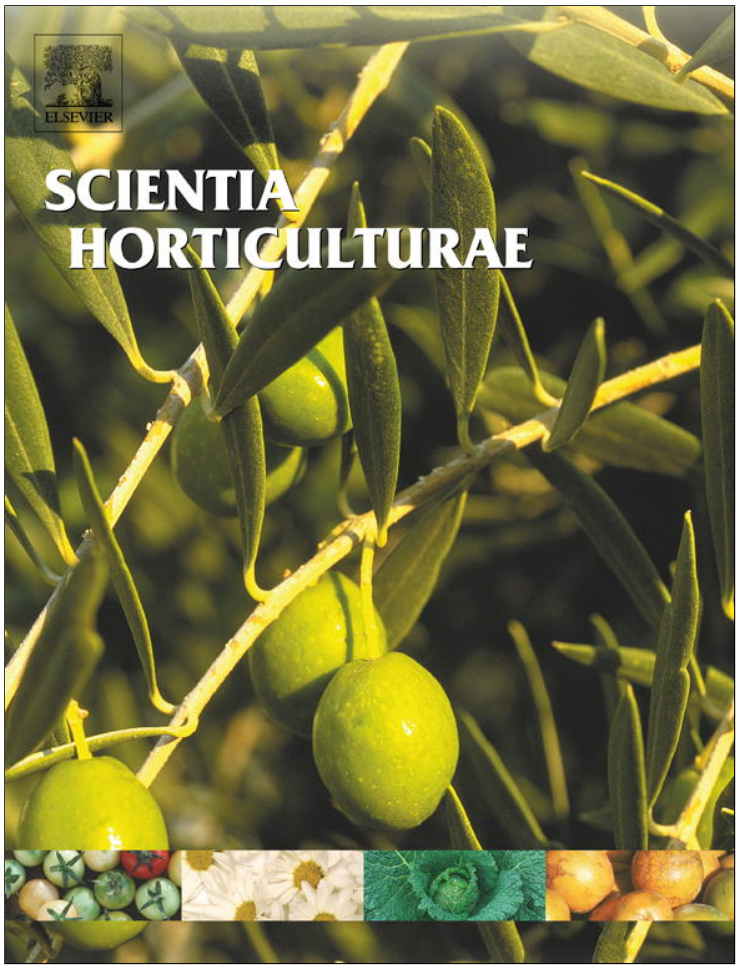

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Relationships of walnut cultivars in a germplasm collection: Comparative analysis of phenotypic and molecular data

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ABSTRACT

Assessment of the genetic relatedness of walnut cultivars with phenotypic data and molecular markers allows progress in conservation and management of the genetic resources, breeding and protection of breeders' intellectual property. In the present study we used 24 morphological traits, 25 random amplified polymorphic DNAs (RAPDs) and 7 simple sequence repeats (SSRs), to study genetic diversity and relationships in walnut cultivars at different levels. The 20 analyzed accessions represent some of the most important Romanian and international walnut cultivars and typify a genetic diversity characteristic of a germplasm collection. The distances based on morphological and SSR markers were significantly correlated. The two DNA marker systems were uncorrelated and RAPD markers failed to describe the pattern of molecular similarity. All marker systems detected polymorphisms that were adequate for the discrimination of all cultivars. Morphological- and SSR-based genetic distances were related to a certain point and differed from RAPD-based genetic distances. Our data indicate that the type and the number of phenotypic traits evaluated can considerably alter the result of the analysis and combination of qualitative and quantitative data needs caution. Moreover, the data imply that the two molecular marker systems are useful for cultivar characterization, but SSR markers are more advisable to investigate genetic relationships. Also, they can be employed to complete and aid the traditional registration of varieties. We propose that, since the information provided by morphological and SSR marker systems in walnut is similar, they should serve for cultivar characterization and assessment in genebanks.

1. Introduction

The walnut (*Juglans regia* L.) is characterized by a wide differentiation and long history of cultivation throughout the temperate regions of the world (McGranahan and Leslie, 1990), and can be found from the northern region of Iran to Japan, from Greenland to Siberia and Burma (Bordeianu et al., 1967), being also a very important species in Romania. Our country is considered one of the main walnut production countries worldwide (Cociu et al., 2003). The total number of Romanian walnut cultivars exceeds 27, most of which have evolved and were selected in order to match local conditions. The presence of this large number of cultivars, adapted to various environments, indicates the abundance of Romanian walnut genetic resources (Cociu et al., 2007).

Evaluation of the genetic diversity is performed by morphological, molecular and biochemical markers (Chahal and Gosal, 2002). DNA markers bypass the setbacks of pedigree data and morphological markers used for biodiversity assessment.

The morphological and agronomic characteristics have been used in different walnut collections for the identification, characterization, and evaluation of cultivars (Amiri et al., 2010; Arzani et al., 2008; Botu et al., 2010). Even though these traits are influenced by environmental factors and their measurements can be ambiguous, leading to an unclear result diagnostic test between genotypes and accessions (Kumar, 1999), they are still considered essential in the management of a germplasm collection (Trentacoste and Puertas, 2011).

Currently, molecular methods are commonly used for identifying and classifying walnut genotypes (Ruiz-Garcia et al., 2011), and are also supporting the classic methods such as morphological traits (Fatahi et al., 2010). Molecular markers were also used in determining the inter- and intraspecific genetic similarity and the relationship between walnut populations (Karimi et al., 2010).

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RAPD markers have the advantages of high usability, low cost of the experiments and the coverage of the entire genome (Williams et al., 1990), although they suffer from a certain lack of reproducibility due to mismatch annealing (Karp et al., 1997). RAPD markers have been used to assess the level of polymorphism in *Juglans* genus by different authors (Qianwen et al., 2010).

SSRs are widely and ubiquitously distributed throughout eukaryotic genomes and can be highly polymorphic, informative and reproducible (Senior and Heun, 1993) and were suggested in order to surpass the limitations associated with RFLP and RAPD (Garcia et al., 2004). The use of microsatellite procedures depends on the availability of suitable SSR markers, which have been previously developed, for species such as *Juglans nigra* (Woeste et al., 2002).

Comparisons of different markers for diversity analysis in walnut have been performed by some authors (Fatahi et al., 2010; Kafkas et al., 2005; Nazeer et al., 2012), but, to our knowledge, none compared in the same study morphological, RAPD and SSR markers, in order to assess the relative efficiencies of the different techniques. Furthermore, Francesca et al. (2010) published the only study using molecular markers to characterize Romanian walnut cultivars and to assess their genetic diversity. Considering the above, evaluation of different marker systems, complementing morphological traits, was considered of high importance for the future conservation and breeding programs of walnut in Romania and worldwide.

In addition the aims of this work were: to compare the level of information provided by morphological traits, RAPD and SSR markers for estimating genetic similarities in walnut (1); to assess the minimum number of SSR *loci* needed to accurately discriminate the accessions and to represent the genetic distance between them (2); to compare the genetic distances (GD) obtained with the different marker systems (3); to compare the usefulness of these three markers in conservation and breeding programs by means of genetic distance estimates (4) and to characterize important Romanian walnut cultivars (5).

