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REVIEW ARTICLE

MOLECULAR MARKERS AND THEIR APPLICATION IN WALNUT IMPROVEMENT

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ABSTRACT

The capacity of molecular markers to permit the assignment of a sample to a particular individual, provenance, stand or species within an allowable livelihood of error has led to wide variety of practical applications. Breeders use markers to understand and monitor levels of genetic diversity and genetic differentiation in breeding population compared to wild relatives. When marker resolution and population genetic structure permit the identification of specific strands or provenances then breeders can potentially make use of untapped genetic diversity located there. The development of molecular markers has become an almost necessary complement to hardwood tree populations for superior growth, form and quality characteristics. Molecular markers are essentially important for determining the reproductive biology and population structure of natural plantations and identify genes affecting quantitative traits .Considerable efforts have been exerted over last forty years in conventional tree improvement programmes through breeding and selection and strategies for breeding and tree improvement of temperate hardwoods have been developed. The long generation and reproductive cycle are some of the problems imposed on conventional tree breeding programmes. Almost any kind of molecular marker can be used for fingerprinting walnut. The most widely used have been RFLPs, RAPDs, AFLPs, SSRs and ISSRs. In this paper we review the work carried out using different molecular marker approaches for improvement of walnut and analyze advantages and disadvantages of various methods.

INTRODUCTION

Walnut improvement has been an important area for over 40 years. Native to central Asia, walnut grows as a wild or semi cultivated tree in a wide area from south Eastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and Eastern Himalayas. Walnut has been cultivated for its nuts and wood for several thousand years. Walnut trees can be raised either from seeds or through budding and grafting method. A young walnut tree starts producing fruits at the age of about 15 years but optimum production starts around 25 years .Considerable effort has been exerted over the last 40 yrs in conventional tree improvement programs through breeding and selection, and strategies for breeding and tree improvement of temperate hardwoods have been developed (Burley and Kanowski, 2005., Michler *et al*. *2005*). The long generation and reproductive cycle, difficulty in conducting controlled pollinations, intermittent or scarce seed crops, and seed recalcitrance of hardwood trees are some of the limitations imposed on conventional tree breeding programs (Lantz, 2007). Moreover conventional breeding still relies on a mainstay of provenance trials to evaluate local adaptation, phenotypic selection to identify potentially superior parents, progeny trials to evaluate those parents, and seed orchards for the production of adapted, improved seed. Nowadays genetic markers have become indispensable tools for understanding, managing, and improving natural and planted populations of walnuts. The discriminatory power provided by molecular markers can be used to resolve and understand hybridization and

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species differentiation. Although each marker system is associated with some advantages and disadvantages, the choice of marker system is dictated to a large extent by the intended application, convenience and the cost involved. An ideal DNA marker should have following properties.

- Easily available.
- Assay is easy and rapid.
- Highly polymorphic and reproducible.
- Co-dominant inheritance and recurrent occurrence in genome.
- Selectively neutral to environmental conditions or management practices.
- Data exchange between different laboratories should be easy.

Depending on the type of study undertaken, a marker system can be recognized that would fulfill the above characteristics. Different types of molecular markers are utilized to evaluate DNA polymorphism and are classified as:

- Hybridization based DNA markers such as Restriction Fragment Length Polymorphisms (RFLPs).
- PCR based DNA markers such as Randomly Amplified Polymorphic DNAs (RAPDs) which can also be converted into Sequence Characterized Amplified Regions (SCARs), Simple Sequence Repeats (SSRs) or Microsatellites\ Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Inter-Simple Sequence Repeats (ISSR) and Cleaved Amplified Polymorphic Sequences (CAPS).

