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Characterization of *Juglans nigra* (L.), *Juglans regia* (L.) and *Juglans* x *intermedia* (Carr.) by SSR markers: a case study in Italy

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Abstract

Juglans nigra and *Juglans regia* are economically important species in Europe, Asia and North America. Natural hybrids between the two species, known as *Juglans* x *intermedia* (Carr), are valued for timber production. We tested ten nuclear microsatellite markers to (1) identify new *J.* x *intermedia* hybrids and characterize their parentage species *J. regia* and *J. nigra* (2) detect *J. nigra* genotypes with a spontaneous crossing ability with *J. regia* in a mixed Italian population. This study was also designed to confirm the transferability of ten black walnut SSR loci to Persian walnut All ten microsatellites amplified in both species, producing fragments of variable size; eight (7.14%) were common, 68 (60.7%) amplified in *J. nigra* and 36 (32.1%) in *J. regia* only (private alleles). Indices of genetic diversity revealed high level of variability. The Principal Coordinate Analysis on the basis of total 112 alleles divided the total sample set into three main groups: *J. nigra*, *J. regia* and *J.* x *intermedia* hybrids. Performing the microsatellite fingerprinting, a triploid hybrid plant with two genome parts of *J. nigra* and one part of *J. regia* was identified. The cytological analysis proved this triploid state showing 48 somatic chromosomes. The mother testing analysis of the 7 diploid hybrids by exclusion method indicated one putative hybridogenic mother plants. The sequence analysis of amplified fragments confirmed the cross-species amplification of SSR. Inter-specific differences between alleles were due not only to simple changes in the number of repeats but also to mutations in the flanking regions.

Key words: Juglans, interspecific hybrids, microsatellites, cross-species amplification, sequencing.

Introduction

Juglans nigra (Eastern black walnut) and *Juglans regia* (common or Persian walnut) are highly economically important species in Europe, Asia and North America. The Persian walnut, an indigenous species in Eurasia from the Balkans to southwest China, is cultivated throughout the temperate regions of the world for its high quality wood and edible nuts. Black walnut, native to the Eastern part of North America, is a fast growing species with a dark-colored wood used in the manufacture of furniture and other wood products. Beginning in the 17th century, *J. nigra* was imported from the Eastern and Central hardwood forests of the United States to the European continent for ornamental purposes, and subsequently for its rapid growth. In Italy the black walnut is usually found in private and public parks of Pianura Padana where is also used for reforestation and recovering degradated areas (FENAROLI, 1975). Both

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common and black walnut are sensitive to soil conditions, developing best on deep, well-drained, moist and fertile soils but they differ in response and to biotic and abiotic factors (McGranahan and Leslie, 1990).

Both species are monoecious and heterodichogamous, with the same number of chromosomes (2n = 32). The mating system of black and Persian walnut is predominantly outcrossing, as they are wind pollinated, although under particular environmental conditions self-pollination is possible. Although phylogenetic analysis based on nuclear RFLP, *mat*K and ITS sequence has demonstrated that black walnut and Persian walnut belong to different sections of genus *Juglans*, *Rhysocaryon* and *Dioscaryon* respectively (Stanford et al., 2000), a hybrid between them, *Juglans* x *intermedia* (Carr), can occur naturally. However *J. nigra* pistillate flowers matures usually later then *J. regia* pollen catkins. Thus, no all plants could produce hybrids and the hybridazation between black and Persian walnut is not common. It requires the overlapping of the bloom time for the two parental trees and an appropriate temperature for pollen germination and penetration though the stigma and the style to the *J. nigra* ovary (Luza et al., 1987). The identification and selection of two hybridogenic parents is the first step to obtain hybrid progeny.

Compared to the parental species, most *J.* x *intermedia* hybrids show increased vegetative vigour, distinct disease resistance, good wood quality, and greater winter-hardiness than *Juglans regia* (Fady et al., 2003). For these reasons there is a great demand for *J.* x *intermedia* for forestry, especially in Northern Europe. The characterization of new *J.* x *intermedia* hybrids and the detection of *J. nigra* genotypes with a spontaneous crossing ability with *J. regia* is useful for selection and breeding programme on *Juglans* spp.

In the past, several methodologies have been used to distinguish between *J. nigra* and *J. regia* and to identify French and German inter-specific hybrids. They were based on morphological traits (Jay-Allemand et al., 1990), biochemical markers such as isozymes (Hussendorfer, 1999) and PCR-markers as RAPDs (Malvolti et al., 1997). Recently Woeste et al. (2002) developed a panel of thirty nuclear microsatellites in *J. nigra* L. as markers for a wide range of genetic investigations. A subset of these markers has been successfully used for clonal identification (Robichaud et al., 2006) and a broad-scale study of the genetic structure of *J. nigra* populations in the Central Hardwood Region of the United States (Victory et al., 2006). At the same time, a subset of microsatellites were also selected and screened in *J. regia* L. as a starting point for the genetic characterization of "Sorrento" variety (Foroni et al., 2005) and some walnut cultivars (Dangl et al., 2005); Pollegioni et al. (2006) carried out a preliminary study of some hybrids (*J. nigra* x *J. regia*). Microsatellite, known as simple sequence repeats (SSRs), are short (1–6 bp long), tandem repeated DNA sequences widely dispersed throughout eukaryotic genomes. These markers require the design of primers for the conserved flanking regions of the microsatellite and the PCR amplification of the repeat region. The single-locus markers are characterized by hypervariability, abundance, high reproducibility, Mendelian inheritance, and co-dominant expression. These positive features make them suitable tools for parentage analysis (Streiff et al., 1999) and molecular fingerprinting of hybrids (Nandakumar et al., 2004). Nevertheless, a detailed study of the inter-species transportability of the microsatellite markers in walnut is not yet available. Peakall et al. (1998) demonstrated that the successful cross-species amplification of SSRs does not prove the maintenance of the repeat motif in the non-source species. Studies employing cross-species amplification should therefore be accompanied by knowledge of the underlying DNA sequence.

This study refers to (1) the identification of new *J.* x *intermedia* hybrids and the characterization of their parentage species *J. regia* and *J. nigra* in a mixed Italian population using SSR markers, (2) the detection of *J. nigra* genotypes with a spontaneous crossing ability with *J. regia*. (3) In particular, this research was designed to confirm the transferability of ten black walnut SSR loci to Persian walnut and check whether interspecific differences between alleles are due to simple changes in the number of repeats or to deletions/insertions in the flanking regions.

