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Juglans regia Provenance Research by Molecular, Morphological and Biochemical Markers: A Case Study in Italy

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ABSTRACT

In the current climate change scenarios, the choice of seed sources is one of the main factors affecting the establishment and productivity of plantations of forest trees. *Juglans regia* L. (walnut) is one of the more valuable hardwood species since it could provide high quality timber and fruits. The aim of this study was to search peculiar Italian walnut provenances as putative biodiversity sources for seed orchards and to establish new hardwood plantations. A multidisciplinary approach, integrating molecular markers (ISSR), seed morphological traits (equatorial and polar diameter, shape, dry weight) and fruit composition (total oil, fatty acids, tocopherol) was applied to analyze samples collected in ten sites (three in Campania and seven in Abruzzo regions) and samples of four varieties (two from Southern and two from Northern Italy). Eleven selected ISSR primers exhibited a strong ability to discriminate walnut provenances. The Principal Coordinate Analysis performed on the Φ_{PT} values divided all germplasm in four distinct groups. The genotyping results were partially confirmed by the morphological and biochemical analysis of fruits. Two walnut provenances, the first from a hilly plateau in Campania region and the second from mountainous zone of Abruzzo, shown to be different both for genetic, morphological and biochemical characters, and can be considered promising source of seed for nurseries and hardwood plantations.

Keywords: agroforestry, ISSR, PUFA, tocopherol, walnut
Abbreviations: **AMOVA**, analysis of molecular variance; **ISSR**, inter simple sequence repeat; **MANOVA**, multivariate analysis of variance; **MSWP**, mean of square deviations within population; **PCR**, polymerase chain reaction; **PUFA(s)**, polyunsaturated fatty acid(s); **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction-fragment length polymorphisms; **SSR**, simple sequence repeat; **UPOV**, Union Internationale Protection Obtentions Vegetables

INTRODUCTION

In European countries as well as in the other countries having forestry resources, great attention is devoted to seed orchard collections as valuable tools for biodiversity conservation, and for the diffusion of high quality biological material. During the last ten years new attention was given to human and environmental safety, eco-sustainable development and protection of the natural resources and, consequently, there was an increasing awareness by consumers that the use of tropical timbers may contribute to deforestation, resulting in a reluctance to buy them. There are, therefore, good reasons to believe that valuable, decorative temperate hardwoods are likely to be in much greater demand as tropical supplies decline (Hemery *et al.* 2005). The choice of seed sources is one of the main factors affecting the establishment and productivity of plantations of forest trees. According to Callaham (1964), besides appropriate silvicultural managements, good fruit production can be mainly obtained using selected cultivar or/and varieties, whereas the identification of suitable seed source is one of the critical point for high quality wood production. Indeed, taking into account the climate change scenarios, from rising temperature and CO_2, to large scale stochastic events such as increased incidences of fire, drought, flood (frequency and severity) and increase of pests and pathogens (distribution and impact), the choice of seed source may be crucial for the success of future plantations (Hemery 2008). Since the second edition of State of the World's Forests (FAO 1997), it was stated that in order to avoid serious problems, it will be useful to acquire information of which seed sources are available, what they were selected for, where they were collected and the ecological conditions prevailing in these areas. Provenance research provides a sound basis for the selection of seed sources and refers to the geographical origin of seeds or trees (Callaham 1964). Nevertheless the current lack of detailed distribution and abundance data for the valuable broadleaved tree species in Europe is relevant.

Among the noble hardwood temperate species *Juglans regia* (Common walnut or Persian walnut) is one of the more valuable since it can provide high quality timber and valuable nuts in a range of latitudes and altitudes (Hemery *et al.* 2005). In Italy, *J. regia* is a naturalized species living from Alps to Sicily at different altitudes, from sea level to over 1500 m elevation. Even if is still debated the walnut survival during the last glaciation era in Balkans and in Italy (Huntley and Birks 1983), the increased finding of walnut pollens allocated to Greek and Roman civilization periods demonstrate the long cultivation experienced by this species. Furthermore, it is also debated if walnut survived the last glaciation era in Balkans and in Italy (Huntley and Birks 1983). Due to this historical tradition and the wide distribution of the species, walnut provenances, naturally selected and adapted to different environments, can be abundant in Italy and represent a valuable source of seeds. However, although *J. regia* is well-known and accepted by farmers and policy makers, compared to other multipurpose tree species walnut is not valorised at the same level, so that in some locations the walnut cultures are still progressively suppressed. In the light of these situations, it is a logical consequence that some local and traditional varieties or provenances were neglected.

The differentiation and characterization of walnut vari-

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eties, as for the other multipurpose species, have been carried out on the basis of morphological and physiological description and in according to the geographical origin (McGranahan and Leslie 1991; UPOV 1999). However, the above characters are often strongly affected by environmental conditions. This limitation had stimulated the development of molecular techniques for detecting DNA polymorphism in walnut species. Biochemical markers as isoenzymes (Solar *et al.* 1994; Fornari *et al.* 1999) and molecular markers as restriction fragment length polymorphisms (RFLP) (Fjellstrom and Parfitt 1994), random amplified polymorphic DNA (RAPD) (Abuin *et al.* 2002), inter simple sequence repeat (ISSR) (Potter *et al.* 2002; Pollegioni *et al.* 2003) and simple sequence repeat (SSR) (Dangl *et al.* 2005; Pollegioni *et al*. 2009) have been useful tools for genetic evaluation of natural and semi-natural populations, cultivars as well as varieties in *J. regia*. ISSR markers offer some advantages: the procedure requires no prior sequence information of locus, being based on polymerase chain reaction (PCR) amplification using random primers composed of simple sequence repeats units (Zietkiewicz *et al.* 1994). The procedure is relatively cheap and less time consuming compared to SSR, with higher reproducibility and polymorphism compared to RAPD markers.

The high lipid content in walnut fruit had relevance as source of carbon and energy during germination and seedling growth (Chenevard *et al.* 1994). During seed germination and seedling development, lipids stored in oil bodies are broken down into fatty acids through the action of lipases. Fatty acids are then β-oxidized by enzymes in peroxisomes. Fatty acid β-oxidation provides carbon for sucrose synthesis in the cytosol and also substrates for energy production in mitochondria (Eastmond and Graham 2001). Other studies describe the oil content in walnut fruits mainly in relationship to nutritional and healthy value. Indeed, walnut fruits are rich in ω-6 (linoleic acid) and ω-3 (linolenic acid) essential polyunsaturated fatty acids (PUFAs), those cannot be produced in the human body and must be taken up through food (Zwarts *et al.* 1999; Cağlarirmak 2003; Amaral *et al.* 2003). In both aspects, germination and human nutrition, important role have the presence in the seeds of antioxidant compounds. Walnut fruits exhibit greater total antioxidant capacity then other nuts (Kornsteiner *et al.* 2006) and in particular walnut contains significant amounts of tocopherols, mainly γ-tocopherol as the dominant isomer (Lavedrine *et al.* 1997; Kornsteiner *et al.* 2006), which has an antioxidant protective function of lipid matrix for both seed germination and consumers (Verardo *et al.* 2009).

In order to improve the knowledge of the *J. regia* species and draw attention of the scientific community and consumers to the revaluation of the natural biodiversity resources, the aim of this study has been the identification of peculiar Italian walnut provenances, naturally adapted to different environment conditions, as putative sources for seed orchards and to establish new hardwood plantations. A multidisciplinary approach, integrating molecular markers (ISSR), morphological and biochemical traits of fruits, was developed to evaluate the ability for discrimination between Italian walnut provenances.