2. Materials and methods

2.1. Plant material and morphological measurements

The 20 accessions used in this study (Table 1) were obtained from the Romanian National Collection of Walnut, maintained at University of Craiova – SCDP Valcea, Romania. Nine morphological descriptors from International Union for the Protection of New Varieties of Plants/International Plant Genetic Resources Institute (UPOV/IPGRI) (nominal and ordinal/multistate) (IPGRI, 1994) (catkins abundance, kernel color, bud break period, fruit ripening period, fruit shape, ease of kernel removal, shell seal, bearing type, dichogamy) and 15 quantitative descriptors (large diameter of the fruit, average fruit weight, fruit size index, average kernel weight, shell thickness, average tree height, average crown diameter, yield per tree (trees in the 4th, 5th and 6th years of production), yield per tree (in the 11th–15th years), area of crown projection on the soil (canopy) in the 15th year, average kernel percentage, share of fruits depending on large diameter, trunk cross sectional area, crown volume, vegetation period), considered as informative, were selected and evaluated on three to five plants per accession. Assessments of nut characteristics were carried out on three samples as replications, each 10–15 nuts and totally 30–45 nuts per tree. In the present study we used only the mean values of the morphological traits recorded among genotypes (Table 2), as an input for the clustering analysis.

2.2. DNA isolation

Young leaves were collected in the spring of 2008 and immediately stored at −80 °C prior to DNA extraction. Total DNA was extracted using the protocol described by Lodhi et al. (1994) and modified by Pop et al. (2003) for the reduction of initial plant tissue and DNA contaminants. The concentration of the extracted DNA was assessed using a Nano Drop ND 1000 spectrophotometer and was later diluted to 50 ng/μl with nuclease-free water (Promega) for PCR amplification using RAPD primers and to 10 ng/μl, respectively, for amplifications using SSR primers.

2.3. DNA amplification and electrophoresis conditions

Twenty five decamer primers (Microsynth) (Table 3) were used for PCR RAPD amplifications. The amplification and electrophoresis were carried out as described by Francesca et al. (2010). Only the consistent, strong amplification products between 200 bp and 2000 bp long were considered for analysis. Each RAPD analysis was repeated in separate experiments twice, and only the uniform and reproducible fragments between replicate PCRs were considered.

PCR amplification reactions using SSR markers were carried out by Microsynth AG (Balgach, Switzerland), according to their protocol, using WGA1, WGA118, WGA276, WGA332, WGA376, WGA69

Table 1
Genotypes used to evaluate morphological and molecular differences among *Juglans regia* accessions.

Name	Geographic origin	Genetic origin
Argesan	Romania	Selection from local populations from Pitesti (Arges) area
Ferjean	France	Grosvert × Lara
Fernor	France	Franquette × Lara
Franquette	France	Old cultivar
Germisara	Romania	Selection from local populations from Hunedoara area
Jupinesti	Romania	Selection from local populations from Arges area
Lara	France	Payne × open pollination
Muscelean	Romania	Selection from local populations from Pitesti (Arges) area
O2	Caucasus	Selection
Roxana	Romania	Selection from local populations from Arges area
Secular	Romania	Selection from local populations from Arges area
Serr	USA – UC Davis	Payne × PI 15968
Unival	Romania	Selection from local populations from Valcea area
Valcor	Romania	Selection from local populations from Valcea area
Valcris	Romania	Selection from local populations from Pausesti–Otasau (Valcea) area
Valmit	Romania	Selection from local populations from Valcea area
Valrex	Romania	Selection from local populations from Valcea area
Valstar	Romania	selection from local populations from Valcea area
Velnita	Romania	Selection from local populations from Iasi area
Vina	USA – UC Davis	Franquette × Payne

Table 2
The mean values of the morphological traits recorded among the 20 walnut accessions used in this study.

Trait/ cultivar	Argesean	Ferjean	Fernor	Franquette	Germisara	Jupinesti	Lara	Muscelean	O2	Roxana	Secular	Serr	Unival	Valcor	Valcris	Valmit	Valrex	Valstar	Velnita	Vina
TCSA	829	530	660	552	531	660	615	881	670	660	690	572	697	683	687	715	730	620	560	552
ACD	7.10	5.30	6.00	5.80	5.20	5.70	5.70	7.30	5.70	5.90	6.10	5.80	5.70	5.60	5.90	5.90	6.10	5.80	5.20	5.90
ACPS	39.60	22.00	28.20	26.40	21.20	25.50	25.50	41.80	25.00	26.70	28.30	26.40	25.40	24.60	26.40	27.30	29.20	26.30	21.20	27.30
ATH	5.90	5.90	6.10	5.90	6.10	5.60	5.80	6.00	5.90	5.60	5.90	5.70	5.80	6.40	6.40	6.80	5.90	5.70	5.70	5.60
CV	194.0	118.8	155.1	142.5	108.2	117.3	135.1	209.0	140.0	124.7	150.2	137.3	132.6	132.8	145.7	158.3	143.1	128.6	99.7	139.2
YPTY	0.93	5.10	4.30	2.33	1.30	3.33	3.47	1.13	1.80	2.37	2.00	4.10	2.40	3.43	2.57	1.93	2.40	2.43	3.07	5.97
YPT	10.74	17.38	13.84	12.24	11.32	13.02	13.44	10.66	12.18	12.76	12.74	12.6	11.32	13.28	13.1	11.63	10.98	11.1	12.42	13.02
SI	40.7	36.8	35.9	35.5	43.8	35.2	39.9	42.1	33.4	36.5	36.9	36.2	39.2	39.9	40.1	39.2	40.6	40.6	40.1	36.7
LDF	37.1	33.4	32.5	33.8	39.6	32.2	36.7	37.9	32.3	35.8	35.9	35.4	35.8	36.4	35.6	35.8	36.6	34.6	36.2	35.6
ST	1.5	1.1	1.2	1.3	1.5	1.4	1.4	1.6	1.7	1.6	1.7	1	1.2	1.2	1.2	1.2	1.3	1.2	1.3	1.1
SF1	75.2	68.3	69.2	81.7	81.7	59.8	79.5	77.5	68.3	72.5	68.9	72.4	79.4	82	79.3	79.1	84.5	83.5	77	78.9
SF2	21.7	24.2	19.8	16.8	17.7	33.7	18.3	20.4	20.8	19.7	20.8	24.1	18.9	16.1	18	18.1	14.8	15.8	19.7	18.6
SF3	3.1	7.5	11	1.5	0.6	6.5	2.2	2.1	10.9	7.8	10.3	3.5	1.7	1.9	2.7	2.7	0.7	0.7	3.3	2.5
AFW	14.3	11	10.9	12	15.1	12.2	12.7	14.6	12.3	14.1	12.5	12.9	14.5	13.3	15	12.6	14.3	12.3	13.1	12.1
AKW	7.36	5.59	5.38	5.8	7.34	6.3	6.39	7.48	5.8	7.05	6.03	6.84	7.2	6.86	7.5	6.58	7.33	6.1	6.65	6.04
AKP	51.5	50.8	49.4	48.3	49.6	51.7	50.3	51.2	47.2	50	48.2	53	51	51.6	49	52.2	51.3	50	50.8	49.9
VP	188.5	182	179	181.5	191.5	186.5	179	188	188	189	189	185	187	187	186	186	188.5	187	190	188.5
FR	10–20/09	20–30/09	20–30/09	1–10/09	20–30/09	10–20/09	20–30/09	20–30/09	10–20/09	10–20/09	20–30/09	20–30/09	10–20/09	10–20/09	20–30/09	10–20/09	1–10/09	10–20/09	20–30/09	20–30/09
BB	9–17/04	18–25/04	17–23/04	19–25/04	9–14/04	8–15/04	17–25/04	8–18/04	2–8/04	11–16/04	9–17/04	07–15/04	09–14/04	9–15/04	08–15/04	7–14/04	9–14/04	8–15/04	9–16/04	11–17/04
D	Pg	Pa	Pa	Pa	Pg	Pg	Pa	Pa	Pa	Pg	Pa	Pa	Pa	Pa	Pg	Pg	Pa	Pg	Pg	Pa
BT	T	La	La	T	Mi	T	La	T	T	T	T	La	T	T	T	T	T	T	T	La
CA	I	I	I	I	H	H	I	I	I	I	I	H	H	H	I	I	I	I	I	I
FS	O	R	LT	BE	BO	E	R	O	O	BO	R	O	BO	BO	R	R	O	R	R	O
SS	I	S	S	S	W	W	I	I	S	S	S	I	W	W	W	W	W	W	I	W
EKR	Mo	Li	Mo	Li	Li	Li	Li	Li	Mo	Li	Mo	Li	Li	Li	Li	Li	Li	Li	Mo	Li
KC	LA	EL	LA	LA	LA	LA	A	LA	Li	Li	Li	EL	EL	EL	EL	EL	EL	EL	LA	A

