ALLOZYME ANALYSIS OF GENETIC DIVERSITY AND DIFFERENTIATION IN EUROPEAN AND ASIATIC WALNUT (*JUGLANS REGIA* L.) POPULATIONS

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ABSTRACT

Genetic structure of 12 anthropised European and 3 natural and naturalised Asiatic populations of walnut (*Juglans regia* L.) was investigated by means of starch gel electrophoresis for 15 enzyme systems. Population genetic parameters and statistics show overall levels of genetic diversity and differentiation lower in *Juglans regia* than in other widespread plant species, outlining a significant amount of genetic erosion suffered by this species, mainly in Europe. The levels of differentiation among European and Asiatic populations, higher than those among European populations, support the thesis of a native origin of European walnut in postglacial times. The closeness to the HW equilibrium and the high levels of heterozygosity and intrapopulation differentiation, found in all the populations, show the capability of the species to avoid self-pollination and inbreeding. The occurrence of some different alleles among European and Asiatic populations provides an interesting item for programs of recovering and conservation of the genetic variability in this endangered widespread species.

Key words: *Juglans regia*, isozymes, genetic diversity, genetic differentiation, widespread plant species, conservation.

INTRODUCTION

Juglans regia L., whose present day distribution ranges from about the 10th to about the 50th parallel northern latitude (Figure 1), plays a considerable role in agroforest economy both for valuable wood and quality seed production.

It belongs to a monoic wind pollinated tree genus characterised by a reproductive strategy based on a nearly complete outcrossing. This is achieved through heterodichogamy, to prevent self pollination (LUZA & POLITO 1988), and through a reduced fertility of female flowers in presence of an excess of pollen grains on pistils (BEINEKE & MASTERS 1976; SZENTIVANYI 1990), likely to hinder inbreeding, as can be gathered considering the limited dispersal distance of walnut seeds.

J. regia is thought primarily differentiate within its genus during Eocene period of Cainozoic Era in mountains of central Asia (MANCHESTER 1987; FJELLSTROM & PARFITT 1995). Presently the species is reported to be native from south-eastern Europe to north-western China (Xinjiang province) through Turkey, Caucasus, northern Iraq, Iran, Pakistan and India, Pamir, Nepal, Himalaya and Tibet (LESLIE & MCGRANAHAN 1988). From north-western China, or from Tibet, *J. regia* was probably introduced into central and eastern China over 4000 years ago, where it still occurs both as cultivated and spontaneous forms (ZHENG 1978). In Europe it is still debated if the species was extinguished during the Pleistocene glaciations or survived in some refugia in peninsular Italy and in Balkans, as suggested by some paleopalynologic studies (HUNTLEY & BIRKS 1983).

In any case during the Holocene, after a prehistoric phase of slow natural propagation, in the last 3000 years the diffusion of *J. regia* in western Europe was ruled by man and presently it is mainly concentrated around human settlements, roads and crop fields, whereas in eastern Europe it is also present as minor component in mixed forests. Human management allowed a fast diffusion of *J. regia*, with an estimated rate of 400 $m \cdot y^{-1}$ (HUNTLEY & BIRKS 1983), but likely affected its genetic structure with a loss of diversity and a decrease of differentiation. At this regard, in a multipurpose species like *J. regia*, besides the genetic erosion caused by the favoured propagation of the best seed producers, the direct dysgenic effect caused by the search of the best quality wood must be considered, which gives rise to the removal of the most valuable trees and the consequent survival of inferior trees left to reproduce.

Since the conservation of a long lived tree species does not depend on its census, but on its chance to adapting to environmental changing conditions, namely on its genetic diversity and differentiation; *J. regia* deserves the inclusion in programs of genetic recovering and protection.

In this paper the genetic structure of a representative

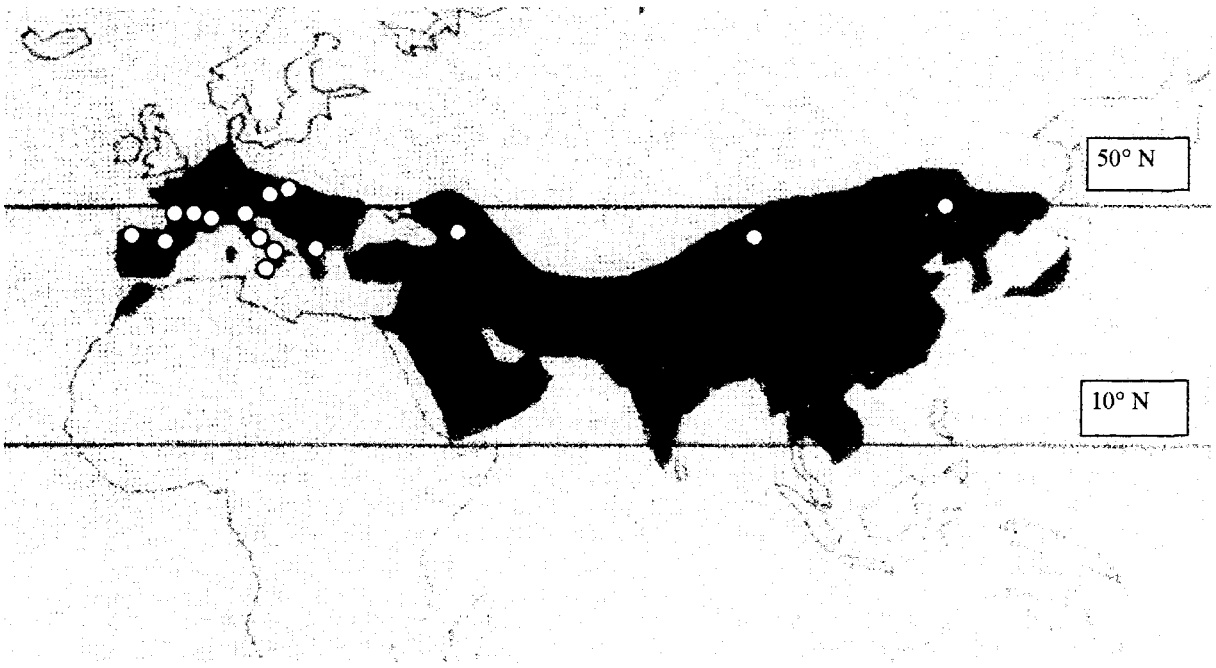

Figure 1. Natural range of *Juglans regia.* in Eurasia and geographic location of the 15 regional populations assayed.

sample of European populations of *J. regia* was investigated to outline and quantify possible genetic erosion in comparison with natural (NW China) and naturalised (Caucasus and NE China) Asiatic populations, with the aim to give a contribution to promote a correct management of the genetic resources of this species both by increasing the number of genotypes for breeding programmes and by encouraging in situ and ex situ conservation interventions.

MATERIALS AND METHODS

Sample collection

It must be considered that at present, because of the human intervention, no totally natural population of *Juglans regia* still exists, except possibly in the Chinese Xinjiang province (ZHENG 1978). In spite of that, wild plants, born from local seeds, are diffused everywhere in European agricultural lands. Thus in this paper the word 'population' is used in the conventional meaning of 'whole of plants'.

In absence of natural reefs, plants within a maximum distance of 10 km from each other were considered as belonging to the same deme. The number of individuals collected is not the same in each population because of the different plant density found in each deme.