Hybridization based DNA markers

RFLPs: Restriction Fragment Length Polymorphisms

Among the various molecular markers developed to date RFLPs were developed first and were initially used for human genome mapping (Botstein *et al*. 1980) .Later these markers were adopted for mapping plant genomes (Helentjaris *et al*.1986., Weber and Helentjaris 1989) RFLPs are DNA fragments obtained from a DNA digestion step followed by a hybridization step, thus resulting in a specific DNA-restriction enzyme-probe pattern. RFLP markers have been used in walnuts to determine parentage (Aly *et al.*1992), to determine phylogenetic relationships in the genus *Juglans* (Fjellstrom and Parfitt, 1995), to determine genetic diversity, and to identify cultivars (Fjellstrom *et al*., 1994; Fjellstrom and Parfitt, 1994a, b).Besides RFLP markers are used for constructing genetic maps. RFLPs are a strong tool for many plant breeding applications,. RFLPs are codominant and reliable markers in linkage analysis and breeding and can be easily determined in homozygous or heterozygous state of an individual. However, their utility has been restricted due to the large amount of DNA required for restriction digestion and Southern blotting, expensive and hazardous, time-consuming, and only one marker may be polymorphic, which is highly inconvenient especially for crosses between closely-related species and their inability to detect point mutations and polymorphism. The main limitation for their utilization in the identification of genotypes in plants lies in the low relation between the degree of polymorphism generated and the complexity of their use. As a consequence, new strategies mainly based on the Polymerase Chain Reaction (PCR), have been used to obtain molecular markers useful in genetic fingerprinting. Among these, RAPDs and microsatellites have been the preferred markers for fingerprinting fruit and nut tree species.

PCR based molecular markers

RAPDs: Randomly Amplified Polymorphic DNA

RAPDs are fragments of genomic DNA amplified through PCR using a decamer primer of random sequence, where polymorphism depends upon the presence or absence of an amplification product. RAPD markers have been used to evaluate the levels of polymorphism at the interspecific level between in the Persian walnut (*J. regia*) and North California black walnut (*J. hindsi*.) (Woeste *et al*.,1996a) and to identify a marker linked to hypersensitivity to the cherry leafroll virus (Woeste *et al*., 1996b). Nicese *et al.* (1998) characterized with eighteen RAPD primers a group of nineteen walnut genotypes that included closely related released cultivars and parents of breeding programs. The cluster analysis separated the genotypes into two groups based on the similarity with their ancestors. Zhang Li *et al.* 2007 generated RAPD marker $T16_{1224}$ using PCR based RAPD technique by bulk segregation analysis (BSA) for proper identification of thick and thin walnut. All these results point out that RAPD marker can be successfully used to establish identification programs for a specific laboratory, but it is difficult to compare or reproduce results among different laboratories. Due to the short length of RAPD primers (10 bp), there can be many complementary sites in the genome, and consequently, the amplification pattern obtained may vary among different assays with the same material (Jones *et al*., 1997). Although RAPD technology has proved very useful but similar to RFLP it has been put to limited use owing to the low level of polymorphism detected and sometimes also owing to lack of reproducibility of results.

AFLPs: Amplified Fragment Length Polymorphisms

These markers combine RFLP and PCR techniques, as they are specific PCR amplified fragments of restriction digests. Their use is more complex than that of RAPDs or SSRs since there are several steps involved besides PCR amplification and marker analysis. They require a genomic restriction digestion, ligation of adapters to the restriction ends and the use of primers that contain the adapter sequence, the enzyme target sequence and selective nucleotides. Amplified Fragment Length Polymorphism (AFLP) technique (Vos *et al.* 1995) was applied to analyze intraspecific diversity in different fruit and nut tree species. The diversity of walnuts in Compania originating either from seed or by vegetative propagation of 'Sorrento' has been studied by AFLP markers (Andreakis *et al*.2002). AFLP analysis for genetic diversity in low chill requiring walnut genotypes was carried out by Bayazit *et al*. 2007 .This technique reveals significant level of DNA polymorphism and appears to be most promising for fingerprinting and genetic diversity studies. In addition one of the main advantages of AFLP technique is its high multiplex ratio which means that a large number of amplified products are generated in a single reaction (Powell *et al.* 1996). Although AFLPs are also dominant markers, they reveal a high level of polymorphism and a great amount of markers per assay,

resulting in a very high discrimination power for germplasm analysis. Despite the numerous reports on the application of AFLPs for fingerprinting purposes, their use in identification of temperate fruit tree genotypes has been scarce, probably due to the availability of other approaches such as microsatellites that are easier to handle.