Note: TCSA (cm^2) = trunk cross sectional area; ACD = average crown diameter; ACPS (m^2) = area of crown projection on the soil (canopy) in the 15th year; ATH (m)= average tree height; CV (m^3) = crown volume; YPTY (kg) = yield per tree (young trees in first years of production); YPT (kg) = yield per tree; SI = size index – calculated as (fruit large diameter + fruit small diameter)/fruit height; LDF (mm) = large diameter of the fruit; ST (mm) = shell thickness; SF1 = share of fruits depending on large diameter >32 mm; SF2 = share of fruits depending on large diameter 30–32 mm; SF3 = share of fruits depending on large diameter <30 mm; AFW (g) = average fruit weight; AKW (g) = average kernel weight; AKP (%) = average kernel percentage; VP (days) = vegetation period; FR (days/month) = fruit ripening period; BB (days/month) = bud break period; D = dichogamy; BT = bearing type; CA = catkins abundance; FS = fruit shape; SS = shell seal; EKR = ease of kernel removal; KC = kernel color. Pg = protogynous; Pa = protandrous; T = terminal; La = lateral; Mi = mixed; I = intermediate; H = heavy; O = ovate; R = round; LT = long trapezoid; BE = broad elliptic; BO = broad ovate; E = elliptic; S = strong; W = weak; Li = light; Mo = moderate; EL = extra light; LA = light amber; A = amber.

and WGA89 primers selected from Dangl et al. (2005). The amplified fragments were analyzed using an ABI 3730.

2.4. Data analysis

2.4.1. Phenotypic data

The data matrix for quantitative variables was standardized according to Corrado et al. (2009) prior to the calculation of the dissimilarity coefficients. For quantitative variables and ordinal qualitative traits, the dissimilarity coefficients between cultivars were computed using Gower distances (Gower, 1971). Non numerical characters were treated as nominal (fruit ripening period, bud break, dichogamy, bearing type, fruit shape and kernel color) and ordinal (catkins abundance, shell seal, ease of kernel removal). For nominal qualitative traits we used Hamming's coefficient (Hamming, 1950) to calculate distances. The analyses were conducted using PAST software (Hammer et al., 2001). The three dissimilarity matrices, generated for each of the morphological traits mentioned above (continuous, ordinal and nominal), were summed term by term, using a weighted approach taking into account the different numbers of variables analyzed to calculate each matrix (Corrado et al., 2009).

2.4.2. Molecular data

For the amplifications using RAPD primers, images were analyzed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Amplified bands were scored present (1) or absent (0) and data entered into a binary matrix. The genetic distance between accessions was calculated using Jaccard's coefficient of similarity (Jaccard, 1901) in PAST software.

For SSR analysis, fragment length data were collected using GENE MAPPER (Applied Biosystems). Genetic distances among genotypes were estimated using the Smouse and Peakall (1999) coefficients in GENALEX (Peakall and Smouse, 2006).

The number of alleles (Na), effective number of alleles (Ne) and allele frequencies were calculated using GENALEX. Observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC) were calculated using CERVUS ver. 3.0 software (Kalinowski et al., 2007).

On the basis of the distance matrices obtained, walnut cultivars were clustered by the unweighted pair-group method with arithmetic averages (UPGMA) using Neighbor module in PHYLIP (Felsenstein, 1989) and the generated dendrograms were visualized using TREEVIEW software (Page, 1996). Also, using PAST software, principal component analyses (PCA) were undertaken for the morphological and RAPD data, to show the relationship between genotypes.

2.4.3. Concordance tests among data

The three dissimilarity matrices, generated with each of the morphological, RAPD and SSR markers were tested in parallel for correlation using Mantel tests in GENALEX with 9999 permutations. PAST software was used to calculate a co-phenetic correlation coefficient (r) between the co-phenetic matrix and the original matrix of similarity to measure the goodness-of-fit of the UPGMA clustering. The correlation coefficient varies from 0 (no correspondence) to 1 (total correspondence).

3. Results

3.1. Phenotypic analysis

According to the UPOV guidelines, all accessions were morphologically distinct. There was no statistical significant correlation between the distances based on nominal and ordinal variables ($p = 0.063$), but the distances based on nominal traits and the distances based on continuous characteristics were correlated ($p = 0.037$) (Table 4). Also, the distances based on ordinal traits and the distances based on continuous traits were correlated ($p = 0.031$), thus yielding related clustering patterns of the cultivars (Fig. 1). The clustering based only on ordinal or nominal traits could not discriminate all the accessions. The clustering based on continuous traits effectively discriminated all the accessions (Fig. 1).

The calculated genetic distance based on morphological characters, using 24 variables, ranged from 0.08 (between Unival and Valcor) to 0.66 (between Argesean and Ferjean), with a mean of 0.36.