MATERIALS AND METHODS

Plant material

Walnut trees in Italy are present throughout the peninsula as the species is well adapted to the temperate pedologic and climatic condition of the country. 'Sorrento' is the most famous Italian walnut variety and growth mainly, such as the 'Malizia' variety, in the Campania region (South Italy); The 'Bleggiana' and 'Feltrina' varieties grow in two different Northern Italian regions (Trentino and Veneto respectively). The Italian varieties should be considered as landraces rather than clonal cultivars. Indeed, they group genotypes having certain genetic integrity and are morphologically recognizable (Foroni *et al.* 2005). In addition, distinctive groups of different walnut genotypes, used especially in the past for local production and consume of the fruits, are still present and distributed in rural Italian areas. These walnut groups are genetically and morphologically variable, and can be considered ad possible sources of conserved biodiversity. The word 'provenance' is normally used to define each group of walnut genotypes and we use it in this paper.

For the present study, the germplasm was sampled in Campania and Abruzzo, two Italian regions in which walnut is traditionally cultivated from centuries in plots, private gardens and to delimitate fields or roads. The collection localities had different ecological and geographical conditions. The description of the provenances, localities and the number of samples are reported in **Table 1**.

According to the appropriate seasons, young leaves and mature fruits were collected directly from the crown of adult walnut trees. Leaves from 276 plants for DNA analysis, fruits from a sub-set of 130 trees (13 per provenance) for morphological and

Table 1 Walnut genotypes investigated, their location with ecological and geographical parameters in Italy.

Provenance Variety	Soil	Annual temp. (°C)	Annual rainfall (mm)	Longitude (°E)	Latitude (°N)	Altitude (m)	Trees sampled (n)	
Trescine[a]	Limestone-pyroclastic	15.8	1137	14°50′	40°42′	280	30[i]	13[j]
Mesanole[a]	Limestone-pyroclastic	14.8	567.3	14°40′	41°03′	284	16	13
Montella[a]	Limestone-pyroclastic	12.8	1341.3	15°00′	40°48′	670	33	13
Prat. Peligna[b]	Calcaric	13.7	615	13°51′	41°08′	330	25	13
Valle Corvo[b]	Calcaric	13.7	615	13°50′	42°03′	430	17	13
Con. Chiuse[b]	Calcaric	13.7	615	13°51′	42°01′	500	52	13
Navelli[b]	Limestone	13.7	615	13°45′	42°12′	685	33	13
Vill. Barrea[c]	Calcaric	7.8	1520	13°47′	41°47′	1000	22	13
C. Alfedena[c]	Calcaric	7.8	1520	14°01′	41°45′	1100	17	13
Pescasseroli[c]	Carbonatic with angular clasts	7.8	1520	13°46′	41°49′	1200	31	13
'Sorrento'[a,f]	Slime sandy	16.0	840	14°48′	40°55′	0-100	20	15
'Malizia'[a,f]	Slime sandy	16.0	840	14°48′	40°55′	0-100	20	15
'Feltrina'[d,f]	Calcaric phaeozem	13.0	1520	11°53′	46°01′	320-500	20	15
'Bleggiana'[e,f]	Limestone-alluvial	9.6	1083	10°50′	46°02′	495-650	20	15
CNR-IBAF[g,h]	Vulcanic	12.5	880	12°02′	42°40′	550		

[a] Campania Region, Southern Italy
[b] Abruzzo Region, Central Italy, Sulmona district
[c] Abruzzo Region, Central Italy, inside the Abruzzo National Park
[d] Veneto Region, Northern Italy
[e] Trentino Region, Northern Italy
[f] Italian model varieties description of native area
[g] Umbria Region, Central Italy
[h] Description of CNR-IBAF Repository
[i] Column gives number of trees sampled for each provenance and variety analysed for ISSR markers
[j] Column gives number of trees on which fruits were sampled for morphological and biochemical analyses

Table 2 ISSR primers used and polymorphism in 356 walnut genotypes including samples of Italian model varieties.

Primer name	Sequence (5′- 3′)	Annealing temperature (°C)	Number total bands	Polymorphic bands (%)	Size range (bp)
UBC-807	AGAGAGAGAGAGAGAGT	55.0	14	57.1	246-1168.5
UBC-810	GAGAGAGAGAGAGAGAT	55.0	12	58.3	467.4-1476
UBC-811	GAGAGAGAGAGAGAGAC	52.0	12	75.0	360-1629.8
UBC-834	AGAGAGAGAGAGAGAGYT	55.0	13	92.3	430.9-1599
UBC-836	AGAGAGAGAGAGAGAGYA	55.0	13	61.5	381.3-1660
UBC-841	GAGAGAGAGAGAGAGAYC	54.0	14	71.4	246-1537.5
UBC-856	ACACACACACACACACYA	54.0	12	75.0	351.4-1968
UBC-888	BDBCACACACACACACA	59.1	9	77.7	430.5-1199.2
UBC-889	DBDACACACACACACAC	59.8	12	58.3	430.5-1230
UBC-890	VHVGTGTGTGTGTGTGT	58.0	14	85.7	338.86-1476
UBC-891	HVHTGTGTGTGTGTGT	58.0	9	100.0	405.9-1291.5
			134	73.8	

Y = (C, T); V = (A, C, G); H = (A, C, T); D = (A, G, T); B = (C, G, T)

biochemical studies were sampled. Several reasons caused the discrepancy in the number of samples: mainly the absence of fruit production for some trees, but also the sudden sawing of some trees during the summer. In addition, 80 genotypes of four traditional walnut varieties, 'Bleggiana', 'Feltrina', 'Malizia' and 'Sorrento' were included in the study for comparison. These genotypes, already analysed by molecular markers (Pollegioni *et al.* 2003), are conserved in the repository of the CNR-IBAF, established in winter 1995 with seeds derived from the native area of each variety: 'Sorrento' and 'Malizia' from Nola (Campania); 'Bleggiana' from Bleggio valley (Trentino) and 'Feltrina' from Feltre (Veneto). Mature fruits were sampled directly from the crown of 15 trees for each variety; a total of 60 genotypes were evaluated for quantitative traits.

Leaves were immediately frozen in liquid nitrogen and then stored at –80°C while the fruits were conserved in open air for three weeks and then stored at +4°C until the analyses. Totally 356 plants were analysed by ISSR markers and 190 were evaluated for morphological and biochemical characters.

DNA extraction

The leaf tissues were powdered in a mortar under liquid nitrogen, then genomic DNA was extracted according to Doyle and Doyle protocol (1987) properly modified for *J. regia* species (Pollegioni *et al.* 2003) and stored at –20°C. DNA quantity were assessed by comparing all samples against six standardized solutions of phage λ DNA (15, 31, 63, 125, 250, 500 ng/µL; Life Technologies), in a 1% agarose gel stained with ethidium bromide and visualized with UV light. The DNA in the samples was brought to a working concentration of 5 ng/µL.

ISSR analysis

Eleven ISSR primers (University of British Columbia series), previously selected for the efficiency discrimination of *J. regia* varieties (Pollegioni *et al.* 2003), were chosen and amplified in all the 356 genotypes (**Table 2**). Polymerase chain reaction (PCR) was done in 12.5 µL of reaction volume containing 20 ng DNA template in 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ reaction buffer, 200 µM dNTP (each), 0.4 µM ISSR primer, 100 µg/mL BSA (final concentrations) and 0.4 U of *Taq* polymerase (Roche Applied Science). For primers UBC-888, UBC-889, UBC-890 and UBC-891 an additional amount of formamide was used (2.5% final concentration). Reactions were performed in a GeneAmp 9700 PCR (Applied Biosystems) apparatus according to the following procedure: initial denaturation at 94°C for 7 min, 45 cycles with denaturation at 94°C for 30 sec, annealing at 50-52°C for 45 sec (depending on the primer), extension at 72°C for 2 min, and a final extension step at 72°C for 7 min. The amplified fragments were separated by electrophoresis, on a 2.0% agarose gel, in 0.5X TBE buffer (pH 8.0), stained with ethidium bromide and visualised under UV light.