A total of 986 individuals were sampled from 29 demes of *Juglans regia* in 15 regional populations of Europe (21 demes, 12 regions), Caucasus (6 demes, 1 region) and China (2 demes, 2 regions) with a mean of 34 plants per deme and 66 plants per regional population. Sampled demes, grouped in the regional populations, are listed in table 1 and their geographic location is sketched in figure 1. In each location wintering buds were collected on the crown of individual trees and stored at –80 °C until analysis. This type of material was chosen to avoid electrophoretic variability due to ontogenetic effect of the tissues age. Caucasus material, kindly provided by Prof. Eric Germain, INRA, Bordeaux (France), came from seedlings grown in a French nursery from seeds collected in the 6 Georgian demes. Spanish samples were collected and analysed, following previously established protocols and enzymes systems, by Dr. Neus Aletà, IRTA, Reus (Spain). Chinese Sunbe population was sampled in the country with the same criterion adopted for European populations. Xinjiang population was sampled in the Chinese germplasm repository of the Institute of Pomology, Tai'an, Shandong, China, by the permission of Prof.s Wang Fencai and Wang Junji and with the help of Dr. Sun Shan.

Electrophoretic procedures

Bud tissues were homogenised in 70 µl of homogenisation buffer (KIM 1979, 1980, modified by MALVOLTI 1993a) containing 1.6 mg Tris, 4 mg soluble PVP, 0.12

Table 1. List of the 29 demes of *Juglans regia* sampled in 15 regions of Eurasia. In the last column the sample size of each regional population is reported.

Subcontin. location	Pop.	Regional location	Demes	Individuals
Europe	1	NW Spain	Ria del Ferrol	53
	2	NE Spain	Baix Ebre	60
	3	SW France	Charente-Poitou	42
	4	SC France	Puy De Dome	43
	5	SE France	Ain and Drome	52
	6	N Italy	Durlo and Friuli	76
	7	C Italy	Polverina and Sabina	58
	8	S Italy	Tardiano and S. Arsenio	57
	9	Italy, Sicily	Bivona and Anapo	68
	10	C Hungary	Dunava	40
	11	E Hungary	Tiszakorod, Nagyar, Vasarosnameny and Milota	124
	12	N Greece	Macedonia 1 and Macedonia 2	86
Caucasus	13	Georgia	Alazani, Lagodekhi, Doucheti, Laponkoury, Batsaraski and Skra	180
China	14	NW China	Xinjiang	23
	15	NE China	Sunbe	24
			Total	**986**

mg EDTA II, 50 mg DTT, 1 ml 2-mercaptoethanol and 5 ml Triton X–100, pH 7.3. The homogenate was then centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was applied on wicks of Whatman 3MM filter paper and used for electrophoresis. Electrophoresis was carried out in 11.5% starch gels as described by MALVOLTI *et al.* (1991) to identify the genotypes of the sample. Separation was obtained by applying the following buffer systems (gel buffer/electrode buffer): Tris-citric acid pH 6.5 and Ashton & Braden (1961) pH 8.00. Gels were assayed for the following 9 enzyme systems: esterase (EST, EC 3.1 .1.2), phosphoglucoisomerase (PGI, EC 5.3.1.9), shiki-mate dehydrogenase (SKDH, EC 1.1.1.25), diaphorase (DIA, EC 1.6.4.3), mannose phosphoglucoisomerase (MPI, EC 5.3.1.8), phosphoglucomutase (PGM, EC 5.4.2.2), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), aromatic alcohol dehydrogenase (AADH, EC 1.1.1.90), glutamate oxaloacetate transaminase (GOT, 2.6.1.1). Genetic analysis of PGI, PGM, EST, PGDH, and SKDH was according to ARUSELKAR *et al.* (1985, 1986) and ALETÀ *et al.* (1993). For the other systems, putative loci were identified on the basis of analysis of half-sib families (MALVOLTI *et al.*, 1993b, 1995, 1997). 15 loci were scored: *Est–1, Pgi–1, Pgi–2, Skdh–1, Skdh–2, Dia–1, Dia–3, Mpi–1, Pgm–1, Pgd–2, Aadh–2, Got–1, Got–3, Got–4* and *Got–5*, where the locus specifying the most anodally migrating allozyme is designed as 1, the next 2 and so on.

Genetic analysis

Genetic structure of the sample was analysed at two classification levels: subcontinental (Europe, Caucasus, northwestern and north / eastern China) and regional population level. According to the supposed phylogenesis of *Juglans regia* (LESLIE & MCGRANAHAN 1988; HUNTLEY & BIRKS *1983*) the two classifications should correspond to preglacial and postglacial phylogenic relationships. Genetic diversity within samples was evaluated with the following parameters, calculated across the polymorphic loci: actual allelic multiplicity N_a, effective allelic multiplicity N_e (KIMURA & CROW 1964; GREGORIUS 1978), observed H_o and HW expected H_e heterozygosities and the fixation index F (WRIGHT 1951, 1965). Within and among populations genetic differentiations were evaluated by means the indices of Gregorius δ_{in} (inner population differentiation, δ_T in Gregorius notation) and δ_{ou} (differentiation of each population from its complement in the whole set, D_j in Gregorius notation; GREGORIUS & ROBERDS 1986; GREGORIUS 1987) and with the WRIGHT (1978) and NEI (1987) statistics, helping a comparison with other analyses. Because of an expected low level of differentiation due to the trimillennial human management, genotype frequencies were used to calculate Gregorius indices, in order to enhance their discrimination. Wright indices were estimated with the Weir and

Table 2. Genotype P_{ij} ($j \geq i$) and allele P_i relative frequencies in polymorphic loci of European population samples. P_{ij} and P_i are reported in the bordered triangular and column matrices respectively. Numbers in columns N are sample sizes, their fluctuations are due to missing data.

	Skdh–1				*Skdh–2*				*Dia–1*				*Dia–3*				*Mpi–1*			
	N	P_{ij}		P_i	N	P_{ij}		P_i	N	P_{ij}		P_i	N	P_{ij}		P_i	N	P_{ij}		P_i
NW	46	.196	.543	.467	46	0	.022	.011	52	.231	.538	.500	53	.491	.415	.698	42	.048	.190	.143
Sp.			.261	.533			.978	.989			.231	.500			.094	.302			.762	.857
NE	60	.083	.383	.275	60	0	0	0	60	.500	.367	.683	60	.117	.383	.308	60	.067	.200	.167
Sp.			.533	.725			1	1			.133	.317			.500	.692			.733	.833
SW	42	.238	.500	.488	42	0	.048	.024	42	.333	.476	.571	42	.452	.500	.702	41	.024	.195	.122
Fr.			.262	.512			.952	.976			.190	.429			.048	.298			.780	.878
SC	43	.233	.326	.395	43	.023	0	.023	43	.372	.465	.605	43	.674	.279	.814	43	0	.047	.023
Fr.			.442	.605			.977	.977			.163	.395			.047	.186			.953	.977
SE	51	.059	.667	.392	52	0	.019	.010	52	.308	.481	.548	50	.480	.500	.730	52	0	.115	.058
Fr.			.275	.608			.981	.990			.212	.452			.020	.270			.885	.942
N	76	.105	.461	.336	50	.040	.020	.050	76	.211	.526	.474	76	.513	.461	.743	76	.105	.263	.237
It.			.434	.664			.940	.950			.263	.526			.026	.257			.632	.763
C	57	.140	.439	.360	58	.017	.190	.112	58	.466	.397	.664	58	.517	.414	.724	58	0	.207	.103
It			.421	.640			.793	.888			.138	.336			.069	.276			.793	.897
S	57	.140	.474	.377	56	.036	.304	.188	57	.211	.614	.518	57	.614	.281	.754	57	0	.123	.061
It.			.386	.623			.661	.813			.175	.482			.105	.246			.877	.939
It.	67	.119	.522	.381	68	0	.103	.051	68	.382	.515	.640	66	.424	.288	.568	68	.103	.353	.279
Si.			.358	.619			.897	.949			.103	.360			.288	.432			.544	.721
C	40	.100	.350	.275	31	.387	.129	.452	37	.081	.297	.230	37	.486	.351	.662	40	.025	.200	.125
Hu.			.550	.725			.484	.548			.622	.770			.162	.338			.775	.875
E	121	.050	.397	.248	122	0	.123	.061	119	.017	.454	.244	124	.540	.435	.758	124	.040	.194	.137
Hu.			.554	.752			.877	.939			.529	.756			.024	.242			.766	.863
N	85	.094	.424	.306	84	.131	.345	.304	86	.023	.767	.407	86	.814	.174	.901	85	.059	.306	.212
Gr.			.482	.694			.524	.696			.209	.593			.012	.099			.635	.788