Materials and Methods

Plant material

In the last six years the C.N.R. Institute of Agro-environmental and Forest Biology (Porano) has been involved in a wide monitoring activity of walnut germplasm in Italy (Project RI.SEL.ITALIA.). In this framework, a mixed population, including *J. nigra*, *J. regia* and some putative *J. nigra* x *J. regia* hybrids, was found in Veneto region, Villa Mezzalira Park, Bressanvido (Northern Italy 45° 39' 0" N, 11° 38' 0" E). In spring 2003, 138 total plants were sampled and the collected mature leaves were stored at –80 °C. Preliminary morphological observations of this germplasm classified 49 individuals as *J. regia*, 82 genotypes as *J. nigra* and 7 plants as "peculiar" trees. These plants, morphologically similar to *J. nigra*, were the only survivors in the dense shade of the undergrowth whereas the other plants from natural regeneration couldn't reach the second year of live. For this reason, without any other information, the owner of the park arbitrary indicated the seven trees as "putative inter-specific hybrids". Sixty samples (49 *J. regia* from 1 to 49, 8 *J. nigra* labelled N3-N4-N5-N17-N18-N21-N22-N23, 3 hybrids labelled H1, H2, H19) were taken from adult trees growing in the Park. To have an overview as more complete is possible of the pollen cloud, we sampled also 15 *J. nigra* adults located outside the garden wall. In absence of information about the origin of the *J. nigra* trees (they were planted during the 19th century by the past owners of Mezzalira country house), these last were labelled "NC" just to recognize the two sub-groups. In addition leaves were picked from 63 young plants (59 *J. nigra* and 4 hybrids labelled IMP3, IMP4, IMP9, IMP18) grown from seeds collected in this Park in autumn 2002 and actually conserved in the Veneto regional nursery (Montecchio Precalcino, Vicenza).

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DNA extraction

Genomic DNA was extracted from leaf tissue using the Qiagen DNeasy96 Plant Kit, and stored at –20°C. DNA presence was monitored by subjecting sample to 1% agarose gel electrophoresis in 0.5 x TBE buffer. The amount of DNA was spectrophotometrically determined and was brought to a working concentration of 5 ng/µL.

SSR analysis

Ten microsatellite loci (WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA276, WGA321, WGA331) already used for the genetic characterization of some walnut cultivars (DANGL et al., 2005) were amplified in all samples. Polymerase chain reaction (PCR) was done in 20 µL of reaction volume containing 20 ng of DNA template, 10 mM Tris-HCl (pH = 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ reaction buffer, 200 µM dNTP (each), 0.2 µM primer (both), 0.008 µg BSA and 0.4U of Taq polymerase (Roche Applied Science). Reactions were performed in a GENEAmp 9700 Thermocycler according to the following procedure: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at the optimum annealing temperature for each couple of primes, and 1 min at 72°C; then a final extension step at 72°C for 7 min. The amplified fragment was checked testing 5 µL aliquot of the amplified reaction by electrophoresis on 1.8% agarose gel in 0.5 x TBE buffer, and stained with ethidium bromide. To determine the exact size of the amplified microsatellite fragments, samples were diluted up to 1:10 in water and 1 µL of the diluted PCR product was mixed with 0.3 (L of a 500bp internal-lane size standard (Gene Scan™ -500 ROX, Applied Biosystem) and 9.7 µL of pure deionized-formamide, denatured in a thermocycler at 95°C for 5 minutes, and immediately chilled on ice. PCR amplification fragments were resolved by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem).

Sequence analysis

A *J. nigra* private allele and a *J. regia* private allele were sequenced at each locus using the corresponding forward and reverse primers. For locus WGA331, two additional alleles of different sizes were analyzed to validate the sequence variation of SSR flanking regions among Persian and black walnut. PCR reactions were performed on homozygous individuals, and the amplification products were subjected to electrophoresis in 1.8% agarose gel. The amplified fragments were directly purified with the QIAquick PCR purification Kit (Qiagen) and used as template for the sequencing reactions. The total reaction volume of 20 µL contained 1.5 µL of template DNA, 2 µL of 5X BigDye Terminator v. 1.1 Buffer, 4 µL of BigDye Terminator v. 1.1 Ready Reaction Mastermix (Applied Biosystem), and 0.2 µM primer. The sequencing thermal profile was 1 min at 96°C, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min on a GENEAmp 9700 Thermocycler. Sequencing reaction products were purified by SpinColumns kits (Princeton/Applied Biosystem) and run on the ABI PRISM 3100 Genetic Analyzer.

Cytological analysis

Developing male flowers from the selected trees were collected in a stage putatively young enough for the cytological examination. The buds were collected and immediately placed into the fixative solution (glacial acetic acid: absolute ethanol = 1:3) for 24 hours at room temperature; the fixative solution was refreshed twice. Subsequently the material was preserved in ethanol 70% and stored at –20°C. Developing pollen mother cells were microscopically checked after traditional aceto-carmine staining.

Data analysis

The SSR amplified fragment data were collected using Gene Scan Analysis version 3.7 Software and genotype profiles were assigned with the aid of Genotyper version 3.7 NT Software (Applied Biosystem). The Simple Match's similarity coefficient (SM- Sokal & Sneath 1963) was calculated between all pairwise combinations of individuals in order to evaluate the genetic relationships between genotypes. The Principal Coordinate Analysis (PcoordA) based on the SM matrix displayed the relative genetic distances of the genotypes in a bi-dimensional plot (ROLF's 2001, NTSYSpc version 2.1 software package).

Two different measures of genetic differentiation among groups were calculated via AMOVA (EXCOFFIER et al., 1992): WRIGHT's (1951) F_{st} coefficient, based on the Infinite Alleles Mutation Model of loci (IAM, KIMURA and CROW, 1964), and SLATKIN's (1995) R_{st} coefficient that takes into account a stepwise mutation model (SMM, KIMURA and OTHA, 1978). SMM model may reflect more accurately the mutation pattern of microsatellites. The indices of genetic diversity, number of alleles per locus (N_a), effective number of alleles (N_e), observed (H_o) and expected (H_E) heterozygosity were calculated for each locus. A Chi-square test (HEDRICK, 2000) was applied to determine whether the observed genotype frequencies were consistent with Hardy-Weinberg expectations, and a g-square test was used to test for linkage disequilibrium between SSR loci. All calcula-

Table 1. – Comparison of the genetic diversity of four *Juglans* populations using F_{ST} (WRIGHT, 1965) and R_{ST} (SLATKIN's, 1995) based on 10 microsatellite loci amplified in 137 diploid genotypes. R_{ST} and F_{ST} below the diagonals, the significance of test for each comparison, based on 1000 permutations, above diagonals.

