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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