	Pgm–1					*Pgd–2*					*Pgm–1*					*Pgd–2*				
	N	P_{ij}			P_i	N	P_{ij}			P_i	N	P_{ij}			P_i	N	P_{ij}			P_i
NW	53	0	0	0	0	47	.085	.426	.064	.330	C 58	.034	.052	.034	.078	58	.224	.259	.052	.379
Sp.			.321	.472	.557			.404	.021	.628	It.		.448	.259	.603			.121	.259	.379
				.208	.443				0	.043				.172	.319				.086	.241
NE	60	0	0	0	0	58	.155	.414	.034	.379	S 57	0	.018	.018	.018	57	.158	.351	.035	.351
Sp			.333	.467	.567			.328	.069	.569	It.		.316	.439	.544			.351	.105	.579
				.200	.433				0	.052				.210	.439				0	.070
SW	42	0	.024	0	.012	41	.122	.171	.244	.329	It. 68	0	.044	.015	.029	66	.106	.288	.106	.303
Fr.			.167	.571	.464			.171	.293	.402	Si.		.353	.412	.581			.333	.136	.545
				.238	.524				0	.268				.176	.390				.030	.152
SC	43	0	0	0	0	43	.233	.256	.233	.477	C 40	0	.050	0	.025	39	.256	.385	.128	.513
Fr.			.279	.535	.547			.140	.093	.314	Hu		.375	.425	.613			.128	.051	.346
				.186	.453				.047	.209	.			.150	.363				.051	.141
SE	50	0	.080	0	.040	48	.021	.250	.104	.198	E 123	.333	.122	.024	.106	119	.437	.252	.092	.609
Fr.			.200	.420	.450			.438	.167	.646	Hu		.496	.285	.699			.168	.050	.319
				.300	.510				.021	.156	.			.041	.195				0	.071
N	76	.039	.079	.053	.105	76	.145	.276	.118	.342	N 86	0	.116	.012	.064	86	.116	.221	.337	.395
It.			.382	.342	.592			.211	.184	.441	Gr.		.581	.256	.767			.093	.209	.308
				.105	.303				.066	.217				.035	.169				.023	.297

Table 3. Genotype P_{ij} ($j \geq i$) and allele P_i relative frequencies in polymorphic loci of European, Caucasian, NW and NE Chines samples. P_{ij} and P_i are reported in the borded triangular and column matrices respectively. Numbers in columns N are sample sizes, their fluctuations are due to missing data.

Region	*Skdh–1*				*Skdh–2*				*Dia–1*				*Dia–3*			
	N	P_{ij}		P_i	N	P_{ij}		P_i	N	P_{ij}		P_i	N	P_{ij}		P_i
Europe	745	.117	.452	.343	712	.041	.124	.103	750	.235	.505	.487	752	.521	.371	.707
			.431	.657			.836	.897			.260	.513			.108	.293
Caucaus	179	.447	.441	.668	179	.212	.374	.399	178	.006	.264	.138	178	.848	.146	.921
			.112	.332			.413	.601			.730	.862			.006	.079
NW China	23	0	.348	.043 .196	23	.261	.565	.174 .630	23	.043	.478	.282	21	.667	0	.667
			.609	0 .783			0	0 .283			.478	.717			.333	.333
				0 .022				0 .087								
NE China	24	0	.500	.250	24	.167	.375	.354	24	.208	.292	.354	23	.696	0	.696
			.500	.750			.458	.646			.500	.646			.304	.304

Region	*Mpi–1*					*Pgm–1*				*Pgd–2*				
	N	P_{ij}			P_i	N	P_{ij}		P_i	N	P_{ij}			P_i
Europe	746	.044	.210		.149	756	.017	.060 .016	.053	738	.191	.289 .130		.400
			.745		.851			.382 .382	.603			.230 .134		.442
								.143	.345			.026		.158
Caucasus	174	.109	.270	.034 .261		180	.028	.272 .006	.167	176	.511	.080 .358		.730
			.494	.080 .670				.622 .067	.792			.017 .023		.068
				.011 .069				.006	.042			.011		.202
NW China	22	0	.409	.091 .250		23	.261	.522	.522	23	0	0 .565		.283
			.364	.091 .614				.217	.478			.043 .348		.217
				.045 .136								.043		.500
NE China	24	.125	.375	.167 .396		22	.364	.636	.682	21	0	0 .619		.310
			.208	.083 .438				0	.318			0 0		0
				.042 .167								.381		.690

Cockerham F, f indices (WEIR & COCKERHAM 1984). Locus averages of Nei and Wright indices were calculated across the whole set of loci examined. The jackknifing method was used to calculate the standard deviations of sample statistics.

Genetics software packages used for data elaboration were: GSED program (v. 1.0) of E. M. Gillet[1] for Gregorius indices, and FSTAT (v. 1.2) program of J. Goudet[2] for Weir and Cockerham's estimators of Wright statistics.

RESULTS AND DISCUSSION

Out of the 15 loci analysed, 7 (*Skdh–1, Skdh–2, Dia–1, Dia–3, Mpi–1, Pgm–1, Pgd–2*) resulted polymorphic and 8 (*Est–1, Pgi–1, Pgi–2, Aah–1, Got–1, Got–3, Got–4, Got–5*) monomorphic for the same allele in the whole sample, polymorphism found in the locus *Est–1* by GERMAIN *et al.* (1993) was likely due to the different ontogenetic stage of the analysed tissue (leaves). The proportion of polymorphic loci $P = 47\%$ was lower than that found on average in widespread species (P = 59 %, HAMRICK *et al.* 1991). Genotype and allele relative frequencies of the polymorphic loci in the 12 European samples and the 4 subcontinental samples are reported in tables 2 and 3 respectively. In each polymorphic locus the alleles are ordered according to the relative migration distances on the electrophoretic gel,

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the first allele corresponding to the shortest one.