Oil extraction

The mature walnut fruits were deshelled and the kernel dehydrated by freeze-dry. The kernels were powdered in a porcelain mortar and mixed with hexane (1.5 mL/g tissue). The solution was energetically shaken for 30 min. The extract was then centrifuged (Sorvall rotor HB4) at 7000 rpm for 20 min and the upper-liquid collected. The extraction procedure was repeated on the sediment for three times. The hexane was evaporated in nitrogen flow until constant volume and weight, to have the pure oil fraction. An aliquot of pure oil (20 µL) was hydrolysed in 1 mL of 1% NaOH in MeOH, at 80°C for 60 min. The solution dried under vacuum and the residue was dissolved in 2 mL of H_2O plus 0.3 mL of 1 N H_2SO_4, and then energetically shaken. The fatty acids, dissolved in 1 mL of hexane, were analysed by the qualitative and quantitative HPLC system.

Analysis of fatty acid

The fatty acids were separated and quantified by HPLC system (Jasco Trirotar VI pump, Jasco UV-975 detector) using a Merck Purospher RP-18 endcapped (250 × 4 mm) column eluted with acetonitrile: isopropanol: water (50: 30: 20, 1 mL/min) for 20 min at 18°C. Finally, to check the total hydrolysis of oil, the column was then eluted for 10 min with acetonitrile: isopropanol: water (50: 45: 5, 1 mL/min). Data at 210 nm wavelength, using UV-975 detector, were recorded, integrated and elaborated by Borwin software program (JMBS Developpements).

Tocopherol analysis

The α, γ, and δ-tocopherol isomers were separated (when present) by HPLC system using the Jasco Tritotar III pump and Jasco Uvidec detector. Five-10 µL of extracted pure oil were loaded into a Merck Chromolith RP-18e column (100 × 4.6 mm), and eluted with 1.5 mL/min MeOH 95%. The data at 280 nm were acquired and elaborated by the Borwin software system.

Morphological analysis

In order to estimate differences in the fruit shapes, the UPOV (1999) guidelines for distinctness and uniformity were followed. The measured nut length (polar diameter), nut thickness (equatorial diameter), the fruit shape, or index of roundness (calculated by diameters ratio), the nut weight were the descriptive morphological traits here considered.

Data analysis

Only repeatable ISSR bands, obtained by two independent amplifications were considered, whereas differences in intensity were not taken in account. The bands were scored on the basis of their presence (1) or absence (0). Analysis of Molecular Variance (AMOVA) was carried out in order to establish the relative percentage of variance displayed among and within provenances. AMOVA permitted to quantify the genetic differentiation among

Table 3 Comparison of the genetic differentiation of provenances and model varieties using Population Φ_{PT} value based on 134 total ISSR markers amplified in 356 walnut genotypes. Φ_{PT} values are below the diagonals, the significance of test for each comparison, based on 1000 permutations, above diagonals.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
2	0.319	-	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
3	0.322	0.058	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
4	0.128	0.282	0.251	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
5	0.289	0.196	0.207	0.265	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
6	0.184	0.201	0.180	0.140	0.116	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
7	0.226	0.140	0.147	0.209	0.089	0.063	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001
8	0.205	0.203	0.181	0.172	0.230	0.131	0.156	-	0.002	0.001	0.001	0.001	0.001	0.001
9	0.178	0.194	0.160	0.136	0.222	0.123	0.161	0.053	-	0.001	0.001	0.001	0.001	0.001
10	0.279	0.213	0.195	0.230	0.222	0.111	0.148	0.133	0.142	-	0.158	0.001	0.001	0.001
11	0.274	0.203	0.197	0.226	0.232	0.138	0.152	0.126	0.121	0.012	-	0.001	0.001	0.001
12	0.298	0.229	0.229	0.244	0.249	0.162	0.193	0.163	0.154	0.096	0.080	-	0.001	0.001
13	0.257	0.213	0.205	0.209	0.206	0.111	0.148	0.147	0.146	0.040	0.060	0.042	-	0.009
14	0.276	0.192	0.189	0.214	0.205	0.124	0.154	0.159	0.164	0.061	0.092	0.056	0.013	-

(**1**) 'Bleggiana'; (**2**) 'Sorrento'; (**3**) 'Malizia'; (**4**) 'Feltrina'; (**5**) Mesanole; (**6**) Montella; (**7**) Trescine; (**8**) Pescasseroli; (**9**) Civitella Alfedena; (**10**) Villetta Barrea; (**11**) Valle Corvo; (**12**) Pratola Peligna; (**13**) Contrada Chiuse; (**14**) Navelli

groups using the Population PhiPT value (Φ_{PT}) (Excoffier *et al.* 1992). AMOVA calculates the analysis of variance on the matrix of the squared distances between all possible pairs of samples [δ^2_{jk}]. In a multidimensional space ($\sum_j \sum_k \delta^2_{jk}/2N$) it is equal to the sum of the squared deviations (SSD) of the samples from the centroid, that is the point having as coordinates the averages of the variables Φ_{PT} value, analogous to F_{st} index (Peakall and Smouse 2006). AMOVA was deliberately studied to analyse the genetic diversity by dominant markers. It corresponds to the ratio of the variance among groups and the total variance. Permuting the original data, e.g. 1000 times, Φ_{PT} tests both the significance of the different variance components and of the Φ_{PT} values. To display the relative genetic distances among the groups in a bi-dimensional plot, the Analysis of Principal Coordinates (PcoorA) was conducted on the matrix of Φ_{PT} values using NTSYS-pc V2.1 software (Rohlf 2001).

Analysis of variance (ANOVA) for morphological and biochemical data was performed using XLStatistics software (Carr 2008). In addition, Canonical Variates Analysis (CVA) was used to study the variation among groups of variables relative to the average variation found within the groups. The CVA is a multivariate technique which is concerned to determining the relationship between groups of variables in a data set. This analysis can be applied to carry out a single classification Multivariate Analysis of Variance (MANOVA). This procedure permits the computation of the Euclidean distances between all pairs of. Moreover, as already applied in walnut (Malvolti *et al.* 1994), *Castanea sativa* (Pigliucci *et al.* 1990, 1991), oak (Kleinschimit *et al.* 1995; Ramírez-Valiente *et al.* 2009), *Nothofagus alessandrii* (Torres-Diaz *et al.* 2007) and in crop species as safflower (Amini *et al.* 2008), the statistical correlation among morphological, biochemical traits and genetic polymorphism was studied using the nonparametric multivariate Mantel's test (Mantel 1967). The Mantel's test calculates a coefficient of correlation between the PhiPT genetic distance (ISSR markers) and each Euclidean distance matrices (morphological and biochemical traits) and tests the significance by randomly permuting rows and columns of a matrix, while keeping the other constant. This randomization process produces an empirical distribution of the matrix correlation coefficient that is evaluated against the observed one (Pigliucci *et al.* 1991; Malvolti *et al.* 1994). Pearson's correlation coefficient (r) was calculated among quantitative characters. Statistical calculations were carried out by GenAlEx 6 software (Peakall and Smouse 2006), NTSYS-pc V2.1 software (Rohlf 2001), and XLStatistics software (Carr 2008).

RESULTS

DNA fingerprinting

The selected eleven ISSR primers amplified in all 356 samples, producing fragments of variable sizes (**Table 2**). A total of 134 bands were detected, an average of 12.2 per primer, ranging from 9 for UBC-888 and UBC-891 to 14 for UBC-807, UBC-841 and UBC-890. These scored fragments accounted for 73.8% of polymorphisms with a minimum value of 57.1% for UBC-807 primer and a maximum value of 100% for UBC-891 primer.