Pairwise Population F_{ST} Values					Pairwise Population R_{ST} Values			
J. nigra	*J. nigra* NC	*J. regia*	*Hybrids 2n*		*J. nigra*	*J. nigra* NC	*J. regia*	*Hybrids 2n*
-	0.001	0.001	0.001	*J. nigra*	-	0.001	0.001	0.001
0.216	-	0.001	0.001	*J. nigra* NC	0.093	-	0.001	0.001
0.432	0.295	-	0.001	*J. regia*	0.958	0.933	-	0.001
0.197	0.174	0.220	-	*Hybrids 2n*	0.735	0.420	0.680	-

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tions were performed using POPGENE version 1.32 programme (YEH and BOYLE, 1997; http://www.ualberta.ca/~fyeh/index.htm) and GenAlEx version 6 software (PEAKALL and SMOUSE, 2005).

Sequences were analyzed and edited with the Sequence analyzing software 3.7 (Applied Biosystem) and aligned using the CLUSTAL W (http://www.ebi.ac.uk/clustalw/) multiple alignment package (THOMPSON et al., 1994). BLAST (blastn) queries were performed against the non-redundant (nr) Viridiplantae database (GeneBank database http://www.ncbi.nlm.nih.gov) to confirm the transferability of microsatellite loci and to assess the sequence variation of SSR flanking regions within *J. nigra* and between *J. nigra* and *J. regia*. Then walnut sequences were compared to the genome database of *Populus tricocharpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) to determine if the flanking regions of the microsatellites had significant homology with any known genes. BLAST searches were conducted using both the DNA sequence and the three possible amino-acid translations. The complete sequence of the corresponding clones available on the public NCBI database was used after filtering low complexity regions. All BLAST hits with expect value (E) > 1.0E -05 were eliminated.

Results

Fingerprinting analysis

All ten microsatellites amplified in both species, producing fragments of variable sizes (*Table 2*). A total of 112 alleles were detected, an average of 11.2 per primer, ranging from eight in WGA69 to 20 in WGA276. The Principal Coordinate Analysis performed on the Simple Match's similarity coefficient on the basis of 112 alleles (binary code) divided the 138 total genotypes into four distinct groups (*Figure 1*). The first principle coordinate, which accounted for 34.23% of the variance, clearly separated the two species. The second principal coordinate, explaining 6.08% of the variance, divided the *J. nigra* trees in two subgroups. *J. nigra*-NC plants, sampled around Villa Mezzalira Park, were in a close but separate group, revealing a definite genetic distinctness from the other eastern black walnut trees planted inside the Park. The putative inter-specific hybrids (*J.* x *intermedia*) H1, H2, H19, IMP3, IMP4, IMP9 IMP18 were not included in the above three groups, but on the plot were located in intermediate position between black and common walnut. Finally, one adult tree located inside park, originally classified as *J. nigra* (N21), was placed between black walnut and the hybrid groups.

Table 2. – List of microsatellite alleles that are common or specific for *J. nigra* (joining the subgroups *J. nigra* and *J. nigra*-NC,) and *J. regia* (percentage in brackets).

.Locus	Number of total alleles	Private alleles (bp)		Common alleles (bp)
		J. nigra	*J. regia*	
WGA1	9	182-184-186-188	181-193-195	191
WGA4	10	240-242-246-248-280-252-257	231-233	237
WGA9	9	229-241-251-255-257-261	239	243-247
WGA69	8	169-171-173	159-161-167	175-179
WGA89	11	190-196-201-203-207-213-222-230	215-221	211
WGA118	12	210-212-215-221-223-226-235-243	183-196-198-206	-
WGA202	11	246-248-250-252-254-258	265-267-275-295	260
WGA276	20	144-147-149-153-157-159-161-163-165-167	171-173-177-179-181-183-187-189-191-195	-
WGA321	12	236-237-242-244-246-254-264	226-239-241-243-245	-
WGA331	10	177-179-181-185-187-189-191-195	270-274	-
Total	*112*	*68 (60.7%)*	*36 (32.1%)*	*8 (7.14%)*

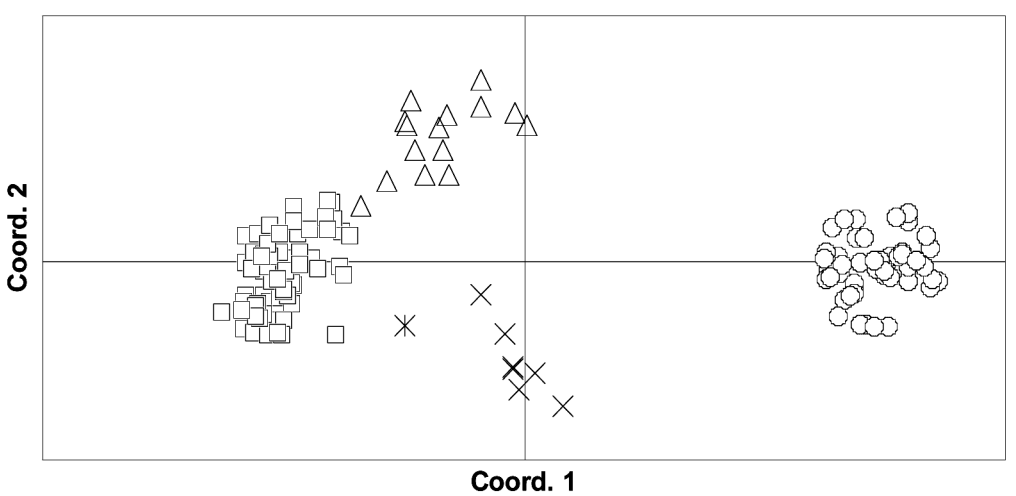

Figure 1. – Principal Coordinate Analysis of 138 *Juglans* individuals based on the genotypic similarities (SM – simple match coefficients) of 10 SSR loci. Labels stand for sample groups: ○ *J. regia*, □ *J. nigra*, △ *J. nigra*NC, ✳ N21 genotype, and × putative hybrids (H1, H2, H19, IMP3, IMP4, IMP9 IMP18).