A glance of tables 2 and 3 outlines the following remarks:

- *Skdh–1* and *Skdh–2* – These loci show two alleles in all but in the NW China sample, which is characterised by the presence, in both loci, of a third allele. In both cases the third allele appears only in combination with the first one. That could be explained supposing, in this population, a negative assortative mating preference of the third allele gametes or to some postzygotic environmental selection. The *Skdh–2* first allele shows a geographic pattern in good agreement with the historical westward spreading of *Juglans regia* in Europe, with $0.304 \leq P_1 \leq 0.452$ in eastern Hungary and northern Greece, $0.051 \leq P_1 \leq 0.188$ in peninsular Italy, Sicily and central Hungary and $P_1 \leq 0.050$ in western Europe (Spain, France and northern Italy).
- *Dia–3* – Chinese samples show no heterozygotes in this locus, suggesting a positive assortative mating behaviour of either or both gametes in these populations.
- *Mpi–1* – This locus is triallelic in Asiatic and biallelic in European samples, where the third allele is missing. The second allele shows an eastward decreasing frequency ($P_2 \cong 0.88\text{-}0.0034 \times longitude$).
- *Pgm–1* – This locus is triallelic in European and Caucasian samples and biallelic in the Chinese ones, where the third allele is missing. The first allele shows an eastward increasing frequency ($P_1 \cong 0.04 + 0.0059 \times longitude$).
- *Pgd–2* – This locus is triallelic in Europe, Caucasus and NW China and biallelic in NE China, where the second allele is missing. An eastward positive gradient is found in the third allele frequency ($P_3 \cong 0.02 + 0.0058 \times longitude$).

Diversity measures

The diversity indices N_a, N_e, H_o, H_e and F are reported in table 4.

The within population actual allele multiplicity ranged from $N_a = 2.00$ in NW Spain to $N_a = 2.57$ in NW China with a mean $N_a = 2.24$ in Europe and $N_a = 2.38$ in Asia. Even if with due reserve, because of the high sensitivity of N_a to the sample size, these values, compared to the value typical of widespread species (HAMRICK *et al.* 1991), evinced some scarcity of alleles, more pronounced in Europe.

Allele diversity, measured by the more meaningful N_e, showed considerable differences among the 4 subcontinental samples. Samples of natural NW China and naturalised NE China populations showed the highest diversities, whereas samples from anthropized European populations showed lower diversities with an overall decreasing trend in the more western ones. The lowest diversity was shown by the Caucasian sample, in spite of its relatively high N_a value. In any case the significance of the deviations of the N_e from their mean across the populations was tested (significance level $\alpha = 5\%$) by comparing the statistics (SOKAL & ROHLF 1995)

$$t_s = \frac{N_{e_i} - \bar{N}_e}{s\sqrt{\frac{n+1}{n}}}$$

with the Student critical t value ($p = 0.05$; d.f. $= n - 1$) $= 2.145$. The test resulted significant only for the NW China population, with $t = 2.186$.

Both the lower level of allele diversity in Europe and its westward decreasing trend clearly point out a more intense and westward increasing effect of human selection on these populations, respect to the Chinese ones. Not very reliable seems the low value of *Ne* found in the Caucasian sample, it is likely attributable to a non-random collection of seeds which could give rise to individuals too much related each other.

Unlike N_a, N_e could not be compared to a typical value in widespread species; however it looks relatively high, as compared to N_a. In order to show that, we measured the deviation between N_a and N_e, *i.e.* the skewness of the allele distribution, by means of the ratio $S_k = (N_a - N_e) / (N_a - 1)$. Since $1 < N_e \leq N_a$, S_k varies from 0 to 1 irrespective of the single locus allele multiplicity, reaching 0 when in each locus the alleles are equifrequent and approaching 1 as the loci tend to the monomorphism. The values of S_k, reported in table 4, ranged from $S_k = 0.26$ in NE China to $S_k = 0.61$ in Caucasus with means $S_k = 0.47$ in Europe and $S_k = 0.43$ in Asia. In some other tree species the values of S_k, calculated with the data found in literature, resulted:

$N_a = 1.63$, $N_e = 1.39$, $S_k = 0.38$ in *Castanea sativa* (VILLANI *et al.* 1991b);

$N_a = 2.78$, $N_e = 1.64$, $S_k = 0.64$ in *Pinus sylvestris* (PRUS-GŁOWACKI & STEPHAN 1994);

$N_a = 2.51$, $N_e = 1.33$, $S_k = 0.78$ in *Fagus sylvatica* (KONNERT 1995);

$N_a = 3.01$, $N_e = 1.50$, $S_k = 0.75$ in *Pinus brutia* (KARA *et al.* 1997);

$N_a = 1.83$, $N_e = 1.20$, $S_k = 0.768$ in *Picea abies* (GIANNINI *et al.* 1991);

$N_a = 2.85$, $N_e = 1.31$, $S_k = 0.83$ in *Quercus ilex* (YACINE & LUMARET 1989).

Table 4. Actual allele multiplicity N_a and averages across the polymorphic loci of effective allele multiplicity N_e (harmonic averages), skewness of allele distribution S_k, observed H_o and expected H_e heterozygosities and fixation index F. * significantly different from the mean ($\alpha = 5\%$).

Subcont. samples	Regional samples	N_a	N_e	S_k	H_o	H_e (sd)	F (sd)
	NW Spain	2.14	1.62	0.46	0.385	0.382 (0.031)	0.022 (0.017)
	NE Spain	2.00	1.58	0.42	0.331	0.365 (0.030)	0.091 (0.016)
	SW France	2.29	1.68	0.47	0.432	0.405 (0.034)	-0.027 (0.017)
	SC France	2.14	1.55	0.52	0.319	0.354 (0.039)	0.052 (0.021)
	SE France	2.29	1.57	0.56	0.400	0.364 (0.035)	-0.064 (0.033)
	N Italy	2.29	1.74	0.43	0.398	0.424 (0.029)	0.137 (0.054)
	C Italy	2.29	1.70	0.46	0.366	0.410 (0.028)	0.107 (0.030)
	S Italy	2.29	1.67	0.48	0.394	0.401 (0.025)	0.013 (0.024)
	Italy – Sicily	2.29	1.76	0.41	0.397	0.432 (0.026)	0.067 (0.031)
	C Hungary	2.29	1.75	0.42	0.338	0.429 (0.020)	0.185 (0.043)
	E Hungary	2.29	1.54	0.58	0.347	0.349 (0.023)	-0.008 (0.031)
	N Greece	2.29	1.70	0.46	0.453	0.412 (0.024)	-0.072 (0.042)
Europe		2.24	1.66	0.47	0.380	0.378 (0.021)	0.066 (0.010)
	Caucasus	2.43	1.56	0.61	0.345	0.364 (0.022)	0.025 (0.023)
	NW China	2.57	1.94*	0.40	0.519	0.482 (0.015)	-0.040 (0.084)
	NE China	2.14	1.84	0.26	0.435	0.457 (0.013)	0.052 (0.088)
Asia		2.38	1.78	0.43	0.433	0.435 (0.013)	0.012 (0.053)
All pop.		2.27	1.68	0.46	0.391	0.402 (0.019)	0.036 (0.012)

Table 5. Inner δ_{in} and outer δ_{ou} genotype differentiations in the 15 populations assayed. Means in the last raw are weighted with the population sample relative sizes. Locus means in the last columns are weighted with the relative genotype multiplicities of the loci.