Microsatellites or SSRs: Simple Sequence Repeats.

Newer PCR-based techniques are being increasingly used for fingerprinting purposes. SSR analysis is a powerful and informative method to fingerprint cultivars and study genetic relationships. SSRs are abundant in most genomes, co dominant and highly reproducible. There multi allelic nature makes them especially useful for the analysis of heterozygous, allogamous species permitting the development of SSR fingerprints for each genotype (Powell *et al*. 1996). Microsatellite markers or SSRs are currently becoming the preferred technique for the molecular characterization of different plant species (Gupta and Varshney, 2000). Microsatellite markers are useful for genetic studies at varietal, species and genus level, due to the high conservation of the flanking regions(Hamza *et al*. 2004). SSRs are tendemly repeated DNA sequences of 2-6 nucleotides. Microsatellites are useful for studying the genetics of populations (Warburton and Horisington, 2001), knowing and understanding the genetic structure of populations useful for developing an optimal strategy for insitu conservation. (Gomez *et al.* 2004) to inform breeding programs. (Protis *et al*. 2004), for denominations of protected origin certification (Gemas *et al*. 2004), or to better understand how the land races adapt to different ecological and environmental stresses (Farid *et al.* 2000). Microsatellite markers have been applied for DNA fingerprinting and parentage analysis of half-sib families when no phenological and morphological data of the trees are available (Pollegioni *et al*. 2009) They exemplify numerous applications in the understanding of the genetic structure of genus *Juglans* (Dangl *et al* 2005., Foroni *et al* 2005., Victory *et al* 2006., Woeste *et al*. 2002., Foroni *et al*.2007) but not perfectly to each sample's geographic origin. Simple sequence repeat markers exhibit hypervariability and are highly informative in nature. Variations on SSRs are ISSRs or Inter Simple Sequence Repeats (Zietkiewicz *et al*., 1994). In this system microsatellites are targeted to take advantage of their abundance but without the need of prior sequence knowledge to design the primers. ISSRs are obtained through the amplification of DNA found between microsatellites, by priming the PCR reaction with a repetitive sequence anchored by arbitrary or degenerate nucleotides. As a result, a large number of bands useful for fingerprinting purposes are obtained .Inter Simple Sequence Repeats (ISSRs) were used to characterize Californian walnut cultivar germplasm (Potter *et al* . 2002).

Selective amplification of microsatellite polymorphic loci (SAMPL) is one of the microsatellite based marker systems and a modification of AFLP methodology .The same template is used as in case of AFLP. Restriction fragments resulting from the digestion of genomic DNA with two endonucleases ligated with adaptors and preamplified using primers digested on the basis of the synthetic adaptor plus the restriction site and carrying one selective base. The selective amplification is achieved using one of the standard AFLP primers with a SAMPL primer. The use of a SAMPL primer in combination with AFLP primer results in the amplification of clear and reproducible fingerprint patterns. (Kafkas *et al.*2005)

Applications of these markers for genetic studies in walnut have been diverse.main uses include:

Genotype identification and genetic diversity.