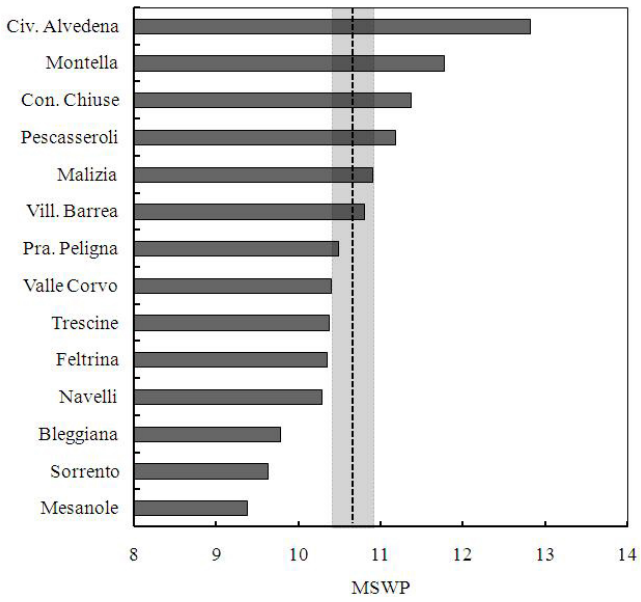

Fig. 1 Mean of Square deviations Within Population (MSWP) in each *J. regia* provenance and model Italian varieties. AMOVA analysis computed on the basis of 134 total ISSR markers. Dotted line and grey vertical area indicate the MSWP mean ± SD (10.67 ± 0.25) through all genotypes.

In order to quantify the genetic diversity among Italian model varieties and provenances, the pairwise values of Population PhiPT index (Φ_{PT}) were computed by the AMOVA analysis (**Table 3**). The greatest inter-population distance detected was between 'Malizia' (**3**) and 'Bleggiana' (**1**) varieties (Φ_{PT} = 0.322) and between Pratola Peligna (**12**) and Mesanole (**5**) provenances (Φ_{PT} = 0.249). Low Φ_{PT} values were detected between Sulmona district provenaces, but the lowest genetic differentiation, statistically non significant, was measured between Valle Corvo (**11**) and Villetta Barrea (**10**) provenances (Φ_{PT} 0.012, p = 0.158). The AMOVA analysis based on 134 ISSR markers attributed 16.4% of the molecular variance among groups and 83.6% within groups using the Φ_{PT} – statistics (**Table 4**). As shown in **Fig. 1**, Civitella Alfedena provenance was characterized by the highest molecular variance (MSWP = 12.81), whereas Mesanole (MSWP = 9.38) and two Italian model varieties, 'Sorrento' (MSWP = 9.62) and 'Bleggiana' (MSWP =

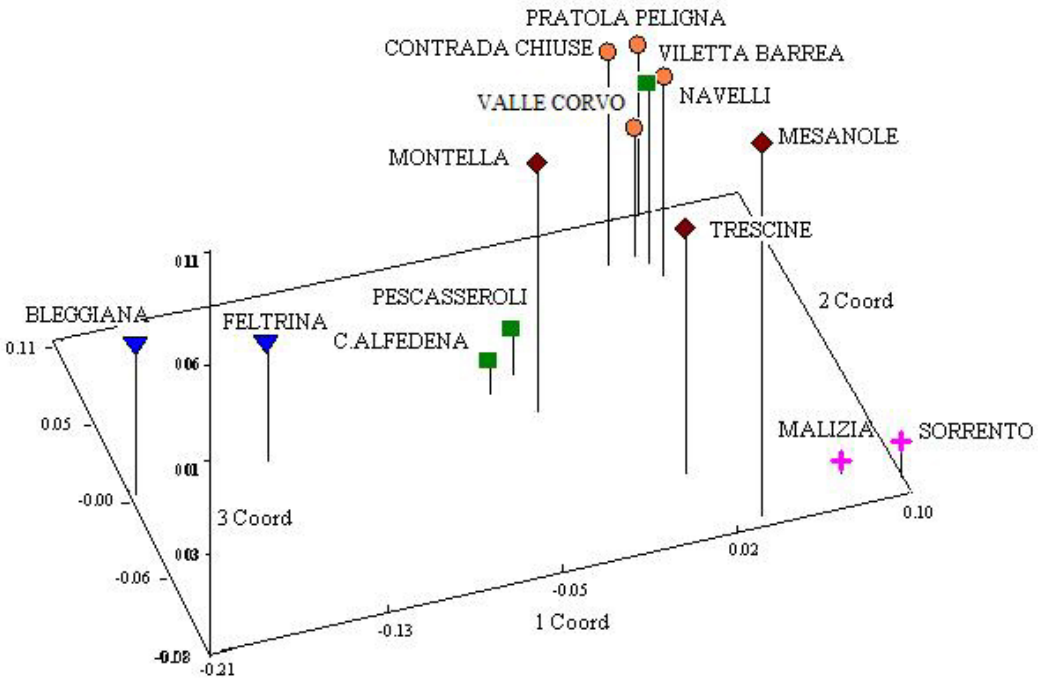

Fig. 2 Principal Coordinate Analysis of provenances and model varieties based on Population Φ_{PT} values using 134 total ISSR markers. Trees from Campania region (♦), and from Sulmona district (●) and National Park of Abruzzo (■) compared with Northern (▼) and Southern (+) Italian model varieties.

Table 4 Summary of Analysis of Variance for genetic markers (AMOVA), morphological and biochemical traits (MANOVA) in walnut genotypes, Italy.

AMOVA	**Φ_{PT} Statistic**			
	df	**Est. Var[a]**	**F-stat**	**P-level**
Among populations	13	2.127	0.1636	0.001
Within populations	342	10.869		
MANOVA	**Wilk's Lambda Statistic**			
(morphological traits)	**df**	**Est. Var[a]**	**F-stat**	**P-level**
Among populations	9	3.19702	51.399	< 0.001
Within populations	432	13.190		
MANOVA	**Wilk's Lambda Statistic**			
(biochemical traits)	**df**	**Est. Var[a]**	**F-stat**	**P-level**
Among populations	36	4.2217	13.596	< 0.001
Within populations	753	4228.50		

[a]Est. Var = Estimated Variance

9.78), were genetically more homogeneous.

The Principal Coordinate Analysis performed on the Φ_{PT} values, on the basis of 134 amplified ISSR bands, displayed the relative genetic distances of the provenances (**Fig. 2**). In a bi-dimensional plot the first and the second principal coordinates, which accounted for 43.35 and 31.72% of the variance, respectively, separated the collected walnut germplasm into four distinct groups (**Fig. 3**). The first cluster clearly included the four provenances collected in the Sulmona area (Pratola Peligna, Valle Corvo, Contrada Chiuse and Navelli) and one of three provenances from Abruzzo National Park (Villetta Barrea). Two out of the three provenances of the Campania region (Trescine and Mesanole) and Southern Italian varieties ('Sorrento' and 'Malizia') were in a close group, revealing a definite genetic distinctness from the Northern Italian varieties ('Bleggiana' and 'Feltrina'). The remaining provenances, one from Campania region (Montella) and two from Abruzzo National Park (Pescasseroli and Civitella Alfedena) were not included in the above three groups, but were placed in the centre of the plot. The third coordinate accounted for 17.15% of the variance and discriminates the provenance Montella from Pescasseroli and Civitella Alfedena.