Population	*Skdh–1*		*Skdh–2*		*Dia–1*		*Dia–3*		*Mpi–1*		*Pgm–1*		*Pgd–2*		Locus means	
	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in}	δ_{ou}	δ_{in} (sd)	δ_{ou} (sd)
NW-SP	.612	.124	.043	.266	.615	.133	.589	.108	.390	.088	.644	.273	.658	.434	.512 (.037)	.220 (.021)
NE-SP	.571	.166	.000	.294	.608	.333	.599	.502	.425	.069	.642	.261	.704	.345	.517 (.040)	.262 (.023)
SW-FR	.640	.122	.093	.238	.641	.174	.556	.196	.361	.107	.603	.406	.801	.235	.535 (.039)	.220 (.017)
SC-FR	.660	.130	.047	.264	.633	.203	.476	.090	.091	.288	.616	.313	.815	.075	.478 (.049)	.203 (.016)
SE-FR	.486	.230	.038	.271	.642	.154	.530	.198	.208	.218	.701	.315	.722	.338	.481 (.043)	.259 (.011)
N-IT	.596	.074	.117	.227	.618	.102	.531	.161	.528	.089	.725	.092	.817	.188	.585 (.037)	.131 (.010)
C-IT	.622	.046	.341	.074	.618	.295	.566	.108	.334	.122	.710	.126	.805	.235	.578 (.030)	.143 (.015)
S-IT	.618	.035	.479	.122	.558	.193	.543	.034	.219	.212	.675	.247	.729	.302	.545 (.028)	.184 (.017)
IT-Si	.593	.079	.187	.186	.587	.273	.664	.202	.577	.182	.683	.181	.776	.241	.597 (.031)	.191 (.019)
C-HU	.579	.179	.619	.315	.533	.275	.631	.106	.368	.101	.671	.160	.768	.192	.598 (.021)	.182 (.013)
E HU	.538	.201	.217	.175	.518	.196	.522	.141	.377	.101	.660	.111	.712	.243	.520 (.028)	.164 (.009)
N GR	.586	.114	.596	.226	.371	.342	.311	.248	.505	.085	.589	.185	.780	.259	.563 (.026)	.197 (.015)
GEOR	.596	.337	.648	.390	.399	.457	.259	.320	.667	.224	.536	.439	.607	.530	.557 (.025)	.387 (.017)
NW CHI	.530	.280	.609	.743	.565	.148	.467	.319	.714	.380	.640	.645	.581	.629	.602 (.013)	.482 (.037)
NE CHI	.522	.177	.649	.278	.649	.168	.464	.320	.797	.482	.489	.869	.464	.793	.579 (.021)	.505 (.048)
Mean (sd)	.585 (.047)	.171 (.085)	.338 (.260)	.260 (.152)	.537 (.085)	.264 (.097)	.479 (.109)	.211 (.122)	.453 (.196)	.163 (.121)	.633 (.065)	.272 (.213)	.717 (.101)	.329 (.189)	.549 (.022)	.242 (.011)

Except in *Castanea sativa*, they are higher than in walnut, showing in this species a more balanced allele distribution and the capability to maintain it even in presence of a long selection pressure.

The mean expected heterozygosities were $H_e = 0.394^{+0.038}_{-0.040}$ in Europe and $H_e = 0.435^{+0.048}_{-0.071}$ in Asia; they obviously confirmed the lower diversity level in Europe respect to the China and the low diversity of Caucasian sample. Moreover the comparison of H_e with the observed heterozygosities H_o showed an overall slight deficiency of heterozygotes: both in Asia and in Europe it was $H_o < H_e$ in 67% and $H_o > H_e$ in 33% of populations. This slight deficiency of heterozygotes may be the result of aggregating populations subdivisions (demes) which differ slightly in allele frequencies (Walund effect).

Anyway it is worthwhile to point out:

- the overall high level of heterozygosity, even in European populations, in comparison with populations of other wind pollinated *latifoliae* namely: *Juglans nigra* (0.217±0.145, RINK *et al.* 1994), *Castanea sativa* (0.274±0.047 VILLANI *et al.* 1991a), *Castanea mollissima* (0.345±0.035, HUANG *et al.* 1994), *Fagus sylvatica* (0.228±0.051, BELLETTI & LANTERI 1996), *Quercus rubra* and *Quercus ellipsoidalis* (0.310±0.027, HOKANSON *et al.* 1993);
- the substantial HW equilibrium found in all populations, as shown by the low level of the mean fixation index F that in Europe and in Asia lies in the intervals $0.042^{+0.143}_{-0.015}$ and $0.012^{+0.040}_{-0.042}$, respectively. In particular, deviations of F from 0 with a 5% significance level were found at the most in 2 loci out of 7 in all the samples, except the eastern Hungary one, where 4 loci resulted significantly in disequilibrium (data not shown). That points out a high rate of outcrossing, consistent with the reproductive strategies of *J. regia* to avoid self-pollination and inbreeding.

Both the substantial HW equilibrium and the considerable heterozygosity levels found in European populations confirmed the relevant capability of *J. regia* to preserve a high level of genetic diversity even in presence of some genetic erosion.

Gregorius differentiation indices

Gregorius inner and outer differentiation indices δ_{in} and δ_{ou} are reported in table 5 with their weighted averages across polymorphic loci and populations. Consistently with the feature of a long-lived outcrossed wind pollinated species, the within population differentiations were systematically higher than the differentiation among populations. The averages across the loci of δ_{in} ranged in the intervals $0.542^{+0.066}_{-0.064}$ and $0.579^{+0.023}_{-0.022}$ in Europe and in Asia respectively, showing high and close together values in all the populations. Corresponding ranges of δ_{ou} were $0.196^{+0.066}_{-0.065}$ and $0.458^{+0.047}_{-0.071}$ respectively, showing, on the contrary, values in Europe systematically lower (about a half) than those found in Asia. The plots of δ_{in} and δ_{ou} are reported in figures 2 and 3, respectively. Figure 2 shows, for δ_{in}, balanced values in all the populations at the loci *Skdh–1, Dia–1, Dia–3, Pgm–1* and *Pgd–2,* whereas high values in Asia and scattered values in Europe at the loci *Skdh–2* and *Mpi–1*. The last plot shows a quite uniform mean inner differentiation across all the populations. Plots in figure 3 show lower and more scattered values of δ_{ou} across the loci and an outlying behaviour of Asiatic populations, quite evident in most of the loci and summarised in the last plot. The clustering of European populations at lower values of δ_{ou} is coherent with a postglacial relationship among them respect to a preglacial relationship with the Asiatic ones. Quite interesting is the unexpected high level of internal differentiation found in Europe. They confirmed, in agreement with the diversity indices, that the long exposure of European *J. regia* to human selection caused a considerable genetic erosion, but seems to have not affected noticeably the internal genotype differentiation. That could be evidence both of the genetic plasticity and reaction of this species.

Wright and Nei indices

The averages across all the loci of the Wright and Nei indices, estimated in 4 national and 2 continental groups of populations, are reported in table 6. Both the statistics confirmed and integrated what came out from the diversity and differentiation indices. In details:

The closeness to the HW equilibrium of all the populations, shown by the fixation indices, was confirmed by the low values of the inbreeding coefficient f in all the groups. Moreover the slightly higher values in Spain and Italy revealed an intrapopulation differentiation, in these groups, more pronounced than in other groups.