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The pairwise values of F_{st} (WRIGHT, 1965) and R_{st} (SLATKIN, 1995) indices determined by the AMOVA analysis (*Table 1*) reflected the genetic diversity among the groups displayed in *Figure 2*. The greatest inter-population distance detected was between *J. nigra* and *J. regia* (R_{st} = 0.958; F_{st} = 0.432). The lowest genetic differentiation was measured between *J. nigra* and *J. nigra*-NC by R_{st} (0.093), and between *J. nigra*-NC and the diploid hybrid genotypes using F_{st} (0.174). The AMOVA analysis attributed 90% of the molecular variance among groups and 10% within groups using the R_{st}-statistic; by contrast, the Fst statistic partitioned 35% of the variation among and 65% within groups.

A detailed analysis of the 112 total alleles revealed that 8 alleles (7.14%) were common to black and Persian walnut and were labelled as "common alleles". In addition, 104 alleles (92.86%), 68 (60.7%) amplifying in *J. nigra* and 36 (32.1%) in *J. regia* only, were classified as species "private alleles". *J. nigra* had more private alleles than *J. regia* at nearly every locus, but these difference decrease considerably when the number of private alleles is normalized by sample size (*Table 2*). The allele frequencies for each locus (data not shown), can be provided.

The microsatellite loci revealed high level of variability in the tested samples. The number of alleles per locus (N_a), effective number of alleles (N_e), observed (H_o) and expected (H_E) heterozygosity were showed in the *Table 3*. In neither species did any of the ten loci exhibit linkage disequilibrium or any significant deviation from Hardy-Weinberg equilibrium. For the loci WGA118, WGA276 and WGA331, the length of the *J. nigra* alleles did not overlap with the corresponding *J. regia* alleles, and large differences in allelic size between species were found.