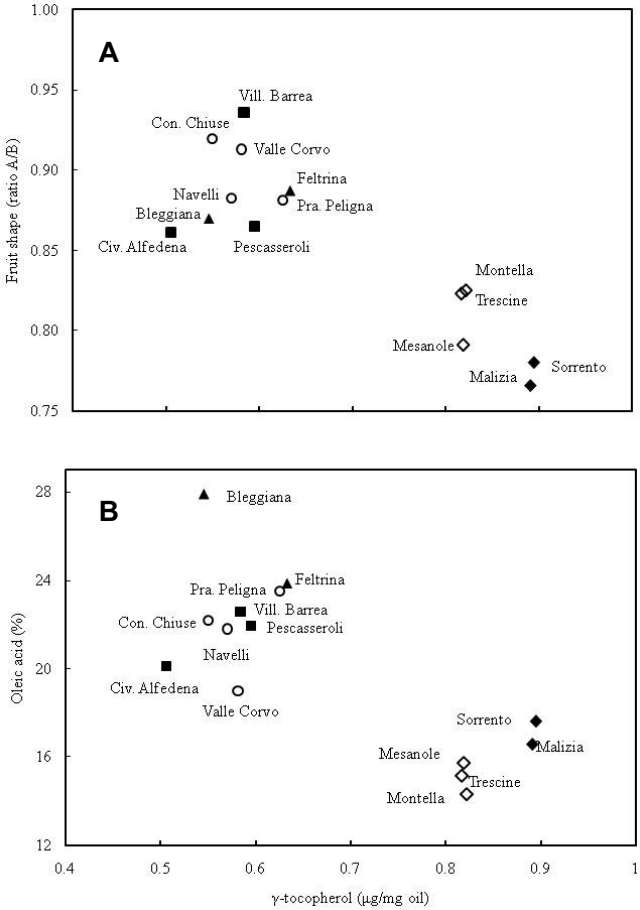

Fig. 3 Distribution of the 4 model varieties and 10 provenances based on the relationship between fruit shape (A) or oleic acid (B) and γ-tocopherol content. Trees from Campania region (◊), and from Sulmona district (○) and National Park of Abruzzo (■) compared with Northern (▲) and Southern (♦) Italian model varieties.

Morphological traits

The morphological traits of the fruits are reported in **Table 5**. The dry weight of the kernels ranged from 6.72 ± 0.88 g for Pescasseroli to 11.48 ± 0.92 g for Southern model variety 'Malizia'. The Northern model variety 'Bleggiana' and Pescasseroli provenance showed small fruit size and weight values but with a high intra-individual weight variability (6.73 ± 1.44 g). From equatorial and polar diameters the ratio was calculated to obtain an indication of the fruit shape (**Table 5**). This character divided all provenances and the model varieties in three distinct groups. The first group included fruits of Northern model variety 'Feltrina'; nuts collected in Sulmona area (Pratola Peligna, Valle Corvo, Contrada Chiuse and Navelli) and in Villetta Barrea site (Abruzzo National Park) exhibited an oblong ovate shape (ratio ranging from 0.936 to 0.881). The second group clustered 'Bleggiana' variety, Civitella Alfedena and Pescasseroli provenances (Abruzzo National Park) showing roundish fruits (ratio from 0.869 to 0.861). Montella, Mesanole, Trescine provenances and the two Southern model varieties, 'Sorrento' and 'Malizia', all from Campania region were characterized by oblong elliptic fruits (ratio from 0.825 to 0.766) and formed the third group.

The analysis of variance for dry weight, equatorial and polar diameters per nut is shown in **Table 6**; significant differences were found for all these characters ($p \leq 0.01$). The MANOVA analysis of morphological data attributed 19.54% of variance among groups and 80.46% within groups, congruent with AMOVA values obtained for genetic ISSR markers (**Table 4**). Mantel test detected low, but significant, statistical correlation between ISSR and morphological data (r = 0.48, $p = 0.004$).

Biochemical traits

Oil, fatty acids and tocopherol were measured in kernels of a subset (190) of walnut genotypes. Data of oil, oleic acid, linoleic acid (ω_6), linolenic acid (ω_3) and γ-tocopherol were reported in **Table 7**. The analysis of variance (**Table 6**) indi-

Table 5 Morphological traits of fruits measured for a subset of 190 walnut genotypes from the provenances and model varieties (mean ± SD).

Provenance	Equatorial diameter (mm)	Polar diameter (mm)	Fruit shape[a]	Dry weight (g)
Prat. Peligna	27.80 ± 1.59	31.53 ± 2.89	0.8816	7.63 ± 1.11
Valle Corvo	30.31 ± 1.58	32.97 ± 2.01	0.9193	9.83 ± 1.3
Con. Chiuse	29.10 ± 2.06	31.75 ± 2.37	0.9165	8.98 ± 1.9
Navelli	29.43 ± 2.92	33.43 ± 2.17	0.8829	10.11 ± 1.14
Vill. Barrea	28.76 ± 2.73	30.72 ± 2.28	0.9361	7.97 ± 1.34
C.Alfedena	28.23 ± 0.99	32.77 ± 2.41	0.8614	8.92 ± 0.74
Pescasseroli	26.84 ± 1.3	31.29 ± 2.17	0.8652	6.72 ± 0.88
'Bleggiana'	26.50 ± 2.49	30.50 ± 2.06	0.8698	6.73 ± 1.44
'Feltrina'	28.60 ± 1.38	32.73 ± 4.58	0.8871	7.47 ± 1.40
'Sorrento'	32.57 ± 2.06	41.82 ± 2.11	0.7804	10.56 ± 1.49
'Malizia'	31.07 ± 1.63	40.96 ± 4.13	0.7660	11.48 ± 0.92
Trescine	29.18 ± 1.6	35.62 ± 3.23	0.8230	9.39 ± 1.46
Mesanole	28.60 ± 1.41	36.62 ± 2.16	0.7910	9.63 ± 1.06
Montella	30.61 ± 2.19	37.21 ± 2.89	0.8250	9.51 ± 1.75
Mean value	29.11 ± 1.63	34.28 ± 3.66	0.857 ± 0.05	8.92 ± 1.43

[a] Equatorial / Polar ratio

Table 6 Analysis of variance for morphological and biochemical traits of walnut fruit measured for a subset of 190 genotypes from provenances and model varieties.

Mean square (morphological traits)				
Source	**df[a]**	**Dry weight**	**Equatorial diameter**	**Polar diameter**
Group	13	32.263	45.003	232.731
	176			
error		1.842	3.427	7.340
P-value		1.75E-25	5.55E-20	2.35E-39

Mean square (biochemical traits)						
Source	**df**	**Total oil**	**ω3 Acid**	**ω6 Acid**	**Oleic acid**	**γ-Tocopherol**
Group	13	136.012	54.063	123.635**	220.082	0.297
	176					
error		17.170	9.569	18.000	23.335	0.011
P-value		2.42E-12	1.38E-0.8	1.24E-10	1.07E-14	2.20E-34

[a] **df** for single provenances and for total genotypes

Table 7 Biochemical traits of fruits for a subset of 190 walnut tree genotypes from the provenances and the model varieties (mean ± SD).