In all the groups H_s resulted close to the values typical of predominantly outcrossed (0.214±0.034), long lived (0.202±0.038) and widespread (0.183± 0.036) plant species (LOVELESS & HAMRICK 1984) confirming, in agreement with δ_{in}, a considerable level of intrapopulation differentiation. Even in Europe the

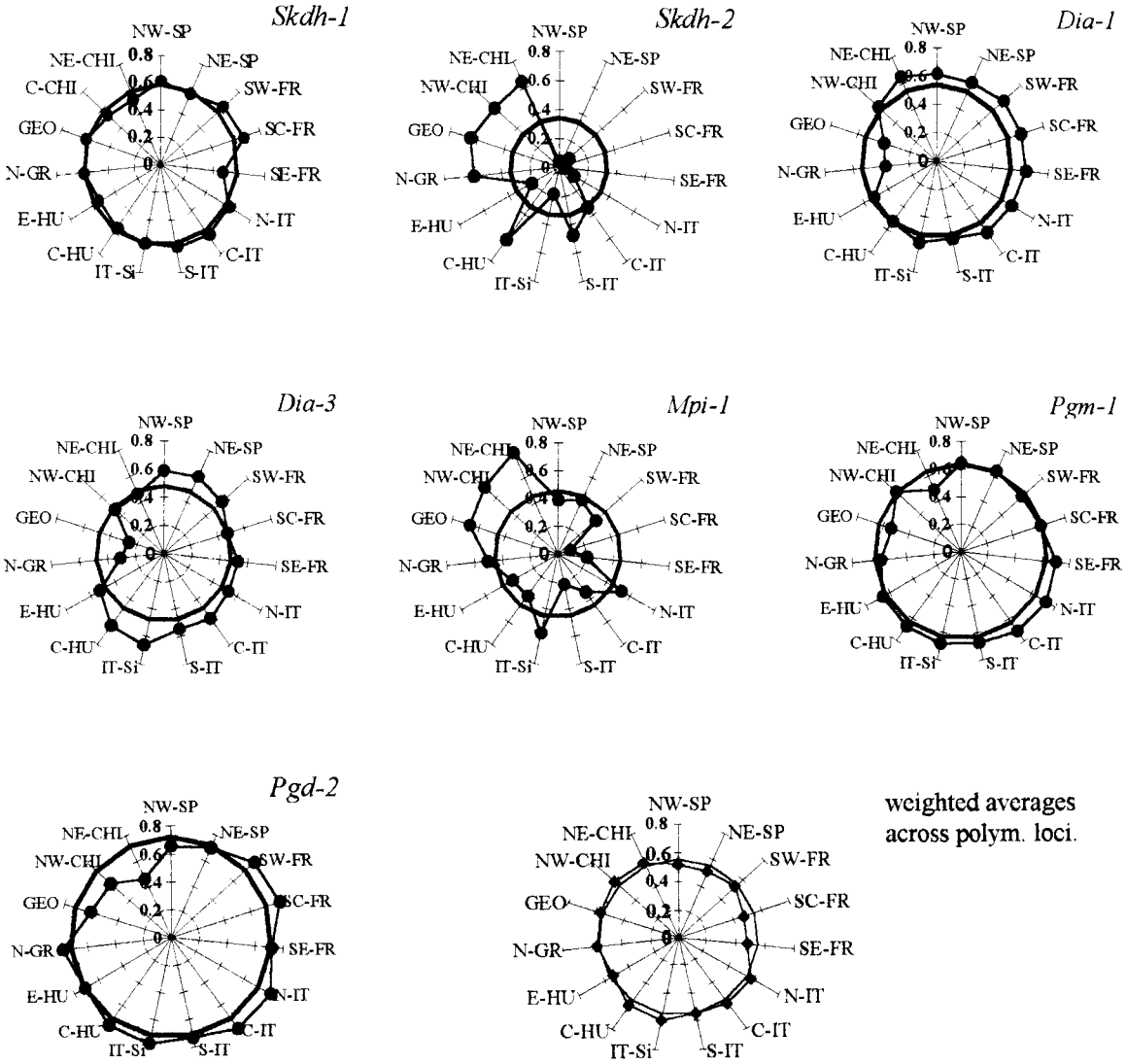

Figure 2. Radar plots of inner population differentiations δ_{in} in the polymorphic loci. The circle in each plot shows the weighted average across the populations; weighted average across the loci is reported in the last plot.

values of H_s, although slightly lower than in Asia, were well higher than those estimated by means of RFLP markers in cultivated selections of congeneric species like *J. cinerea* (H_s = 0.088) and *J. nigra* (H_s = 0.111) (FJELLSTROM & PARFITT 1994).

The comparison of θ, D_{st} and G_{st} among Europe, Asia and all the populations outlined, in agreement with δ_{ou}, the relative closeness of European populations respect to the Asiatic group and the relevant differentiation among Caucasus, NW China and NE China populations. This is particularly evident comparing the values of G_{st} in Europe (G_{st} = 0.066) and in Asia (G_{st} = 0.106) with the value $G_{st} \equiv F_{st}$ = 0.108, reported by FJELLSTROM & PARFITT (1994) using RFLP markers for the analysis of natural populations of *J. regia.*

CONCLUSIONS

The comparative analysis of the genetic structure of 12 European and 3 Asiatic populations of *Juglans regia* suggests the following considerations:

The mean number of alleles per polymorphic locus N_a = 2.27 and the total differentiation index H_t = 0.214, lower than the typical values reported for widespread plant species (N_a = 3.70 and H_t = 0.380 respectively, HAMRICK *et al.* 1991, MILLAR & LIBBY, 1991), together