The genotypic profile of the hybrids showed one *J. nigra* and one *J. regia* private allele at the loci WGA4, WGA118, WGA202, WGA276, WGA321, WGA331, whereas in three loci, WGA1, WGA9, WGA89, one or two private alleles were replaced by one or two common alleles (*Table 4*). This fingerprinting analysis also clarified the genetic constitution of the N21 genotypes genotype. The amplification of 1 common/*J. regia* and 2 common/*J. nigra* private alleles at 6 loci, 1 common/*J. nigra* and 1 common/*J. regia* private alleles at 3 loci indicated the triploid hybrid nature of this plant, characterised by two genome parts of *J. nigr*a and one part of *J. regia*. For the primer pair WGA69, no *J. regia* and only one *J. nigra* private allele was amplified in each hybrid tree. The observed allele (range size 169–180 bp for *J. nigra* and 159–180 bp for *J. regia*) overlapped, an amplification advantage for the shorter fragments cannot explain the presence of a null allele in the hybrids. Probably the lack of the amplification was due to a mutation in the SSR flanking regions that changed the affinity of the primer pairs in the annealing sites. In addition the mother testing analysis of the diploid hybrids by exclusion method (based on Mendelian rules of inheritance)

```
WGA331_AY479963     TCCCCCTGAAATCTTCTCCTCCCATCAGAATGTTCTCTCATTTCTTCTTCCATGAATACC 60
WGA331_J.nigra      TCCCCCTGAAATCTTCTCCTCC-----GAATGTTCTC-CATTTCTTCTTCCATGAATACC 54
WGA331_J.nigra      TCCCCCTGAAATCTTCTCCTCC-----GAATGTTCTC-CATTTCTTCTTCCATGAATACC 54
WGA331_J.regia      TCCCCCTGAAATCTTCTCCTCC----AGAATGCTCTC-CATTTCTTCTTCCATGAATAAC 55
WGA331_J.regia      TCCCCCTGAAATCTTCTCCTCC----AGAATGCTCTC-CATTTCTTCTTCCATGAATAAC 55
                    ********************     ***** **** ******************** *

WGA331_AY479963     ACTTTTTACCCACCCCCTTTCAGTTTATTTTTCTTTTCTTCACCCTCAACTTATCTTCTC 120
WGA331_J.nigra      ACTTTTTACCCACCCCCTTTCAGTTTATTTTTCTTTTCTTCACCCTCAACTTATCTTCTC 114
WGA331_J.nigra      ACTTTTTACCCACCCCCTTTCAGTTTATTTTTCTTTTCTTCACCCTCAACTTATCTTCTC 114
WGA331_J.regia      ACTTTTTACCCACCCACTT-CAGTTTATTTTTCTTTTCTTCACCCTCAAATTATCTTCTC 114
WGA331_J.regia      ACTTTTTACCCACCCACTT-CAGTTTATTTTTCTTTTCTTCACCCTCAAATTATCTTCTC 114
                    *************** *** ***************************** **********

WGA331_AY479963     AGTAGACGTGCTCTCTCTCTCTCTCTCTCTCTCT------------------------- 156
WGA331_J.nigra      AGTAGACGTGCTCTCTCTCTCTCTCTCTCTCTCT------------------------- 150
WGA331_J.nigra      AGTAGACGTGCTCTCTCTCTCTCTCTCTCTCTCT---------------------- 152
WGA331_J.regia      AATAGA--TGCTCTCTCTCTCTCTCT------------CGGTCTCTTTTTCAATTTTA 160
WGA331_J.regia      AATAGA--TGCTCTCTCTCTCTCTCT--------CTTGGTCTCTTTTTCAATTTTA 164
                    * ****  **********************

WGA331_AY479963     ------------------------------------------------------------ 156
WGA331_J.nigra      ------------------------------------------------------------ 150
WGA331_J.nigra      ------------------------------------------------------------ 152
WGA331_J.regia      TTTTCACCTCCTTGTGTACCATACTCAACCGCTAGCCATGAGAACCACCATGCTCATACA 220
WGA331_J.regia      TTTTCACCTCCTTGTGTACCATACTCAACCGCTAGCCATGAGAACCACCATGCTCATACA 224


WGA331_AY479963     -------------------CACTCACTCACGCATTTGCCTTACACCACCG 187
WGA331_J.nigra      ---------------------CTCACTCACGCATTTGCCTTACACCACCG 179
WGA331_J.nigra      ---------------------CTCACTCACGCATTTGCCTTACACCACCG 181
WGA331_J.regia      CGACAGAAGCCGCTGGGGCCACTAACTCAAGCATTTGCCTTACACCACCG 270
WGA331_J.regia      CGACAGAAGCCGCTGGGGCCACTAACTCAAGCATTTGCCTTACACCACCG 274
                                  ** ***** ********************
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Figure 2. – CLUSTAL W alignment of two private alleles of *J. nigra* (179bp; 181bp), two private alleles of *J. regia* (270bp; 274bp) and the database sequence (GeneBank accession number AY479963) at locus WGA331. The forward and reverse primers are indicated in bold, gaps are indicated with dashes, stars indicate sequence identity.

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Table 3. – Values of genetic variability observed in two subgroups of black walnut (*J. nigra* and *J. nigra* NC), *J. regia* species and their hybrid: Number (N_a), size range and the effective number (N_e) of the alleles per locus, observed (H_o) and expected heterozygosity (H_E).

Source	Locus	N_a	Size range (bp)	N_e	H_o	H_E
Juglans nigra (N =66)	WGA1	5	182-191	2.703	0.652	0.630
	WGA4	8	237-257	1.834	0.439	0.455
	WGA9	6	229-261	2.901	0.667	0.655
	WGA69	2	169-171	1.163	0.152	0.140
	WGA89	7	190-213	3.337	0.758	0.700
	WGA118	3	215-226	2.297	0.697	0.565
	WGA202	5	248-260	1.618	0.424	0.382
	WGA276	6	147-167	1.924	0.500	0.480
	WGA321	6	236-264	1.265	0.212	0.210
	WGA331	6	177-195	2.921	0.682	0.658
	Mean (SE)	*5.4 (0.56)*		*2.196 (0.24)*	*0.518 (0.21)*	*0.487 (0.06)*
Juglans nigra NC (N = 15)	WGA1	4	182-197	1.891	0.533	0.471
	WGA4	6	237-252	4.891	0.800	0.796
	WGA9	6	229-257	3.435	0.800	0.709
	WGA69	4	171-180	2.332	0.467	0.571
	WGA89	7	190-230	3.689	0.800	0.729
	WGA118	7	210-243	2.744	0.667	0.636
	WGA202	6	246-260	5.625	0.800	0.822
	WGA276	8	144-167	5.172	0.600	0.807
	WGA321	6	236-264	5.357	1.000	0.813
	WGA331	7	179-195	4.787	0.800	0.791
	Mean (SE)	*6.1 (0.41)*		*3.992 (0.43)*	*0.727 (0.16)*	*0.714 (0.04)*
Juglans regia (N = 49)	WGA1	4	181-195	2.853	0.612	0.650
	WGA4	3	231-237	1.782	0.429	0.439
	WGA9	3	239-247	2.441	0.531	0.590
	WGA69	5	159-180	3.435	0.816	0.709
	WGA89	3	211-221	2.823	0.653	0.646
	WGA118	4	183-206	3.120	0.776	0.680
	WGA202	5	260-295	3.482	0.735	0.713
	WGA276	10	171-195	3.161	0.531	0.684
	WGA321	5	226-245	3.349	0.633	0.701
	WGA331	2	270-274	1.979	0.571	0.495
	Mean (SE)	*4.4 (0.70)*		*2.843 (0.19)*	*0.629 (0.12)*	*0.631 (0.03)*
Hybrid (N = 7)	WGA1	5	181-193	4.083	1.000	0.755
	WGA4	3	231-250	2.513	1.000	0.602
	WGA9	3	239-247	2.649	1.000	0.622
	WGA69	1	171	1.000	0.000	0.000
	WGA89	4	201-215	2.882	1.000	0.653
	WGA118	4	196-226	3.920	1.000	0.745
	WGA202	3	252-267	2.513	1.000	0.602
	WGA276	3	147-189	2.279	1.000	0.561
	WGA321	4	226-244	2.970	1.000	0.663
	WGA331	3	181-274	2.513	1.000	0.602
	Mean (SE)	*2.732 (0.86)*		*1.021 (0.40)*	*0.900 (0.31)*	*0.581 (0.21)*

indicated one of the eight *J. nigra* trees growing in the Park, *J. nigra*-N17, as the only putative hybridogenic mother plant in this walnut population (*Table 4*).

Somatic chromosome count was carried out on pollen mother cells from hybrids, confirming the genotyping results. The number of somatic chromosomes was verified as diploid (2n = 32) for 7 hybrids (H1, H2, H19, IMP3, IMP4, IMP9 IMP18) and triploid (3n = 48) for N21 plant.

SSR sequence comparison

Sequence analysis was carried out to confirm the identity of the amplified fragments and to assess the sequence variation of SSR flanking regions within *J. nigra* (joining the subgroup *J. nigra* and *J. nigra* NC) and between *J. nigra* and *J. regia*. In this way it was possible to explore the reasons for the large differences in the allelic size between *J. nigra* and *J. regia* observed at WGA118, WGA276, WGA331, as well as to investigate the presumed null allele at locus WGA69 in the hybrids.

