci analysed allowed discrimination of the genotypes studied, and 16 alleles were either present only in 'Sorrento' landrace or in other cultivars (Table II). The 242 and 250 bp alleles of locus WGA5 were found at high frequency in the 'Sorrento' landrace. Only one of 15 'Sorrento' samples (S2) had neither allele; the majority had both. S4 and S5 were identical in SSR allele composition for all loci analysed. S7 and SO could be discriminated only at the locus WGA9. The 196 bp allele of locus WGA27 was present in seven out of 17 'Sorrento' landrace genotypes, and absent from all other cultivars analysed.

The allelic richness (calculated as: number of alleles for each locus / number of individuals tested) was 3.8 for 'Sorrento' seedlings, 2.8 for 'Sorrento' clones, and 4.2 for other varieties (Table I). The six primer pairs detected a total of 33 putative alleles (product sizes) of which nine were unique to single individuals. The most common allele (196 bp, WGA32) had a frequency of 0.71, while the least common (136 bp, WGA71) had a frequency of 0.02. The most polymorphic loci were WGA5 and WGA71, each with seven alleles. To compare the number of alleles and the expected heterozygosity for each SSR locus among the three groups of samples, SO and S7 were included in the 'Sorrento' seedling group because no information exists concerning their origin. The data indicated that the three groups had different levels of allelic richness and different levels of observed heterozygosity per locus (Table I). An average heterozygosity of 0.47 ± 0.26 was observed for the 'Sorrento' clones, whereas the 'Sorrento' seedlings and the other cultivars group were more heterozygous (0.60±0.11 and 0.71±0.08, respectively). There was no difference among the three groups in the number of loci that were heterozygous or homozygous as determined by chi-square analysis (data not shown). No correlation was found between numbers of alleles and SSR repeat length, in agreement with data presented by other authors (Li *et al.*, 2001; Rongwen *et al.*, 1995). However, such correlations have been reported in plants (Bryan *et al.*, 1997; Smulders *et al.*, 1997).

TABLE II

Distribution of the putative alleles for six polymorphic microsatellite loci in walnut

			SSR markers and Allele size (bp)					
Group	Name	Code	WGA1	WGA5	WGA9	WGA27	WGA32	WGA71
'Sorrento' seedlings	MU 6	S1	181/195	ND	240/248	210	120/196	136/210
	MA 1	S2	193	252	238/244	205	166/196	200/210
	'Sorrento' XI	S3	181/195	242/250	240	196/210	166/196	200/210
	CC1 pt1	S4	195	242	240/244	205/210	166/196	198/208
	CC1 pt2	S5	195	242	240/244	205/210	166/196	198/208
	VR3 pt2	S6	181/195	242/250	240/248	196/210	196	200/210
	VR3 pt3	S8	181/195	242/250	240/248	210	196	202/210
	'Sorrento' IV	S9	181/195	242/250	238/248	196/210	196	202/210
	'Casolla'	SO	195	240/250	238	205/210	196	202/210
	'Sorrento' 30	S7	195	240/250	240	205/210	196	202/210
'Sorrento' clones	NOLA 6	G1	181/195	242/250	238	210	196	206
	NOLA 13	G2	181	242/250	238	196/210	196	198/208
	NOLA 16	G3	181/195	242/250	ND	210	196	198/208
	NOLA 19	G4	181	240/250	236/240	196/210	196	198/206
	NOLA 26	G5	181	242	238/240	196/210	196	202/210
	NOLA 36	G7	181	242/250	238/244	196/210	196	198/208
Other genotypes	'Blegiana'	B	195	266	240/246	205/210	170	200/210
	'Franquette'	F	193	242/256	240/246	192/205	170	198/208
	'Hartley'	H	193	240	238/244	203	196	200/210
	'Malizia'	M	181/195	240/248	238	210	196	200/208
	'Parisienne'	P	195	240/250	238/244	205/207	170/196	200/210
	'Serr'	SR	181/195	242	240/246	203	166/196	198/208

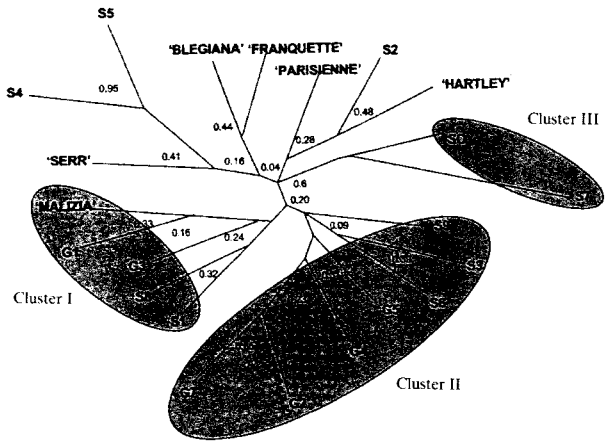

FIG. 1
Neighbour-joining dendrogram based on Nei's (1972) genetic distance showing relationships among 22 samples, 10 'Sorrento' seedlings (S), 6 'Sorrento' clones and 6 cultivars. The dendrogram was calculated from SSR data using CONVERT, MICROSAT and PHYLIP softwares.