Plant genetic resources are one of the most valuable assets available to mankind. Protection and conservation of these resources for future generations therefore assume great significance. An important component for effective and efficient management of plant genetic resources as well as their utilization is characterization of germplasm. Such a characterization is important not only for identification but also to determine genetic relatedness among them. Improvement of any crop depends upon extent of diversity present in the population. Greater the diversity higher is the heterosis in F_1. Efforts have been made to predict the prospects of developing superior genotypes from a cross by the measurement of genetic similarity or genetic distance between the parents. RFLPs were successfully used to investigate the genetic diversity among 48 *J. regia* cultivars and germplasm introductions (Fjellstrom, 1993., Fjellstrom *et al*., 1994). Cluster analysis of genetic differences among accessions along with principal component analysis of allelic genotypes revealed presence of two major groups of walnut domestication. The California germplasm was associated with germplasm from France, central Europe and Iran and had less genotypic similarity with germplasm from Nepal, China, Korea and Japan. This information was used for making breeding decisions and establishing germplasm collection priorities. A patent has been obtained for the first Chinese x California cultivar. RFLPs were also used to begin mapping the walnut genome (Fjellstrom, 1993).Fjellstrom and Parfitt (1994b) also used RFLP's to estimate the genetic diversity of 13 *Juglans* species worldwide. Though no linkage maps exist specifically for the eastern black walnut, Fjellstrom and Parfitt (1994a) used the inheritance and linkage of 48 RFLP loci to establish a linkage map for Persian walnut. In 1995, Fjellstrom and Parfitt used species-level phylogenetic trees based on RFLP's (Restriction Fragment Length Polymorphisms) to detect clear distinctions between the sections of *Juglans* (e.g., old world walnuts vs. new world walnuts). Abuin *et al.* 2006 used the PCR-RFLP analysis of chloroplast DNA to determine detect inter-specific variation in the genus *Juglans* (family *Juglandaceae*). Different chloroplast DNA regions were amplified with specific primers in five species of *Juglans: J. regia, J. nigra, J. major, J. hindsii* and *J. australis*, and digested with several restriction enzymes in order to detect interspecific polymorphisms. Species-specific restriction fragment length polymorphisms patterns were determined for three species, *J. regia, J. major* and *J. hindsii*. Intra-specific variation was also found in two of the species analysed, *J. australis* and *J. nigra*. Analysis of different Juglans hybrids specimens indicated that the chloroplast genome was maternally inherited in this genus and therefore appropriate to determine maternal phylogenetic relationships. From a UPGMA and NJ cluster analysis based on a simple matching similarity matrix the Persian

walnut species was clearly distinguished from the four taxa of American walnuts. Within black walnuts, *J. nigra* from Northwest America showed a haplotype that differed substantially from *J. major, J. hindsii* and *J. australis*, which suggest that these last species may have a common maternal origin and may be distinct from *J. nigra*.

Randomly Amplified Polymorphic DNA (RAPD) loci from a walnut backcross population, [(*J. hindsii* x *J. regia*) x *J. regia*], were used to improve the genetic map. (Woeste *et al*., 1996a). Segregation data from these polymorphisms were joined to the RFLP marker data set to expand the genetic map of walnut to 107 markers in 15 linkage groups. RAPD markers were also used for molecular characterization and confirmation of genetic relatedness among walnut cultivars with known pedigree (Nicese *et al*., 1998). To evaluate the genetic diversity in 'Sorrento'walnut, Foroni *et al* . 2007 analyzed 16 'Sorrento' plants grown in Caserta (10 originated from seeds and six from grafts), and 26 grafted 'Sorrento' clones grown in the Sorrento peninsula. Their genotypes along with six other walnut cultivars using 12 microsatellite (SSR) markers. A total of 66 putative alleles were detected, 16 of which were unique to one individual. Two loci, WGA9 and WGA71, were particularly useful for distinguishing Caserta samples from Sorrento peninsula clones. The phylogenetic and structure analysis highlighted the genetic distance between the Sorrento peninsula and Caserta groups, assigning the samples to two different clusters corresponding closely, but not perfectly, to each sample's geographic origin.

The utility of intersimple sequence repeat (ISSR) markers was examined (Potter *et al*., 2002a). Like RAPD markers, ISSR markers are a quick, relatively inexpensive method for analyzing variability and developing fingerprints. They have been considered more reliable than RAPD markers due to higher reproducibility. Eight ISSR primers were found in combination to provide a unique fingerprint for each of the 48 cultivars and germplasm accessions tested. In a dendrogram developed from these data some of the groupings corresponded to expected relationships from known pedigrees but others did not, suggesting that there is a limitation in using ISSRs for inferring genetic relationships. A very useful study for rootstock breeding and selection involved DNA sequence markers (Potter *et al*., 2002b). Representatives of the five black walnut species were screened for variability 113 in the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA and in three noncoding regions from the chloroplast genome. Unique sequence markers were identified for each species. Total DNA extracts from 27 nursery source trees were tested for those markers. Chloroplast DNA profiles were used to trace the maternal linkages of the source trees, the ITS data provided evidence as to whether the source trees were themselves hybrids. The results indicated that among industry 'Paradox' sources, there is a considerable genetic contribution from black species other than *J. hindsii*. 14 microsatellite (SSR) markers were successfully used to characterize the germplasm collection at the University of California (Dangl *et al*., 2005). Primer pairs originally designed to amplify microsatellites in eastern black walnut were used (Woeste *et al*., 2002). Among the 48 accessions, there were 44 unique multilocus profiles. The accessions with identical profiles were assumed to be either synonyms or bud sports. One French cultivar was also identified as a selection and one grafting error was detected. This microsatellite method appears to be the method of choice for fingerprinting cultivars and germplasm.