When the priming sites, SSR flanking regions, and repeat motifs of black and common walnut were compared to the *J. nigra* consensus sequences available on the NCBI database using BLASTn, large interlocus differences in the number of transition and transversion events were observed (*Table 5*). In every case, the microsatellite repeat region was found and could be further classified into three categories: pure (WGA9, WGA202, WGA276, WGA321, WGA331) compound pure (WGA4, WGA89, WGA118) or interrupted (WGA1, WGA69). In *J. regia*, WGA1 contained pure $(GA)_n$ repeats instead of the interrupted microsatellite found in *J. nigra*. In both species, the flanking regions at all ten loci displayed significant homology with the corresponding *J. nigra* consensus sequences from which the primers were originally derived (E -value ≤ $3e^{-27}$). Particularly the microsatellites WGA1, WGA4, WGA9,

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WGA69, WGA89, WGA118, WGA276 and WGA321 revealed a high percentage of sequence identity with the consensus sequences at the SSR flanking regions (from 100% to 95% for *J. nigra* and from 98% to 90% for *J. regia*). In these loci, few gaps and point mutations were observed. The differences in allele size reflected mainly variation in the repeat region.

Among the eight loci mentioned above, results for WGA1, WGA118, WGA276 and WGA69 were particularly interesting. The WGA1 locus exhibited the highest percent homology in the 155 bp flanking regions, 100% for *J. nigra* with no gaps or base mutations, and 98% for *J. regia*, with gaps (1.27%) and point mutations (1.27%). At this locus the interrupted microsatellite, $(GA)_5GCA(GA)_3GCA(GA)_3$ of the *J. nigra* consensus sequences (*Table 5*) changed, becoming $(GA)_{15}G(GA)$ in the *J. nigra* private-allele 188 (bp), and a pure microsatellite $(GA)_{12}$ in the 181 bp private allele of *J. regia*. Although the SSR flanking regions were highly conserved, wide differences in allele size between *J. regia* and *J. nigra* were found at WGA118 and WGA276 (*Table 5*). The difference in length between *J. regia* 189 bp and *J. nigra* 153 bp alleles at the WGA276 locus was probably due to a large contraction of the repeat region, with the loss of 16 (GA) units. On the other hand, expansion of the repeat region by the addition of nine (GA) and 17 (GT) units occurred at the WGA118 locus, giving rise to the 226 bp allele in *J. nigra*, instead of the 196 bp allele observed in *J. regia*. The alleles of identical size (180 bp) that amplified in black and common walnut at the WGA69 locus showed different percent homology in the flanking regions (97% and 90% respectively), and several mutations in the corresponding interrupted microsatellite. In the *J. regia* sequence of WGA69 a short indel of 3 bp was observed 5 bp downstream of the forward primer. This result cannot explain the absence of a *J. regia* allele at locus WGA69 in the hybrids (*Table 2*). A dis-

Table 4. – SSR genotypes for seven diploid hybrid one triploid hybrid plant and one *J. nigra* hybridogenic plant. Allele length in base pairs (bp), – marks stand for null allele. Shading: 50% grey for *J. nigra*-private alleles, 25% grey for *J. regia*-private alleles, unshaded cells for common alleles.

		Hybrids 3n	Hybrids 2n							H. mother
Locus		N21	H1	H2	H19	IMP3	IMP4	IMP9	IMP18	N17
WGA1	Allele1	188	186	188	188	186	186	186	186	188
	Allele2	186	181	181	193	193	191	181	193	186
	Allele3	181								
WGA4	Allele1	250	250	250	250	250	250	250	250	250
	Allele2	248	233	233	233	233	233	231	231	250
	Allele3	233								
WGA9	Allele1	247	247	247	247	247	247	247	247	247
	Allele2	247	243	243	239	239	239	243	239	247
	Allele3	239								
WGA69	Allele1	171	171	171	171	171	171	171	171	171
	Allele2	171	-	-	-	-	-	-		171
	Allele3	-								
WGA89	Allele1	207	201	201	201	201	201	201	201	201
	Allele2	201	221	215	221	221	215	211	215	201
	Allele3	211								
WGA118	Allele1	221	221	226	226	226	221	221	221	221
	Allele2	226	198	196	198	196	198	196	196	226
	Allele3	198								
WGA202	Allele1	252	252	252	252	252	252	252	252	252
	Allele2	246	267	265	265	265	265	267	265	252
	Allele3	260								
WGA276	Allele1	153	147	147	147	147	147	147	147	147
	Allele2	149	189	189	189	189	189	189	179	153
	Allele3	189								
WGA321	Allele1	244	244	244	244	244	244	244	244	244
	Allele2	244	243	243	239	226	239	226	239	244
	Allele3	226								
WGA331	Allele1	181	181	181	181	181	181	181	181	181
	Allele2	181	270	274	270	270	274	270	270	181
	Allele3	270								

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Table 5. – Summary of sequence analysis of 10 microsatellite loci in the studied samples belonging to *J. nigra* and *J. regia* species. Comparison with *J. nigra* consensus sequence for the SSR flanking regions available on public NCBI database (BLASTn; WOESTE et al., 2002).

Locus SSR	Allele Size (bp)	GeneBank Accession Number	N° of (AG) repeats	*J. nigra* consensus sequence N° of (AG) repeats	BLASTn-Search for SSR flanking regions				
					GeneBank A. number	E-value	% homology	% gaps	Point mutations
									% trans[a] / % transv[a]
WGA1-*J.nigra*	188	EF640283	$(GA)_{15}G(GA)$	$(GA)_5GCA(GA)_3GCA(GA)_3$	AY465952	1e-68	100	0	0 / 0
WGA1-*J.regia*	181	EF640294	$(GA)_{12}$			1e-62	98	1.27	0.63 / 0.63
WGA4-*J.nigra*	250	EF640284	$(GT)_5(GA)_{17},(GA)_{11}$	$(GT)_5(GA)_{15},(GA)_{11}$	AY465953	1e-41	95	3	1 / 1
WGA4-*J.regia*	231	EF640295	$(GT)_5(GA)_{10},(GA)_{10}$			3e-51	95	2	2 / 1
WGA9-*J.nigra*	229	EF640285	$(GA)_7$	$(GA)_{16}$	AY465954	1e-75	99	0	1 / 0
WGA9-*J.regia*	239	EF640296	$(GA)_{12}$			1e-53	95	2	0.5 / 2.5
WGA69-*J.nigra*	180	EF640286	$(GA)_4ATATAA(GA)_{15}$	$(GA)_4ATATAA(GA)_{16}$	AY333953	1e-47	97	0	3 / 0
WGA69-*J.regia*	180	EF640297	$(GA)_4ATATAAGC(GA)_2GC(GA)_{13}$			3e-29	90	3	5.5 / 0.5
WGA89-*J.nigra*	201	EF640287	$(GT)_{12,}(GA)_{13}$	$(GT)_{13}(GA)_{21}$	AY352440	1e-47	97	0	1.35 / 1.65
WGA89-*J.regia*	215	EF640298	$(GT)_{20,}(GA)_{13}$			1e-62	97	0.06	1.5 / 0.5
WGA118-*J.nigra*	226	EF640288	$(GA)_{23}(GT)_{18}$	$(GA)_{18}(GT)_{11}$	AY479958	4e-63	98	0	1 / 1
WGA118-*J.regia*	196	EF640299	$(GA)_{14}(GT)_1$			3e-66	97	1.2	1.2 / 0.6
WGA202-*J.nigra*	252	EF640289	$(GA)_{11}$	$(GA)_{20}$	AY479959	2e-34	69	17	9.5 / 4.5
WGA202-*J.regia*	265	EF640300	$(GA)_{18}$			2e-28	68	17	10 / 5
WGA276-*J.nigra*	153	EF640290	$(GA)_{14}$	$(GA)_{14}$	AY479961	7e-36	95	2.5	2.5 / 0
WGA276-*J.regia*	189	EF640301	$(GA)_{30}$			3e-32	94	2	2.5 / 1.5
WGA321-*J.nigra*	244	EF640291	$(GA)_{18}$	$(GA)_{14}$	AY479962	5e-72	100	0	0 / 0
WGA321-*J.regia*	230	EF640302	$(GA)_{15}$			2e-43	92	4.8	0.5 / 2.7
WGA331-*J.nigra*	179	EF640292	$(GA)_{13}$	$(GA)_{13}$	AY479963	2e-45	95	5	0 / 0