Provenance	γ-Tocopherol (µg/mg oil)	Total oil (% dry weight)	ω3 Acid (%)	ω6 Acid (%)	Oleic acid (%)
Prat. Peligna	0.62 ± 0.11	54.95 ± 4.35	15.93 ± 2.26	60.56 ± 5.15	23.50 ± 4.36
Valle Corvo	0.58 ± 0.13	52.09 ± 7.55	16.67 ± 3.38	64.33 ± 3.56	18.99 ± 5.57
Con. Chiuse	0.55 ± 0.09	54.46 ± 3.73	15.11 ± 2.54	62.69 ± 4.23	22.19 ± 4.43
Navelli	0.57 ± 0.06	52.01 ± 3.39	16.40 ± 2.93	61.80 ± 3.32	21.79 ± 4.37
Vill. Barrea	0.58 ± 0.07	54.12 ± 3.92	17.95 ± 3.20	59.47 ± 3.68	22.56 ± 4.10
C. Alfedena	0.50 ± 0.06	53.35 ± 4.43	16.94 ± 1.55	62.95 ± 4.14	20.10 ± 4.52
Pescasseroli	0.59 ± 0.11	53.49 ± 3.18	17.91 ± 2.68	60.16 ± 3.00	21.91 ± 3.13
'Bleggiana'	0.54 ± 0.12	55.35 ± 4.94	14.44 ± 3.37	57.42 ± 6.33	27.80 ± 8.24
'Feltrina'	0.63 ± 0.13	53.38 ± 1.80	15.58 ± 2.64	60.53 ± 7.03	23.87 ± 9.35
'Sorrento'	0.89 ± 0.01	61.05 ± 3.19	19.43 ± 3.43	62.42 ± 3.79	17.62 ± 2.46
'Malizia'	0.89 ± 0.01	58.66 ± 1.52	20.65 ± 3.16	62.47 ± 3.12	16.59 ± 1.82
Trescine	0.81 ± 0.11	60.70 ± 4.82	19.99 ± 4.28	64.85 ± 2.19	15.15 ± 4.40
Mesanole	0.81 ± 0.14	57.53 ± 5.95	17.98 ± 2.87	66.27 ± 5.23	15.73 ± 5.76
Montella	0.82 ± 0.21	52.42 ± 4.24	16.52 ± 3.27	69.15 ± 4.69	14.31 ± 5.90
Mean value	0.67 ± 0.14	55.25 ± 3.04	17.25 ± 1.83	62.51 ± 2.98	20.15 ± 3.90

Table 8 Pearson's coefficient calculated among morphological and biochemical traits of walnut fruits of 190 genotypes from provenances and model varieties.

Component	1	2	3	4	5	6	7	8
1 - Eq. diameter								
2 - Polar diameter	0.83**							
3 - Dry weight	0.86**	0.80**						
4 - Shape	-0.46 ^{ns}	-0.86**	-0.54*					
5 - γ-tocopherol	0.68**	0.91**	0.62*	-0.86**				
6 - ω3-Acid	0.51^{ns}	0.70**	0.57*	-0.66**	0.72**			
7 - ω6-Acid	0.53*	0.51^{ns}	0.58*	-0.40^{ns}	0.53*	0.25^{ns}		
8 - Oleic acid	-0.67**	-0.75**	-0.73**	0.64*	-0.77**	-0.67**	-0.88**	
9 - Total oil	0.38^{ns}	0.65*	0.38 ^{ns}	-0.71**	0.73**	0.71**	0.07^{ns}	-0.41^{ns}

* indicates significance at $P \leq 0.05$
** indicates significance at $P \leq 0.01$
^{ns} no significance

cated large significant differences among groups for all these biochemical traits of fruit ($p \leq 0.01$).

The oil content per kernel dry weight ranged between 52.01% for Navelli provenance to 61.05% for 'Sorrento' variety. The composition of fatty acids, stored in the seeds as triglyceride esters, revealed that the oleic and ω_6 acids content varied from 14.31% (Montella) to 27.80% ('Bleggiana') and from 57.42% ('Bleggiana') to 69.15% (Montella), respectively. The provenance Trescine (19.99 ± 4.28) and the varieties 'Sorrento' (19.43 ± 3.43) and 'Malizia' (20.65 ± 3.16) genotypes, all growing in Southern Italy, produced nuts with the highest percent levels of ω_3 acid (**Table 7**).

The γ-tocopherol content accounted for over 90% of total tocopherols in kernel oils. The highest tocopherol concentration was found in 'Sorrento' (0.895 ± 0.008 µg/mg) and 'Malizia' (0.891 ± 0.008 µg/mg) varieties, whereas the lowest levels were measured in the provenance Civitella Alfedena (0.506 ± 0.06 µg/mg) and 'Bleggiana' variety (0.546 ± 0.12 µg/mg) (**Table 7**).

The MANOVA analysis of biochemical data (**Table 4**) attributed 0.06% of variance among and 99.94% within the groups. These results can be, at least partially, expected considering the high standard deviation values within each provenance and variety. Furthermore, relative biochemical homogeneity was observed between the provenances collected inside a region, either for Abruzzo and Campania. Mantel's test, based on Φ_P value and Euclidean distance was performed to test a possible correlation among genetic markers and biochemical traits. It showed a weak but significant statistical correlation between ISSR markers and biochemical traits (r = 0.39; $p = 0.008$), whereas the correlation computed on the averaged distances (Euclidean distances) based on morphological and biochemical data showed strong significance (r = 0.70, p=0.001). Pearson's correlation coefficients between morphological traits and biochemical components were given in **Table 8**. In general, a negative coefficient between nut shape and some biochemical traits (γ-tocopherol, linolenic acid, total oil) were found. In particular, the γ-tocopherol component was negatively correlated with shape of fruit (r = -0.86; $p \leq 0.01$) and with oleic acid (r = -0.77; $p \leq 0.01$) and positively correlated with linolenic (r = 0.73; $p \leq 0.01$) and linoleic acid ((r = 0.53; $p \leq 0.05$) (**Table 8**). Nevertheless, these correlation values between γ-tocopherol, nut shape and fatty acids may be biased by the clustering of provenances and model varieties in two distinct groups as shown in **Fig. 3A** and **3B**.

DISCUSSION

The tested 11 ISSR primers, in consequence of their high sequence variability, exhibited a strong ability to discriminate inside the walnut provenances and the four Italian varieties, even if the detected polymorphism level (73.8%) was lower than the values of 95.9% and 100% observed in *Prunus* (Goulão *et al*. 2001) and *Olea europea* (Hess *et al*. 2000), respectively. However, the degree of polymorphism (73.8%) computed in this study was higher than 57% and 65.7% previously reported for *J. regia* cultivars (Potter *et al.* 2002; Pollegioni *et al.* 2003).

Molecular markers divided all germplasm in four distinct groups. Two of three Campania region provenances (Trescine and Mesanole) showed high genetic closeness with the two Southern Italian model varieties ('Sorrento' and 'Malizia') both originated in the Sorrentina peninsula. The morphological and biochemical parameters of fruits were also relatively similar. The Southern model varieties were famous for their quality of fruits with oblong elliptic shape (ratio of equatorial/polar diameters: 0.75-0.78), high dry weight of kernel and elevated amount of total lipids (55-65% of dry weight), the major fatty acids of the Southern varieties were linoleic (~62%), linolenic (~20%) and oleic (~17%) acids (Limongelli 1993; Vergano *et al.* 1995). The data presented here for the 'Sorrento' and 'Malizia' varieties were congruent to the previously data above reported. These characteristics were also all present in Trescine and Mesanole provenances. Since Trescine and Mesanole provenances showed high genetic, morphological and biochemical similarity with Italian Southern varieties, it can be easily hypnotize that trees in Trescine and Mesanole were originated from seeds (from free pollination) related to the Southern varieties. The Montella provenance was also located in Campania region, but was genetically distinct from other provenances and varieties from the same region. However, Montella showed a combination of morphological and biochemical fruit traits (oblong elliptic shape of nuts, dry kernel weight, γ-tocopherol and PUFAs content) similar to Mesanole and Trescine provenances and 'Sorrento' and 'Malizia' varieties. Moreover, Montella fruits contained a high level of linoleic acid (69.15 ± 4.69% of total fatty acids) that exceeded the values previously detected in other walnut genotypes (Vergano *et al.* 1995; Zwarts *et al*. 1999; Cağlarirmak 2003; Verardo *et al.* 2009). It is well known that, aside from genetic background, factors as environment and/or agricultural practices can affect the fatty acid composition. It has been proved that a general inverse relationship between PUFAs and growth temperature exists: PUFAs increase with decreasing temperature in membrane as well as in seed storage lipids (Neidleman 1987; Rennie and Tanner 1989). This relationship was the result of microsomal ω_6-desaturase enzyme activity increase at low growth temperature (Tocher *et al.* 1998). Nevertheless, as reported recently by Verardo *et al.* (2009), excess nitrogen fertilization can lead to a higher level of linoleic acid in the walnut kernel. Although Montella is located in Southern Italy, it is an Apennine plateau (altitude: 670 m) characterized by abundant rainfall (1341.3 mm), relatively low temperature (annual mean 12.8°C) and limy soil, with pyroclastic and alluvial deposit, reach in mineral nutrients including nitrogen, phosphorous and potassium. The linoleic acid content in Montella provenance fruits may be influenced by both cold temperature and/or nitrogen availability in soil either during the trees growing season and fruits development and filling period.