Marker assisted selection

This is one of the most important applications of molecular markers. MAS is being increasingly adopted in breeding programmes since this approach permits the breeder to make earlier decisions about his selections while examining fewer plants .Several disease resistant genes have been identified using gene tagging .Walnut black line disease caused by the cherry leafroll virus causes a fatal necrosis at the graft union between *J. regia* which can be systemically infected without exhibiting symptoms and 'Paradox' or black rootstock. This virus is pollen-borne and is transmitted through flowers of the Persian walnut scion in the spring. Over the years it moves down the stem to the graft union. The hypersensitive response of *J. hindsii*, *J. nigra*, and *J. major* is conferred by a single dominant gene (Dosba and Germain, 1993). Woeste and McGranahan 1995 identified an RAPD marker in *juglans* of approximately 720 bp length at an estimated map distance of 8.45 cM from the hypersensitivity locus . The use of this marker permits rapid identification of putative hypersensitive trees in a backcross population, reducing the number of trees that need to be graft tested by 50% or more .Woeste *et al*. (1996b) published the most useful molecular tool. He identified a marker for hypersensitivity through bulked segregant analysis of backcross populations that were either tolerant or hypersensitive The hypersensitive response of the rootstock to the virus kills the scion, and appears to be governed by a single dominant gene for hypersensitivity (McGranahan *et al*., 2007).

Conclusion

Walnuts are economically important tree species cultivated throughout the world for their timber and nutritious nuts. These are valuable resources that must be managed and enhanced for productivity in a sustainable fashion. The different works reviewed in this paper reveal that the studies to identify *Juglans* species with different molecular DNA markers have succeeded in distinguishing among accessions, clarifying synonyms, identifying mislabeled cultivars and establishing genetic similarities or geographical origins. Each type of marker system has advantages and disadvantages and it is necessary to evaluate the usefulness of each marker before its application. Among the DNA markers developed in the last two decades, probably microsatellites are currently the marker of choice for fingerprinting purposes. One of the advantages is the high level of polymorphism they reveal due to the high mutation rates associated to the repetitive sequences. Another advantage is the fact that they are mostly codominant markers which allows to distinguish between heterozygous and homozygous individuals, at the same time they are inherited in a Mendelian fashion which permits to carry out paternity analyses. The main disadvantage of microsatellite markers is the large amount of effort that has to be dedicated to isolate them. Nevertheless the possibility to transport microsatellite loci among species and genera, makes a whole set of isolated

microsatellites readily available for germplasm characterization. None of the other kind of currently widespread markers used for fingerprinting purposes (RAPDs, RFLPs, ISSRs or AFLPs) meets those advantages at the same level than microsatellites. New DNA technologies are constantly being developed, SNPs (Single Nucleotide Polymorphisms) (Wang *et al*, 1998) seem to be of growing interest due to their high frequency. Although SNPs are just beginning to be used in plants (Coryell *et al*. 1999) and their application in fruit breeding schemes will not take place immediately, fruit tree identification will surely benefit from them in the future. Phenotypic observations must still complement the results obtained using molecular markers to identify clones that differ in one or few genes at least until new molecular methods become available. In fact, molecular identification is just another tool that will be added to the battery of approaches used to identify fruit tree cultivars.. There has also been extensive work on fingerprinting walnut varieties, such that now all the old varieties can be identified through DNA analysis and fairly soon all the selections in the breeding programmes will have their own unique published fingerprint. Over the next few decades, these new technologies promise to enhance and expand the toolkit available to the tree improvement specialist.