3.1.1. Principal component analysis

Factor analysis was used based on principle components to provide a reduced dimension model indicating differences measured among groups (Fig. 2). The first principle component (PC) represented a total variance of 29.20% (Eigenvalue of 7.592), while the second and third PCs contributed each to total variance with 17.53 and 11.54%, respectively (Eigenvalues of 4.559 and 3.000, respectively). The PC loadings for traits in PC1 showed that average kernel weight (0.316), average fruit weight (0.315), size index (0.272), large diameter of the fruit (0.268) and trunk cross sectional area (0.230) were major differentiators. The variables that mostly defined PC 2 were: shell thickness (0.326), average crown diameter (0.317), area of crown projection on the soil (canopy) in the 15th year (0.306), crown volume (0.301) and ease of kernel removal (0.295). Bud break (days/month) (0.337), crown volume (0.292), bearing type (0.267), share of fruits depending on large diameter >32 mm (0.259), and dichogamy (0.256) had the highest loadings in PC3.

3.1.2. Cluster analysis

The UPGMA analysis of morphological measurements corresponded well to the evaluated accessions' origins with a few exceptions (Fig. 3A). Due to the sufficient variability, all varieties were discriminated and clustered into two main groups. A cophenetic correlation coefficient of 0.73, measured between the similarity matrix and the co-phenetic matrix originated from the dendrogram, indicated a fair fit.

Cluster analysis of the selected genotypes detected a relationship between the morphological diversity and the geographical location of the accessions. All the Romanian accessions, except from Roxana and Secular, were clustered together. These last two were grouped with O2 accession, of Caucasian origin, as a subgroup in the cluster containing all the foreign accessions, where, also all the accessions of French and American origin formed a subgroup.

Within the Romanian origin group, all the accessions created at SCDP Valcea were clustered together in a subgroup. The close relationship between these accessions is supported mainly by the similarity of quantitative characters, like shell thickness, size index and large diameter of the fruit. The results of the cluster analysis partially confirmed the results of PCA on the genotypes (Fig. 2).

3.2. Molecular analysis

3.2.1. RAPD analysis

All the primers yielded scorable amplification patterns. The total number of polymorphic bands obtained was 206, with a mean of 8.2 polymorphic bands per primer. The most polymorphic bands (15) were obtained with OPF10 primer (Table 3). The polymorphism ranged from 33% (primer OPH15) to 100% (primers OPF10 and OPE14) with a mean of 76.3%.

3.2.1.1. Principal component analysis. In order to analyze the information specificity yielded by each RAPD primer, we assessed correlations of the genetic similarity estimates obtained from each

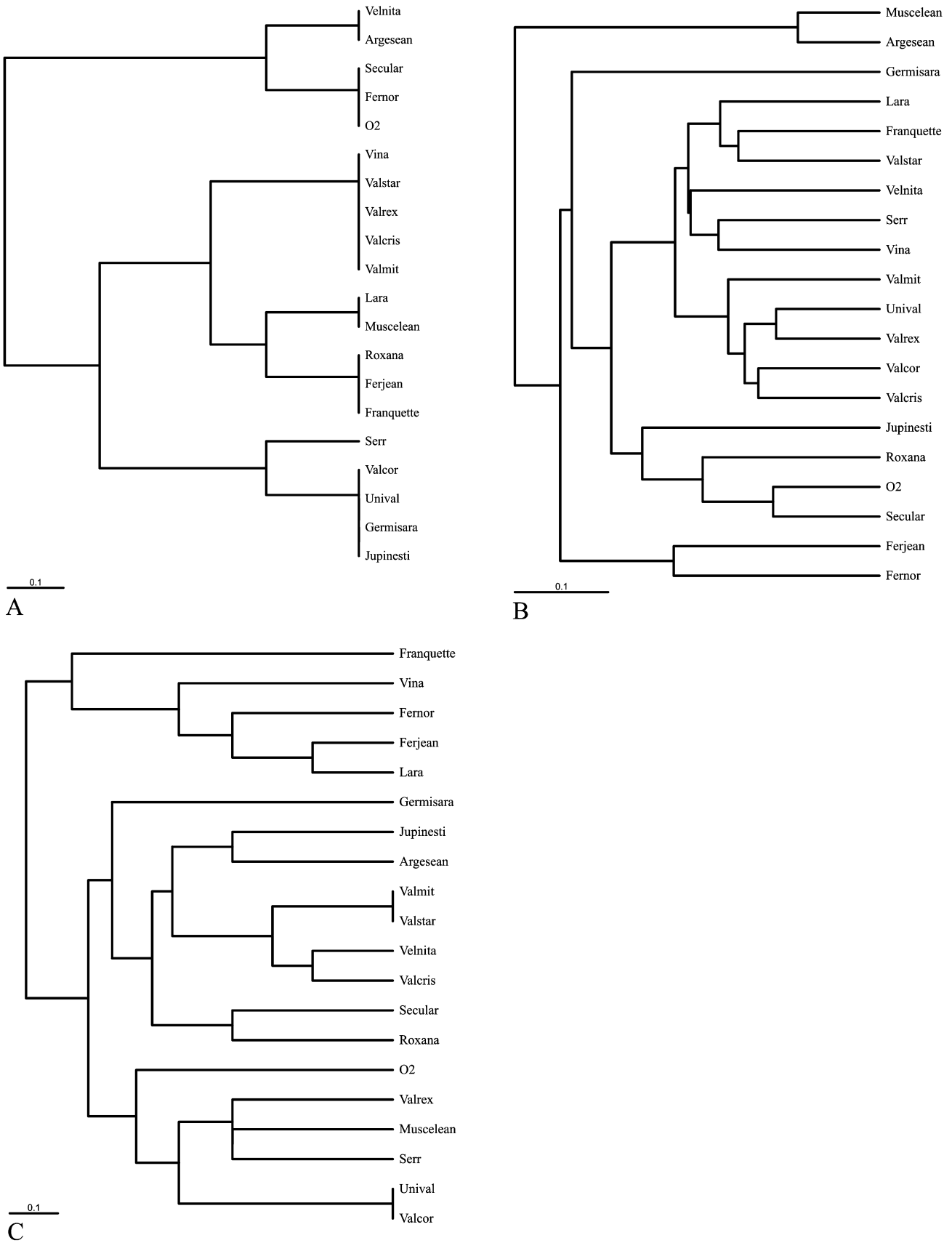

Fig. 1. UPGMA clustering of the accessions based on genetic distances calculated using ordinal traits (A), continuous traits (B) and nominal traits (C). Distances were obtained using the Gower coefficient for ordinal and continuous data (after standardization) and Hamming distances for nominal traits.