WGA331-*J.nigra*	181	EF640293	$(GA)_{14}$			1e-50	95	5	0 / 0
WGA331-*J.regia*	270	EF640303	$(GA)_9$			3e-27	55	42	1 / 2
WGA331-*J.regia*	274	EF640304	$(GA)_{10}$			4e-50	55	42	1 / 2

[a] Number of transition mutations, [b] Number of transversion mutations.

tinct mutation in the annealing site of the primer may have taken place. This hypothesis could be verified only by cloning and then sequencing WGA69 from *J. regia*.

Despite the conservation of primer sequences, WGA202 and WGA331 showed the lowest sequence identities in the flanking regions. At the WGA202 locus, a substantial sequence diversity, not only among species (homology = 68%) but also within *J. nigra* genotypes (homology = 69%), was observed. In both species an indel of 37 bp was found 38 nucleotides downstream of the forward primer when compared with the expected product. There was 95% homology between the flanking region of our *J. nigra* and *J. regia* samples. At the WGA331 locus, *J. regia* alleles displayed the lowest sequence identity (55%) in comparison with *J. nigra*. The multiple sequence alignment generated by cross-species amplification of the WGA331 locus showed the complex nature of polymorphism at some microsatellite loci (*Figure 2*). Locus WGA331 had alleles of 179 bp and 181 bp in black walnut and 270 bp and 274 bp in common walnut. A large insertion of 100 bp was detected in the *J. regia* alleles between the pure (AG) microsatellite and the reverse primer; the SSR region was actually shorter than in J. nigra. We further determined that WGA331 has a putatively paralogous locus, WGA24, which is one of the thirty microsatellites identified in *J. nigra* by WOESTE et al. (2002) but not tested in this study. A subset of samples from both species was amplified using the WGA24 primer pair (data not showed). Analysis of the sequence variation for WGA24 locus revealed high levels of homology (87%) between *J. nigra* and *J. regia*. Surprisingly, the sequence of WGA331 and WGA24 showed 70% similarity at the SSR flanking regions in *J. regia* and 54% homology in *J. nigra*.

In light of the high level of conservation at the SSR flanking regions between the two species, BLAST-searches for nucleotide similarity were performed. Among others, the genome database of *Populus tri-*

Table 6. – Summary of BLASTn similarity searches between the flanking regions of three microsatellites highly conserved in *J. regia*, *J. nigra*, and *Populus tricocharpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).

Sequence ID	Size (bp)	BLASTn – Search vs. Populus tricocharpa database				Gene	Match description		
		Alignment Length (bp)	E-value	% Identity	Map Location		Putative Function	Location1	MS2
AY465952 (WGA1)	487	35	1e-7	98	LG_VIII 15395426:15395460	Fgenesh1_pg_C_LG_VIII001943	Haem peroxidase	E Secretory peroxisade domain	-–
AY465953 (WGA4)	484	35	1e-15	100	LG_I 7467113:7467167	–	–	INTG	+
		45	1e-15	91.1	LG_I 7467322:7467366	–	–	INTG	
AY479958 (WGA118)	500	125	1.9e-19	83	LG_XII 345718:345842	Eugene3.00120042	Transcription factor ZF-HD protein dimerisation region	E ZF-HD homeobox protein Cys/His-rich dimerisation region	-

[1] Location of sequences highly similar to the microsatellite flanking regions as determined by BLAST search-poplar database. E, exon; INTG, intragenic region. [2] Indicates conservation of microsatellite repeat between walnut and poplar.)

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chocarpa (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was employed to determine whether these regions had significant homology with any genes. The BLAST-search results based on the full-length sequences from which the primer pairs were designed revealed that the flanking region of two of the ten microsatellites showed significant similarity with a known gene of California poplar (*Table 6*). Upstream (215 bp) of the forward primer the WGA1 sequence (AY465952) we found a short (35 bp) stretch of high nucleotide identity (98%) to Haem peroxidase. Using the translated amino-acid sequence, we detected a region of 76.92% homology ($E = 3e^{-08}$) corresponding to a longer stretch of 78 bp located in poplar at position LG VIII:15395401-15395478. This segment of sequence, which included the 35 bp stretch described above, corresponded to the secretory peroxidase domain of the class III plant heme-dependent peroxidase superfamily. A 125 nucleotide stretch of the *J. nigra* WGA118 sequence (AY479958), immediately preceding the SSR region, matched a Transcription factor ZF-HD protein dimerisation region, with 83% nucleotide homology (E-value = $1.9e^{-19}$). By comparing the translated amino-acid sequence of the fragment, an identity of 90% was detected (E value =$7e^{-22}$). This part of the sequence encoded a ZF-HD homeobox protein Cys/His-rich dimerisation region.

Discussion

All ten primer pairs isolated from *J. nigra* also amplified microsatellites in *J. regia*. In this study the tested microsatellite markers, because of their high variability, exhibited a strong ability to discriminate walnut species and interspecific hybrids, in this case, *J.* x *intermedia* and to identify some putative *J. nigra* genotypes with a spontaneous crossing ability with *J. regia* (hybridogenic mother N17). They can also be used to verify the ploidy of hybrids as N21 genotype and for the alignment of a common *Juglans* genetic map. By the use of morphology the identification of the hybrids was not so easy. In general, traits as bark colour and ruggedness, shape of leaves, apical dominance, are not universally valid and any way appear only in adult trees. In our case both *J. nigra* plants and hybrids presented the same above characters. The N21 plant, in particular, appeared as a pure *J. nigra* and only the SSR analysis discovered its triploid nature. In addition, the microsatellite displayed high levels of genetic diversity both within *J. nigra* and between the species. A low level of ascertainment bias was observed; the average number of alleles per locus was higher for black walnut than for Persian walnut, an expected result for microsatellites discovered in one species but applied in non-source species. The mean number of alleles per locus and the effective number of alleles in each group were higher than the levels of variability detected in black and Persian walnut using other molecular markers such as allozymes (FORNARI et al., 1999) RFLP (FJELLSTROM and PARFITT, 1994) and RAPDs (MALVOLTI et al., 1997). The range of allelic richness and observed heterozygosity across the SSR loci was similar to the values reported for other domesticated tree species, black poplar (VAN DER SCHOOT et al., 2000), and *Castanea sativa* (MARINONI et al., 2003).