The genotypes located in the Sulmona district, at different altitudes (Navelli 685 m, Contrada Chiuse 500 m,

Valle Corvo 430 m, Pratola Peligna 330 m) and in Villetta Barrea (Abruzzo National Park, 1000 m) clustered together and were genetically differentiated from the other walnut genotypes collected. This was also noticeable for the oblong ovate shape of the fruits. Nevertheless, the high individual heterogeneity hindered to identify any genetic structure within the Sulmona group. The large difference in local environmental conditions confirmed the high adaptability of walnut species, as noted for other tree species (Schlichting 1986). The genetic differentiation of Sulmona group could be due to the selection and propagation, in the past centuries, of local genotypes for wood or/and fruit production. The closeness of the traits noticed in the Villetta Barrea provenance to the Sulmona genotypes suggested that the origin of the former lies within the Sulmona valley. In the last centuries, every summer the local shepherds moved their herds from the Sulmona valley to the high pastures of the mountains, taking with them the necessary supplies and foodstuffs, including walnut nuts for their high nutritive value. It could be supposed that some of these seeds abandoned along the road have generated trees genetically similar to the Sulmona genotypes in the Villetta Barrea pasture areas, traditionally recognized as one of the summer pasture locality (Paone 2002).

The Pescasseroli and Civitella Alfedena provenances, sampled in the Abruzzo National Park at 1100-1200 m of altitude, with an annual mean temperature of 7.8°C and abundant rainfall (annual mean 1520 mm), resulted genetically distinct from the other *J. regia* germplasm collected in Abruzzo region. Plants surveyed in Pescasseroli and Civitella Alfedena showed small and roundish fruits, having a relatively high oleic and low linoleic acids content such as 'Bleggiana' variety (Alpine valley). The expansion rate of fruit is mostly related to the temperature in the post setting and development. The number and the size of cells in fruits growing in warmer conditions are greater than those on plants cultivated in cooler condition. This response to temperature has been observed in several herbaceous and arboreal species such as tomato (Adams *et al.* 2001), melon (Higashi *et al*. 1999), maize, pea and sunflower (Francis and Barlow 1988), apple (Bergh 1990), and banana (Jullien *et al*. 2001).

Although in this study a high variance within provenances was observed using biochemical traits of fruits, it is interesting to note that the oleic acid content significantly declined, while the essential PUFAs and γ-tocopherol content were increasing in all provenances of Campania region and in the Southern model varieties. The opposite trend was detected in the nuts derived from the Sulmona area, the Abruzzo National Park and the two Northern model varieties. Tocopherols were correlated negatively to oleic acid and positively to linoleic and linolenic acid content in seeds of several species (Kamal-Eldin and Andersson 1997; Dolde *et al.* 1999; Richards *et al.* 2008).

Tocopherols are a major lipid soluble antioxidant present in the PUFA enriched tissues. Between tocopherol isomers the highest vitaminic activity was attributed to α-tocopherol, nevertheless γ- and δ-tocopherol act as more effective antioxidants to protect the fats (Lehmann *et al.* 1994). Tochopherol levels increase in the responses to a variety of abiotic stresses and this was often cited as evidence of its protective role (Munne-Bosch and Alegre 2002). In walnut nuts γ-tocopherol as the dominant isomer (80-97%) has been reported by several authors (cited in Lavedrine *et al.* 1997). Comparing two walnut varieties cultivated in two geographic areas, California (USA) and Dauphine (France), Lavedrine *et al.* (1997) suggested that the geographical origin influence seemed to be more important than the varietal one for the tocopherol content. The data presented here for Italian walnut provenances and model varieties confirmed the relationship between tocopherol content and oil in seeds, and tocopherol and nut shape, giving further indication of the influence of both genotype and environment on tocopherol content and its putative protective antioxidant role.

The results prove that the multidisciplinary analysis combining molecular, morphological and biochemical markers, is a valuable approach to increase the basic knowledge of *J. regia* species, to evaluate walnut biodiversity and to recover the neglected germplasm. The Montella and Pescasseroli provenances, showing peculiar genetic, morphological and biochemical characters, can be promising sources of seed for nurseries and hardwood plantations. Till now fruit traits had been considered; in the future some wood traits such as wood density or homogenous annual ring, and/or sensitivity to pests and pathogens can be integrated in the multidisciplinary approach for biodiversity identification and selection of seed sources. The presented data can support the idea that the search of walnut provenances in different area along Italy can be profitable and may have a positive effect either on biodiversity identification or on the economical and practical exploitation.

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REFERENCES

Abuin M, Díaz R, Fernández-Lopez J (2002) Identification of *Juglans regia* L. genotypes using RAPDs molecular markers. *Forest Genetics* **9**, 31-37

Adams SR, Cockshull KE, Cave CRJ (2001) Effect of temperature on the growth and development of tomato fruits. *Annals of Botany* **88**, 869-877

Amaral JS, Casal S, Pereira JA, Seabra RM, Oliveira BP (2003) Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. *Journal of Agriculture and Food Chemistry* **51**, 7698-7702

Amini F, Saeidi G, Arzani A (2008) Study of genetic diversity in safflower genotypes using agro-morphological traits and RAPD markers. *Euphytica* **163**, 21-30

Bergh O (1990) Effect of temperature during the first 42 days following full bloom on apple fruit growth and size at harvest. *South African Journal of Plant Soil* **7**, 11-18

Cağlarirmak N (2003) Biochemical and physical properties of some walnut genotypes (*Juglans regia* L.). *Nahrung/Food* **47**, 28-32

Callaham RZ (1964) Provenance research: Investigation of genetic diversity associated with geography. *Unasylva* **18**, 40-50

Carr R (2008) XLStatistics 08.05.12, XLent Works, Australia. Available on line http://www.deakin.edu.au/~rodneyc/XLStatistics

Chenevard D, Frossard JS, Lacointe A (1994) Lipid utilization and carbohydrate partitioning during germination of English walnut (*Juglans regia*). *Annals of Forest Science* **51**, 373-379

Dangl GS, Woeste K, Aradhya MK, Koehmstedt A, Simon C, Potter D, Leslie CA, McGranahan G (2005) Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut. *Journal of the American Society for Horticultural Science* **130**, 348-354

Dolde D, Vlahakis C, Hazebroek J (1999) Tocopherols in breeding lines and effects of planting location, fatty acid composition, and temperature during development. *Journal of the American Oil Chemists' Society* **76**, 349-355

Doyle JJE, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11-15

Eastmond PJ, Graham IA (2001) Re-examining the role of the glyoxylate cycle in oilseeds. *Trends in Plant Science* **6**, 72-77

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491

FAO (1997) State of the World's Forests. Food and Agriculture Organization Rome Italy. Available online: http://www.fao.org/docrep/W4345E/W4345E00.htm

Fjellstrom RG, Parfitt DE (1994) Walnut (*Juglans* spp.) genetic diversity determined by restriction-fragment length polymorphisms. *Genome* **37**, 690-700

Fornari B, Cannata F, Spada M, Malvolti ME (1999) Allozyme analysis of genetic diversity and differentiation in European and Asiatic walnut (*Juglans regia* L.) populations. *Forest Genetics* **6**, 115-127