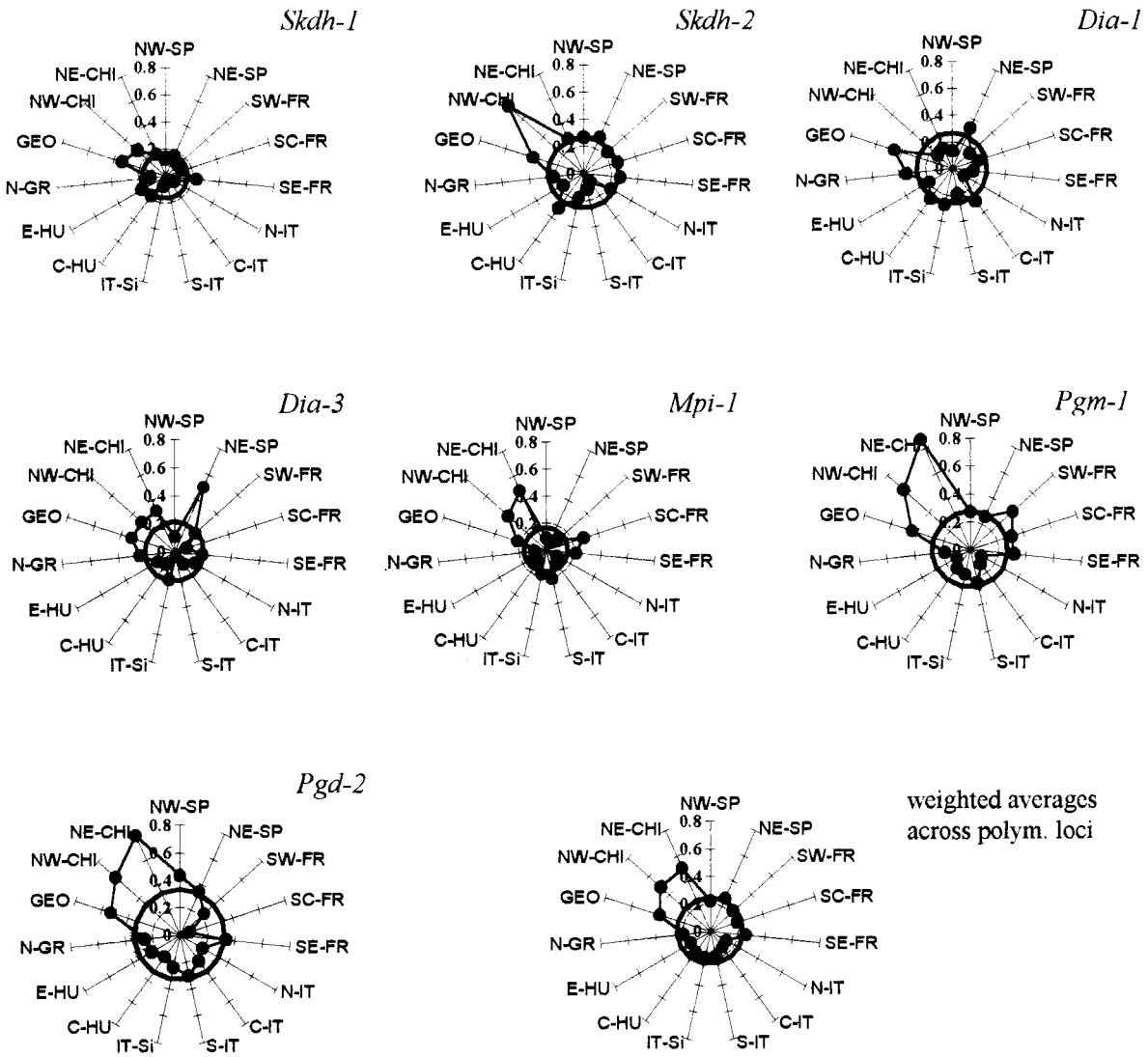

Figure 3. Radar plots of outer population differentiations δ_{ou} in the polymorphic loci. The circle in each plot shows the weighted average across the populations; weighted average across the loci is reported in the last plot. The same scale of fig. 2 was used to help a comparison with δ_{in}.

with the fact that the polymorphic loci and the alleles of the monomorphic ones coincided in all the samples, revealed a reduced level of genetic variability of *J. regia* and confirmed the soundness to number this tree species among the widespread endangered ones.

About the debated origin of European walnut, the considerable level of differentiation found among the 4 subcontinental groups of populations (Europe, Caucasus, NW China and NE China), the presence of different allelic pools in a few polymorphic loci (*Skdh–1, Skdh–2, Mpi–1, Pgm–1*) and the lower levels of differentiation among European populations, seem to give credit to a survival of *J. regia* in Europe during Pleistocene glaciations. On the other hand the thesis of the extinction of the species and subsequent recolonisation from Asia (BOTTEMA 1980), entails the arduous acceptance that 100 centuries elapsed from the last glaciation were sufficient to give rise, in a long lived tree species, to the amount of differentiation found among European and Asiatic populations.

As for the glacial refugia in Europe, the relatively higher level of diversity in Italy, in Hungary and in Greece and its westward decreasing trend (see δ_{in} and H_s) suggest, in agreement with some paleopalynologic

Table. 6. Estimates of the Wright and Nei indices in national and continental groups of populations (standard deviations in brackets). In the last column the Gregorius outer differentiation . δ_{ou} is reported for comparison.

Groups	Popul.	F	θ	f	H_t	H_s	D_{st}	G_{st}	δ_{ou}
Spain	1–2	.123 (.058)	.071(.052)	.057 (.029)	.182 (.031)	.174 (.030)	.008	.044	.242
France	3–5	.001 (.036)	.018 (.019)	-.018 (.027)	.178 (.037)	.175 (.036)	.003	.017	.229
Italy	6–9	.092 (.039)	.020 (.008)	.073 (.039)	.199 (.025)	.195 (.025)	.004	.020	.161
Hu and Gr	10–12	.058 (.076)	.054 (.024)	.004 (.068)	.193 (.018)	.185 (.017)	.008	.041	.178
Europe	1–12	.096 (.032)	.064 (.014)	.034 (.034)	.197 (.026)	.184 (.025)	.013	.066	.191
Asia	13–15	.225 (.037)	.191 (.051)	.042 (.064)	.227 (.014)	.203 (.013)	.024	.106	.409
All pop.	1–15	.156 (.031)	.125 (.015)	.036 (.032)	.214 (.021)	.188 (.019)	.026	.121	.242

data (HUNTLEY & BIRKS 1983), their location in penin sular Italy and in Balkans, where the presence of native residual germplasm could contribute to the diversity. The lower diversity levels in western Europe is attributable to the human propagation that, leading to the selection with the most suitable traits, negatively affects the genetic variability along the way of diffusion.

Unlike to the negative effects on the genic pool, the human forced propagation did not seem to significantly affect, in Europe, the within population differentiation levels which resulted comparable with those found in Asiatic populations and close to the levels found in other broad-leaves cross-pollinated species. The capability of *J. regia* to maintain a noticeable intrapopulation differentiation, even in presence of selection pressures, is attributable to its reproductive strategies to prevent self-pollination and inbreeding.

In conclusion this study pointed out an evident genetic erosion suffered by the species, mainly in Europe, and, despite the incomplete description of Asiatic walnut, the presence of populations well adapted in areas with different environmental conditions and the occurrence of different rare alleles within populations from Asia and Europe. These items could be useful to organise conservation programmes aiming to preserve the genetic variability still present in different countries and to succeed in identifying genotypes for future walnut breeding programs.

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REFERENCES

ALETÀ, N., ROVIRA, M., NINOT, A. & ARUS, P. 1993: Inheritance of four isozymes in walnut. *Acta Horticulturae* **311**: 62–65.

ARULSEKAR, S., PARFITT, D. E. & MCGRANAHAN, G. 1985: Isozyme gene markers in Juglans species. Inheritance of GPI and AAT in *Juglans regia* and *Juglans hidsii*. *J. of Heredity* **76**: 103–106.

ARULSEKAR, S., PARFITT, D. E. & MCGRANAHAN, G.1986: Inheritance of phosphoglucomutase and esterase isozymes in Persian walnut. *J. of Heredity* **77**: 220–221.

ASHTON, G. C. & BRANDEN, W. H. 1961: Serum globulin polymorphism in mice. *J. Biol. Sci.* **14**: 248–253.

BEINEKE, W. F. & MASTERS, C. J. 1976: Controlling pollination in black walnut. *In* Proc. 10th Central States Forest Tree Improvement Conf., Purdue Univ., W. Lafayette, Indiana. p. 66–72.

BELLETTI, P. & LANTERI, S. 1996: Allozyme variation among European beech (*Fagus sylvatica* L.) stands in Piedmont, North Western Italy. *Silvae Genet.* **45**(1): 33–37.

BOTTEMA, S. 1980: On the history of the walnut (*Juglans regia* L.) in south-eastern Europe. *Acta Bot. Neerl.* **29**(5–6): 343–349.

FJELLSTROM, R. G. & PARFITT, D. E. 1994: Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. *Genome* **37**: 690–700.

FJELLSTROM, R. G. & PARFITT, D. E. 1995: Phylogenetic analysis and evolution of the genus *Juglans* (*Juglan-*

daceae) as determined from nuclear genome RFLPs. *Pl. Syst. Evol.* **197**: 19–32.

GERMAIN, E., HANQUIER, I. & MONET, R. 1993: Identification of eight *Juglans* spp. and their interspecific hybrids by isoenzymatic electrophoresis. International Walnut Meeting, 21–25 October 1991, Tarragona, Spain. *Acta Horticulturae* **311**: 86–91.

GIANNINI, R., MORGANTE, M. & VENDRAMIN, G. G. 1991: Allozyme variation in Italian populations of *Picea abies* Karst. *Silvae Genet.* **40** (3–4): 160–166.

GREGORIUS, H. R. 1978: The concept of genetic diversity and its formal relationship to heterozygosity and genetic distance. *Math. Biosci.* **41**: 253–271.

GREGORIUS, H. R. 1987: The relationship between the concepts of genetic diversity and differentiation. *Theor. Appl. Genet.* **74:** 397

GREGORIUS, H. R. & ROBERDS, J. H. 1986: Measurements of genetical differentiation among subpopulations. *Theor. Appl. Genet.* **71**: 826–834.