Nei's genetic distance among samples was calculated on the basis of allele frequency data, and the results of the similarity analysis were represented by a Neighbor-joining dendrogram (Figure 1). The dendrogram organized most of the 'Sorrento' landrace samples into three major clusters. The first included two of ten 'Sorrento' seedlings (S1, and S8), two of seven 'Sorrento' clones (G1 and G3), and the cultivar 'Malizia'. The inclusion of 'Malizia' in this cluster was expected because it originated from an open pollination of 'Sorrento'. The second cluster included three 'Sorrento' seedlings (S3, S6 and S9) and four 'Sorrento' clones (G2, G4, G5 and G7). The third cluster included only the two plants with traits associated with ancient origins (S7 and SO). Cultivars 'Franquette', 'Serr', 'Blegiana', and 'Hartley', and three 'Sorrento' seedlings (S4, S5 and S2) remained outside the clusters. The association of S2 with 'Hartley' and S4/S5 with 'Serr' indicates that they are distinct from other 'Sorrento' genotypes, and raises the possibility that there has been recent gene flow between 'Sorrento' and 'Hartley' or 'Serr', or that S2 and S4/S5 share some common ancestry with 'Hartley' and 'Serr'.

DISCUSSION

Walnut has a long juvenile period, therefore it is important to identify desired genotypes as early as possible. DNA fingerprinting can be used to validate the identity of scion sources, and to test for propagation errors, reducing the risk of mixing valuable genotypes with less valuable ones. SSR analysis is a powerful and informative method to fingerprint cultivars and study genetic relationships. SSRs are abundant in most genomes, are generally distributed across the whole genome, and are hypervariable, co-dominant, and highly reproducible. Their multi-allelic nature makes them especially useful for the analysis of heterozygous, allogamous species, permitting the development of SSR fingerprints for each genotype (Powell *et al.*, 1996). Here, we demonstrate that SSRs developed in *J. nigra* (Woeste *et al.*, 2002) proved to be effective for fingerprinting *J. regia* genotypes and closely-related members of a landrace. This represents the first SSR data set for discriminating the 'Sorrento' landrace.

Two loci, WGA5 and WGA27, were particularly polymorphic in these populations, so may be useful more widely for identifying and distinguishing walnut germplasm. Some alleles of locus WGA5 (248, 256 and 266) were present only in non-'Sorrento' varieties. The 196 bp allele of locus WGA27 was present in 7 out of 16 of the 'Sorrento' landraces and in no other cultivar tested.

Neighbour-joining clarified the large genetic distance between most of the 'Sorrento' plants and some genotypes labelled as 'Sorrento' (e.g. S2). The level of similarity found among 'Serr', S5 and S4 indicates gene flow, homoplasy, or relatedness among these genotypes. The same is true for 'Hartley' and S2. Gene flow into 'Sorrento' may be explained by the high level of outcrossing that occurs in all *Juglans* species as a result of heterodichogamy (McGranahan and Leslie, 1990). It is possible that the S4, S5, and S2 genotypes originated from crosses of 'Sorrento' with 'Serr' and 'Hartley', respectively.

Compared to 'Sorrento' seedlings and other cultivars, 'Sorrento' clones showed a higher level of homogeneity. Indeed they shared most alleles. The genetic similarity of the 'Sorrento' clones group made these plants a good reference. They represent a cluster of genotypes that can be labelled 'Sorrento' with greater confidence than the seedling population. On the other hand, alleles present in the 'Sorrento' seedling group that were absent from 'Sorrento' clones represent a source of genetic diversity which may be useful for future breeding and research. They also provide an opportunity to integrate molecular data with agronomic observations. As with AFLP-based genetic diversity observed in an analogous set of samples, (Andreakis *et al.*, 2002), the SSR diversity data reported here on the 'Sorrento' clones confirm that they originated from different plants that, although closely related, had some genetic differences. Genetic differences among 'Sorrento' plants were also confirmed by RAPD fingerprinting of two genotypes grown in different areas of Campania (Malvolti *et al.*, 1994).

Although the relatively small number of molecular markers used in this study might not be able to distinguish all genotypes included in 'Sorrento' plantations, our results do contribute to the establishment of genetic boundaries circumscribing the 'Sorrento' walnut-tree, an important pre-requisite for a "label of origin" for 'Sorrento' nuts. This has already occurred for olive oil produced by specific varieties (Claros *et al.*, 2000). A more complete picture of the genetic and agronomic variability present in the 'Sorrento' landrace will require more extensive comparisons of walnuts within and outside the Campania Region.

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