Foroni I, Rao R, Woeste K, Gallitelli M (2005) Characterization of *Juglans regia* L. with SSR markers and evaluation of genetic relationship among cultivars and the Sorrento landrace. *Journal of Horticultural Science and Biotechnology* **80**, 49-53

Francis D, Barlow PW (1988) Temperature and the cell cycle. In: Long SP, Woodward FI (Eds) *Plants and Temperature , 42nd Symposium of the Society*

for Experimental Biology, Company of Biologist, Cambridge, UK, pp 181-201

Goulão JE, Monte-Corvo L, Oliveira CM (2001) Phenetic characterization of plum cultivars by high multiplex ratio markers: amplified fragment length polymorphisms and inter-simple sequence repeats. *Journal of the American Society for Horticultural Science* **126**, 72-77

Hemery GE, Savill PS, Thkur A (2005) Height growth and flushing in common walnut (*Juglans regia* L.): 5-year results from provenance trials in Great Britain. *Forestry* **78**, 121-133

Hemery GE (2008) Forest management and silvicultural responses to projcted climate change impacts on European broadleaved trees and forests. *International Forestry Review* **10**, 591-607

Hess J, Kadereit JW, Vargas P (2000) The colonisation history of *Olea europea* L. in Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and inter simple sequence repeats (ISSR). *Molecular Ecology* **9**, 857-868

Higashi K, Hosoya K, Ezura H (1999) Histological analysis of fruit development between two melon (*Cucumis melo* L. *reticulatus*) genotypes setting a different size of fruit. *Journal of Experimental Botany* **50**, 1593-1597

Huntley B, Birks HJB (1983) *An Atlas of Past and Present Pollen Maps for Europe: 0-13000 Years Ago*, Cambridge University Press, New York, USA, 667 pp

Jullien A, Munier-Jolain NG, Malezieux E, Chillet M, Ney B (2001) Effect of pulp cell number and assimilate availability on dry matter accumulation rate in a banana fruit (*Musa* sp. AAA group "Grande naine" (Cavendish subgroup)). *Annals of Botany* **88**, 321-330

Kamal–Eldin A, Andersson R (1997) A multivariate study of the correlation between tocopherol content and fatty acid composition in vegetable oils. *Journal of the American Oil Chemists' Society* **74**, 375-380

Kleinschmit JRG, Bacilieri R, Kramer A, Roloff A (1995) Comparison of morphological and genetic traits in pedunculate oak (*Q. robur*) and sessile oak (*Q. petraea* (Matt.) Liebl.). *Silvae Genetica* **44**, 256-269

Kornsteiner M, Wagner KH, Elmafda I (2006) Tocopherols and total phenolics in 10 different nut types. *Food Chemistry* **98**, 381-387

Lavedrine F, Ravel A, Poupard A, Alary J (1997) Effect of geographic origin, variety and storage on tocopherol concentrations in walnuts by HPLC. *Food Chemistry* **58**, 135-140

Lehmann JW, Putman DH, Qureshi AA (1994) Vitamin E isomers in grain amaranths (*Amaranthus* spp.). *Lipids* **29**, 177-181

Limongelli F (1993) A new cultivar "Malizia". *Acta Horticulturae* **311**, 46-48

Malvolti ME, Fineschi S, Pigliucci M (1994) Morphological integration and genetic variabilità in *Juglans regia* L. *Journal of Heredity* **85**, 389-394

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**, 209-220

McGranahan G, Leslie C (1991) Walnuts (*Juglans*). *Acta Horticulturae* **290**, 907-974

Munne-Bosch S, Alegre L (2002) The function of tocopherols and tocotrienols in plants. *Critical Reviews in Plant Sciences* **21**, 31-57

Neidleman SL (1987) Effects of temperature on lipid unsaturation. *Biotechnology and Genetic Engineering Reviews* **5**, 245-268

Paone N (2002) La transumanza appenninica: storia di un efficente sistema economico. In: *Agricoltura e Paesaggio*, Accademia dei Georgofili, Florence, Italy, pp 11-18

Peakall R, Smouse PE (2006) GenAlEx 6: Genetic Analysis in Excel. Available on line: http://www.anu.edu.au/BoZo/GenAlEx/index.php

Pigliucci M, Villani F. Benedettelli S (1990) Geographic and climatic factors associated with the spatial structure of gene frequencies in *Castanea sativa* Mill. from Turkey. *Heredity* **69**, 141-149

Pigliucci M, Paoletti C, Fineschi S, Malvolti ME (1991) Phenotipic integration in chestnut (*Castanea sativa* Mill.): leale versus fruits. *Botanical Gazzette* **152**, 514-521

Pollegioni P, Bartoli S, Cannata F, Malvolti ME (2003) Genetic differentiation of four Italian walnut (*Juglans regia* L.) varieties by inter simple sequence repeat (ISSR). *Journal of Genetics and Breeding* **57**, 231-240

Pollegioni P, **Woeste K, Major A, Scarascia Mugnozza G, Malvolti ME** (2009) Characterization of *Juglans nigra* (L.), *Juglans regia* (L.) and *Juglans x intermedia* (Carr.) by SSR markers: a case study in Italy. *Silvae Genetica* **58**, 68-78

Potter D, Gao F, Aiello G, Leslie C, McGranahan G (2002) Inter simple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars. *Journal of the American Society for Horticultural Science* **127**, 75-81

Ramirez-Valiente JA, Lorenzo Z, Soto A, Valladares F, Gil L, Aranda I (2009) Elucidating the role of genetic drift and natural selection in cork oak differentiationregarding drought tolerance. *Molecular Ecology* **18**, 3803-3815

Rennie BD, Tanner JW (1989) Fatty acid composition of oil from soybean seeds grown at extreme temperatures. *Journal of the American Oil Chemists' Society* **66**, 1622-1624

Richards A, Wijesundera C, Salisbury P (2008) Genotype and growing environment effects on the tocopherols and fatty acids of *Brassica napus* and *B. Juncea*. *Journal of the American Oil Chemists' Society* **85**, 159-168

Rohlf FJ (2001) NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System. V 2.1. Exeter Software, Setauket, NY, USA

Schlichting CD (1986) The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* **17**, 667-693

Solar A, Smole J, Stampar F (1994) Characterization of isozyme variation in walnut (*Juglans regia* L.). *Euphytica* **77**, 105-112

Tocher DR, Leaver MJ, Hodgson PA (1998) Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Progress in Lipid Research* **37**, 73-117

Torres-Diaz C, Ruiz E, Gonzalez F, Fuentes G, Cavieres LA (2007) Genetic diversity in *Nothofagus alessandrii* (Fagaceae), an endangered endemic tree species of the Coastal Maulino Forest of Central Chile. *Annals of Botany* **100**, 75-82

UPOV (1999) Guidelines for the conduct of tests for distinctness, uniformity and stability. Walnut (*Juglans regia* L.). TG/125/6, Geneva, Switzerland, 34 pp

Verardo V, Bendini A, Cerretani L, Malaguti D, Cozzolino E, Caboni MF (2009) Capillary gas chromatography analysis of lipid composition and evaluation of phenolic compounds by micellar electrokinetic chromatography in Italian walnut (*Juglans regia* L.): irrigation and fertilization influence. *Journal of Food Quality* **32**, 262-281

Vergano G, Botta R, Radicati L (1995) Preservation of *Juglans regia* L. nuts under different storage conditions. *Acta Horticulturae* **379**, 451-457

Zietkiewicz E, Rafalsky JA, Labua D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20**, 176-183

Zwarts L, Savage GP, McNeil DL (1999) Fatty acid content of New Zealand-grown walnuts (*Juglans regia* L.). *International Journal of Food Sciences and Nutrition* **50**, 189-194