HAMRICK, J. L., GODT, M. J. W., MURAWSKI, D. A. & LOVELESS, M. D. 1991: Correlations between species traits and allozyme diversity: implications for conservation biology. *In*: Genetics and Conservation of Rare Plants, edited by D. A. Falk and K. E. Holsinger. Oxford University Press. New York – Oxford, p. 75–86.

HOKANSON, S.C., ISEBRANDS, J. C., JENSEN, R. J. & HANCOCK, J. F. 1993: Isozyme variation in oak of the Apostle islands in Wisconsin: genetic structure and levels of inbreeding in *Quercus rubra* and *Quercus ellipsoidalis* (Fagaceae). *American Journal of Botany* **80**: 1349–1357.

HUANG, H., DANE, F. & NORTON, J. D. 1994: Allozyme diversity in Chinese, Seguin and American chestnut (*Castanea* spp.). *Theor. Appl. Genet.* **88:** 981–985.

HUNTLEY, B. & BIRKS, H. J. B. 1983: An atlas of past and present pollen maps for Europe: 0–13000 years ago. Cambridge University Press. N. Y.: p. 238–242.

KARA, N., KOROL, L., ISIK, K. & SCHILLER, G. 1997: Genetic diversity in *Pinus brutia* Ten.: altitudinal variation. *Silvae Genet.*, **46**(2–3): 155–161.

KIM, Z. S. 1979: Inheritance of leucine aminopeptidase and acid phosphatase isozymes in beech (*Fagus sylvatica* L.). *Silvae Genet.* **28**: 68–71.

KIM, Z. S. 1980: Veränderung der genetischen Struktur von Buchenpopulationen durch Viabilitätsselektion in Keimlingstadium. *Göttingen Res. Notes in For. Genetics*, 3 (Phd thesis).

KIMURA, M. & CROW, J. F. 1964: The number of alleles that can be maintained in a finite population. *Genetics* **49**: 725–738.

KONNERT, M. 1995: Investigation of the genetic variation of beech (*Fagus sylvatica* L.) in Bavaria. *Silvae Genet.* **44** (5–6):346–351.

LESLIE, C. A. & MCGRANAHAN, G. 1988: Native populations of *Juglans regia*. A draft. *In*: Proceedings of the International Conference on Walnut, Yalova, Turkey, September 19–23, p. 111–124.

LOVELESS, M. D. & HAMRICK, J. L. 1984: Ecological determinants of genetic structure in plant populations. *Ann. Rev. Ecol. Syst.* **15**: 65–95.

LUZA, J. G. & POLITO, V. S. 1988: Microsporogenesis and anther differentiation in *Juglans regia* L.: a developmental basis for heterodichogamy in walnut. *Bot. Gaz.* **149** (1): 30–36.

MALVOLTI, M. E., TEISSIER DU CROS, E., FINESCHI, S. & PACIUCCI, M. 1991: Biochemical markers in eastern cottonwood (*Populus deltoides* Bartr.). *In*: Biochemical markers in the population genetics of forest trees. SPB Academic Publishing bv, The Hague, The Netherlands, p. 31–40.

MALVOLTI, M. E., CANNATA, F. & SPADA, M. 1993a: Contract U.E AIR1–CT92–0142; Project: European Development of Walnut Trees for Wood and Fruit Production as an Alternative and Extensive System to Agricoltural Crops. Second report of activity, (07/01/1993–12/31/ 1993); working group C–2.4.1.

MALVOLTI, M. E., PACIUCCI, M., CANNATA, F. & FINESCHI, S. 1993b: Genetic variation of Italian populations of *Juglans regia* L. International Walnut Meeting, 21–25 October 1991, Tarragona, Spain. *Acta Horticulturae* **311**: 86–91.

MALVOLTI, M. E., FINESCHI, S., MORGANTE, M. & VENDRAMIN, G. G. 1995: Mating system of naturalised *Juglans regia* L. population in Italy. *In*: Population genetics and genetic conservation of forest trees. SPB Academic Publishing, Amsterdam, The Netherlands, p. 305–308.

MALVOLTI, M. E., BERITOGNOLO, I., SPADA, M. & CANNATA, F. 1997: Ricerche sulle risorse genetiche e sulla biologia riproduttiva di *Juglans regia* L. in Italia mediante marcatori molecolari. *Ann. Ist. Sper. Selv.* **25 & 26**: 9–34.

MANCHESTER, S. R. 1987: The fossil history of the *Juglandaceae*. Monog. Syst. Bot. *Missouri Bot. Gard.*, **21**: 1–137.

MILLAR, C. I. & LIBBY, W. J. 1991: Strategies for conserving clinal, ecotypic and disjunct population diversity in widespread species. *In* Genetics and Conservation of Rare Plants, D. A. Falk and K. E. Holsinger eds. Oxford University Press. New York – Oxford, p. 149–170.

NEI, M. 1987: Molecular Evolutionary Genetics. Columbia University Press, New York, chapt. 8.

PRUS-GLOWACKI, W. & STEPHAN, B. R.. 1994: Genetic variation of *Pinus sylvestris* from Spain in relation to other European Populations. *Silvae Genet.* **43**(1): 7–14.

RINK, G., ZHANG, G., JINGHUA, Z., KUNG, F. H. & CARROLL, E. R. 1994: Mating parameters in *Juglans nigra* L. seed orchard similar to natural population estimates. *Silvae Genet.* 43(4): 261–263.

SOKAL, R. R. & ROHLF, F. J. 1995: Biometry; third edition. W. H. Freeman and Company Ed., N. Y., USA, chapt. 9.

SZENTIVÁNYI, P. 1990: Effect of fertility auto-regulation on productivity of walnut. First International Symposium on Walnut Production. Budapest, Hungary, 25–29 September 1989. *Acta Horticulturae* **284**: 251–256.

VILLANI, F., PIGLIUCCI, M., BENEDETTELLI, S. & CHERUBINI, M. 1991a: Genetic differentiation among Turkish chestnut (*Castanea sativa* Mill.) populations. *Heredity* **66:**131 –136.

VILLANI, F., BENEDETTELLI, S., PACIUCCI, M., CHERUBINI, M. & PIGLIUCCI, M. 1991b: Genetic variation and differentiation between natural populations of chestnut (*Castanea sativa* Mill.) from Italy. *In*: Biochemical markers in the population genetics of forest trees, p. 91–103. Edited by

S. Fineschi, M. E. Malvolti, F. Cannata and H. H. Hattemer. 1991 SPB Academic Publishing bv, The Hague, The Netherlands.

YACINE, A. & LUMARET, R. 1989: Genetic diversity in holm-oak (*Quercus ilex* L.): insight from several enzyme markers. *Silvae Genet.* **38**(3–4): 140–148.

WEIR, B. S. & COCKERHAM, C. C. 1984: Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**(6):1358–1370.

WRIGHT, S. 1951: The genetical structure of populations. *Ann. Eugen.* **15**: 323–354.

WRIGHT, S. 1965: The interpretation of population structure by *F*-statistics with special regard to systems of mating. *Evolution* **19**: 395–420.

WRIGHT, S. 1978: Evolution and Genetics of Populations, vol. 4: Variability within and among Natural Populations. Chicago III. University of Chicago Press, USA.

ZHENG, W. C. 1978: *Juglans regia* L. *In*: The planting technology of the main forest trees in China. The editorial commission of "Chinese Flora of Trees" Eds., p. 1342.