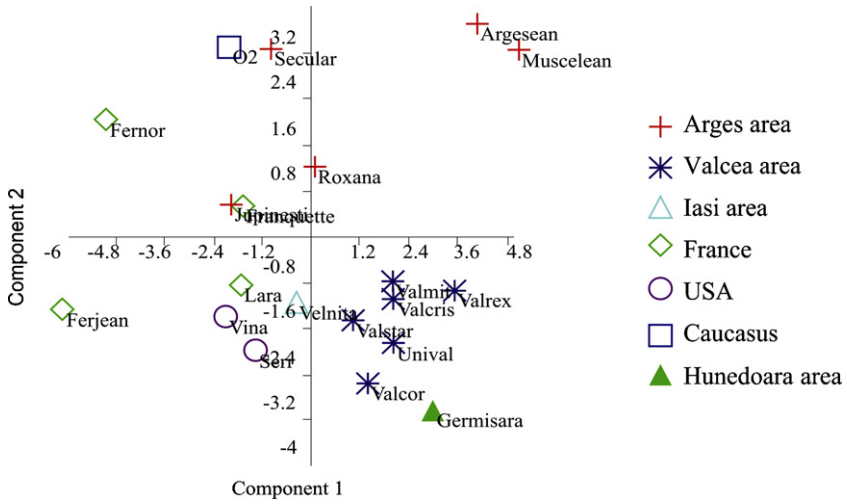

Fig. 2. PCA of the selected walnut accessions based on 24 morphological traits.

primer with the genetic similarity obtained from all the remaining primers. Sixteen out of 25 primers were positively correlated with the remaining primers, with values ranging from 0.22 to 0.68, suggesting that the redundancy of information content in the selected RAPD primers is primer-specific. Also, a PCA analysis was carried out, that supported the abovementioned affirmation, as a lack of clustering of primer pairs for the first 3 components (the summed total variance equaling 45.10%) was observed (data not shown).

3.2.1.2. Cluster analysis. Each accession could be clearly distinguished from the others. The dendrogram constructed using PHYLIP software is presented in Fig. 3B. A high co-phenetic correlation coefficient of 0.91 was obtained, showing a good fit. The used RAPD primers did not cluster the accessions according to their origin.

Table 3
RAPD primers used for differentiation of the twenty analyzed walnut accessions and their polymorphism.

Primer	Total no. of bands	No. of polymorphic bands	Polymorphism (%)
OPA 01	10	8	80
OPA 03	14	12	85.7
OPA 04	12	9	75.0
OPA 06	10	9	90.0
OPA 09	9	8	88.9
OPA 11	13	10	76.9
OPA 20	11	9	81.8
OPAB 11	14	11	78.6
OPAL 20	9	7	77.8
OPB 08	10	9	90.0
OPB 10	9	6	66.7
OPB 11	12	8	66.7
OPB 17	8	3	37.5
OPC 02	10	6	60.0
OPC 08	11	10	90.9
OPC 14	11	9	81.8
OPC 15	11	8	72.7
OPD 16	11	7	63.6
OPE 14	12	12	100.0
OPF 02	8	4	50.0
OPF 20	15	11	73.3
OPF 10	15	15	100.0
OPH 02	13	10	76.9
OPH 12	6	3	50.0
OPH 15	6	2	33.3
Total	270	206	76.3

Table 4
Pairwise comparison of morphological and molecular data for 20 walnut accessions.

	Morphological data			Molecular data	
	Nominal	Continuous	Ordinal	SSR	RAPD
Nominal	–	0.037	0.063	0.000	0.305
Continuous	0.102	–	0.031	0.048	0.427
Ordinal	0.413	0.185	–	0.104	0.241
SSR	0.366	0.201	0.111	–	0.438
RAPD	0.307	0.021	0.078	−0.034	–

Note: the results below the diagonal compare the similarity matrices. Numbers above the diagonal indicate the statistical significance (p) (Mantel test, 9999 permutations).

3.2.2. SSR analysis

All the tested loci were polymorphic. All primer pairs produced a maximum of two bands per genotype, in accordance with the diploid structure of walnut. Genotypes with a single amplified fragment were considered as homozygous for that particular locus, because segregation analysis is necessary to detect the presence of putative null alleles (Callen et al., 1993). The number of scored alleles ranged from 3 in WGA332 to 13 in WGA276, with a total of 47 and an average of 6.71 alleles/locus. The number of effective alleles represented 54.65% of the total number of alleles and was strongly correlated to the number of alleles/locus ($r = 0.89$, $p = 0.008$). The alleles' frequencies ranged from 2.5% to 57.5%, with an average of 14.89%. The most frequent allele was 214 bp at locus WGA89. Fifteen alleles, private to single individuals were observed, the most being generated by primer WGA276 (Table 5). PIC ranged from 0.512 (WGA89) to 0.826 (WGA276) (Table 6), proving that all markers were highly informative (PIC > 0.50) and useful for genetic diversity studies. The 3-primers combination WGA 001, WGA069 and WGA089 allowed the unequivocal differentiation of all the cultivars studied (Table 5).

The average observed (Ho) and expected (He) heterozygosity were 0.650 (Sd = 0.112) and 0.718 (Sd = 0.094), respectively (Table 6). The large Sd values mainly resided in the large variation in the number of alleles per locus and allele frequency distribution detected among accessions. Loci having a smaller number of alleles tended to have also lower heterozygosity and vice versa.

3.2.2.1. Cluster analysis. The data showed that the selected SSR loci were useful for depicting the presence of a good level of diversity among the analyzed samples. An UPGMA dendrogram was