To determine the purity of our parental species, we compared the detected genetic variability with the results obtained on *J. nigra* natural populations (VICTORY et al., 2006), *J. regia* cultivars (DANGL et al., 2005) and varieties (FORONI et al., 2005). Moreover, preliminary unpublished data still in progress, obtained by the application of the same SSR primers set on natural and naturalised *J. regia* populations collected in France, Italy, Hungary, Greece, Caucasus, 3 Chinese Regions and Cile, were also considered. As expected, the genetic diversity detected in this study for *J. nigra* was lower than values obtained using twelve SSR loci in 43 indigenous populations of the same species (VICTORY et al., 2006), whereas it was representative of the genetic diversity observed in *J. regia* germplasm.

In black walnut, ROBICHAUD et al. (2006) demonstrated that the high levels of allelic richness and observed heterozygosity reduce the probability of pairwise genetic identity and increase the exclusion probability in the parentage analysis. These features make these markers an excellent tool for fingerprinting and parentage analysis not only in black walnut but also in Persian walnut.

A focal point of this study was the confirmation of the cross-species amplification of ten nuclear microsatellites within *Juglans* germplasm by sequence analysis. The results presented here demonstrate not only the conservation of the flanking regions of the microsatellites, but the microsatellites as well. This is a pre-requisite for using SSRs as markers in the non-source species *J. regia*. The DNA sequence analysis revealed high homology (90%) at the SSR flanking regions of the tested loci between walnut species. These data confirmed that the detected fragments were really alleles of one locus and not of closely related loci, although the observed changes in the microsatellites were sometimes more complex than changes in the number of repeat units. Interruption of the dinucleotide repeat by indels and base substitutions was observed in loci WGA1 and WGA69. The WGA69 locus exhibited the lowest total number of alleles per locus and the highest percentage of common alleles between species (25%). Several studies report that the interruption of perfect microsatellites is related to DNA stability in the region (TAYLOR et al., 1999; DAVIERWALA et al., 2000). These authors suggest that the purity of a repeat region influences the mutation rate and, consequently, the levels of polymorphism in SSR loci. Interrupted microsatellite repeats appear to have lower mutation rates than perfect repeats. This could explain the low level of polymorphism found at WGA69 in walnut. It was also interesting that the number of effective alleles of WGA1 in *J. nigra* was lower than that observed in *J. regia*. At this locus the interrupted microsatellite region of *J. nigra* was a pure repeat in *J. regia*. Microsatellite mutation rate may be affected by the nature of the flanking sequences, by the position of a microsatellite in the chromosome, and by "self-accelerating" (MORGANTE et al., 2002).

The analysis of the DNA sequences carried out in this study also proved that mutation at the SSR loci was not restricted to hypervariable regions. Insertion and deletion events in the flanking regions contributed to the

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variation in allelic size among and within *Juglans* species. Relatively low identity at the flanking regions (55%) was found at WGA331 when *J. nigra* and *J. regia* private alleles were compared. We detected the presence of two putative paralogous loci WGA331 and WGA24. We conclude that WGA331 and WGA24 might be the result of a duplication event that evolved differently in the two species. The deletion of 100 bp at WGA331 occurred in *J. nigra* only. This deletion reduced within black walnut the percentage of identity between WGA24 and WGA331 and was responsible for the low homology at WGA331 between black and common walnut.

Size homoplasy was observed at WGA69, in which alleles from *J. nigra* and *J. regia* had the same size (180 bp) but different underlying sequences. Complex mutational processes and the possibility of size homoplasy complicate the interpretation of the SSR variation and bias the interpretation of F_{ST} and R_{ST} estimates of genetic differentiation among populations. F_{ST} and R_{ST} coefficients are based on two extreme mutation models. F_{ST} is calculated assuming the Infinite Alleles Mutation Model of loci (IAM, KIMURA and CROW, 1964) that does not allow for homoplasy. R_{ST} is computed using the sum of squared number of repeat differences and is based on Stepwise Mutation Model (SMM, KIMURA and OTHA, 1978). Although more realistic mutation models could be developed, SMM seems to reflect more accurately the mutational mechanism of microsatellites than IAM. We believe the R_{ST} coefficient better estimated genetic differentiation between black and common walnut, as was reported in several studies (BALLOUX and LUGON-MOULIN, 2002). In the case of these two *Juglans* species, the SMM model appears to overstate their level of genetic differentiation (R_{st} = 0.958) because sequence analysis proved that large indels (e.g., WGA331) occurred in the SSR flanking region. According to ANGER and BERNATCHEZ (1997), strong biases in the extent of divergence among populations are likely to be generated using R_{ST} when large indels in SSR flanking regions are misinterpreted as size differences in the SSR motif. In practice, the best measure to use is far from clear, and the interpretation of data should be made very cautiously. For all these reason we reported results using F_{ST} and R_{ST}.

The usefulness and inter-specific transferability of microsatellites depends not only on their level of conservation but also on their origin and their dispersion in the genome. MORGANTE and et al. (2002) observed that dinucleotide microsatellite have been found in the non-coding portion of the genome as well as in translated (exons) and untranslated regions of genes (introns, 5'- and 3'-UTR). Genic SSRs derived from ESTs (Expressed Sequence Tags) are expected to possess high inter-specific transferability, as they belong to relatively conserved genic regions. In this study, two of eight SSR loci exhibited a high level of conservation in the flanking region within *Juglans* and even matched a locus in *Populus trichocarpa*. Specifically, WGA1 and WGA118 showed significant similarity with Haem peroxidase, highly conserved in plants, and a transcription factor ZF-HD protein dimerisation region.

In conclusion the success of cross-species SSR amplification can be increased by using EST databases as a source of SSR markers (Genic SSRs). These markers, which are likely to become more available as the cost of sequencing falls, together with anonymous SSRs derived from enriched genomic DNA libraries, are increasingly important tools for genetic studies in walnut.

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