REFERENCES

Abuín1, M., Díaz1, R., Alfonsín1, J., Aleta, N., Ninot and Fernández-López1, J. 2006. PCR-RFLP Analysis of Cp-DNA in the genus Juglans. Proc. Vth Int. Walnut Symp. Eds. M.E. Malvolti and D. Avanzato *Acta Hort*., 705.

Aly, M. Fjellstrom, R. G., Mc Granahan, G. H. and Parfitt, D.E. 1992. Origin of walnut somatic embryos determined by RFLP and isozyme analysis. *Hortscience.*27:61-63.

Andreakis, N., Piccirillo, P., Santangelo, I., DiVaio, C., Monti, L.M. and Rao, R. 2002. Diversita molecolare di biotipi di noce provenient da semenzali e dell acv. 'Sorrento'. *Frutticoltura*.LXIV: 71-4.

Botstein, D., White, R.L., Skolnick, M. and Davis, R. W.1980. Construction of a genetic linkage map in man using Restriction Fragment Length Polymorphisms. *Am. J. Hum. Genet*., 32:314-341.

Burley, J. and Kanowski, P.J. 2005. Breeding strategies for temperate hardwoods. *Forestry,* 78:199–208.

Dangl, G .S. Woeste. M. K., Aradhya, M. K., Koehmstedt, A. and Simon, C. 2005. Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut. *J. Am. Soc .Hort.Sci*.,130: 348-354.

Dosba, F. and Germain, E. 1993. Behavior of the progeny of *Juglans* interspecific hybrids towards cherry leaf roll virus (CLRV). *Acta Hort*., 311:68.72.

Farid, A., O'Reilly, E., Dollard, C and Kelsey, C.R.Jr. 2000. Genetic analysis of ten sheep breeds using microsatellite markers. *Can. J. Anim.Sci*., 80 (1):9-17.

Fjellstrom, R.G and Parfitt, D.E. 1995. Phylogenetic analysis and evolution of the genus *Juglans* (Juglandaceae) as determined from nuclear genome RFLPs. *Plant Syst. Evol.,* 197(1-4):19-32.

Fjellstrom, R.G. and Parfitt, D.E. 1994a. RFLP inheritance and linkage in walnut. *Theor Appl. Genet* .,89(6): 665-670.

Fjellstrom, R.G. and Parfitt, D.E. 1994b. Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. *Genome*.37 (4): 690-700.

Foroni, I ., Woeste, K., Monti, L. M. and Rao, R. 2007 Identification of 'Sorrento' walnut using simple sequence repeats (SSRs). *Genet. Resour. Crop. Evol.,* 54:1081–109.

Foroni, I., Rao., Woeste, K. and Gallitelli, M. 2005. Characterization of *Juglans regia* L. with SSR markers and evaluation of genetic relationship among cultivars and sorento landrace. *J. Hort. Sci. Biotechnol*., 80:49-53.

Gemas, V. J. V., Almadanim, M.C., Tenreiro, R., Martins, A. and Fevereiro, P. 2004. Genetic diversity in the olive tree (*Olea europapae* L. Subsp. Europapae) cultivated in Portugal revealed by RAPD and ISSR markers. *Genet. Resour. Crop Evol*., 51:501-511.

Gomez, O.J., Blair, M.W., Frankow-Lindberg, B.E. and Gullberg, U. 2004. Molecular and phenotypic diversity of common bean landraces from Nicaragua. *Crop Sci*., 44:1412-1418.

Gupta, P.K. and R.K. Varshney, 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica* .113: 163–185.

Hamza, S., Hamida, W. B. Rebai, A. and Harrabi, M. 2004. SSR based genetic diversity among Tunisian Barley and relationship with morphological traits. *Euphytica*. 135:107-118.