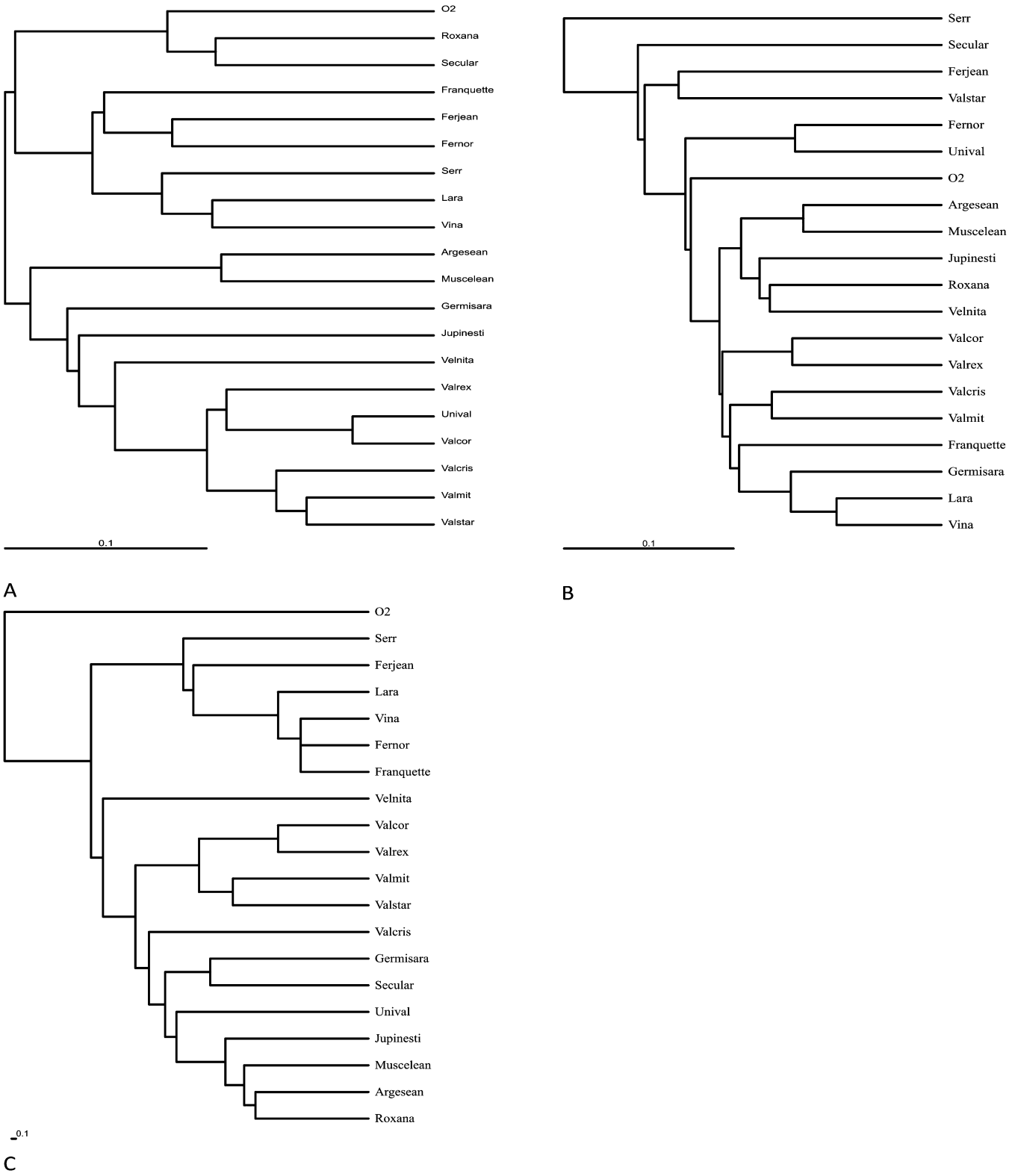

Fig. 3. UPGMA dendrograms of walnut accessions based on genetic distances calculated using morphological data (A), RAPD data (B) and SSR data (C). Distances were obtained using a weighted approach for morphological data, Jaccard's coefficient for RAPD data and Smouse and Peakall's coefficient for SSR data.

further built using the genetic distance matrix obtained, showing a cophenetic correlation coefficient of 0.79, which indicated a good fit. The dendrogram organized all of the samples into two main clusters, which generally agreed with their geographic origins and pedigree (Fig. 3C). "O2" accession, of Caucasian origin, separated itself as an outgroup. The first cluster, from "Serr" to "Franquette," consists of the cultivars with origin in France and USA. In the first cluster, a discrete sub-cluster can be defined, and for its sub-groups, a close relationship with their pedigree can be established. The second cluster, from "Velnita" to "Roxana," includes all the

Table 5
Allelic profiles of the 20 walnut accessions using 7 SSR loci.

	WGA1		WGA69		WGA89		WGA118		WGA276		WGA332		WGA376	
Argesan	188	188	158	176	214	218	182	194	168	174	219	222	239	245
Ferjean	186	190	158	158	210	214	196	196	172	188	219	222	253	253
Fernor	186	188	158	174	214	214	196	196	188	188	219	219	245	245
Franquette	188	188	158	174	214	218	196	196	188	188	219	219	245	253
Germisara	188	190	176	176	214	218	194	194	170	170	213	219	237	251
Jupinesti	188	188	158	168	210	218	182	194	172	178	219	219	237	253
Lara	186	188	158	158	214	214	182	196	188	188	219	219	245	253
Muscelean	186	188	158	176	214	218	182	190	172	186	219	219	237	245
O2	186	186	174	174	218	218	184	194	152	160	213	213	229	237
Roxana	178	188	158	176	214	218	182	194	172	174	213	219	231	237
Secular	188	190	174	176	214	218	182	194	172	188	213	213	237	255
Serr	178	190	176	178	214	216	182	196	170	188	213	219	245	253
Unival	182	188	158	158	214	218	182	196	172	186	213	213	229	237
Valcor	188	190	158	160	214	214	190	194	172	178	213	213	253	253
Valcris	178	188	168	168	214	218	194	194	166	170	213	219	231	253
Valmit	188	188	158	158	214	220	190	190	178	184	213	222	237	253
Valrex	178	188	158	176	214	214	194	194	172	178	213	222	253	253
Valstar	188	190	158	158	214	218	182	190	166	180	213	222	253	253
Velnita	174	188	160	160	212	214	182	182	172	176	219	222	231	253
Vina	188	190	174	178	214	214	196	196	188	188	219	219	245	253

Romanian accessions. Within this cluster, "Velnita" is separated from the other cultivars, as it is the only one with the geographic origin in Iasi. The other cultivars are clustered into two subgroups, one consisting of cultivars only from Valcea region ("Valcor," "Valrex," "Valmit" and "Valstar") and one including mostly cultivars from Pitesti and Arges regions ("Secular," Jupinesti," "Muscelean," "Argesan" and "Roxana"), but also from Valcea region ("Valcris" and "Unival") and the only selection from Hunedoara region ("Germisara").

3.3. Comparison of marker systems

A cluster analysis to identify possible uniform subclasses of markers was made using data reported in Table 4. As shown in Fig. 4, SSR and morphological markers fell in the same cluster, while RAPD markers represented a different cluster. The co-phenetic correlation coefficient ($r = 0.68$) measured between the similarity matrix and co-phenetic matrix originated from dendrogram indicated a modest fit. The highest correlation coefficient was found between the distances based on ordinal and nominal traits, although this correlation was not statistically significant. The most statistically significant correlation was seen between distances based on nominal traits and SSR markers. There was a low correlation between the distance matrix obtained with RAPD primers and the distance matrix obtained with morphological markers ($R = 0.048$). Moreover, a negative correlation was found between the distances generated with RAPD and SSR primers ($R = -0.034$), but was not statistically significant.

Table 6
Genetic characterization of seven microsatellite loci for 20 walnut accessions.

Locus	Na	Ne	Ho	He	PIC
WGA1	6	2.941	0.750	0.677	0.622
WGA69	6	3.587	0.550	0.740	0.687
WGA89	6	2.353	0.700	0.590	0.512
WGA118	5	3.883	0.550	0.762	0.696
WGA276	13	6.349	0.750	0.864	0.826
WGA332	3	2.572	0.500	0.627	0.531
WGA376	8	4.000	0.750	0.769	0.716
Mean	6.714	3.669	0.650	0.718	0.656
Sd	3.15	1.34	0.11	0.09	0.11

Note: for each locus total number of alleles (Na), effective number of alleles (Ne), observed (Ho), and expected heterozygosity (He), polymorphic information content (PIC).

4. Discussion

4.1. Phenotypic data

Although morphological traits can be greatly influenced by environment, they can be taxonomically informative (Green, 1969). The morphological attributes showed considerable variability among the accessions, especially average kernel weight, average fruit weight, large diameter of the fruit, size index and trunk cross sectional area, as having major loadings for the first principal component in the PCA analysis. The structure of the first three principal components, as well as the loadings' values are in agreement with previously published studies by Arzani et al. (2008) and Fatahi et al. (2010), employing criteria with high heritability, such as leafing date and nut characteristics. Due to the high diversity in the assessed traits, it is advisable to use the conserved accessions also for future breeding programs, in order to obtain improved genotypes.

In Fig. 2 it is not surprising that Lara plots near the US cultivars, since it is a well known "secret" that Lara was, in fact, a US-derived

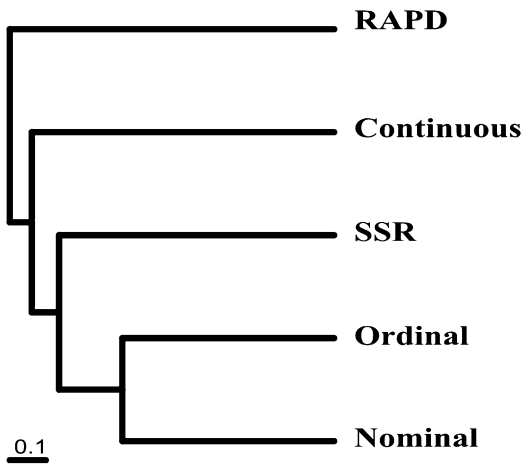

Fig. 4. Dendrogram of the markers used in this study. Correlations between distance matrices were used to perform the UPGMA clustering algorithm. A cophenetic value of the UPGMA clustering was also used to test for goodness-of-fit of the clustering to the similarity matrix on which it was based.

clone that was "borrowed" and re-labeled by the French (K. Vahdati, personal communication).

The UPGMA analysis resulted in two distinct clades with most of the Romanian accessions forming a separate clade from the foreign ones. This is not surprising, since they are visibly distinct from the foreign accessions evaluated here. Noticeable was the clustering of the two Romanian accessions "Roxana" and "Secular" with the "O2" accession from Caucasus, in a subgroup of the foreign accessions' cluster. This can be due to the similar morphological traits, especially nominal traits.

The clustering based only on ordinal or nominal traits could not discriminate all the accessions, as the accessions fell into a few unresolved classes, but the clustering based on continuous traits efficiently differentiated the accessions. Moreover, the correlation of the distances based on qualitative and quantitative variables was weak, yet significant, and lower than that observed with the SSR markers. However, the phenotypic data yielded three unrelated clustering patterns of cultivars, similar results being reported by Corrado et al. (2009). Our data imply that the type and the number of phenotypic characteristics scored can greatly affect the result of the analysis, particularly when qualitative and quantitative data are combined.

4.2. RAPD data

The RAPD primers used produced a unique fingerprint for each of the walnut genotypes included in this study, allowing unambiguous identification of each genotype. This is important for the characterization of cultivars, since each cultivar is defined by a set of markers (Nicese et al., 1998). The high level of polymorphism obtained is characteristic for a germplasm collection that includes accessions from different countries of origin and is correlated with results published by Erturk and Dalkilic (2011) and Fatahi et al. (2010). Also, it might reflect the outcrossing nature of walnut, since our results agree with previous studies using RAPDs in other outcrossing fruit tree species such as pistachio (Wang et al., 2010) or olive (Belaj et al., 2003). Previous studies using RAPDs in walnut (Nicese et al., 1998) reported a lower degree of polymorphism detected, but this could be explained by the strict criterion adopted to score the markers and the genetic origin of the tested cultivars.

Nicese et al. (1998) found a cophenetic correlation coefficient of 0.65 for 19 walnut genotypes with 23 RAPD primers, while we report here a higher correlation coefficient (0.91). They suggested that a higher number of markers are required to acquire a dendrogram that accurately reflects the similarity matrix. Erturk and Dalkilic (2011) obtained a cophenetic correlation of 0.91 using 37 primers on eight walnut genotypes. In their study, a lack of clustering according to the geographic origin was seen, as the eight cultivars of different geographic origin clustered together. The same lack of clustering according to geographical origin was reported by Hagidimitriou et al. (2005) regarding 34 olive cultivars studied with twelve RAPD, four AFLP markers and ten morphological traits.

The lack of clustering according to the geographical origin or pedigree can be due to the occurrence of nonparental bands, which has been described in previous studies with RAPDs (Pooler and Scorza, 1995) and diverse reasons have been suggested, such as formation of heteroduplex molecules between alternate RAPD alleles, mutations or recombination events within the primer binding sites or inside the amplified fragments, competition for primer binding sites or somatic rearrangements in perennial plants (Nicese et al., 1998).

The primer-specific redundancy of information content in the selected RAPD primers was also presented by Corrado et al. (2009), in regard to AFLP primers, explaining why the addition of highly polymorphic SSR markers increases the reliability of relationship inference based on a high number of AFLP bands.

4.3. SSR data

The results support that SSR markers developed initially in *J. nigra* can be used to determine the level of genetic variability in *J. regia*, in accordance with previous studies (Gunn et al., 2010). All the studied cultivars presented a unique SSR fingerprint, confirming the high efficiency of these markers. Moreover, the average number of alleles detected per locus (7) and the mean polymorphic information content of the markers (0.6), sustain that SSRs are a more suitable tool for walnut identification than other molecular marker systems previously used (Erturk and Dalkilic, 2011; Fatahi et al., 2010).

The effective number of alleles was lower than that observed in all samples, differences that may be due to the presence of private alleles that exist in a few genotypes, which could be employed for their identification, the same situation being reported by Ruiz-Garcia et al. (2011). Observed heterozygosity was lower than expected for five loci out of seven and thus the population inbreeding coefficient values were positive. These results are not surprising, given the artificial nature of the population. The observed genetic heterozygosity of the assessed walnut collection was high, with an average value of 0.65, comparable to previous studies (Dangl et al., 2005; Foroni et al., 2007; Ruiz-Garcia et al., 2011). The presence of private alleles in some cultivars, as explained by Ruiz-Garcia et al. (2011), could be due to a mutation in the microsatellite sequence that would create longer or shorter new alleles. The average number of alleles per locus, higher than previously reported with the same markers (Dangl et al., 2005; Ruiz-Garcia et al., 2011) and the high level of heterozygosity are indicative of the large DNA diversity of the walnut accessions studied.

The comparison of the allele sizes (in base pairs) for the "Franquette" and "Serr" cultivars obtained with the seven SSR markers studied in common by Dangl et al. (2005) and Ruiz-Garcia et al. (2011) and in the present analysis detected small changes that may have resulted from differences in methodology (Ruiz-Garcia et al., 2011) and the equipment used (Wunsch and Hormaza, 2002). There was no marker with identical results across the three studies, although similarity between two studies taken separately exists (e.g. WGA001 and WGA089 between Dangl et al. (2005) and Ruiz-Garcia et al. (2011), WGA376 between Ruiz-Garcia et al. (2011) and the present study). All markers showed alleles either smaller or larger with 1–4 bp across the three studies, but the differences between consecutive alleles were identical. As indicated by Brownstein et al. (1996), cited by Ruiz-Garcia et al. (2011), most of these differences might be interpreted as stutter due to extra base additions that appear with some *Taq* polymerases.