Helentjaris, M., Slocum, S., Wright, A .,Schaefer and Nienhuis, J. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. *Theor. App. Genet*., 72,761-769.

Jones, C. J., Edwards, K.J., Castaglione, S., Windfield, M.O., Sala, F., van de Wiel, C. G., Bredemeijer, B., Vosman M., Matthes, A., Daly, R., Brettschneider, P., Bettini, M., Buiatti, E., Maestri, A., Malcevschi, N., Marmiroli, R., Aert, G., Volckaert, J., Rueda, R., Linacero, A., Vazquez and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Mol Breeding,* 3: 381–390.

Lantz, C.W. 2007.Genetic improvement of forest trees. In: Bonner, F.T. (tech. coord.) and Nisley, R.G. (man. ed.), Woody Plant Seed Manual. USDA Forest Service, Washington, DC.

Mc Granahan, G. 2007. Molecular Genetics of Walnuts – a Breeder's Perspective Proc. On Biotechnology. Temp. Fruit Crops and Trop. Species Eds. R.E. Litz and R. Scorza *Acta Hort*., 738, ISHS

Michler, C.H., Meilan, R., Woeste, K.E., Pijut, P.M., Jacobs, D., Aldrich, J., Glaubitz. 2005. Hardwood genetics and tree improvement. A Midwest USA perspective. In: Colombo. S.J. (comp.), Proc.: The thin green line: A symposium on the state-of-the-art in reforestation. Ont. Min. Nat. Resour., Ont. For. Res. Inst., Sault Ste. Marie, ON. For. Res. Inf. Paper. No. 160. 69–74.

Nicese, F.P., Hormaza, J.I., McGranahan, G.H. 1998. Molecular characterization and genetic relatedness

among walnut (*Juglans regia* L.) genotypes based on RAPD markers. *Euphytica*.101:199–206.

Pollegioni, P., Woeste, K., Scarascia, M. G. and Malvolti, M.E. 2009. Retrospective identification of hybridogenic walnut plants by SSR fingerprinting and parentage analysis. *Mol. Breeding*. 24:321-335.

Portis, E., Acquadro,A.,Comino,C. and Lanteri, S. 2004.Effect of farmers seed selection on genetic variation of a landrace population of pepper (*Capsicum annum* L.) grown in north west Italy. *Genet .Resour. Crop. Evol*., 51: 581-590.

Powell. W., Machray, G.C. and Provan, J. 1996. Polymorphism revealed by simple sequence repeats .*Trends in Plant Sciences*., 1.215-22.

Victory, E. R., Glaubitz, J.C., Rhodes, O.E., and Woeste, K.E. 2006. Genetic homogeneity in *Juglans nigra* at nuclear microsatellites. Amer. *J. Bot.*, 93:118-126.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Fritjters, A., Pot, Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucl. Acids Res*., 23: 4407–4414.

Warburton, M. and Hoisington, D. 2001. Applications of molecular marker techniques to the use of international germplasm collections. P.15-27. In: Henry R.J. (eds.), Plant Genotyping: the DNA fingerprinting of plants.

Weber, D., and T. Helentjaris. 1989. Mapping RFLP loci in maize using B A translocations. *Genetics*. 121,583-590.

Woeste ,K., McGranahan, G.H. and Bernatzky, R. 1996. A Randomly amplified polymorphic DNA Loci from a walnut backcross [(*Juglans hindsi* × *Juglans regia*)]. *J. Am .Soc. Hort. Sci*., 121:358-361.

Woeste K., McGranahan, G.H. and Bernatzky, R.1996b. The identification and characterization of a genetic marker linked to hypersensitivity to the cherry leaf roll virus in walnut. *Mol. Breed*., 2(3):261-266

Woeste, K., Burns, R., Rhodes, O., Michler, C. 2002. Thirty polymorphic nuclear microsatellite loci from black walnut. *J. Hered.,* 93:58–60.

Zhang, Li., Lanying, Z.and Qianwen,X. 2007. Identification of RAPD markers linked to thickness gene of shuck in walnut. *Advances in Biological Res.*1(5-6):137-140.

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