Considering the different results published by Ruiz-Garcia et al. (2011), Dangl et al. (2005) and our study, the analysis of identical samples and the comparison of walnut SSR data among laboratories working on walnut genetic resources could help at developing a consistent method for genetic fingerprinting using reference SSR loci, as has previously occurred in grape (This et al., 2004). We confirm, as recommended by Ruiz-Garcia et al. (2011), this set of reference alleles could be employed in order to code the data from multiple laboratories and would enable a very easy comparison of data and/or genetic resources. Also, for a better and more-rationalized management of walnut collections, the establishment and feeding of a uniform database with confirmed microsatellite profiles for true-to-type walnut cultivars would be of interest.

A definite clustering of the cultivars was established according to their geographical origin. For the French and American cultivars, a close relationship between the known pedigree and the

genetic similarity was observed with SSRs. Thus, among all the genotypes tested, "Fernor" showed a similarity value of 3 with the cultivars "Franquette," "Lara" and "Vina," and also, "Franquette" showed a similarity value of 3 with the cultivar "Vina." This can be explained by the fact that "Fernor" cultivar comes from a cross "Franquette" × "Lara" and "Vina" comes from a cross "Franquette" × "Payne" (Table 1). The clustering is also consistent with previous results (Dangl et al., 2005; Foroni et al., 2007; Ruiz-Garcia et al., 2011).

The Romanian accessions assessed in this work are cultivars selected from local populations. As expected, most of the cultivars representing selections from Valcea region clustered together. Some cultivars, however, were set outside of their regions. The other cluster, including cultivars not only from Arges region, but also from Valcea and Hunedoara region, might be explained by the movements of plant material from one region to another.

The number of polymorphic markers to use is important to detect true associations among accessions and developing cost efficient conservation strategies and breeding programs. Up to now, there are no firm guidelines as to the number or type of markers necessary for a particular use. The use of this particular set of SSR markers allowed us to differentiate 100% the analyzed cultivars; the same result being obtained also by using just three markers (WGA 001, WGA069 and WGA0897), confirming the high efficiency of this type of markers for cultivar identification. These results correspond to some extent to those reported by Galli et al. (2005), who were able to discriminate 66 apple cultivars (excluding cultivars derived from clonal mutations, such as those of "Fuji" or "Gala") using just 4 SSR markers; also, in sweet cherries, 68 cultivars (89.5% of the studied population) were discriminated using 9 SSR markers (Wunsch and Hormaza, 2002). A possible conclusion can be drawn: careful selection of the SSR markers leads to a differentiation of walnut accessions with yields similar to other fruit crops.

4.4. Comparison of marker systems

All marker systems detected polymorphisms that were adequate for the discrimination of all cultivars. Morphological- and SSR-based genetic distances were related to a certain point and differed from RAPD-based genetic distances. The data based on morphological traits and RAPD markers were different in the study by Fatahi et al. (2010), reporting a few correlations between the molecular and the morphological traits. Comparably weak correlation coefficients between RAPD markers and morphological characteristics were also reported in banana (Uma et al., 2004), pomegranate (Zamani et al., 2007) and Italian olive cultivars (Hagidimitriou et al., 2005). Given this, it may be deduced that the RAPD primers used in this study screened the intronic regions of the genome; therefore, undesirable fragments represented the molecular categories (Solouki et al., 2008).

The lack of correlation between the genetic distances obtained by the different types of molecular markers employed here was previously observed by Garcia et al. (2004). Also, Leao and Motoike (2011) showed that microsatellites were more efficient than RAPDs to study genetic relationships. Differences between these two types of markers reflect the intrinsic features of the molecular assays (Powell et al., 1996). Moreover, the formation of non-parental bands and the dominant nature of RAPD primers (Weising et al., 2005) could explain why they performed less than expected in comparison to SSR primers.

We found a correlation coefficient of 0.345 ($p = 0.000$) between morphological and SSR data. Similar results were obtained by Fufa et al. (2005) in red winter wheat and Hamza et al. (2004) in winter barley. SSR markers clustered effectively the accessions according to their geographic origin and pedigree. The topologies of the dendrograms based on morphological and SSR data were somewhat similar, but no correspondence to the pedigree could be established based on morphological traits only. These confirm that phenotypic characteristics are not as reliable as SSR markers in representing genetic relationships, even when numerous traits are sampled. Studies by Rebourg et al. (2001) and Maccaferri et al. (2007), using a lower number of morphological characteristics, implied the potential of a higher number of phenotypic markers rendering a better representation of genetic distances. As stated by Corrado et al. (2009), for cultivated plants, the discrepancy between morphological and molecular diversity may result from similar selection pressure oriented toward the same traits, which conducts to similar phenotypical forms with different genetic backgrounds. Due to the scarcity of knowledge regarding the walnut genome, the number of genes involved in the morphological traits that we analyzed is very hard to predict. Qualitative traits are expected to be under monogenic or oligogenic control, while quantitative traits are governed by multiple genes and their interactions, having a more complicated genetic base (Lynch and Walsh, 1998). Studies involving comparison of distances based on morphological and molecular data have also showed that these two methods may lead to variable and often low correlations (Dias et al., 2008). Hence, in the future, it could be of interest to employ different types of molecular markers in order to assess the way they reflect morphological diversity (Reale et al., 2006). A high correspondence between molecular and morphological relationships is awaited if there is little effect of the environment on a limited gene pool (Roldan-Ruiz et al., 2001).

5. Conclusions

Studies of the relationship between phenotypic and molecular distances have implications for germplasm conservation and management, besides being of use for the assessment of genetic similarities. Our results show that morphological markers based on widely used descriptors and different types of molecular markers provide different information and may be employed for different purposes.

Each type of the three marker approaches could identify the different walnut genotypes. In general, clusters observed in both dendrograms, based on morphological descriptors and SSR markers, fit expected results. Instead, RAPD markers did not cluster the accessions according to their geographic origin or pedigree. The clusters and dendrograms showed variations depending on the type of markers used, but SSRs reflect better the genetic relationships between accessions, their geographical origin and pedigree. Also, the advantage of microsatellites is that they allow for comparison between different laboratories. Therefore, we could conclude that a protocol of accession identification and characterization would preferably include morphological and SSR markers, in order to make best decisions for conservation and breeding. Additionally, since clonally propagated species have been insufficiently studied, especially those grown in traditional farming systems, this study could also help us understand the correlation between the morphological and molecular diversity of this type of trees. According to present results, it can be recommended that both morphological and SSR markers should be used in describing the diversity in the walnut genotypes.

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